The microbiology of chronic suppurative otitis media

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Abstract

Chronic suppurative otitis media (CSOM) is characterised by otorrhoea and chronic inflammation of the middle ear and mastoid. It contributes to a significant disease burden worldwide.

The aim of the thesis was to update the current understanding of the microbiology of CSOM. To do this a scoping review of the literature on CSOM microbiology was performed. Majority of studies identified a single microbe that was isolated from the ear canal using swabs and culture-based techniques.

An understanding of the demographics of patients with CSOM in New Zealand was obtained by performing a 10-year retrospective study. This showed that the majority of CSOM patients had a low socioeconomic background, minority ethnic groups were overrepresented and hearing loss was common.

To investigate the microbiology of CSOM, traditional culture-based and molecular methods were compared in a prospective study using swabs. Bacterial 16S rRNA sequencing identified the polymicrobial nature of CSOM and the presence of a microbiome in healthy ears, previously thought to be sterile. The results from molecular methods did not support culture-based findings.

The inflammatory response and the presence of intramucosal bacteria in tissue specimens was investigated for the same cohort. Chronic inflammatory cells and intramucosal bacteria were identified in CSOM subjects, in keeping with the persistent inflammatory state of these patients.

Very few studies have investigated the microbiology of CSOM of tissue derived from the primary site of infection in the middle ear. For this reason, tissue specimens of CSOM patients and healthy controls were analysed prospectively to investigate the microbial composition and absolute abundance of clinically relevant bacteria. Droplet digital PCR (ddPCR), amplicon sequencing and multiplex PCR were utilised in this study. The distribution of the bacteria within the tissue was examined using histology and Gram staining.

Genera *Staphylococcus* and *Pseudomonas*, the main pathogens reported in culture studies, had very low relative abundance. ddPCR also measured very low absolute numbers of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Large groups of Gram-positive cocci

were identified and not believed to be genus *Staphylococcus*. Instead, it is possible that these Gram-positive bacteria were members from the genus *Anaerococcus*.

Conclusion: This thesis challenges some aspects the current understanding of the microbiology of CSOM, which may have some implications for the optimal treatment of this condition.

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Glossary

Abbreviation	Definition
ANOVA	analysis of variance
AOM	acute otitis media
CD20	cluster of differentiate 20, a protein expressed on B cells
CD3	cluster of differentiate 3, a protein expressed on T cells
CD68	cluster of differentiate 68, a protein expressed on monocytes, including macrophages
CI	cochlear implant, here: patient who received a cochlear implant
COME	chronic otitis media with effusion
CSOM	chronic suppurative otitis media
CWD	canal wall down procedure
CWU	canal wall up procedure
DAMP	damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole, blue-fluorescent DNA stain
DNA	deoxyribonucleic acid
EAONO	European academy of otology and neurotology
GPAC	gram-positive anaerobic cocci
HL	hearing level
HPV	human papilloma virus
JOS	Japanese Otological Society
MALT	mucosa-associated lymphoid tissue
ME	middle ear
MESNA	sodium 2-mercaptoethanesulfonate
MIC	minimum inhibitory concentration
MRSA	methicillin resistant S*taphylococcus aureus*
NGS	next generation sequencing
NHL	normal hearing level
NIH	National Institutes of Health
OM	otitis media
OME	otitis media with effusion
ORL	otorhinolaryngology
OTU	operational taxonomic unit
PA	postauricular
PAMP	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate ANOVA
PRISMA	preferred reporting items for systematic reviews and meta-analysis
RNA	ribonucleic acid
SD	standard deviation
STROBE	strengthening the reporting of observational studies in epidemiology
TM	tympanic membrane
WHO	World Health Organisation

Chapter 1

Introduction to chronic suppurative otitis media

* This chapter briefly outlines the current understanding of CSOM and introduces the research questions that form the basis of this thesis. A more detailed and focussed introduction to the studies performed in this thesis is provided at the beginning of each chapter.

1.1. General introduction

Chronic suppurative otitis media (CSOM) is one of the most common childhood diseases worldwide(1). It causes a significant disease burden, estimated at 2 million disability-adjusted life years(2, 3). CSOM is characterised by chronic ear discharge through a perforated tympanic membrane for more than six weeks, with some authors defining CSOM as discharge for two or more weeks (3–5). Its prevalence is related to poor socio-economic conditions and, accordingly, it is relatively uncommon in developed countries. Estimates suggest that between 65 and 300 million cases occur worldwide, with 60% of these cases suffering significant hearing impairment(2, 6, 7). Globally, 28,000 deaths per year due to complications of CSOM have been reported(3). There is an enormous financial burden (around $5 billion per annum in the US) associated with otitis media and its sequelae, including chronic suppurative otitis media(8, 9)

CSOM often begins as an acute infection of the middle ear, acute otitis media (AOM), which occurs in up to 80% of children by the age of three(8, 10). While most cases resolve

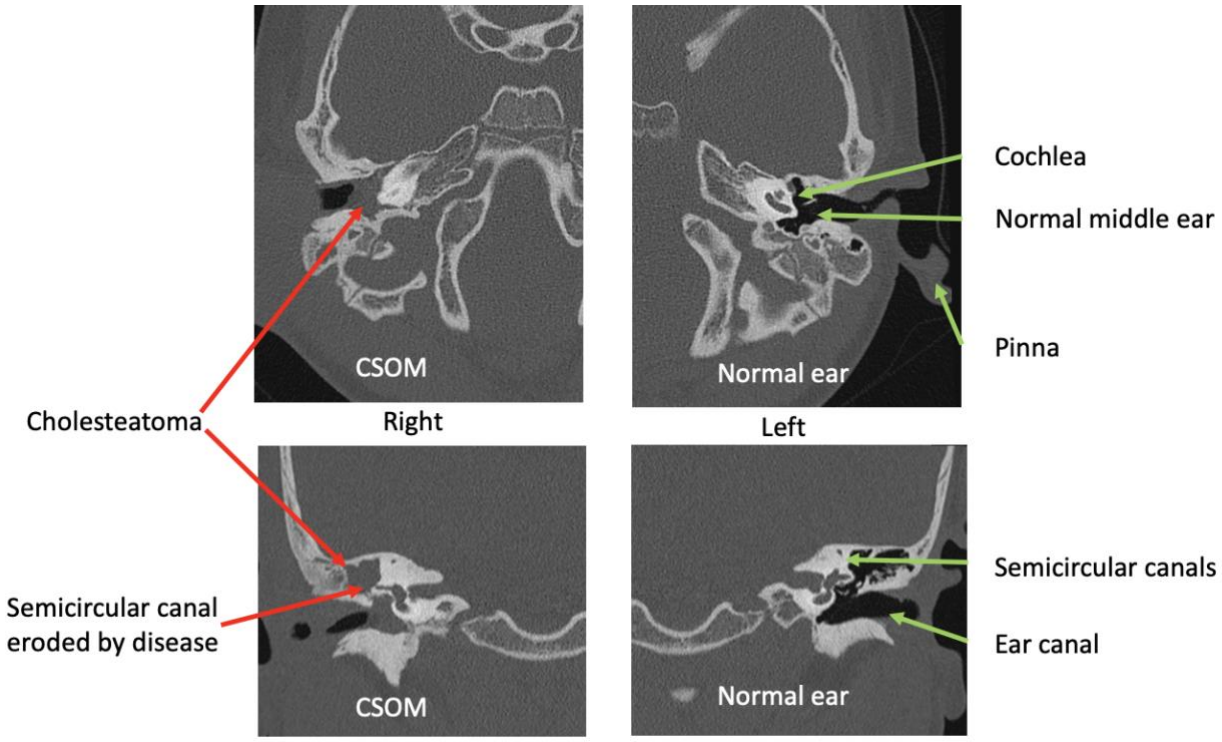


Figure 1*: Computed tomography (CT) scan, bony windows, axial (top) and coronal (bottom) sections of a patient's petrous temporal bones at the level of the middle ears. Right CSOM compared to left normal ear. Right middle ear and mastoid opacification due to cholesteatoma. Note partial erosion of right lateral semicircular canal. (Original image, anonymised CSOM patient, Auckland City Hospital).*

spontaneously, a small minority of patients progress to a chronic phase characterised by chronic purulent ear discharge through a perforated tympanic membrane with associated inflammation of the mastoid and middle ear mucosa, and hearing loss.

CSOM can occur with or without cholesteatoma (epithelial inclusion cyst) (Figure 1), but the presence of cholesteatoma does not necessarily alter the clinical symptoms. Intracranial complications such as brain abscess and meningitis contribute to the morbidity and occasional mortality of this condition(2, 3).

The pathogenesis of CSOM remains poorly understood. Complex interactions between the environment, microbes, and host are thought to lead to the development of this multifactorial disease(2, 10, 11). Topical and oral antibiotics are prescribed to patients empirically or based on bacterial culture results when available, but the clinical benefit of antibiotic therapy is not always clear(2). Surgery may prevent local, regional or systemic complications but some patients may continue to have ear discharge postoperatively(12). Most of the research into the putative microbial causes of CSOM has so far been reliant on culture-based techniques. In these studies *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most commonly isolated bacteria, with methicillin-resistant *S. aureus* (MRSA) isolated in some cases(10, 13–21). However, there are treatment failures even when antibiotics are chosen to target these specific organisms(22, 23). Microbiome studies have increased the understanding of the polymicrobial nature of CSOM (24–27).

It has previously been assumed that the middle ear of healthy individuals is sterile(28, 29). Infections in the middle ear typically occur when pathogens enter the middle ear through the external ear canal or Eustachian tube. Other theories that have been put forward to explain the persistent nature of this disease include toxin production by *P. aeruginosa*, microbes embedding within dead or poorly vascularised tissue (cholesteatoma), through the formation of biofilms, recurrent bacterial infection from the nasopharynx not covered by antibiotics prescribed, or development of antibiotic resistance(16, 18, 20, 21, 30–33). Fluorescence in situ hybridization has previously shown *S. aureus* biofilms to be present within the tissue of CSOM patients(34). Biofilm have also been demonstrated on the middle ear mucosa of children with chronic OM, and in middle ear effusion from children with recurrent acute OM which is a likely risk factor for CSOM(29, 35, 36). These evade the host immune response and antibiotic therapy

by growing in a protective biofilm matrix. The identification of biofilm requires specific techniques and pathogenically significant biofilm-associated infections, such as CSOM will be missed using conventional culture-based(29). Eradication of biofilm by surgical cleansing such as mastoidectomy in chronic ear disease has been suggested as a treatment strategy to improve graft survival(37). Using antibiotic pallets biofilm has been eradicated in in vitro studies(38). In animal studies rinsing the middle ear with saline or baby shampoo has resulted in a reduction of biofilm(39). No definitive medical treatment to eradicate biofilm in human chronic ear disease has been reported.

1.2. Thesis overview and research aims

The scope of this thesis was to investigate the microbiological profile using molecular techniques in patients with CSOM. Comparisons were made between normal and diseased middle ears. A correlation between bacteria and the resulting host inflammatory response was made. The following is a summary of the chapters.

1.2.1. A scoping review of the microbiology and treatment of CSOM (Chapter 2)

The 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) was followed to complete this review. The current state of knowledge of the microbiology and the antibiotic treatment of CSOM was assessed in this chapter. It summarises the results of studies which were mostly culture-based. A comparison to a few molecular based studies was made. This chapter and chapter 3 set the stage for chapter 4, the molecular study of the microbiology of CSOM.

1.2.2. CSOM at Starship Children's Hospital, Te Whatu Ora, Health NZ (Chapter 3)

Chapter 3 is a retrospective cohort study assessing chronic suppurative otitis media trends at a children's hospital in New Zealand over the last 10 years. In particular an association to low socioeconomic status and ethnic inequities were highlighted. The negative impact of the disease on hearing was described. This study highlights the importance of addressing the current diagnosis and management of CSOM at a local level.

1.2.3. Molecular microbiological profile of chronic suppurative otitis media (Chapter 4)

In this chapter molecular methods are used to describe the microbiology of the normal middle ear and mastoid, and the middle ear and mastoid in patients with CSOM. The results are compared with culture based results on the same controls and subjects. The polymicrobial nature of diseased ears is described and discussed in contrast to mostly monomicrobial results derived from traditional culture-based methods. The microbiome in normal middle ears is identified and discussed.

1.2.4. Host-microbe interactions in the middle ear of healthy and CSOM subjects. Local inflammatory cell response. Role of intramucosal bacteria in CSOM (Chapter 5)

This chapter examined the local immune cell response in patients with chronic suppurative otitis media, describing the interaction between host inflammatory response and microbes. The presence of microbes in tissue using gram staining and antibody-based *P. aeruginosa* and *S. aureus* staining techniques was then reviewed.

1.2.5. The tissue microbiome of CSOM patients (Chapter 6)

In this chapter sequencing techniques, ddPCR and multiplex PCR were used to compare normal tissue samples of normal middle ear mucosa with cholesteatoma tissue samples from patients with CSOM. This study describes absolute abundance at the primary site of infection. Molecular findings are also compared to conventional swab results in the same group of patients.

1.2.6. Thesis summary and future directions

In Chapter 7 the findings of the thesis are summarised. The implications of the findings on diagnosis and management of CSOM, and possible future directions of research into this condition are discussed.

Chapter 2

A scoping review of the microbiology and antibiotic sensitivity of chronic suppurative otitis media

2.1. Introduction

2.1.1. Rational

Chronic suppurative otitis media (CSOM), with or without cholesteatoma, is a polymicrobial infection(2, 3). Most patients respond to conservative measures (suction and water precautions) but topical or systemic antibiotics are added to the regimen if the infection does not settle(4, 40). In most cholesteatoma cases, and also in a small proportion of patients without cholesteatoma, otorrhoea persists despite appropriate medical treatment with antibiotics(4, 19). The antibiotic treatment of persistent CSOM empirically targets *Pseudomonas aeruginosa* and *Staphylococcus aureus*(7, 30, 41). Antibiotic treatment may later be rationalised, guided by individual microbiology swab results.

2.1.2. Objectives

This scoping review will help identify gaps in knowledge on the microbiology of CSOM and answer some persisting questions in this area. We plan to investigate (1) prevalent organisms reported from samples taken from patients with CSOM, (2) antibiotic sensitivities where available, and (3) geographic variability in microbes and antibiotic sensitivity. The microbiological techniques used to detect bacteria in CSOM will be compared and discussed.

2.2. Methods

The 'Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) extension for scoping review (PRISMA-ScR) methodology was followed while performing this review(42).

2.2.1. Eligibility Criteria

To be included in this scoping review, published articles on CSOM in adults or children were included. Only articles published in English were included. Reviews and conference abstracts were excluded, as were all animal studies.

2.2.2. Information Sources

Medline, Scopus, PubMed, and Web of Science databases were searched. The search included publications over a ten-year period to 23 May 2022. The final list of papers was entered into an Excel spreadsheet for evaluation.

2.2.3. Search

The term “chronic suppurative otitis media or CSOM” AND “bacteria”, “culture”, “microbiome”, “microbiota”, “microflora” were used and then filtered for “Human” and “English”. (Table 1) The number of recorded, identified, included, and excluded studies were illustrated in a PRISMA flow diagram (Figure 1)(43).

***Table 1**: Search summary*

(chronic suppurative otitis media Or CSOM) AND (microbiota OR culture OR bacteria OR microbiome) (TITLE-ABS-KEY (chronic AND otitis AND media AND suppurative) AND TITLE-ABS-KEY (microbiota OR culture OR bacteria OR microbiom* OR microflora) OR TITLE-ABS-KEY (csom)) AND (LIMIT-TO (EXACTKEYWORD , "Human")) AND (LIMIT-TO (LANGUAGE , "English")) AND (LIMIT-TO (DOCTYPE , "ar")) AND (LIMIT-TO (PUBYEAR , 2022) OR LIMIT-TO (PUBYEAR , 2021) OR LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013))

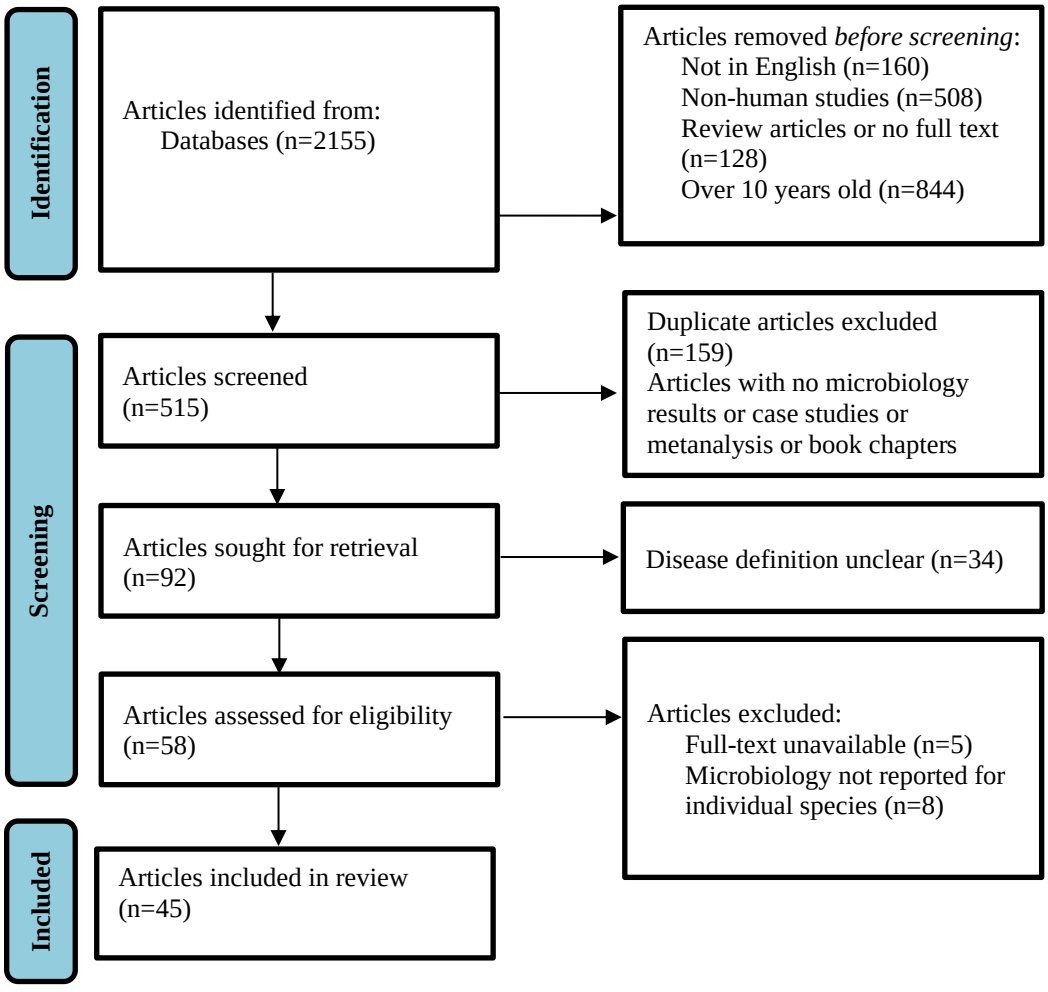


***Figure 1**: Flow diagram of article selection*

2.2.4. Selection of sources of evidence

Articles were screened for eligibility based on the title and abstract. Full text articles were retrieved only for studies deemed relevant. Two reviewers completed the search and screened the articles. Two reviewers agreed on articles that were appropriate to be included for the scoping review.

2.2.5. Data charting process

Key variables were identified, and all relevant data collated. All reviewers extracted data from an allocated set of articles independently, avoiding duplication of data. Key variables were based on the primary and secondary outcomes described below (Data items).

2.2.6. Data items

Baseline characteristics (including country of origin, number of patients, number of microbial isolates) were collated and listed in Table 2. The primary outcome was microbiology of CSOM. The secondary outcome was microbial antibiotic sensitivity.

***Table 2**: Baseline characteristics of CSOM studies.*

Authors	Publication Year	Country	Age	Gender	Micro method	Subjects	Controls	Isolates	Bacteria, fungi, viruses	Antibiotic sensitivity data
Frank, DN et al (26)	2022	Philippines	15-73 years	49% male	16S amplicon and viruses (9 viruses-qPCR)	98 patients	none	218 (103 bacteria and 115 viruses)	Bacteria and viruses	no
Xu, J et al (44)	2021	China	0-60 years	55% male	culture, swabs	289 patients	none	223	Bacteria	yes
Agarwal, A et al (45)	2021	India	23 children, 31 adults	51.9% male	culture, swabs	54 patients	none	50	Bacteria and fungi	yes
Wan Draman, WNA et al (46)	2021	Malaysia	1 to 90 years, 40% children	34.1% male	culture, swabs	88 patients	none	91	Bacteria and some fungi	yes
Khatun, MR et al (47)	2021	Bangladesh	10 to 70 years	fewer male	culture, swabs	96 patients	none	73	Bacteria	yes
Xu, F et al (48)	2020	China	4 to 78 years	not documented	culture, swabs	1087 patients	none	657	Bacteria	yes
Jamil, R et al (49)	2020	Pakistan	5-62 years	46% male	culture, swabs	85 patients	none	85	Bacteria	yes
Abed, AY et al (50)	2020	Iraq	40-70 years	47% male	culture, swabs	88 patients	none	70	Bacteria	yes
Malagutti, N et al (51)	2020	Italy	Adults less than 65 years	not documented	qPCR from DNA	52 patients	56	52	HPV	no
Waqas, K et al (52)	2020	Pakistan	Children and adults	48% male	culture, swabs	200 patients	none	442	Aerobic bacteria and candida	yes
Alia, D et al (53)	2020	Japan	1.7-62 years, 29 adults, 10 children	not documented	culture, swabs, and PCR (HIRA-TAN)	39 patients	none	39	Bacteria	no
Molla, R et al (54)	2019	Ethiopia	1-74 years	51.6% male	culture, swabs	62 patients	none	74	Bacteria	yes
Abraham, ZS et al (55)	2019	Tanzania	7m-82 years	54.4% male	culture, swabs	79 patients	none	79	Bacteria, fungi	yes
Toman, J et al (56)	2019	South Africa	Children and adults	64% male	culture, swabs	14 patients	none	18	Bacteria and fungi	yes

Shamim, R, Gopi, A (57)	2018	India	Children and adults	50% male	culture, swabs	100 patients	20	100	Bacteria and fungi	yes
Kalcioglu, MT et al (25)	2018	Turkey	not documented	not documented	PCR, Sanger and NGS, tissue	37 patients	none	37	Bacteria	no
Rath, PP et al (58)	2018	India	not documented	not documented	culture, swabs	105 patients	none	105	Bacteria	yes
Samanth, T et al (59)	2017	India	Children and adults	63.3% male	culture, swabs	60 patients	none	60	Bacteria	yes
Minami, S (27)	2017	Japan	Children and adults	42% male	PCR, 16s rRNA, tissue	88 patients	67	155	Bacteria	no
Hydri, AS et al (60)	2017	Pakistan	7-80 years	71.4% male	culture, swabs	140 patients	none	160	Bacteria	yes
Juyal, D et al (61)	2017	India	Children and adults	56.5% male	culture, swabs	571 patients	none	583	Bacteria	yes
Rath, S et al (62)	2017	India	not documented	not documented	culture, swabs	number of patients not clear	none	1230	Bacteria and fungi	yes
Sharan, H (63)	2016	India	72% younger than 20 years	52.7% male	culture, swabs	150 patients	none	146	Bacteria	yes
Kumar, S et al (64)	2016	India	7-64 years	54% male	culture, swabs	202 patients	none	238	Bacteria	yes
Neeff, M et al (24)	2016	NZ	Children and adults	male and female	16s rRNA and culture, swabs	26 patients	none	26	Bacteria	no
Mushi, MF et al (65)	2016	Tanzania	27.6% younger than 20 years	45.2% male	culture, swabs	301 patients	none	301	Bacteria and fungi	yes
Kim, SH et al (66)	2015	Korea	6.9% 20 years or younger	fewer male	culture, swabs	2256 patients	none	1764	Bacteria and fungi	yes
Metri Basavaraj, C et al (67)	2015	India	52.8% 20 years or younger	53.5% male	culture, swabs	159 patients	none	159	Bacteria and fungi	yes
Poorey, VK, Thakur, P (68)	2015	India	10.8% younger than 11 years, most were 16-30 years	56.7% male	culture, swabs	120 patients	none	127	Bacteria	yes
Chirwa, M et al (69)	2015	Malawi	2-64 years	61.5% male	culture, swabs, aspirate via pipette	104 patients	none	118	Bacteria and fungi	yes
Si, Y et al (70)	2014	China	40 years (mean)	51.4% male	culture, swabs	72 patients	none	72	Bacteria and fungi	no
Shaikh, AA (71)	2014	India	10-82 years	58.7% male	culture, swabs	150 patients	none	137	Bacteria	yes
Effat, KG, Madany, NM (72)	2014	Egypt	9-45 years	72.2% male	culture, swabs, cholesteatoma tissue	18 patients	none	17	Fungi	no
Adebola, SO et al (73)	2014	Nigeria	4-50 years	fewer males	culture, swabs	104 patients	none	154	Bacteria, anaerobes, and aerobes	yes
Juyal, D et al (74)	2014	India	42.1% 20 years or younger	60% male	culture, swabs	107 patients	none	83	Fungi	no
Shyamala, R, Sreenivasulu, RP (75)	2013	India	73% 20 years or younger	57.4% male	culture, swabs	100 patients	none	100	Bacteria	yes
Prakash, M et al (76)	2013	India	not documented	45% male	culture, swabs	80 patients	none	75	Bacteria	yes
Aduda, DSO et al (77)	2013	Kenya	5-18 years	not documented	culture, swabs	300 patients	none	336	Aerobic bacteria	yes
Agrawal, A et al (78)	2013	India	62.4 % 20 years or younger	53.6% male	culture, swabs	125 patients	none	135	Bacteria and fungi	yes
Prakash, R et al (14)	2013	India	0-80 years	46.1% male	culture, swabs	204 patients	none	191	Bacteria and fungi	yes
Tiedt, NJ et al (79)	2013	South Africa	1-12 years	52.3% male	culture, swabs	86 patients	none	153	Bacteria and fungi	no
Olajide, TG et al (80)	2012	Nigeria	3m-85 years	59% male	culture, swabs	78 patients	none	99	Bacteria	yes
Cheong, CSJ et al (81)	2012	Singapore	5-96 years	52.7% male	culture, swabs	91 patients	none	118	Bacteria and fungi	yes

Ahn, JH et al (16)	2012	South Korea	7–73 years	46.3% male	culture, swabs	244 patients	none	252	Bacteria and fungi	no
Afolabi, OA et al (82)	2012	Nigeria	5-64 years	53.7% male	culture, swabs	134 patients	none	134	Bacteria and fungi	yes

2.2.7. Critical appraisal of individual sources of evidence

All articles were assessed using the National Heart, Lung, and Blood Institute (NHLBI) NIH study quality assessment tool(83) and STROBE checklist(84). Three reviewers independently assessed the papers, reaching consensus on the quality of selected articles after detailed discussion (Table 3)(80, 81).

Table 3*: Critical appraisal of selected papers, NIH*(83)*, and STROBE*(84).

Authors	Year	NIH	STROBE
Frank DN et al (26)	2022	Fair	17/22
Xu J et al (44)	2021	Fair	13/22
Agarwal A et al (45)	2021	Fair	12/22
Wan Draman WNA et al (46)	2021	Fair	12/22
Khatun MR et al (47)	2021	Fair	12/22
Xu F et al (48)	2020	Good	18/22
Jamil R et al (49)	2020	Fair	12/22
Abed AY et al (50)	2020	Poor	11/22
Malagutti N et al (51)	2020	Fair	16/22
Waqas K et al (52)	2020	Good	16/22
Alia D et al (53)	2020	Good	17/22
Molla, R et al (54)	2019	Good	18/22
Abraham ZS et al (55)	2019	Fair	13/22
Toman J et al (56)	2019	Fair	19/22
Shamim R, Gopi A (57)	2018	Fair	12/22
Kalcioglu MT et al (25)	2018	Fair	17/22
Rath PP et al (58)	2018	Fair	12/22
Samanth T et al (59)	2017	Fair	15/22
Minami, S (27)	2017	Good	19/22
Hydri AS et al (60)	2017	Fair	14/22
Juyal D et al (61)	2017	Fair	13/22
Rath S et al (62)	2017	Poor	06/22
Sharan H (63)	2016	Poor	10/22
Kumar S et al (64)	2016	Fair	13/22
Neeff M et al (24)	2016	Good	19/22
Mushi MF et al (65)	2016	Good	19/22
Kim SH et al (66)	2015	Fair	18/22
Metri Basavaraj C et al (67)	2015	Poor	10/22
Poorey VK, Thakur P (68)	2015	Poor	10/22
Chirwa,M et al (69)	2015	Good	18/22
Si Y et al (70)	2014	Good	16/22

Shaikh AA (71)	2014	Fair	15/22
Effat KG, Madany NM (72)	2014	Fair	14/22
Adebola SO et al (73)	2014	Good	19/22
Juyal D et al (74)	2014	Fair	14/22
Shyamala R, Sreenivasulu RP (75)	2013	Fair	14/22
Prakash M et al (76)	2013	Fair	14/55
Aduda DSO et al (77)	2013	Fair	15/22
Agrawal A et al (78)	2013	Poor	12/22
Prakash R et al (14)	2013	Fair	14/22
Tiedt NJ et al (79)	2013	Good	19/22
Olajide TG et al (80)	2012	Fair	16/22
Cheong CSJ et al (81)	2012	Fair	17/22
Ahn JH et al (16)	2012	Fair	17/22
Afolabi OA et al (82)	2012	Fair	15/22

2.2.8. Synthesis of results

The included studies were grouped and compared by type of microbial identification method used (culture-based versus molecular methods). Results of microbes and antibiotic sensitivities identified were collated.

2.3. Results

2.3.1. Selection of sources of evidence

Figure 1 is a PRISMA 2020 flow diagram illustrating the process of articles selected for this scoping review(43). 2155 articles were identified from the databases and after screening, 45 articles were included for evaluation.

2.3.2. Characteristics of sources of evidence

The majority of studies were from the Indian subcontinent, Africa, East Asia, and South East Asia. There were single studies from Italy, Turkey, Egypt and NZ. All were prospective observational studies. The aim of the culture-based studies was to identify common bacteria and their sensitivities to commonly used antibiotics, to help guide subsequent empirical treatment of CSOM. Forty studies reported the microbiology of CSOM using conventional culture techniques. Most results were based on ear swabs, apart from one study which took middle ear aspirates, and two studies which used tissue-based culture(16, 69, 72). Pus swabs were taken from the ear canal or from the middle ear through a tympanic membrane perforation using sterile techniques. The majority of swabs were collected in the outpatient setting. A few studies collected samples intraoperatively(16, 24–26, 48, 51). Two studies focused on fungal growth

alone(72, 74). Aerobic bacterial pathogens were reported in the majority of studies. Two culture based studies specifically targeted anaerobic bacteria (Table 2)(14, 79).

Six studies used molecular methods to identify microbes (one study(53) used molecular and culture techniques), all but one of these studies(24) used tissue samples(24–27, 51, 53).

2.3.3. Critical appraisal within sources of evidence

See Table 3 for the NIH(83) and STROBE(84) assessments. The included studies were assessed as fair (n=28), good (n=11), or poor (n=6).

2.3.4. Results of individual sources of evidence

Bacteria were the main focus in the majority of the studies. 17 studies reported on bacteria and fungi, two on fungi alone, one on bacteria and viruses, and one on viruses alone. Anaerobes were reported in three studies (see below).

2.3.4.1. Culture-based studies

a) Aerobic bacteria

The majority of culture-based studies identified *P. aeruginosa* and *S. aureus* as the most common pathogens (Table 4). The median reported rate of *S. aureus* was lower in African studies (15.1%) compared to rates reported in the Indian subcontinent (28.9%) and East Asia (34.8%). The reported median of *Pseudomonas* spp. (including *P. aeruginosa*) was lower in Africa (20.0%) and East Asia (20.2%) compared to the median in the Indian subcontinent (33.1%). In contrast, the reported median of the genus *Proteus* was higher in Africa (24.3%) compared to the Indian subcontinent (4.1%) and East Asia (2.45%). Median rates of the genus *Klebsiella* were fairly similar between the geographical regions (7-7.5%). Other potential aerobic pathogens frequently reported at lower rates included Coagulase negative *Staphylococci, E. coli, Streptococci, H. influenzae* amongst others.

Table 4*: Regional differences of reported microbes (median %, interquartile range (IQR) %, mean % where fewer than 4 studies were included) from culture swabs.*

		Africa	**South-East Asia**	**Indian Subcontinent**	**East Asia**	**Arabia**
Number of studies:		10	2	19	6	1
S. aureus	All	**Median 15.1** **Interquartile range (IQR) 4.8**	**16.85** (mean of 2 studies)	**Median 28.9** **IQR 13.8**	**Median 34.8** **IQR 31**	**30.9** (single study)

	MRSA	7.4 (mean of two studies)	not reported	**Median 6.4** IQR 5.7	**19.35** (mean of 2 studies)	not reported
Pseudomonas		**Median 20** **IQR 10.2**	**34.35** (mean of 2 studies)	**Median 33.05** **IQR 5.7**	**Median 20.2** **IQR 9.41**	**33** (single study)
Proteus		**Median 24.3** **IQR 21.2**	**14.9** (mean of 2 studies)	**Median 4.1** **IQR 8.29**	**Median 2.45** **IQR 3**	**15.9** (single study)
Klebsiella		**Median 7** **IQR 13.1**	**2.8** (mean of 2 studies)	**Median 7.5** **IQR 4.8**	**7.6** (mean of 3 studies)	**2.3** (single study)

b) Anaerobic bacteria

Table 5 shows results of bacteria identified in studies which specifically targeted anaerobic bacteria by both culture techniques and molecular techniques. It shows the diversity of anaerobic bacteria, acknowledging that amplicon sequencing results were reported at the family level. Reporting at a higher taxonomic resolution such as species-level would likely have resulted in larger diversity. Results of only three studies reporting on anaerobic bacteria could be included in this review preventing generalisability of these findings(14, 25, 79).

Table 5*: Anaerobes identified using either culture or molecular techniques were compared (results in %).*

Swab, culture-based	**Tiedt**(79)	**Prakash R**(14)	Tissue, molecular-based	**Kalcioglu**(25)
Clostridium spp.		26.1	***Clostridiales***	23.4
Peptococcus spp.		23.2	***Peptococcaceae***	2
Peptostreptococcus spp.		23.2	***Peptoniphilaceae***	9.2
Peptostreptococcus tetradius	4.4			
Peptostreptococcus anaerobius	0.9			
Peptostreptococcus prevotii	0.9			
Prevotella melaninogenica		16		
Bacteroides spp.		12	***Bacteroidaceae***	2
Bacteroides ovatus	3.5			
Bacteroides thetaiotaomicron	5.3			
Fusobacterium necrophorum	0.9		***Fusobactericeae***	3.1
Porphyromonas asaccharolytica	1.8		***Porphyromonadaceae***	5.1
			Ruminococcaceae	3.1

c) Fungi

Several studies reported the presence of *Candida* spp. in their culture results. The two studies that specifically focused on fungal culture, found a diverse range of fungal isolates. These included the genera *Aspergillus*, *Candida*, *Penicillium*, *Mucor*, and *Alternaria* (Table 6)(72, 74).

***Table 6**: Studies focused on fungal culture (results in %).*

	Effat(72)	**Juyal**(74)
Aspergillus spp.	16	3.6
Aspergillus flavus		20.5
Aspergillus niger	16	14.5
Aspergillus fumigatus		8.4
Candida albicans	21	23
Candida tropicalis		10.8
Candida spp.		7.2
Penicillium spp.	5	9.6
Mucor spp.	11	2.4
Alternaria spp.	5	
Epidermophyton spp.	16	
Microsporum canis	5	

d) Specimen type, monomicrobial versus polymicrobial

Of the 40 culture-based studies, culture swabs were used to collect samples in all but two. Specimens were taken of the mucus discharge from the ear canal, or from the middle ear through a perforation, in most studies.

In 32 studies, the majority of culture-positive specimens were monomicrobial with a median of 92.7%. Eleven studies only had monomicrobial results. In three studies 50% or more of the culture positive results were reported as polymicrobial (median 52.5% specimens polymicrobial), with up to four different microbes present. Five studies did not provide information on the split between monomicrobial and polymicrobial cultures(16, 48, 56, 79, 82). Thirty-six of 40 culture-based studies provided a rate for culture negative specimens (range 0-56.0%) with a median of 9%.

One study compared the microbiology of pre-operative culture swabs with granulation tissue taken from the antrum intraoperatively(16). The microbes in swab and tissue specimens were similar, including *S. aureus* (45.6% and 47.7% respectively) and *P. aeruginosa* (25.8% and 34.8%). In this study the culture negative rates were 34.1% of preoperative and 76.6% of intraoperative cultures.

The microbes from aspirated ear discharge from the ear canal has also been investigated(69). A 50% split in monomicrobial and polymicrobial cultures, were reported, a higher rate compared to the majority of studies. However, the diversity of microbes identified were similar compared to other studies (*S. aureus* 20.1% and *P. aeruginosa* 20.8%).

e) Age and CSOM

Thirty-eight studies enrolled children and adults but did not provide sufficient detail to analyse potential differences according to age.

2.3.4.2. Molecular-based studies

In contrast to studies using culture-based methods, results from molecular-based studies on CSOM samples show significantly more microbial diversity and highlight that CSOM is a polymicrobial chronic infection(24–27).

A New Zealand-based study in 2016 investigating CSOM also used bacterial amplicon sequencing(24). Compared to previous reports (culture-based) a high bacterial diversity was observed. Over 300 operational taxonomic units (OTUs) were found from 46 swabs that sampled the cholesteatoma and mucosa of patients with CSOM. *Pseudomonas* spp. represented 9% of the average relative abundance of the bacterial community composition of all samples and *Staphylococcus* spp. 39% (range 1-99%). Following on from this, in 2018 a group in Turkey investigated the bacterial community composition in tissue samples from CSOM patients using molecular tools(25). A positive detection of bacteria was reported in 93.2% of samples containing at least 2-8 different bacterial taxa. Bacteria were reported at the family-level, with average relative abundance of *Clostridiales* at 23.4% and *Staphylococcaceae* at 17.3% being the most abundant. In 2020, a Japanese study used quantitative polymerase chain reaction (PCR) and in addition 'Human cell-controlled identification of the respiratory agent (HIRA-TAN method) to exclude colonizing bacteria and identify pathogens found in specimens of patients with CSOM(53). Compared to standard bacterial culture techniques, they were able to detect numerous other bacteria such as *P. aeruginosa* (29% of samples) and *Proteus* spp. (54% of samples). However, *Staphylococcus* spp. was not detected. (53).

Recently (2022), tissue samples (cholesteatoma and mucosa), and discharge from cholesteatoma and non-cholesteatoma patients was assessed for bacterial composition using bacterial 16S rRNA gene sequencing (26). The dominant genera were *Prevotella* (relative

abundance 75%), *Propionibacterium, Corynebacterium* (relative abundance 11.5%), and *Streptococcus, Haemophilus, Pseudomonas, Staphylococcus* (relative abundance 8%), a mixture of commensal and potentially pathogenic bacteria. The sample type (mucosa, discharge, cholesteatoma), as well as older patient age, had an influence on the diversity and relative abundance of bacteria identified. CSOM was stated to be a bacterial infection based on this study(26). These results were similar to a 2017 study that also investigated the bacterial composition of tissue samples from CSOM patients. In patients with CSOM with and without cholesteatoma, *Firmicutes* were frequently identified on intraoperative tissue samples(27). In the ten most abundant OTUs genera *Staphylococcus* (relative abundance 34%), *Corynebacterium* (relative abundance 23%), *Peptoniphilus* (relative abundance 3%), *Anaerococcus* (relative abundance 2%), and *Acinetobacter* (relative abundance 2%), were detected amongst others. *Peptoniphilus* was dominant in patients with CSOM with cholesteatoma(27).

The role of viruses in the pathogenesis of CSOM was examined by one study (51). In this study, quantitative PCR was used to investigate the abundance of human papilloma virus (HPV) in diseased tissue (mucosa and cholesteatoma) of 52 patients with CSOM and mucosa of 56 healthy control patients. The viral load quantified by qPCR was higher in older cholesteatoma patients compared to healthy controls, but the significance of this finding was not clear(51). Another molecular study used qPCR screening for HPV and a range of other viral pathogens seen in otitis media. 45 patients with CSOM were screened and115 ME samples obtained (80 tissue samples (cholesteatoma, mucosa, granulation tissue) and 35 pus swabs). No viruses were identified in tissue or discharge from patients with CSOM(26).

2.3.4.3. Antibiotic susceptibility

Bacterial sensitivities were investigated in 29 of 40 culture-based studies included in this review. None of the molecular studies provided information on bacterial sensitivities. Only aerobic bacteria were considered. In general, results were reported as a percent of bacterial isolates sensitive or resistant to the antibiotics being tested. Most studies tested sensitivities of commonly reported bacteria reported in CSOM. These included *P. aeruginosa* (n=29 studies), *S. aureus* (n=26), *Proteus* spp. (n=21), and *Klebsiella* spp. (n=17). Information on the minimum inhibitory concentration (MIC) of antibiotics for these isolates was not provided. The median rate of antibiotic susceptibility has been reported (Table 7). Where there were less than three

studies of a region, sensitivity results were disregarded. Antibiotic sensitives for bacterial species identified from samples of patients with CSOM will be discussed further below.

Table 7: *Antibiotic sensitivity of common bacteria per region is displayed (median %, interquartile range % (IQR), mean %, where less than 4, n=number of studies).*

S. aureus		**Amoxicillin clavulanic acid**	**Cephalosporin (1st, 2nd, 3rd generation)**	**Macrolide**	**Fluoroquinolone**	**Vancomycin**
	Africa	***Mean 46.8 n=3**	**Median 54.5 IQR 38.9 n=4**	**Median 69.0 IQR 36.9 n=5**	**Median 69.0 IQR 6.7 n=6**	**97.0 n=1**
	South-East Asia	no data	**93.3 n=1**	**80.0 n=1**	**66.7 n=1**	no data
	Indian Subcontinent	**Median 88.3 IQR 43 n=8**	**Median 87.8 IQR 36.5 n=10**	**Median 61.7 IQR 26.2 n=4**	**Median 65.3 IQR 20 n=10**	**Median 100 IQR 0 n=6**
	East Asia	**80.2 n=1**	**81.0 n=1**	**Mean 36.2 n=3**	**Mean 43.6 n=3**	**Mean 100 n=3**
	Arabia	**75.0 n=1**	**54.0 n=1**	no data	**65.0 n=1**	**29.0 n=1**
Pseudomonas		**Fluoroquinolone**	**Cephalosporin (2nd, 3rd, 4th generation)**	**Aminoglycoside**	**Piperacillin/Tazobactam**	**Carbapenem**
	Africa	**Median 100 IQR 16.1 n=5**	**Median 53 IQR 39.8 n=5**	**Median 64.9 IQR 46 n=6**	**100 n=1**	**86.5 n=2**
	South-East Asia	**Mean 87.5 n=2**	**100 n=1**	**Mean 81.1 n=2**	**100 n=1**	**100 n=1**
	Indian Subcontinent	**Median 63.0 IQR 25.5 n=14**	**Median 69 IQR 42.6 n=13**	**Median 71 IQR 11.3 n=16**	**Median 90.5 IQR 9.6 n=10**	**Median 97.0 IQR 10 n=11**
	East Asia	**Mean 59.8 n=3**	**Mean 69.6 n=3**	**Mean 81.9 n=3**	**Mean 87.5 n=3**	**Mean 92.9 n=3**
	Arabia	**43.0 n=1**	no data	**85.0 n=1**	**51.0 n=1**	**70.0 n=1**
Proteus		**Amoxicillin clavulanic acid**	**Aminoglycoside**	**Piperacillin/Tazobactam**	**Fluoroquinolone**	**Carbapenem**
	Africa	**Median 30 IQR 32.5 n=**	**Median 78.9 IQR 32.9 n=7**	**50.0 n=1**	**Median 87.1 IQR 50 n=7**	**96.0 n=1**
	South-East Asia	no data	**100 n=1**	**100 n=1**	**75.0 n=1**	**100 n=1**
	Indian Subcontinent	**Median 45 IQR 34.7 n=7**	**Median 72.5 IQR 20 n=10**	**Mean 75.0 n=2**	**Median 80 IQR 23.0n=9**	**Median 77 IQR 18.2 n=4**
	East Asia	**87.2 n=1**	**94.7 n=1**	**97.8 n=1**	**95.6 n=1**	**98.9 n=1**
	Arabia	no data	**93.0 n=1**	**54.0 n=1**	**86.0 n=1**	**69.0 n=1**
Klebsiella		**Amoxicillin clavulanic acid**	**Cephalosporin (3rd, 4th generation)**	**Fluoroquinolone**	**Aminoglycoside**	**Carbapenem**
	Africa	**Median 25 IQR 22 n=4**	**Median 56.3 IQR 31 n=6**	**Median 73.5 IQR 35.16 n=6**	**Median 56 IQR 56 n=6**	**75.0 n=1**
	South-East Asia	**80 n=1**	**80 n=1**	**80 n=1**	**100 n=1**	**100 n=1**
	Indian Subcontinent	**Median 20 IQR 48.9 n=6**	**Median 55.7 IQR 47.5 n=9**	**Median 80 IQR 27.35 n=7**	**Median 75 IQR 28.3 n=9**	**Median 87.85 IQR 17.5 n=6**
	East Asia	no data	no data	no data	no data	no data
	Arabia	no data	**100 n=1**	**43.0 n=1**	**73.0 n=1**	**91.0 n=1**

*The mean was calculated if results included three studies or less, the median was calculated when four or more studies were included.

a) Staphylococcus aureus

Sensitivities to amoxicillin/clavulanic acid and cephalosporins were lower in African-based studies compared to studies from the Indian subcontinent. In studies from East Asia sensitivities to macrolides and fluoroquinolones were lower when compared to studies from other regions. High sensitivity to vancomycin was reported in studies from East Asia (100%) and the Indian subcontinent (100%). Methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in 11 studies, mostly from the Indian subcontinent (n=7). In this region, the rate of detecting MRSA in the bacterial cultures from ear swabs of CSOM patients remained stable over a study period of 8 years (range 1.7-10.5%). Similar rates were reported from two African studies (4.3% and 10.5%). Two studies from East Asia reported rates of 30.5% and 8.2%.

b) Pseudomonas aeruginosa

Sensitivity to fluoroquinolones was higher in African studies (median 100%) compared to those from the Indian subcontinent (median 63%) and East Asia (mean 59.8%). In contrast, sensitivity to cephalosporins was lower in African studies (median 53%) compared to those from East Asia (mean 69.6%) and the Indian subcontinent (median 69%).

c) Proteus spp.

Amoxicillin/clavulanic acid had low sensitivity in studies from Africa (median 30%) and Indian subcontinent (median 45%). Sensitivity to aminoglycoside, fluoroquinolone or carbapenem ranged from 72.5-96% in these studies.

d) Klebsiella spp.

Sensitivity to amoxicillin/clavulanic acid and aminoglycosides was low in African studies (median 25% and 56% respectively). Studies from the Indian subcontinent had low sensitivity to amoxicillin/clavulanic acid (median 20%) but better sensitivity to aminoglycosides (median 75%). Fluoroquinolones and carbapenem sensitivity ranged from 73.5-87.9%.

2.3.5. Synthesis of results

(1) Prevalent organisms reported

Most culture-based studies identified aerobic bacteria, including *S. aureus*, *Pseudomonas* spp., *Proteus* spp. and *Klebsiella* spp.. Less frequently reported were coagulase-negative *Staphylococci*, *E. coli*, *Streptococcus* spp., and *H. influenzae* amongst others. The involvement of anaerobes and fungi was examined in only five studies.

In molecular studies, a polymicrobial mix of commensal and potentially pathogenic bacteria were identified. *S. aureus* and *P. aeruginosa* were frequently identified but were not as prevalent as indicated in culture-based studies. An explanation for this could be that diagnostic laboratories are selecting for known pathogens such as *S. aureus* and *P. aeruginosa*. Other cultivable organisms from samples can be overlooked. Molecular methods offer a better insight into the bacterial community composition of a given sample(85). *S. aureus* and *P. aeruginosa* are part of this community in CSOM which is a polymicrobial infection.

(2) Geographic variability in antibiotic sensitives of prevalent microbes

Sensitivities to many antibiotics remained high for most bacterial isolates from CSOM patients and empirical treatment covering the reported bacteria was suggested repeatedly. In Africa, sensitivity of *S. aureus, Klebsiella spp. and Proteus spp.* to amoxicillin/clavulanic acid was low compared to most other regions, but *Pseudomonas spp.* were more frequently sensitive to fluoroquinolones compared to the Indian subcontinent or East Asia. Some studies reported MRSA detection and fluoroquinolone resistance in *Pseudomonas* spp. infection to be more common in the Indian subcontinent or East Asia compared to other regions.

(3) Microbiology techniques

Majority of studies on CSOM were based on microbiology swabs from the ear canal, generally producing monomicrobial results. There were a small number of molecular studies of the microbiology in CSOM, which revealed the diversity of bacteria in this polymicrobial infection.

2.4. Discussion

2.4.1. Summary of evidence

Traditional culture-based methods from microbiology swabs of the discharge from the ear canal or middle ear was most commonly used. Monomicrobial infections of *S. aureus* and *P. aeruginosa* were most commonly reported in most culture-based studies. Anaerobes and fungi were rarely investigated.

a) Sample type, culture

Several shortcomings have been reported when identifying microbes by culture-dependent methods(86, 87). Only about 1% of microbes can be identified using traditional methods as only microbes that can grow *in vitro* will be identified(86, 87). Some microbes, such as fungi or

strictly anaerobic bacteria, may be extremely hard to culture even under optimal sampling, transfer, and cultivation methods(87, 88). When antibiotics have been administered before sampling occurred, cultures can be negative in the presence of overt infection. Efforts to improve culture media and culture conditions are ongoing(86). Bacteria in biofilm can be difficult to grow in culture as they can mutate into culture-impaired variants. The mutation alters outer lipopolysaccharide structure of bacteria providing them with a survival advantage(89). To increase the yield of culture-dependent methods, tissue samples and aspirates have been found to be superior to the swabs used at other sites(87, 88). Tissue samples are a more intrusive sampling method but may be more representative of the site of the infection and may include intracellular and interstitial microbes that swabs will miss(87, 88). This is likely also true in CSOM, but the results from the small number of studies that cultured from tissue specimens in this review did not suggest an increase in detection rate. It is also not practical to obtain intraoperative samples, including tissue, as most CSOM patients are treated in the outpatient setting where sampling from the ear canal is performed(86, 87).

b) Sampling site

The sampling site may influence bacterial growth. The ear canal and the immediately adjacent middle ear through a perforation are in continuity with the infection but do not represent its central focus. The sampling environment here will have a higher oxygen concentration than the centre of an infected cholesteatoma or infected mastoid air cell system(90). Aerobic bacteria found in the ear canal could have a growth advantage over other bacteria, including anaerobic bacteria. However, the bacteria identified may not be the main causative pathogen(4). It is likely that the bacteria identified in the studies reviewed were from a polymicrobial ecosystem and not from the true site of infection.

c) Non-culture dependent methods

Studies using non-culture dependent methods have deepened awareness of the polymicrobial nature of CSOM(24–27). The middle ear and mastoid in CSOM were demonstrated to have an ecosystem of multiple microorganisms, a more diverse community of bacteria. The relative abundance of bacteria differed compared to bacteria described in traditional studies, in particular *Pseudomonas spp.* did not seem to be prominent in these studies. However, it is difficult to tell which bacteria were involved in the pathogenesis from these studies alone, as the list of microbes reported also included bacteria known to be commensal bacteria and no absolute abundance data were available. Molecular methods can rapidly detect bacterial, viral,

and fungal DNA, even in patients who received antibiotics(91). Another advantage of molecular methods is the ability to detect difficult to culture anaerobes, as reported previously in CSOM patients(24). Despite the potential advantages of molecular methods over traditional approaches, culture-based methods remain the main method in a hospital setting. They are more cost-effective compared to molecular techniques. It is mostly not practical to obtain intraoperative samples, including tissue, as most CSOM patients are treated in the outpatient setting where sampling from the ear canal is performed.

It has been suggested that in the clinical setting, culture-dependent methods often suffice(91). Broad spectrum antibiotics are aimed at *S. aureus* and *P. aeruginosa* but would also cover most other unidentified or uncultured bacteria, which have an uncertain role in the pathogenesis of this disease. However, this therapeutic approach can lead to failures and the growth of antibiotic resistant organisms.

d) Treatment failures

In a subgroup of patients with CSOM, treatment with broad spectrum antibiotics does not work. Some patients continue to have otorrhoea despite culture-guided management(19). Some of these patients have a cholesteatoma, for which surgery is almost always indicated. But in patients with CSOM without cholesteatoma, who do not respond to antibiotic therapy, antibiotic resistance, or biofilms may be the underlying cause(92–94).

Bacteria in biofilms could include highly antibiotic resistant persister cells, which have been described in chronic and recurrent infections(95, 96). These may be resistant to or highly tolerant of antibiotics and cause recurrent infections once antibiotics have been stopped. This phenomenon has been described in bacterial and fungal biofilm infections and could play a role in persistent CSOM(95). The involvement of biofilms specifically in the pathogenesis of CSOM warrants further study.

e) Geographic variation in microbes

The median report of *S. aureus* was 15% in African studies, but higher in the Indian Subcontinent and East Asia (29-35%). In the Indian Subcontinent the median rate of *P. aeruginosa* was higher (33%) than in Africa and East Asia (20%). *Proteus* was more frequently identified in African studies (median 24%) compared to Indian Subcontinent and East Asia (median 3-4%). *Klebsiella* was reported in African studies and studies from the Indian

Subcontinent (median 7-7.5%) at a lower rate than the microbes above (see table 4). Some of these geographical and racial variations could be due to differences in genetics and immunity(97). Differences in diet, hygiene, environmental exposure, exposure to different antibiotics will also contribute(97).

f) Antibiotics and resistance

Antibiotic therapy continues to be directed against commonly reported bacteria, although it is unlikely that a sole pathogen is responsible for CSOM. This approach may contribute to the formation of multi-drug resistant organisms (MDROs). The overuse of antibiotics will select for resistant strains of bacteria over time(96).

World-wide MDROs are increasing and in CSOM the current empirical treatment approach may be a contributor to this(98, 99). The WHO developed a priority list of the pathogens developing resistance, dividing microbes into critical, high, and medium priorities. Some of these bacteria have been reported in CSOM. Carbapenem-resistant *P. aeruginosa* is on the critical list and methicillin-resistant *S. aureus* (MRSA) is a high priority(100). Drug resistance found in this review is likely related to antibiotic prescribing patterns (misuse and overuse), lack of clean water, and poor infection prevention and control(101). The exact factors contributing to regional differences in antibiotic resistance could not be determined in this review.

None of the molecular studies in this review looked for antibiotic resistance genes. The current molecular methods for detecting antibiotic resistance rely on PCR(102). This method to assess for antibiotic resistance or sensitivity are fast and precise but are technically complex and so are not commonly used in the hospital setting currently(102).

2.4.2. Limitations

The articles reviewed were mostly judged to be either good or fair, but some were poor. Only a small number of molecular papers covering CSOM were reviewed. Most papers were from developing countries, which reflects the higher incidence of CSOM in these countries(40).

2.4.3. Conclusions

Using current culture techniques to identify the pathogens involved in the polymicrobial infection usually identifies only one bacterial species. The current treatment approach of using

antibiotics sensitive against this one microbe identified is successful in many patients as other species present will either be covered by this antibiotic or not have a pathogenic role. However, this approach can lead to treatment failures and the development of MDROs.

Molecular techniques are better at describing the polymicrobial nature of CSOM. It is also possible to detect bacterial resistance genes using these techniques. Further studies are required to identify the absolute abundance of bacteria and to better differentiate pathogens from commensals. Because of the technical complexity and expense of molecular techniques, they are not yet widely available in most hospital settings.

Chapter 3

CSOM at Starship Children's Hospital
Te Whatu Ora, Health NZ

3.1. Introduction

Chronic suppurative otitis media (CSOM) is a rare sequela of acute otitis media. It is defined as chronic ear discharge for six weeks or longer(2, 3). The prevalence of CSOM is influenced by socioeconomic status and therefore reported to be low in developed countries(2, 3). The prevalence of CSOM in New Zealand is unknown. However, poorer health outcomes have been reported for Māori and Pacific People in related ear and other conditions(103). Many patients with CSOM who do not respond to medical treatments have an underlying cholesteatoma. Others continue to have otorrhoea without an obvious cause, despite appropriate therapy. Children in this group require tympanomastoid surgery, with the primary aim of creating a safe and dry ear to prevent complications, and a secondary aim of maintaining or improving hearing.

If untreated, CSOM can result in complications including hearing loss, balance disturbance, facial palsy, acute mastoiditis, venous sinus thrombosis, subperiosteal/subdural/extradural or cerebral abscess, meningitis, and sepsis. Of these complications hearing loss is common and often disabling(103). It affects speech and language development, cognition, education and future employment. The aim of this study was to evaluate potential health disparities of children with CSOM undergoing primary mastoid surgery. The patients' hearing outcomes were also evaluated(2, 3)(100–102).

3.2. Material and Methods

3.2.1 Patient selection

A retrospective review of electronic clinical records was undertaken at Starship Children's Hospital, a tertiary paediatric centre in Auckland, New Zealand. Children up to the age of 15 coded to have had primary mastoid surgery at Starship Children's Hospital, Paediatric Otorhinolaryngology between January 2012 and December 2021 were identified. Only children with CSOM undergoing surgery were included. Ethical approval was obtained from the Auckland Health Research Ethics Committee (AHREC) (AH 24570).

3.2.2 Data collection

Ethnicity was grouped into European, Māori, Pacific, Asian, and other children. Ethnicity data were compared with the most recent New Zealand Census data(104). Gender and age at the time of surgery were recorded. Socioeconomic status was determined using the New Zealand Index of Deprivation (NZDep) based on the child's home address at the time of surgery. The NZDep derives a decile score by considering individuals access to communication (e.g.

internet), income, employment, educational qualifications, home ownership, living space and living condition. Index deciles from 1 (least deprived) to 10 (most deprived) were recorded(105, 106).

The type of surgery was recorded after reviewing the operating notes. The ear disease was staged using the EAONO/JOS staging system(107). This staging system was introduced to assess disease severity (based on location and complications of cholesteatoma in the middle ear and mastoid), and likelihood of disease clearance and achieving normal function. Stage I (the least severe) is defined as cholesteatoma limited to one site, stage II involving two or more sites, stage III cholesteatoma with extracranial complications, and stage IV cholesteatoma presenting with intracranial complications(107).

Pre-operative and postoperative audiograms at nine to 12 months were recorded for the operated and contralateral ear. The four-frequency pure tone hearing average air conduction was calculated for 0.5, 1, 2 and 4 kHz. Pre-operative and postoperative results were calculated. The values in dB HL obtained for the operated and non-operated ear were plotted in a benefit plot, based on the Glasgow benefit plot(108) generated in Excel based on patients' audiograms. The values for the operated ear were plotted on the x-axis and the values for the non-operated ear on the y-axis. In this plot vertical and horizontal lines at 35 dB HL represent the cut-off for bilateral hearing disability as defined by the World Health Organisation (WHO)(109). Results were separated by socioeconomic status and ethnicity, compared and evaluated.

3.2.3. Statistical analysis

All analyses and data visualisation were carried out using Microsoft Excel and R statistical program version 4.1.2. and GraphPad Prism (v9.0.2, GraphPad Software Inc., San Diego, CA, USA)(110). Patient demographics and clinical characteristics were summarised using descriptive statistics. Overall significant differences between study groups were evaluated using the Kruskal-Wallis test. Pairwise comparisons were conducted using Wilcoxon test with Benjamini-Hochberg *p*-value adjustment for multiple comparisons. Statistical significance was defined as $p < 0.05$.

When evaluating pre- and postoperative audiograms, a 10 dB deterioration in the pure tone average and a 5 dB improvement in the pure tone average was regarded as significant, as defined by the Occupational Safety and Health Administration (OSHA)(111).

3.3. Results

3.3.1. Demographics

A total of 160 children undergoing primary mastoid surgery were identified. Thirty-five children were excluded (surgery coding not performed, data error, second look or revision procedure), leaving data of 125 children to be analysed. Eighty-seven (69%) of the children were male and 38 (31%) female. The median age of the children at the time of surgery was similar between European (8 years, IQR 4)), Māori (10 years, IQR 4) and Pacific (9 years, IQR 5) but was significantly lower in Asian children (6 years , IQR 4) (see Figure 1).

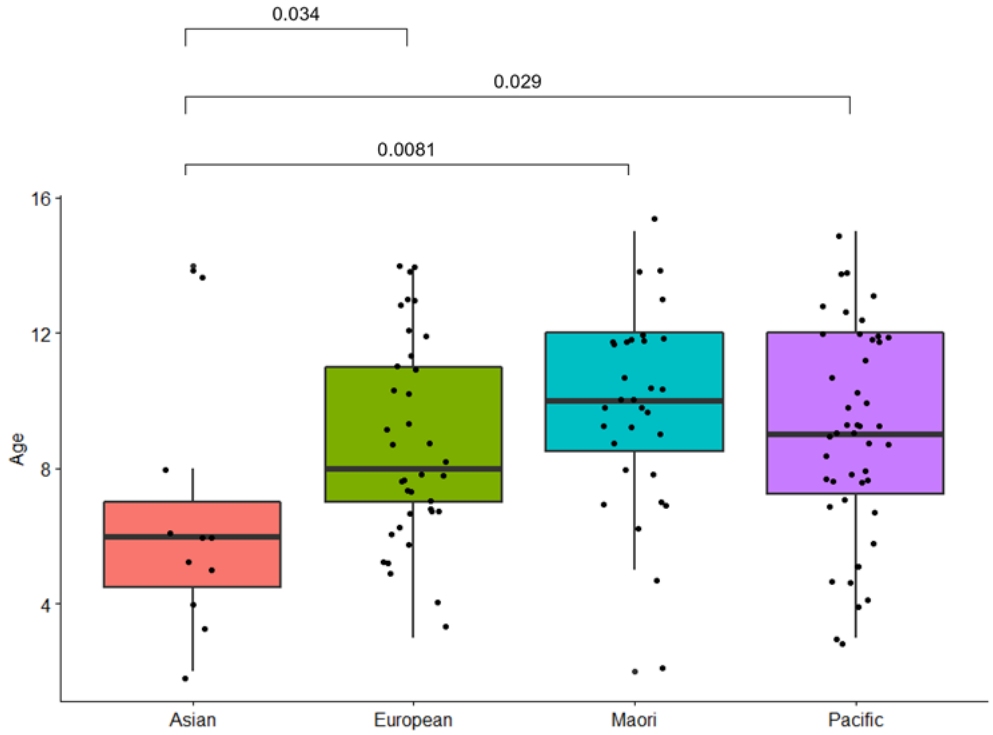


Figure 1*: Median and distribution of age of children at the time of surgery. Data have been grouped by ethnicity. Overall comparison between groups was performed using Kruskal-Wallis test. Comparisons between groups were performed using Wilcoxon test with BH adjustment for multiple comparisons.*

The proportion of Māori (26%) and Pacific (36%) children was higher in this study compared to the Auckland paediatric population as per Census 2018 (Māori children 16.9%, Pacific children 23.5%).(104) Thirty percent of the study population were European and 9% Asian, compared to

50% European and 28% Asian in the Auckland paediatric population as per Census 2018 (Figure 2).

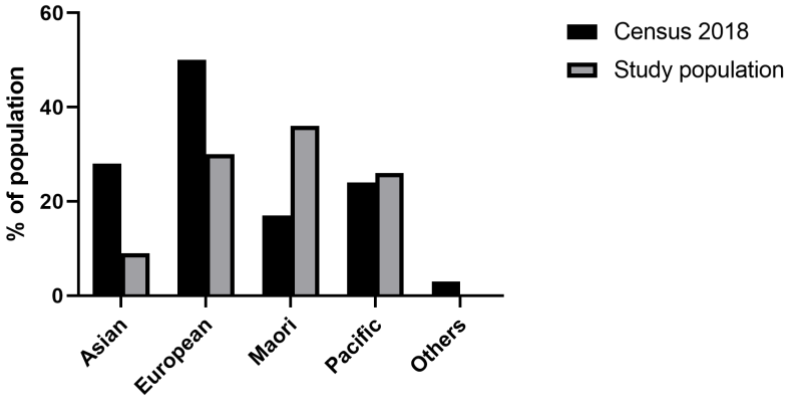


***Figure 2**: The distribution of ethnicity. Study population of CSOM children over 10 years in the Auckland area compared to population census 2018.*

The majority of our study population (70%) were decile 7 or higher as measured by the NZ Index of Deprivation (NZDep). The distribution was as follows: NZDep 1, 2: n=14, 11%; NZDep 3, 4: n=11, 9%; NZDep 5, 6: n=12, 10%; NZDep 7, 8: n=35, 28%; NZDep 9, 10: n=53, 42%. There was a significant difference in the NZDep scores based on ethnicity (Figure 3).

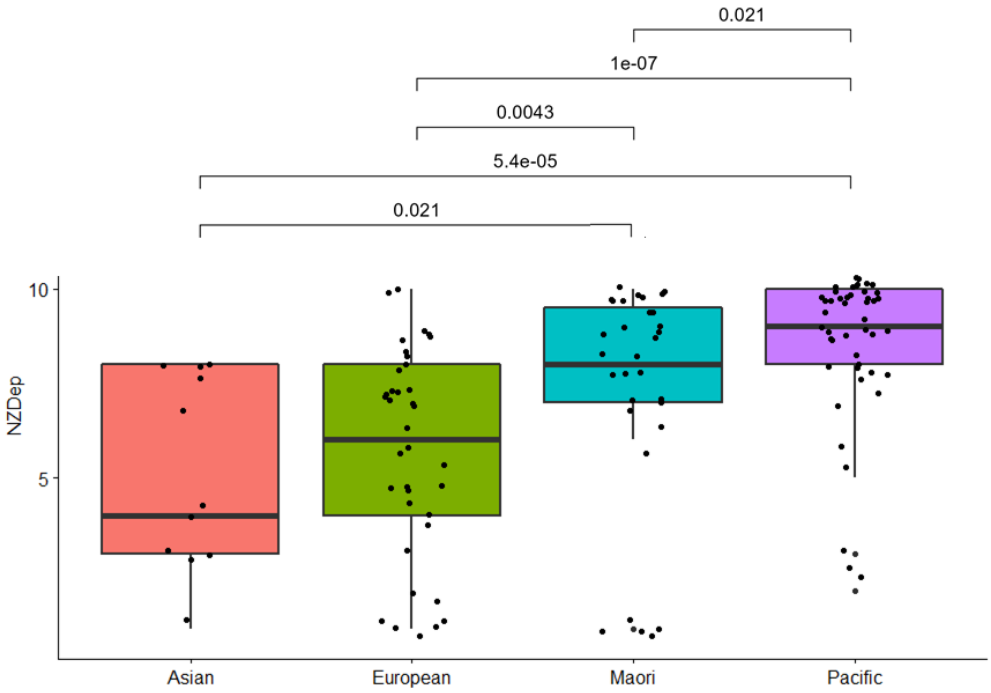
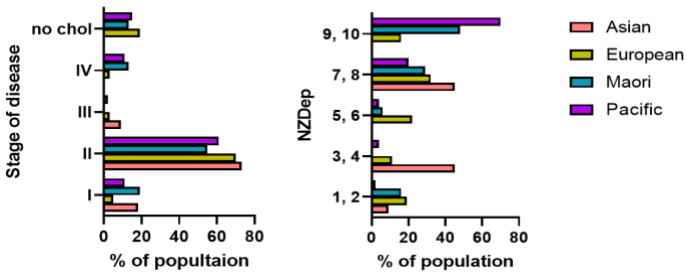


***Figure 3**: NZDep of children at the time of surgery. Data have been grouped by ethnicity. Overall comparison between groups was done using Kruskal-Wallis test. Comparisons between groups were done using Wilcoxon test with BH adjustment for multiple comparisons.*

Pacific children averaged 8.7 on the decile scale, followed by Māori children (7.4) which was significantly higher compared to decile averages for European (5.7) and Asian children (5.2). Over 75% of Māori and 90% of Pacific children were identified as decile 7 or higher (Figure 4 B).



A **B**

Figure 4*: The population distribution grouped by ethnicity. (A) Stage of disease; (B) NZDep.*

3.3.2 Surgery and CSOM

Most children (107 out of 125, 85.6%) underwent surgery for the removal of a cholesteatoma. EAONO/JLO stage II disease was the most common in this group (n=79, 63%) (Figure 4A). Fifteen children had stage I disease and 3 had stage III disease. There were 10 children (8%) with stage IV disease consisting almost entirely of Māori (n=4) and Pacific (n=5) children. The average age of children with stage IV disease was 9.4 years and 80% of them were male. There were no ethnic differences for the type of surgery performed (Table 1).

Table 1*: Type of surgery performed grouped by ethnicity.*

	European	**Pacific**	**Māori**	**Asian**
CWU	51 (19)	57 (26)	42 (13)	64 (7)
CWD	46 (17)	41 (19)	58 (18)	36 (4)
Other	3 (1)	2 (1)	0	0

CWU-canal wall up procedure, CWD-canal wall down procedure, in % (n=).

The two most common surgical procedures were canal wall up (n=65) or canal wall down (n=58) mastoidectomy. Two patients underwent an atticotomy.

3.3.3 Hearing outcomes

Complete pre-operative and postoperative pure tone audiograms were available for 98 of the 125 children in this study. The 27 children with incomplete audiological data were either too

unwell to have a pre-operative audiogram (n=3), too young or developmentally unable to complete the test (n=4), had no postoperative audiograms (n=18), or had a pre-operative audiogram in the sound field only (n=2). The ethnicity of the children with incomplete audiological data were mostly Pacific (n=14) and Māori (n=8) children.

Pre-operatively, most children had a cochlear reserve of 20 dB HL or better, apart from four children who had a mixed hearing loss (i.e. associated sensorineural hearing loss) in the operated ear. All children had a normal cochlear reserve in the contralateral ear. Twenty-eight children had acceptable pre-operative hearing levels (air conduction) of less than 35 dB HL pure tone average (range 7.5 to 33.75 dB HL) in the operated ear. The mean pure tone average of the remaining 70 children was elevated at 52 dB HL, with a range from 35 to 87.5 dB HL.

Following surgery, the hearing was worse by more than 5 dB HL (range 6.25 to 35 dB HL) in 34 children. The hearing was unchanged (average 0.2 dB HL change) in 29 children (range -5 to 5 dB HL). In 35 children the hearing improved, on average by 21.6 dB HL (range of improvement 7.5 to 48.75 dB HL). In children with good hearing (<35 dB HL) before surgery, it was more likely that the hearing was significantly worse after surgery (Figure 5). In children with poor hearing (≥35 dB HL) before surgery, it was more likely that the hearing improved significantly after surgery.

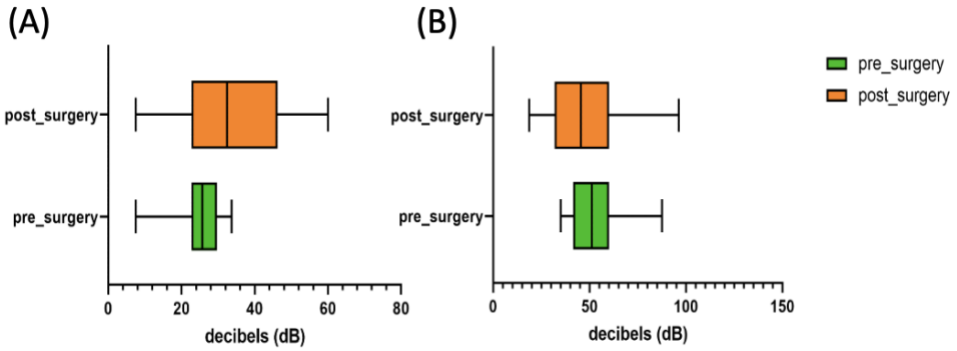


Figure 5*: Pre- and postoperative hearing (air conduction, four-frequency pure tone average) in dB HL. The median hearing in dB HL for each group is represented by a black line in a coloured box. (A) in children with pre-operative hearing <35 dB HL, (B) in children with pre-operative hearing ≥35 dB HL.*

There were no ethnic differences between pre- and postoperative pure tone hearing averages between Māori (pre 42.5 dB HL, post 41.4 dB HL, difference 1.1dB HL worse), Pacific (44.3,

42.3, 2dB HL better) and European (47.2, 45.7, 1.5dB HL worse) children, but a small improvement in hearing in Asian children (48.4, 42.7, 5.7 dB HL better).

There was also no significant difference comparing pre- and postoperative pure tone hearing averages between children in decile 1-6 (n=31, pre 44.5 dB HL, post 39.8 dB HL, non-significant improvement of 4.7 dB HL) and decile 7-10 (n=67, 44.3, 44.9, non-significant deterioration of 0.6). Thirteen children had bilateral hearing loss with hearing at 35 dB HL or worse in the better hearing ear at presentation (Figure 6, top right quadrant). This number increased to 16 children postoperatively. Of these, 5 (31%) were European, 1 (6%) was Asian, 3 (19%) were Māori and 7 (44%) were Pacific. 19 children had bilateral hearing levels better than 35 dB HL pre-operatively (Figure 6, bottom left quadrant). There were 30 children in this group postoperatively.

There was no significant difference in pre-operative hearing between socioeconomic groups NZDep 1-6 (pure tone average 44.5 dB) and NZDep 7-10 (44.3 dB). Overall, there was also no significant change in hearing between these groups as a whole postoperatively (NZDep 1-6 39.5 dB; NZDep 7-10 44.9 dB).

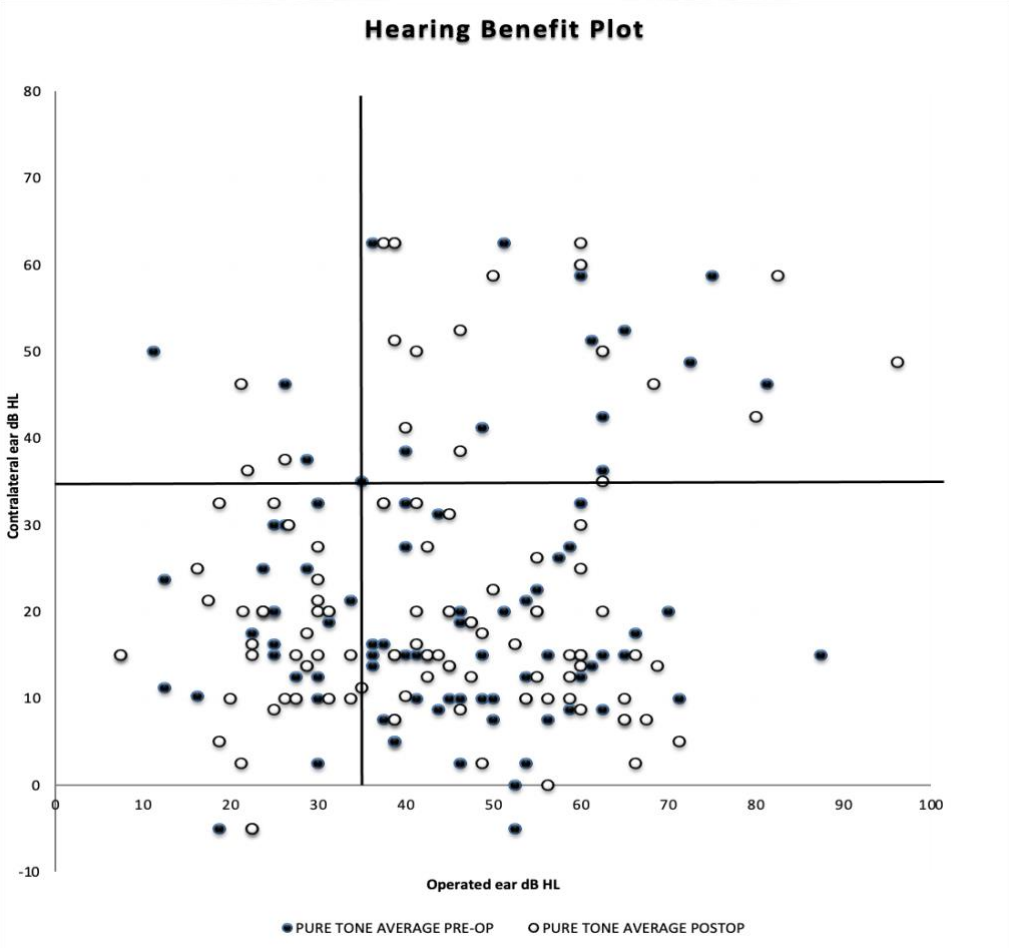


Figure 6*: Benefit plot of pre-operative and postoperative) four-frequency pure tone average (in dBHL), n=98. The operated ear (x-axis) is plotted against the non-operated contralateral ear (y-axis).*

3.4. Discussion

This study highlights pre- and postoperative challenges facing children with CSOM requiring mastoid surgery. It confirms the relationship between low socioeconomic status and CSOM, and shows health disparities between ethnicities.

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Significant health disparities between Māori and non-Māori children have also been reported for other surgical conditions. This includes Māori children undergoing adenotonsillectomy in NZ and presenting with peritonsillar abscess(112–114). Higher rates of middle ear disease in Māori children undergoing adenotonsillectomy have also been identified(112). The influence of ethnicity and socioeconomic status on other general surgical conditions in children in NZ has also been reported(114).

3.4.1 Socioeconomic status

The majority of children with CSOM came from a low socioeconomic background (70% NZDep 7 or higher). The WHO reported on multiple factors associated with low socioeconomic status which can contribute to CSOM in children, including poor access to medical care and antibiotics, poor housing and overcrowding, poor hygiene and nutrition, bottle-feeding, passive smoking, and a family history of middle ear disease(3). An association between CSOM and low socioeconomic status has also been reported by others(115–117).

3.4.2 Ethnicity and socioeconomic status

Māori and Pacific children were more likely to come from low socioeconomic backgrounds, and were the majority in the group presenting with stage IV disease. Ear disease in general is recognised as one of the leading causes of hospital admissions among Māori children and the rate of middle ear disease is higher in young Pacific children(118–121). Our findings are consistent with these results.

Some reported no significant differences in the incidence of CSOM between different socioeconomic groups, but others had similar results to ours(122, 123). A higher proportion of complicated cholesteatoma (stage IV disease) has been reported among children from lower

socioeconomic backgrounds with minority populations such as Pacific and Native American populations often overrepresented in this group in the US(122). Socioeconomic disadvantage in children presenting with frequent ear infections has also been described(124, 125).

Socioeconomic status and ethnicity did not influence the type of surgery performed, but socioeconomic differences in the type of surgery offered to patients with middle ear infections has been reported(126). Delays in surgery are also more common in children of poor socioeconomic backgrounds undergoing tympanostomy tube insertions for middle ear infections(127).

The fact that Asian children presented at an earlier age (6.6 years) and were under-represented in this study compared to the ethnic census data may reflects the 'healthy immigrant effect'(128). Many groups of immigrants frequently arrive in better health compared to the local population and are less likely to present with chronic conditions(129). Our study did not explore if these children were from families who recently immigrated to NZ.

The importance of addressing health inequities in NZ has been described as an important measure to improve the health and wellbeing of this country(130). Initiatives that have been implemented to address these inequities such as the inclusion of Māori health-care providers, cultural competence training for healthcare providers and providing community-led programmes with health literacy focus, have reported some success(130–132). However, inequities persist and the lasting effectiveness of such policies in creating equity has been questioned(133). Organisational structures have been identified as the root of inequities among Māori patients(134). The recent introduction of a Māori Health Authority (Te Aka Whai Ora) into the NZ health system hopes to address systemic shortcomings by making Māori equal partners in the health system, with the aim to improve health outcomes.

3.4.3 Hearing Loss

Most children with CSOM presented at an older age for surgery (mean age 9-10 years), independent of socioeconomic status, compared to Asian children presenting at an earlier age (6.6 years). It is likely that many of these children had chronic infection and unrecognised associated hearing loss for several years before the diagnosis was made.

Delay in age of presentation for surgery is likely to be multifactorial. However, we propose that limited access to hearing screening is central to this. In NZ, a hearing check is completed at the age of four, prior to commencement of school(129). If a child passes, further screening is not undertaken. As such, diagnosing CSOM relies on the patient being seen multiple times by a General Practitioner. Yet there is a critical shortage of primary healthcare workers in NZ.

The proportion of children with significant unilateral hearing loss did not change significantly postoperatively. However children whose hearing was worse pre-operatively were more likely to have a significant improvement in hearing postoperatively, and those whose pre-operative hearing was <35 dB HL were more likely to have worse hearing postoperatively. The hearing outcome after tympanomastoid surgery depends on the extent of disease, presence or absence of ossicles, tympanoplastic surgery performed and Eustachian tube function.

Thirty children had normal hearing (<35 dB HL) bilaterally postoperatively, an improvement compared to 19 children with normal hearing before surgery. But there was a small (n =16), but significant proportion of children with disabling hearing loss, defined by the WHO as a hearing loss of 35 dB HL or worse in the better hearing ear(109). Hearing loss is common in patients with CSOM with or without cholesteatoma due to the disease itself or as a result of subsequent surgery(135). If untreated, children with disabling hearing loss could face significant speech and language challenges(109, 136).

Hearing loss could also have negative follow on effects on cognitive function (e.g. memory), with consequences on long term educational and occupational achievements; the worse the hearing loss, the lower the level of education achieved(137). Once aided, speech and language performance in children improve, but remained below those of normal hearing peers(138, 139). Even a mild hearing loss can have a negative outcome on speech and language development in children who remain unaided(140).

Non-attendance of clinic appointments by minority populations in NZ has been highlighted as an issue preventing optimal healthcare(118). Measures have been put in place to engage with families to improve the postoperative follow up for children with CSOM.

3.5. Conclusion

CSOM has a significant effect on the general health and hearing of affected children. Ethnicity is a likely socioeconomic marker for inequities seen in this group of children. Addressing health inequities is an important strategy to improve health outcomes.

Chapter 4

Molecular microbiological profile of chronic suppurative otitis media

A version of this chapter has been published in the Journal of Clinical Microbiology:

Michel Neeff, Kristi Biswas, Michael Hoggard, Michael W. Taylor, Richard Douglas. Molecular microbiological profile of chronic suppurative otitis. *J Clin Microbiol*. 2016 Oct;54(10):2538-46.

4.1. Introduction

The aim of this study was to characterize the bacterial communities within the middle ear and mastoid of patients with and without CSOM. Bacterial community profiles obtained using culture and molecular-based techniques were compared, with the latter in particular providing novel insights into the microbiota of CSOM patients. While previous studies have described the microbiota of otitis media with effusion, there is limited information on the microbiota associated with CSOM(141–143).

4.2. Materials and Methods

4.2.1. Patient information

This is a prospective study of 24 patients undergoing mastoid surgery for CSOM, and 22 patients with healthy middle ears undergoing either cochlear implantation (CI) or benign brain tumour (vestibular schwannoma) (T) removal via the mastoid and middle ear. None of the study patients received antibiotics within two months before surgery, but all patients (subjects and controls) received intravenous Cefazolin on induction as part of the institutional perioperative protocol. The CSOM patients were subdivided based on the presence or absence of cholesteatoma. Patients undergoing surgery for CSOM were also categorized according to the extent of surgery (canal wall-up or canal wall-down procedure). Patients with cholesteatoma were graded by disease severity, but there is no such disease severity scale currently available for CSOM without cholesteatoma. Patients with anatomical temporal bone abnormalities or immune deficiencies were excluded. The study was approved by the New Zealand Health and Disability Ethics Committee (NTX/12/03/024)(144).

Mastoid surgery was performed under general anaesthetic and sterile conditions. The ear canal was left intact in more limited disease (canal wall up, CWU) but taken down, and the mastoid air cell system externalised (canal wall down, CWD), in more extensive disease. Assessment of disease severity was based on clinical and imaging findings that were evaluated by an otolaryngologist (M.N.). Intra-operatively, tissue samples and two sterile rayon-tipped swabs (Copan, #170KS01) were taken from the mastoid and middle ear. Collected swab samples were placed immediately on ice, and then frozen (-20^{o}C) within two hours of surgery.

Conventional microbiology swabs were also collected during surgery and sent to a hospital laboratory for bacterial culture analysis. Swabs were inoculated on the following culture media (Fort Richard Laboratories Ltd, New Zealand): Columbia Sheep Blood, Supplemented

Chocolate with Bacitracin, MacConkey, Colistin Naladixic and Sabouraud Dextrose agars. The former two were incubated at 37°C in ambient air supplemented with 5% CO_2, while the latter three were incubated at 37°C in ambient air. Swabs collected from patients with chronic infections were also plated on Brain Heart Infusion media and incubated anaerobically. Significant bacterial species were identified using the Vitek MS (bioMérieux) and antibiotic sensitivity testing was performed on the Vitek 2 (bioMérieux) or by using disc diffusion criteria as appropriate and interpreted using the Clinical and Laboratory Standards Institute guidelines. Samples that led to cultivation of a single colony or more were considered to be positive.

4.2.2. DNA extraction

Swab samples were thawed on ice and placed into a sterile Lysing Matrix E tube (MP Biomedicals, Australia). Genomic DNA was extracted from the paired swabs using the AllPrep DNA/RNA Isolation Kit (Qiagen), as described previously(145). A negative extraction control, to test for contamination originating from the DNA isolation kit, was carried out in triplicate using 200 µL sterile water. In no case did these extraction controls yield measurable DNA, and subsequent PCR reactions including this DNA were invariably negative.

4.2.3. 16S rRNA gene sequencing and bioinformatics

A nested PCR approach was used to amplify the bacterial 16S rRNA genes from genomic DNA due to difficulties in obtaining PCR products from most samples after 35 cycles of amplification. Primers 616V and 1492R were used to amplify nearly the full length (targeting *Escherichia coli* positions 8 to 1513) of the bacterial 16S rRNA gene, as described previously(146, 147). After 12 initial PCR cycles, 1 µL from the first PCR reaction was used as template for the second PCR amplification. In this second reaction, the V3-V4 region of the bacterial 16S rRNA gene was amplified (35 cycles) using primers 341F and 806R (read length of 465 bp)(148, 149). The PCR reaction mixture included the following: genomic DNA template (~100 ng), equimolar concentrations (0.2 µM) of each primer, dNTPs (0.2 mM), HotStar PCR buffer (x1), $MgCl_2$ (2 mM), 0.5 U HotStar DNA polymerase (Qiagen) and PCR-grade water to a final volume of 25 µL. PCR reactions were conducted under the following conditions: 95^{o}C for 15 min, followed by 35 cycles of 95^{o}C for 30 s, 55^{o}C for 30 s, and 70^{o}C for 40 s, with a final step of 3 min at 70^{o}C. Duplicate PCR reactions were conducted for each sample. Negative and positive controls were run for all PCR reactions. In addition, 1 µL of the initial PCR negative control was used as a template in the second PCR, to ensure that the final PCR products obtained were not due to contamination. Amplicons were purified using

Agencourt AMPure beads (Beckman Coulter Inc.), and then subjected to both quality (Agilent Bioanalyzer DNA High sensitivity assays) and quantity (Qubit dsDNA HS assay kit) checks.

Sequencing on an Illumina MiSeq was performed by a commercial sequencing provider (Macrogen, South Korea) and raw sequences were uploaded to the NCBI Sequence Read Archive (project number PRJNA320336).

Raw sequences were analyzed using a combination of UPARSE and QIIME software packages(150, 151). In brief, raw sequences were merged, trimmed, and quality checked using UPARSE. Unique sequences were clustered in UPARSE into operational taxonomic units (OTUs) at a threshold of 97% 16S rRNA gene sequence similarity. Chimera checks were performed by checking the sequences against a chimera database (SILVA gold chimera reference database). Taxonomic assignment of the sequences was subsequently performed in QIIME using RDP Classifier 2.2 and SILVA 16S rRNA gene database (v. 119) as reference(152). Sequences were aligned using PyNAST, and a phylogenetic tree generated via FastTree 2.1.3(153, 154). Samples were rarefied to even sequencing depth of 1168 sequences per sample. Alpha and beta diversity analyses (including UniFrac) were conducted in QIIME(155).

4.2.4. Statistical analysis

The software Prism 6 and PRIMER6 were used to statistically analyse the data sets. Permutational ANOVA (PERMANOVA) was performed in PRIMER6 (version 6.1.13) using Jaccard, Bray-Curtis, and weighted and unweighted UniFrac distances. Multidimensional scaling plots were constructed from the weighted UniFrac distance matrix in PRIMER6. Kruskal-Wallis tests were performed with Dunn's multiple comparison corrections to measure significant differences ($p<0.05$) between variables.

4.3. Results

4.3.1. Clinical characteristics of the cohort

A total of 24 patients with CSOM were recruited, along with 22 healthy controls. Most patients with cholesteatoma had extensive middle ear and mastoid disease (grade 3-5). The patients' demographic information is presented in Table 1.

TABLE 1 Patient demographics

Group	Surgery[a]	Patient[b]	Sex[c]	Age[d]	Side[e]	Stage[f]	Ethnicity
Controls	Cochlear implant	C_CI_1	F	12	L		European
		C_CI_2	M	18 m	L		European
		C_CI_3	F	5	R		Chinese
		C_CI_4	F	4	L		European
		C_CI_5	M	4	L		African
		C_CI_6	F	14	L		European
		C_CI_7	F	14	R		Indonesian
		C_CI_8	M	70	L		European
		C_CI_9	F	85	R		European
		C_CI_10	M	15	R		Maori
		C_CI_11	M	10	R		European
		C_CI_12	F	7 m	R		Polynesian
		C_CI_13	M	6	R		Maori
		C_CI_14	M	53	R		European
		C_CI_15	F	6 m	R		Chinese
	Tumor	C_T_1	M	65	L		European
		C_T_2	M	52	R		European
		C_T_3	M	72	R		Indian
		C_T_4	M	57	L		European
		C_T_5	M	61	R		European
		C_T_6	M	36	L		Vietnamese
		C_T_7	M	63	R		European
Cholesteatoma	CWD	CWD_Ch_1	F	71	L	4	European
		CWD_Ch_2	M	74	R	2	Chinese
		CWD_Ch_3	M	45	R	4	Indian
		CWD_Ch_4	M	4	R	4	Indian
		CWD_Ch_5	M	12	R	4	Polynesian
		CWD_Ch_6	F	55	L	4	Maori
	CWU	CWU_Ch_1	M	6	R	4	Polynesian
		CWU_Ch_2	M	19	R	3	Chinese
		CWU_Ch_3	M	5	R	2	Maori
		CWU_Ch_4	F	66	R	2	European
		CWU_Ch_5	F	7	R	4	European
		CWU_Ch_6	M	29	L	4	Polynesian
		CWU_Ch_7	F	7	L	2	European
		CWU_Ch_8	M	40	R	4	European
		CWU_Ch_9	M	7	L	2	European
		CWU_Ch_10	M	17	R	1	Polynesian
No cholesteatoma	CWD	CWD_NC_1	F	75	R		European
		CWD_NC_2	M	30	R		European
		CWD_NC_3	M	46	R		Maori
		CWD_NC_4	M	52	L		European
		CWD_NC_5	F	73	R		Maori
	CWU	CWU_NC_1	M	7	R		Indian
		CWU_NC_2	F	44	L		Polynesian
		CWU_NC_3	M	1	L		Maori

[a] CWD, canal wall down; CWU, canal wall up. [b] CI, cochlear implant; T, tumor; Ch, cholesteatoma; NC, no cholesteatoma. [c] F, female; M, male. [d] Age in years or months (m). [e] L, left; R, right. [f] Stage: 1 to 5 (5 represents the most extensive disease).

4.3.2. Culture-based description of the bacterial communities

Swabs collected from control (CI and T) and non-cholesteatoma patients with CWU procedure, led to no microbial growth on culture plates. In contrast, patients with cholesteatoma (CWU and CWD) had positive growth in both middle ear (40-60%) and mastoid (20-50%) samples (Figure 1(A)). None of the negative controls produced any amplification products after the nested PCR amplification process and were considered to be effectively sterile (or at least beyond the limits of our detection strategy). A range of organisms was identified as Gram-positive or anaerobic, and included *Corynebacterium jeikeium* and *Shewanella algae*. In addition, non-cholesteatoma patients undergoing a CWD procedure had higher rates of positive cultures from the middle ear (80%) compared with the mastoid (20%) swab samples. Microbes identified in these patients

included *Klebsiella oxytoca*, *P. aeruginosa*, *C. jeikeium*, *Aspergillus flavus* and methicillin-resistant *S. aureus*.

4.3.3. Molecular characterisation of the bacterial communities

16S rRNA gene sequences could be obtained from 50-80% of cholesteatoma patients, 35-80% of non-cholesteatoma patients, 43% of control CI patients and 14% of control tumour patients (Figure 1(B)). The success in obtaining amplifiable DNA was not consistent between sample sites (middle ear and mastoid) for each patient. The number of sequences in samples varied between 1168 and 86952. The total numbers of OTUs obtained after quality trimming of the sequences

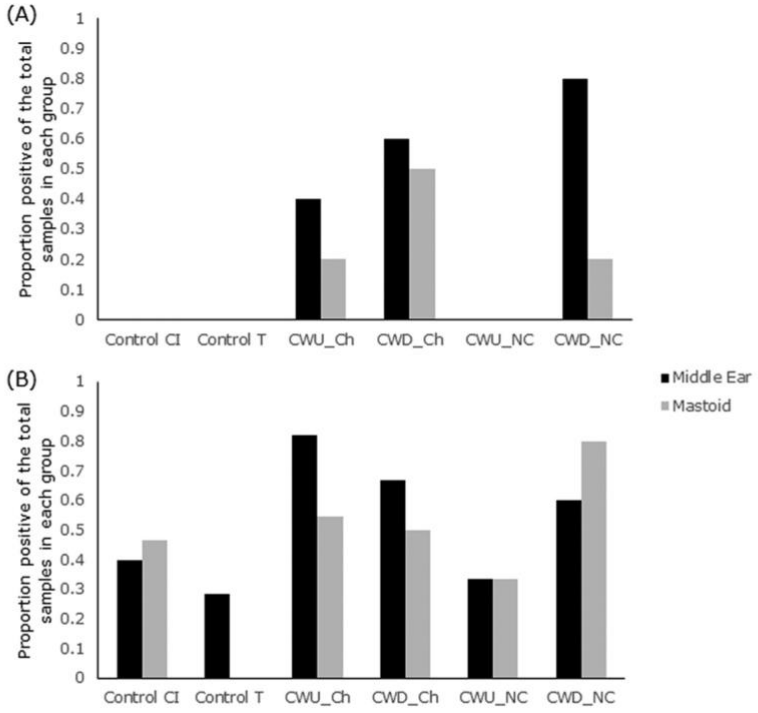
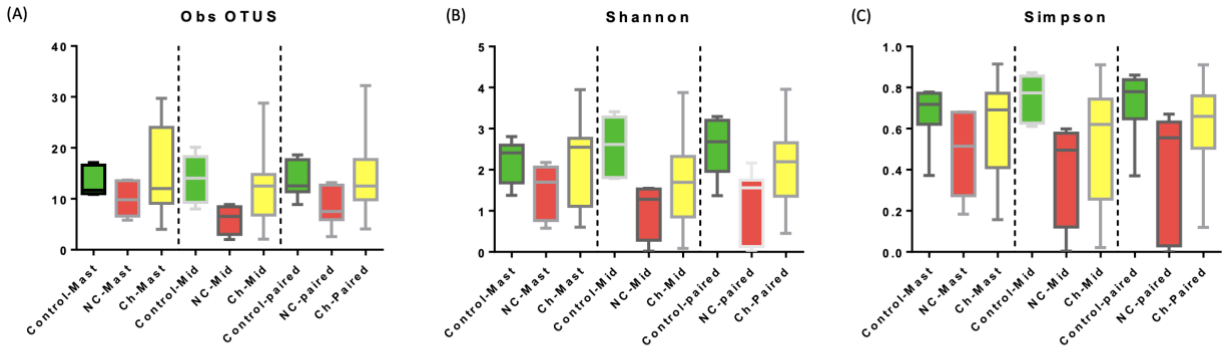


FIG 1 Relative proportion of samples that had positive results for bacteria from either culture (A) or molecular techniques (B). Middle ear (black) and mastoid (gray) samples are shown in the graphs. Samples were grouped into control with cochlear implant (CI) or tumor (T) surgeries, cholesteatoma (Ch) with canal wall up (CWU) or canal wall down (CWD) procedures, and noncholesteatoma (NC) with CWU or CWD.

across all mastoid (n=21) and middle ear (n=25) samples were 156 and 163, respectively. Non-cholesteatoma patients had the lowest bacterial diversity compared with cholesteatoma and control groups, as measured by the number of observed species, Shannon and Simpson indices (Suppl. Figure 1). However, these observed differences were not significant (Kruskal-Wallis test).



Supplementary Fig 1: Box and whisker plots to compare bacterial community diversity between 3 groups: control, non-cholesteatoma (NC) and cholesteatoma (Ch) patients. The mastoid, middle ear and both combined (paired) were compared within each of the 3 groups. **(A)** Observed OTUs-97%, **(B)** Shannon-Wiener, and **(C)** inverse-Simpson indices were calculated for the samples.

Inter-patient differences explained the majority of the variation in bacterial composition observed in the dataset of CSOM patients (R^2 =34.4%, p=0.002). Interestingly, such high variation was not observed in the control group. In addition, no significant differences were measured between sampling site (mastoid vs middle ear) or disease status or sub-groupings or age.

The microbiota of the healthy ear was composed of a range of bacteria including members of the genera *Novosphingobium*, *Staphylococcus*, *Streptococcus*, *Escherichia-Shigella*, and *Burkholderia* (Figure 2). The bacterial composition of CSOM was also variable between

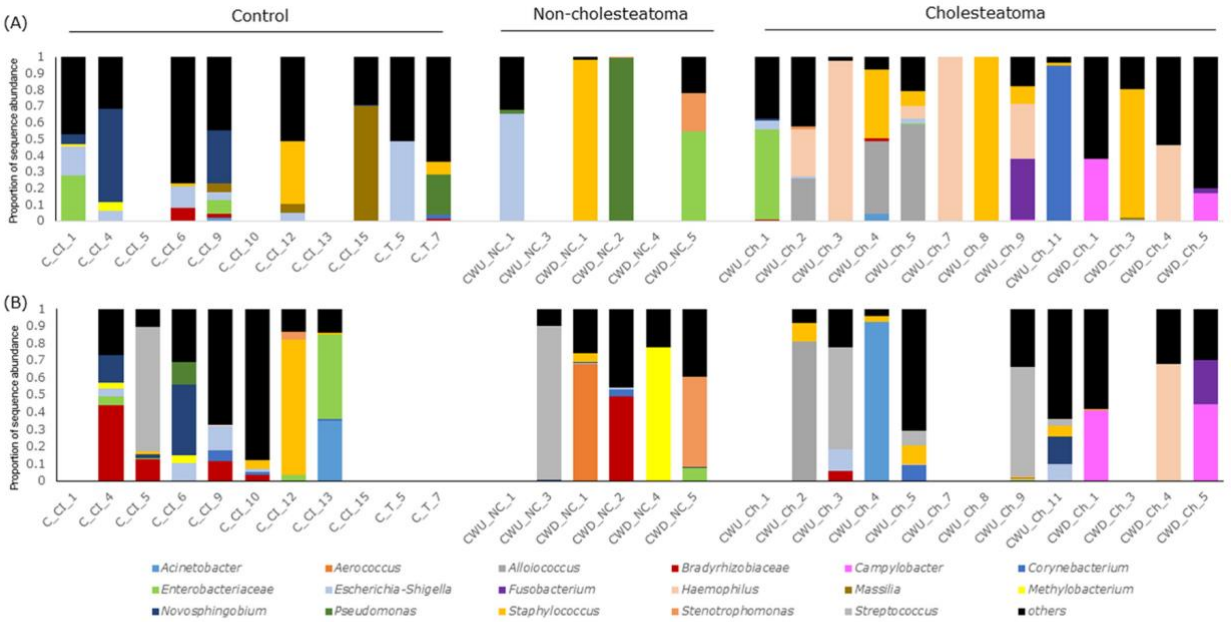


FIG 2 16S rRNA gene-based bacterial community compositions of the middle ears (A) and mastoids (B) of CSOM subjects and healthy individuals. CSOM patients have been grouped into cholesteatoma (Ch) and noncholesteatoma (NC) patients. Types of surgery (canal wall up [CWU] or canal wall down [CWD]) are also indicated on the graph. Bacterial community sequence data are displayed at the genus level, with data for each taxon expressed as a proportion of sequence reads for a given sample. CI, cochlear implant; T, tumor.

patients, comprising the genera *Haemophilus*, *Staphylococcus*, *Alloiococcus*, and *Streptococcus*. The genus *Pseudomonas* was found in a minor proportion (4/46) of samples and with low relative abundance (<10%), with two exceptions: sample CWD_NC_2 (99%) and C_T_7 (24%). *Staphylococcus*, another commonly cultured organism from ear discharge, was detected in 39% (18/46) of the samples including healthy controls, with relative abundance ranging from 0.01 to 99%. *Propionibacterium* was found in the middle ear and mastoid of both control and CSOM patients (<11% relative sequence abundance). In addition, *Alloiococcus* and *Haemophilus* were detected largely in cholesteatoma patients (Figure 3).

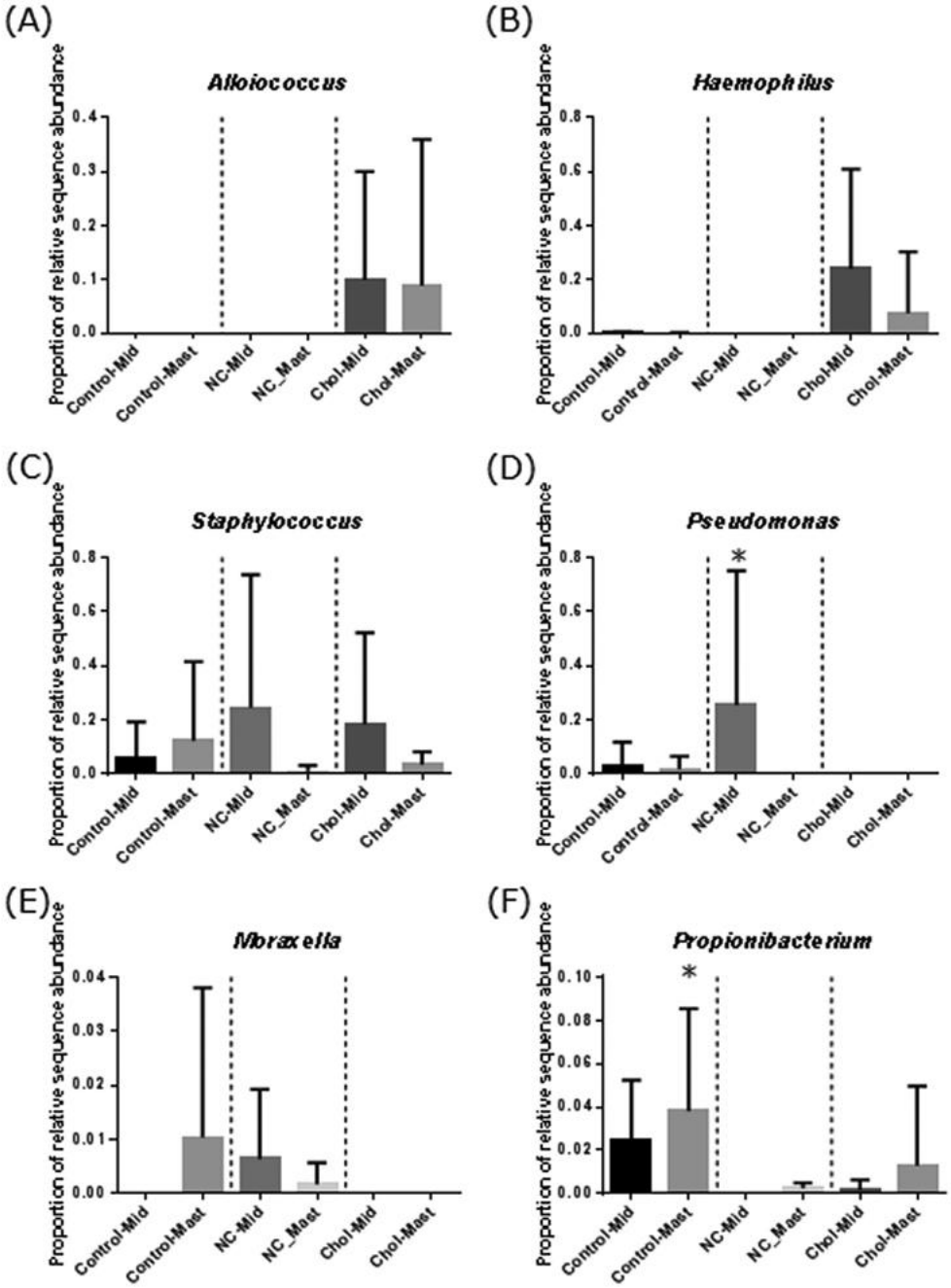


FIG 3 Relative sequence abundance of six prominent ear bacterial genera. These include *Alloiococcus* (A), *Haemophilus* (B), *Staphylococcus* (C), *Pseudomonas* (D), *Moraxella* (E), and *Propionibacterium* (F). The data shown represent the middle ears (Mid) and mastoids (Mast) of control, noncholesteatoma (NC), and cholesteatoma (Chol) patients. An asterisk represents a significantly different ($P < 0.05$) group compared with the 5 other groups in this study. Error bars are standard deviations.

All samples from this study with sequence information were plotted on a multidimensional scaling (MDS) plot using weighted UniFrac distances to visualise clustering of sample types (Figure 4). However, no clustering was observed based on disease status (with and without CSOM) or sampling site (mastoid vs middle ear) or CWU or CWD procedure.

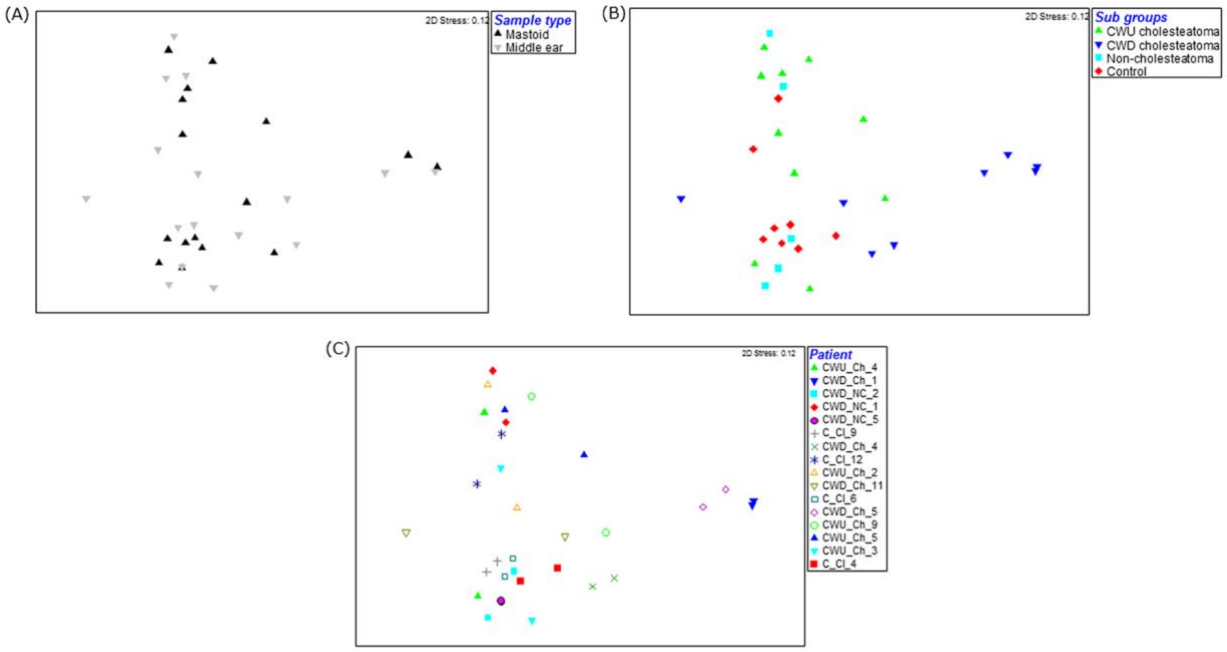


FIG 4 Nonmetric multidimensional scaling plots based on weighted UniFrac values of the bacterial communities. Only paired mastoid and middle ear samples from patients were chosen for this analysis. Bacterial communities were grouped based on sample type (mastoid versus middle ear) (A), study subgroups (cholesteatoma [CWU and CWD], noncholesteatoma, and controls) (B), and patients (C).

4.4. Discussion

Our current understanding of the microbiology of the middle ear and mastoid mucosa in healthy and diseased states has been largely derived from bacterial cultivation studies. These results are inevitably skewed due to the failure of some bacteria to grow in standard culture media(85). By contrast, cultivation-independent molecular techniques are able to provide a more accurate assessment of the microbial communities growing on the mucosa. Here, we apply molecular techniques to determine the microbiota in both patients with CSOM and healthy middle ear mucosa, and our findings are very different from those of previous culture-based studies.

4.4.1. Molecular evidence that the healthy middle ear is not sterile

It is a long-held belief that the middle ear of healthy individuals is sterile(28, 29). In contrast, the results from this study show a diverse bacterial community being detected in 45% of healthy mastoids and middle ears. Notably, our cultivation-based data, which are targeted towards suspected pathogens, detected no bacteria in either the middle ear or mastoid of healthy

individuals. Only with the application of cultivation-independent, molecular biology techniques were bacteria detected in these patients. Future studies should focus on confirming the findings from this study by using alternative approaches such as fluorescence in situ hybridisation. Even then, a highly permissive PCR protocol (comprising >45 PCR cycles) was required, suggesting that bacterial load on the mucosa of healthy individuals is low.

CSOM is thought to result from bacteria ascending from the nasopharynx via the Eustachian tube, or invading from the external auditory meatus (ear canal) through a perforation of the tympanic membrane(156). This ultimately causes irreversible mucosal changes that produce chronic otorrhoea (ear discharge) and lead to hearing loss. However, our demonstration that many healthy middle ears do in fact contain bacteria demands consideration of a potential new concept for initiation of CSOM. Infections within the middle ear and mastoid may need to be viewed as a consequence of disturbance to the normal balance of commensal bacteria (i.e. dysbiosis), at least in some individuals. Under such a model, pathogenesis would likely still involve invasion of bacteria from the nasopharynx or ear canal, but after a perturbation of the resident bacterial community rather than invasion of a previously sterile space.

Alternatively, CSOM may arise from middle ear or mastoid commensal bacteria alone, without contribution from adjacent anatomical sites. Indeed, potential pathogens such as *Staphylococcus*, *Pseudomonas*, *Streptococcus* and *Moraxella*, which have been implicated in ear disease using cultivation techniques, were also detected among healthy controls, supporting the notion of the resident microbiota as a potential source of infection from within. Perturbations to the commensal microbiota could conceivably occur due to, for example, excessive antibiotic usage and/or compromised immunity.

At other mucosal sites, commensal organisms play important roles in defence against opportunistic bacterial invasion. In the gut, for example, communication and regulation between bacteria can have positive or negative effects on bacterial growth. Such interactions appear to be mediated through signalling molecules released by bacteria and reabsorbed by the host cell(157, 158). It is possible, but remains to be demonstrated, that similar interactions occur in the middle ear.

4.4.2. Disease status and inter-personal differences account for much of the variation in the microbiota of the middle ear and mastoid

The majority of the variation in the bacterial communities of CSOM patients was accounted for by inter-personal differences. As this observation was not seen for controls, it further supports the suggestion that the bacterial communities of CSOM patients are disrupted, leading to an imbalance in community structure. Such observations have been reported previously in the gut and paranasal sinuses(159–161). Interestingly, sample site (mastoid vs middle ear) within an individual had no influence on bacterial community variation, suggesting that similar communities were found at both sites. Large inter-patient variation has been observed in a number of human sinus, gut, and skin microbiomes(145, 162, 163). Ethnicity has been shown to contribute to some of this variation(164). Due to the small patient numbers in this study no such associations were observed (Table 1). A study with more patients might show ethnicity related variations.

4.4.3. Identification of prominent CSOM microbiota

The diversity of pathogens/bacteria identified in swab samples by hospital cultivation techniques is underestimated. Routine diagnostic hospital laboratories are selecting for known pathogens, therefore, often other cultivable organisms from samples can be overlooked. Notwithstanding this caveat, given that much of the human microbiota is uncultivable or extremely hard to grow, molecular tools are able to offer a better insight into the bacterial community composition of a given sample(85). Based on the results of this study we did not observe a specific microbial profile typical of CSOM. Putative pathogens such as *Staphylococcus*, *Pseudomonas* and *Haemophilus* were also observed in healthy control microbiota. The next step for this research is to elucidate the influence of the microbiota on the host.

We demonstrated the presence of *Propionibacterium* in normal mastoid and middle ear mucosa. The relative sequence abundance of *Propionibacterium* was significantly higher in controls compared to patients with CSOM. Members of the genus *Propionibacterium* are part of the normal microbiota of the skin, the mouth and gut(162, 165–167). They are believed to play a role in stabilizing the normal microbiota by occupying niches which then cannot be invaded by pathogens(165, 166). *Moraxella* is a known pathogen of the human respiratory tract, typically causing pneumonias in adults and acute otitis media in children(156). It is not a classic pathogen reported in CSOM. The occurrence of *Moraxella* was variable between groups, but it

was mostly found in control and non-cholesteatoma patients at relatively low abundance. Our findings thus support it being part of the commensal microbiota in normal middle ears and mastoids, but not a significant player in CSOM.

There has been debate about whether members of the bacterial genus *Alloiococcus* play a role in the pathogenesis of otitis media in children, or if they are part of the commensal microbiota(168, 169). Using molecular techniques we found higher relative abundance in CSOM patients with cholesteatoma compared to controls or other subject groups. To better understand the potential role of *Alloiococcus* in CSOM, further study into host interaction and colonisation of adjacent sites in normal and diseased patients will be necessary. Of note, *Staphylococcus* was identified in the microbiota of many patients in our study, but in relatively few patients was *Pseudomonas* detected. These results contrast with many culture-based studies in which *P. aeruginosa* and *S. aureus* were the dominant bacteria(10, 13–16, 18). Differences in the capability of certain species to grow in culture media may account for much of the disparity between culture-based and molecular techniques(85). The preliminary findings of this small study will need to be further validated in the future with larger cohort sizes.

4.4.4. Clinical implications

In light of our findings, the importance of *Pseudomonas* as a pathogen in CSOM requires further investigation. Patients in this study would not have benefited from antibiotics targeting *Pseudomonas* in CSOM. This is clinically important, as over-use of anti-pseudomonal antibiotics can promote bacterial resistance(10, 17, 30). More generally, the use of broad-spectrum antibiotics may have an unfavourable effect on commensal bacterial communities, potentially affording pathogens a competitive advantage by eliminating competing commensals. Antibiotics administered early in life may alter the biomass and composition of the normal microbiota and reduce the exposure of potential pathogens to the immune system. In studies on gut microbiota these mechanisms have been reported to result in dysbiosis(170). It is possible that antibiotic use for CSOM early in life may cause dysbiosis and may have a detrimental effect on a patient's long-term ear health. Further studies are needed to examine the effect of antibiotics on the middle ear microbiota in health and disease.

In conclusion, the healthy middle ear and mastoid mucosa is dependent on normal Eustachian tube function and mucosal immunity, and an absence of allergy and infection(2). In human beings, commensal bacteria have been shown to co-exist to maintain a healthy environment in a

variety of body sites, and this study suggests that a bacterial community is often present in the normal human middle ear and mastoid. There is a disparity between culture and molecular assessment of the middle ear and mastoid, which may lead to inappropriate prescribing of antibiotics. The use of molecular techniques to describe the microbiota should further refine our understanding of the role of the middle ear and mastoid microbiota in the pathogenesis of chronic inflammatory ear diseases.

Chapter 5

Host-microbe interactions in the middle ear of healthy and CSOM subjects

5.1 Local inflammatory cell response

5.2 Role of intramucosal bacteria in CSOM

5.1 Local inflammatory cell response

5.1.1 Introduction

Most cases of chronic suppurative otitis media (CSOM) are preceded by recurrent bacterial or viral acute otitis media (AOM). Resolution of inflammation is an active process that involves factors that normally clear the acute infection. But if microbes are not cleared, or if pro-resolution factors are deficient, chronic inflammation ensues(171, 172).

The resultant CSOM is characterised by ongoing, potentially destructive interactions between the host immunity and microbial pathogenicity(172–174). It is not clear what triggers the transition from the acute to the chronic inflammatory phase. Regulation by mucosal mediators as well as inflammatory cell mediators are involved(171, 172).

In chronic middle ear infections, immune cells are increased in number in the middle ear mucosa(175). Apart from microbes, other potential factors including genetics, environmental exposure such as exposure to tobacco smoke, and previous surgical interventions (ventilation tubes) are thought to contribute to the increased inflammatory response and the subsequent development of CSOM, due to their impact on the immune response and an increased risk of exposure to pathogens(171, 176–178).

The aim of this study was to describe local cellular immune responses in the mucosa of healthy middle ears and of patients with CSOM. Associations between the local immune cell response and ear microbiota (results from Chapter 4) will be explored.

5.1.2. Methods

The same cohort of patients and controls described in Chapter 4 was used, but tissue specimens rather than swabs were analysed. Middle ear and mastoid mucosa of 24 subjects with CSOM (with and without cholesteatoma) undergoing tympanomastoid surgery and 22 controls with healthy middle ears undergoing cochlear implantation or translabyrinthine surgery was collected prospectively by a single ORL surgeon from Auckland City Hospital, New Zealand.
Patients received intravenous antibiotics (cephazolin) on induction of anaesthesia. Mucosal samples from the middle ear and mastoid were harvested under sterile conditions and fixed in Carnoy's fixative (6:3:1 of ethanol:chloroform:glacial acetic acid) for one week before being transferred to 70% ethanol prior to paraffin embedding. The study was approved by the New

Zealand Health and Disability Ethics Committee (NTX/12/03/024). Written consent was obtained from all participants or their guardians prior to sampling.

5.1.2.1. Enumeration of host inflammatory cells

Tissue sections (4 µm) were prepared from paraffin embedded tissue as described previously(179). In brief, sections were processed separately for each of the following: 1. Eosinophil and neutrophil cell counts (using routine hematoxylin-eosin (H&E) staining procedures); 2. Plasma cell counts (methyl green-pyronin staining); 3. $CD3^+$ T cells (anti-CD3 monoclonal antibody (mAb)); 4. $CD20^+$ B cells (anti-CD20 mAb); and 5. $CD68^+$ macrophages (anti-CD68 mAb). Immunohistochemistry was used to enumerate $CD3^+$ T cells, $CD20^+$ B cells, and $CD68^+$ macrophages, following antigen retrieval using a pressurized heat-retrieval method (2100 retriever) with citrate buffer (pH 6). Sections were incubated with either mouse anti-CD3 (IgG1), anti-CD20 (IgG2a), or anti-CD68 (IgG2a) (Leica Biosystems, Newcastle Upon Tyne, UK) and Novocastra IHC Diluent (Leica Biosystems, Newcastle Upon Tyne, UK) at the following dilutions: 1:400; 1:200; and 1:50. Sections were then processed with the Novolink Polymer Detection System Kit (Leica Biosystems, Newcastle Upon Tyne, UK) as per the manufacturer's instructions. Negative controls (omitting the antibody) were included for all samples. Five representative high-power field images (63x magnification) were taken for each section using an epifluorescence Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) with a SPOT camera (Diagnostic Instruments, MI) and analysis FIVE software (Olympus, Japan). Cell counts were conducted independently by two separate observers who did not know subject diagnoses at the time of counting. Cells were counted using ImageJ (NIH, Bethesda), with replicates for each cell type averaged for subsequent analyses.

5.1.2.2. Data analyses

Immune cell numbers were compared for the following groups: 1) CSOM versus controls; 2) mastoid versus middle ear; 3) cholesteatoma versus non-cholesteatoma. Differences between groups were tested using Kruskal-Wallis test or Dunn's test with False Discovery Rate (FDR) corrections for multiple comparisons.

Associations between the local immune cells and our previously published ear microbiota data for the same cohort were investigated using Spearman correlation analysis along with significance testing (p <0.05) for each pairwise correlation. The following variables were included in the analysis: immune cells counts, bacterial OTUs, bacteria community alpha

diversity (Shannon index) and disease status. The key variables from the correlation analysis have been illustrated in a heat map. Spearman's correlations were performed on immune cell counts and relative abundance of bacterial communities. Mastoid and middle ear samples were analysed separately for cholesteatoma and non-cholesteatoma patients.

5.1.3. Results

5.1.3.1. Characterisation of the immune cells based on disease status

T cells (CD3), B cells (CD20) and macrophages (CD68) were identified in significantly elevated numbers in CSOM patients compared with controls where numbers were negligible (Table 1). In addition, no significant differences were found between T cells, B cells and macrophages for both middle ear and mastoid samples.

Table 1*: The mean cell counts in tissue specimens for T cells (CD3), B cells (CD20), and macrophages (CD68) in CSOM and control subjects are shown. Mastoid and middle ear tissue samples were analysed.*

	CD3	**CD20**	**CD68**
	Mastoid		
Controls	0.7 (SD 0.2)	0	0.015 (SD 0.05)
Cholesteatoma	48.7 (SD 76.3)	54.2 (SD 88.9)	25.5 (SD 27.9)
Non-cholesteatoma	53.5 (SD 49.2)	61.4 (SD 92.6)	9.3 (SD 10.6)
	Middle ear		
Controls	0.1 (SD 0.3)	0 (SD 0)	0.03 (SD 0.1)
Cholesteatoma	28.9 (SD 34.4)	62.3 (SD 89.9)	8.2 (SD 10.6)
Non-cholesteatoma	52.3 (SD 80.0)	68.4 (SD 102.8)	6.6 (SD 9.3)

SD–standard deviation. Values are means. All statistical comparisons with controls and disease (cholesteatoma and non-cholesteatoma) samples were significant $p<0.01$.

Representative images for T cells (CD3), B cells (CD20), and macrophages (CD68) are shown in Figure 2.

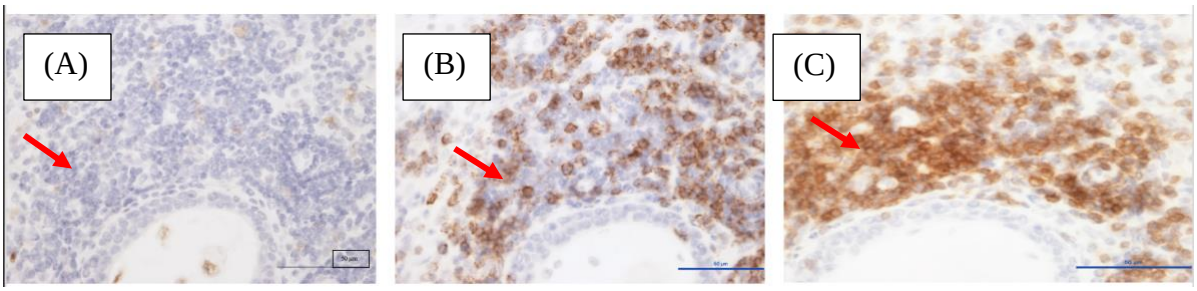


***Figure 2**: Mucosa CSOM; (A) $CD3^+$ T cells (anti-CD3 monoclonal antibody (mAb)), (B) $CD20^+$ B cells (anti-CD20 mAb), (C) $CD68^+$ macrophages (anti-CD68 mAb)*

A similar pattern was observed for lymphocytes and histiocytes, with higher numbers in CSOM compared with controls (Table 2). However, the number of neutrophils, eosinophils and plasma cells were low for all subjects.

***Table 2**: The mean cell counts in tissue specimens for lymphocytes, histiocytes, eosinophils, neutrophils and plasma cells in CSOM and control subjects are shown. Mastoid and middle ear tissue samples were analysed.*

	Lymphocytes	**Histiocytes**	**Eosinophils**	**Neutrophils**	**Plasma cells**
			Mastoid		
Controls	2.3 (SD 4.8)	16.3 (SD 34.1)	<1	<1	<1
Cholesteatoma	45.9 (SD 35.3)	39.7 (SD 34.1)	<1	<1	<1
Non-cholesteatoma	49.1 (SD 363.3)	33.2 (SD 28.6)	<1	1.4 (SD 1.7)	<1
			Middle ear		
Controls	0	0	<1	<1	<1
Cholesteatoma	48.4 (SD 35.5)	32.2 (SD 31.9)	<1	<1	<1
Non-cholesteatoma	38.9 (SD 36.6)	35.7 (SD 34.5)	<1	<1	<1

SD – standard deviation. Values are means. All statistical comparisons with controls and disease (cholesteatoma and non-cholesteatoma) samples for lymphocytes and histiocytes were significant $p < 0.01$. There were negligible cell counts for eosinophils, neutrophils and plasma cells in all tissue specimens.

5.1.3.2. Characterisation of the host immune cells based on sampling location (mastoid vs middle ear).

There were no significant differences in the number of immune cells (CD3, CD20, CD68) in the mastoid compared with middle ear of CSOM patients (Figure 3).

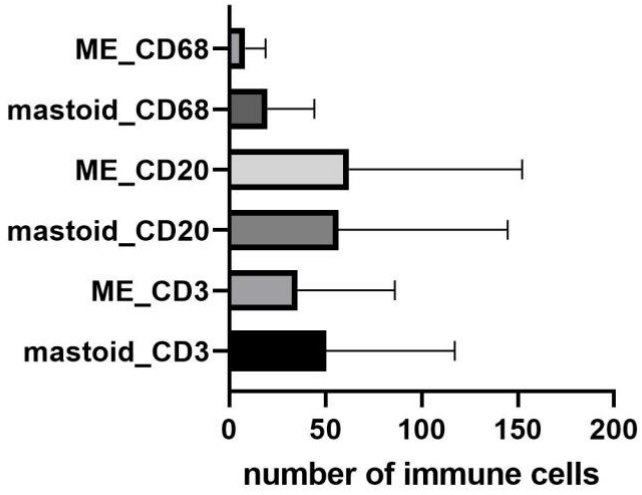


Figure 3*: The number of immune cells (CD3, CD20, CD68) detected in the mastoid of middle ear (ME) of CSOM patients have been shown. No statistical differences were found in the study variables between middle ear and mastoid of CSOM patients.*

5.1.3.3. Characterisation of the host immune cells based on the presence of a cholesteatoma

There were no significant differences found between cholesteatoma and non-cholesteatoma samples for CD3, CD20 and CD68 cell counts. However, lymphocytes and histiocytes were elevated within each sub group compared to neutrophils, eosinophils, and plasma cells, which is in keeping with a chronic, non-allergic immune response. In middle ears of controls no lymphocytes or histiocytes were identified in the middle ear, and less than 10 lymphocytes and 20 histiocytes were seen in mastoid samples. CD3, CD20 and CD68 cells were not detected in controls.

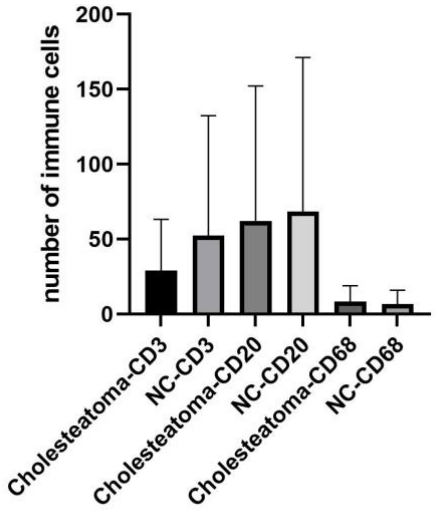


Figure 4*: Number of inflammatory cells (CD3, CD20, CD68) for cholesteatoma compared to non-cholesteatoma (NC) samples in CSOM patients.*

5.1.3.4. Correlation between inflammatory cells and microbiota of CSOM patients and controls.

The immune cell counts (lymphocytes, histiocytes, neutrophils and eosinophils) for control patients were negligible, and so were not included in the analyses. The correlation co-efficient values with significant *p*-values (<0.05) for mastoid and middle ear samples are illustrated in Figure 5 and 6.

In the mastoid, the relative abundance of *Haemophilus* had a significantly negative correlation with CD20 (B cells) (and overall lymphocyte counts) for CSOM patients with cholesteatoma. In contrast, genus *Alloiococcus* had a significantly negative correlation with CD3 (T cells) and CD68 (macrophages) counts in the middle ear samples with cholesteatoma. In addition, genera *Moraxella* and *Propionibacterium* were found to be negatively correlated to CD68 and histiocytes, while members from the family *Enterobacteriaceae* were positively correlated with lymphocytes of middle ear samples from cholesteatoma patients.

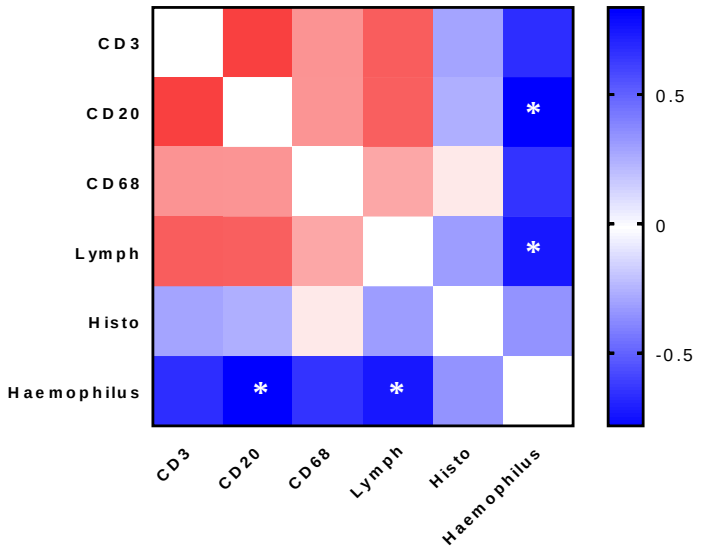


***Figure 5**: Spearman values for mastoid samples (cholesteatoma) with significant correlations (p<0.05) (*****).*

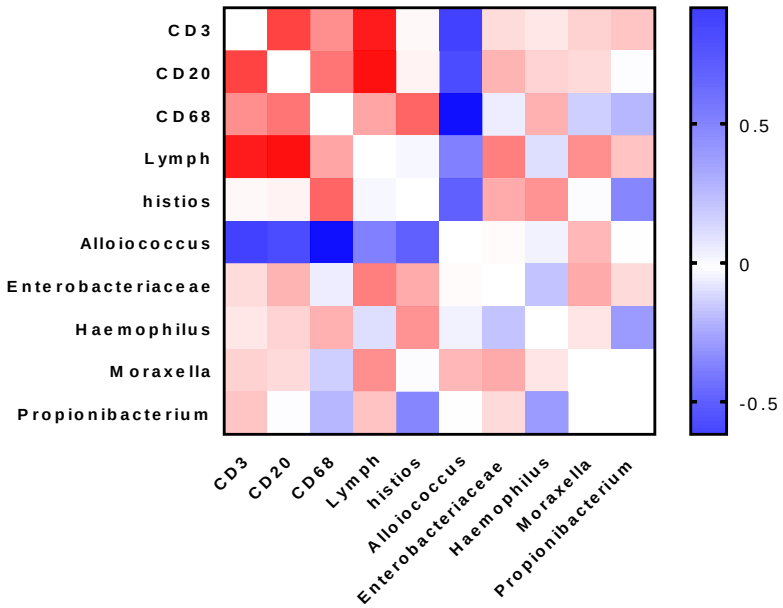


***Figure 6**: Spearman values for middle ear samples (cholesteatoma) with significant correlations (p<0.05) (*****).*

Non-cholesteatoma patients had no significant correlation between the microbiota and immune cell response for both mastoid and middle ear samples.

5.1.4. Discussion

Acute infection usually settles as a result of pro-resolution mediators. The failure of these mediators, and sustained exposure to pathogens results in a chronic inflammatory response(180). Macrophages are involved in the mediation of resolution of inflammation but are also key to the initiation and maintenance of chronic inflammation(181). There is sustained recruitment of mononuclear leukocytes (monocytes and lymphocytes) and associated tissue injury and repair in response to the chronic inflammatory response(182, 183). The significant elevation of immune cells in the CSOM patients compared to controls is typical for a chronic inflammatory response seen in CSOM and other chronic inflammatory conditions(171, 183). A T cell mediated immune response has been described in CSOM, and T cells were also elevated in the current study(175). The systemic inflammatory response was not examined in this chapter but the inflammatory response in CSOM has been reported to be due to a local response only(184). The sampling location (mastoid versus middle ear) made no difference to inflammatory cell numbers, in keeping with the anatomical continuity of the middle ear and mastoid air cell system.

Pathogen derived antigen (pathogen-associated molecular patterns (PAMPs)) and antigen derived from damaged tissue such as cholesteatoma (damage associated molecular patterns (DAMPs)) activate the expression of a variety of cytokines and growth factors via antigen binding to toll-like receptors (TLRs), which foster inflammation. These are assumed to be higher in cholesteatoma compared to non-cholesteatoma patients, but the numbers of immune cells between these disease states counted in the current study were similar(185).

Potential host-microbial associations were identified in patients with CSOM. Genera *Haemophilus* and *Alloiococcus* had a negative correlation with T cell (CD3) and B-cell (CD20) markers which may suggest a suppressed immune response and has been described in other chronic ear conditions(186–188).

In otitis media with effusion (OME), extracellular proteins of genus *Alloiococcus* have been reported to have an indirect pro-inflammatory effect on the middle ear mucosa, and a more chronic course of OME in patients in whom *Alloiococcus* was isolated has been described(189–191). *Alloiococcus* has been isolated more commonly in OME as opposed to acute otitis media (AOM), and may therefore contribute to the chronicity of polymicrobial middle ear infections(192). But the role of *Alloiococcus* in the pathogenesis of otitis media is debated. It

has been identified as a commensal organism of the ear canal, but controlling for contamination when collecting middle ear specimens through the ear canal still identified *Alloiococcus* in middle ear fluid of patients with OM. *Alloiococcus* has been shown to trigger innate and adaptive immune responses in the middle ear(190, 191) and its ability to form biofilm in vitro has been demonstrated(193). Unlike other pathogens involved in OM believed to cause middle ear infections by ascending from the nasopharynx to the middle ear, *Alloiococcus* has not been identified in the nasopharynx using conventional microbiology methods and has been reported at only low abundance in molecular studies(192), concluding that the source of *Alloiococcus* found in middle ear infections is likely the ear canal(192). All specimens in our CSOM study were collected intraoperatively under sterile conditions. The finding of *Alloiococcus* in our specimens is therefore not considered a contaminant. There are no studies exploring the effect of genus *Alloiococcus* in patients with CSOM but our findings would suggest a possible growth advantage for members from this genus, or a suppressive function on the host's immune cells. Further study of *Alloiococcus* has been proposed to explore their effect of middle ear microbial growth and their role in chronicity and recurrence of infection(192).

Genus *Haemophilus* has been implicated in mucin overproduction in patients with acute otitis media(194). This action is believed to play a role in the transition of acute to chronic infection, in which both mucosal hyperplasia and mucin overproduction are prominent features(171, 194). In *in vitro* otitis media models, *Haemophilus influenzae* fosters progression and severity of disease(187). This has also been shown in other chronic infections (lung), where *Haemophilus* displays pro-inflammatory activity leading to increased disease severity(188). Members of the genus *Haemophilus* can evade host defences by attaching to and invading mucosa or inflammatory cells, and by binding and surviving in biofilms(186, 195). These findings could support a negative association of genus *Haemophilus* and immune cell numbers in the mastoid of patients with CSOM found in this study. Genus *Alloiococcus* can induce an increase in resistance in genus *Haemophilus*, providing it with a growth advantage in OME, which has not yet been explored in CSOM(193).

A limitation of this study was the use of short read sequencing in the molecular identification of microbiota in CSOM as this was only and most economical method available at the time of this study. This provided microbial information only to the family or genus-level but a higher resolution may be required when studying pathogens involved in otitis media. For example, *Enterobacteriaceae* has limited sequence divergence and short read sequencing provides little resolution to differentiate between species with numerous pathogens in this group known to

cause human infections(196). Without further differentiation, meaningful comparisons between this group and inflammatory cells were not possible. Since completion of this study long-read sequencing has become more readily available and has been shown to provide improved taxonomic resolution(197).

As discussed in chapter 4, *Staphylococcus* was identified in the microbiota of many patients in this study, but in relatively few patients was *Pseudomonas* detected. These results contrast with many culture-based studies in which *P. aeruginosa* and *S. aureus* were the dominant bacteria(10, 13–16, 18). Differences in the capability of certain species to grow in culture media may account for much of the disparity between culture-based and molecular techniques(85).

5.1.5. Conclusion

The inflammatory response we observed in our CSOM cases is typical of chronic mucosal inflammation, as expected. The site and sub-type of disease did not influence the inflammatory counts significantly. Further study is required to better define the factors that cause the persistence of the inflammatory process. Our results suggest the possibility that *Alloiococcus* and *Haemophilus* may have significant roles in this process.

5.2 The role of intramucosal bacteria in chronic suppurative otitis media

5.2.1. Introduction

Bacteria have developed strategies to avoid elimination by host defenses and antimicrobial agents(198). These strategies include developing antimicrobial resistance, persisting in communities such as biofilms, secreting bacterial toxins and invading the host tissue(17, 30, 92, 199).

Using a combination of confocal laser scanning microscopy, transmission electron microscopy, and FISH, intracellular pathogenic bacteria have been demonstrated in middle ear infections (AOM, OME) and are believed to contribute to the chronicity and recurrence observed in these infections(35, 200). The ability of bacteria to invade the epithelial layer has also been described in chronic rhinosinusitis and is a known mechanism of bacterial survival evading host defenses but has not been previously investigated in CSOM(201–204).

P. aeruginosa and *S. aureus* are two most reported bacteria from swabs of CSOM patients(45–47), even though there are differences prevalence of bacteria reported between different countries as reported in Chapter 2, Table 4. Accordingly, the aim of this study was to investigate the presence of intramucosal *P. aeruginosa* and *S. aureus* in patients with CSOM, compared to a control group of patients with healthy middle ears.

5.2.2. Materials and Methods

The same cohort of patients and controls described in Chapter 4 was used. This was a prospective study of 24 subjects with CSOM who had failed medical therapy and underwent tympanomastoid surgery, and 21 patients with healthy middle ears and mastoids who required cochlear implantation or translabyrinthine surgery. All patients were given intravenous cephazolin on induction of anaesthesia. No other antibiotics were given within at least one month pre-operatively. Middle ear and mastoid mucosa from subjects and controls were harvested intraoperatively under sterile conditions.

Harvested specimens were immediately placed in Carnoy's fixative (6:3:1 of ethanol:chloroform:glacial acetic acid) before being transferred to 70% ethanol prior to paraffin embedding. The study was approved by the New Zealand Health and Disability Ethics

Committee (NTX/12/03/024).

5.2.2.1. Histology

Tissue sections of 4 µm thickness were prepared from paraffin-embedded tissue.
Prepared sections were processed separately for the following: (1) Gram stain (*S. aureus only)*; (2) immunofluorescence targeting *P. aeruginosa* and *S. aureus*.

a) Gram staining

Gram staining and H&E staining were performed as per routine procedures. A subset of nine cases was stained with Gram stain for middle ear and mastoid samples. Furthermore, all the remaining mastoid samples were also Gram stained. Between 11 and 25 serial sections per case were screened using a Leitz camera (Leica) DMR upright microscope (Leica Microsystems, Wetzlar, Germany) x63 oil objective. A Gram stain was considered positive when there were Gram-positive cocci in the same location within the mucosa on three consecutive sections.

b) Antibody-based P. aeruginosa and S. aureus staining

Sections were blocked in normal goat serum for 30 min, followed by HIER (heat induced epitope retrieval) in citrate buffer (10 mmol/L, pH 6) in the 2100 Retriever System (PickCell Laboratories, Amsterdam, The Netherlands) for the tissue that was stained with *S. aureus* antibody. Sections were stained overnight at 4^{o}C with either monoclonal antibody for *S. aureus* clone staph 11-248.2 (1:500 dilution, MAB930, Merck Millipore, Temecula, CA, USA) or polyclonal *P. aeruginosa* (1:1500 dilution, Abcam 74980 Cambridge, UK). The secondary antibody Goat anti-mouse (1:400 dilution, IgM 488, A21042 Invitrogen Karlsbad CA USA) or Goat anti-chicken (1:400 dilution, IgY 594 Abcam 150176 Cambridge, UK) were applied for 1 hour at room temperature to fluorescently label bacteria. 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) was used to stain DNA. The sections were cover-slipped using Citifluor AF1 (Hatfield, PA, USA). Ten serial sections per case were stained with each antibody. These were visualized under fluorescence using a Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) with 100x oil objective. Photos were taken with the SPOT camera (Diagnostic Instruments, MI), and analysis FIVE software (Olympus, Japan) was used to capture the IHC fluorescent images. Image J (NIH, Bethesda, MD) software was used to merge photos. A section was considered positive if the bacterial DNA stained with DAPI and this was surrounded with the antibody stain. A tonsil specimen containing either *S. aureus* or *P. aeruginosa* was used as a positive control. When positive staining was seen on three

consecutive sections, a result was called positive for intracellular/interstitial bacteria. Any three, non-consecutive sections with positive stains were called uncertain. A negative result was one with fewer than three positive stains.

5.2.3. Results

Of the 24 patients undergoing mastoid surgery for CSOM and 21 patients with healthy middle ears undergoing either cochlear implantation (CI) or translabyrinthine surgery, 32 yielded sufficient mucosa to allow histological examinations (22 subjects and 15 controls). In two subjects and six controls insufficient mucosa was available for analysis.

5.2.3.1. Gram stain

The middle ear mucosal samples were negative for Gram staining. In three cases with mastoid tissue Gram-positive bacteria were detected (Figure 1). No inflammatory cells were identified in the vicinity of these bacteria. All controls were negative on gram stain.

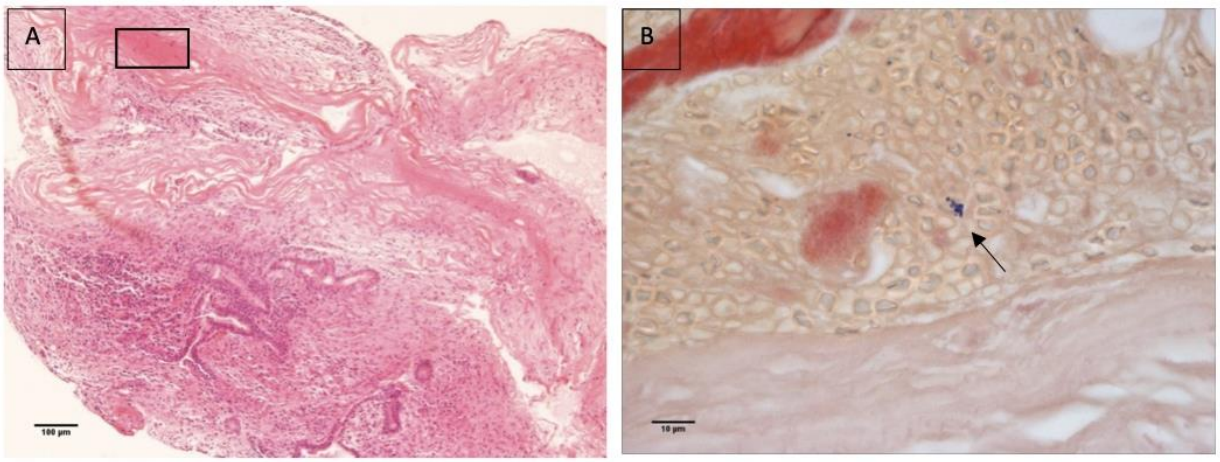


Figure 1*: A: H&E section x10, B: The area from the rectangle in A as seen in a Gram stain. The arrow points to gram-positive cocci x100. Taken on the Leica DMR microscope with Nikon Digital Sight DS-5Mc-U1 cooled colour camera.*

5.2.3.2. Antibody-based S. aureus and P. aeruginosa staining

All CSOM subjects yielded sufficient mucosa to allow antibody-based staining for *S. aureus* and *P. aeruginosa*. For five controls insufficient material was available for *S. aureus* staining, and for nine controls *P. aeruginosa* staining could not be performed due to insufficient mucosa. Of 24 CSOM subjects only three stained positive for *P. aeruginosa* (n=24, 13%) (Figure 2). Five CSOM subjects were positive for *S. aureus* (n=24, 21%)

(Figure 2) and five were considered uncertain (n=24, 21%).

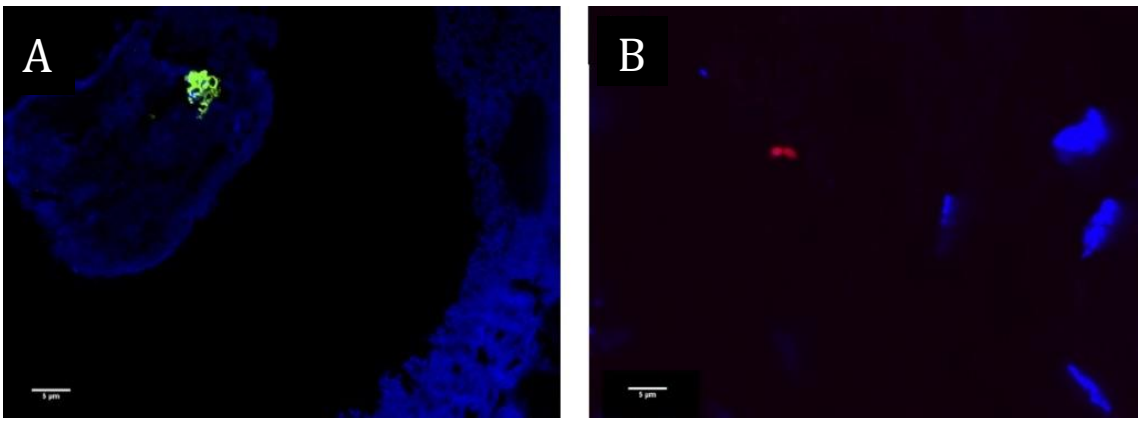


Figure 2*: A: S. aureus fluorescing green, B: P. aeruginosa fluorescing red x100. Taken on the Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) with 100x oil objective.*

None of the controls had positive results for either stain, but two controls had uncertain positivity (n=16, 13%) for *S. aureus* stains only.
All bacteria identified on histological sections and on antibody-based staining appeared to be beneath epithelial surfaces, but it could not be determined from the images if they were within cells.

5.2.4. Discussion

Studies using culture and molecular techniques have shown that bacteria are present in middle ear and mastoid samples of CSOM patients(14, 18). In this study the spatial distribution of bacteria in the tissue was investigated. Antibody-based staining demonstrated the presence of intramucosal *S. aureus* in 21% (positive result only) of patients with CSOM. Intramucosal *P. aeruginosa* was observed in 13% of patients with CSOM. Considering the abundance of these two bacteria reported in the literature using culture-based methods, this finding of low abundance in tissue was surprising(45–47). There were no inflammatory cells in the vicinity of intramucosal bacteria suggesting that these bacteria exist unrecognized by host immune defenses.

The focus in this study was on *P. aeruginosa* and *S. aureus* as they are the most reported pathogens in culture-based studies in CSOM(45–47). But other pathogens have also been demonstrated and warrant further study, particularly including anaerobic bacteria that are more suited to grow in these environments. It was assumed that the gram-positive cocci

identified on gram stain represented *S. aureus*, but other gram-positive cocci can look similar. *Alloiococcus* are gram-positive cocci and their potential involvement in middle ear disease was discussed in chapter 5.1. above. There have also been reports of the role of gram-positive anaerobic cocci (GPAC) causing soft tissue infections in polymicrobial infections(205). The involvement of GPAC in CSOM warrants further investigation.

Bacteria can invade from the mucosal surface to the stroma through a disrupted epithelial barrier. Once within the cell, they can evade host responses(206). Bacteria can trigger their own release from the cell, re-infecting the host. Host cells damaged in the process are digested and serve as nutritional substrate for invading bacteria(207–215). No mucosal bacteria were identified in control patients. In healthy mucosa, bacterial invasion of the cells is prevented by a protective layer of epithelial cells, mucus, immunoglobulin A and immune cells(216).

5.2.4.1. Limitations

The limitations of this study include the small sample size and limited amount of normal mucosal specimens available. The viability of intramucosal bacteria identified in this study was not determined.

5.2.4.2. Future directions

The treatment of intracellular bacteria has been investigated, in which an anti *S. aureus* B-cell antibody was linked to an antibiotic(217). This complex was opsonized into the host cell. Intracellular cleavage activated the antibiotic and killed intracellular *S. aureus in vitro*. This complex was more effective than the use of an antibiotic alone (vancomycin)(217). This approach may be helpful in treating recalcitrant CSOM in the future.

5.2.5. Conclusion

The two species of bacteria most commonly cultured from CSOM were found in low abundance and frequency within mucosal samples from CSOM patients. Our technique of investigation was species specific, and bacterial species, including GPAC warrant further investigation. Intracellular bacteria can evade host defenses and may be less susceptible to systemic antibiotics. Intracellular bacteria can be released from cells, reinfecting the host which could contribute to treatment failures seen in CSOM.

Chapter 6

The tissue microbiome of CSOM patients

6.1. Introduction

Most studies reporting on the microbiology of chronic suppurative otitis media (CSOM) are based on the culture of swabs taken from or through the ear canal. When cultured, aerobic bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus* spp. and *Klebsiella* spp. are generally assumed to play a pathogenic role. To date, there has been little reported use of molecular methods and tissue-based studies to identify pathogens associated with CSOM(16, 24–26, 69).

Anaerobes form a significant part of the commensal mucosal flora, and can also cause infections(218). Few studies have specifically investigated anaerobic bacteria as a potential cause of CSOM(14, 25, 79) This is because anaerobes are difficult to be detect by conventional culture due their slow growth *in vitro*. The most common anaerobic bacteria associated with infection in CSOM patients is *Bacteroides* spp.. Less frequently reported anaerobic bacteria include members from the genera *Clostridium*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Peptostreptococcus* and *Peptococcus*(14, 25, 79).

Histopathology studies of CSOM microbiology often report the identification of Gram-positive cocci(34). However, the identification of these organisms is uncertain, and most are assumed to be *S. aureus* because of this species predominance in culture(209). Gram-positive anaerobic cocci (GPAC) have been reported as part of the normal human microbiome but have also been cultured from a number of infection sites(205, 219–223). In the head and neck region, they are usually regarded as part of the polymicrobial community on mucocutaneous surfaces, from where they are able to invade deep tissues at sites of breakdown of the mucocutaneous barrier(218). This group of bacteria is often under-reported in culture-based studies because of their requirement for fastidious growth conditions, and difficulty in their identification when grown(222, 223). Tissue specimens or an aspirate of pus may improve the ability to identify these bacteria compared to swabs(218).

The aim of this study was to investigate the microbial composition of mastoid and cholesteatoma tissue from CSOM patients and compare this to healthy controls using both molecular and culture-based methods. The absolute abundance of clinically relevant bacteria in the tissue specimens was also measured.

6.2. Methods

6.2.1 Patient information

Eleven adult patients undergoing mastoid surgery for CSOM with cholesteatoma, and ten adults with healthy middle ears undergoing either cochlear implantation (CI) or translabyrinthine vestibular schwannoma surgery (TL) via the mastoid and middle ear were included in this study. Participants were recruited from Auckland City Hospital between August 2021 and May 2022. The study was approved by the New Zealand Health and Disability Ethics Committee (NTX/12/03/024). None of the study patients received antibiotics within two months of surgery. All participants (including controls) received intravenous cefazolin on induction. Patients' disease status was staged according to the European Academy of Otology and Neurotology and Japanese Otological Society (EAONO/JOS) staging system(107). The socioeconomic status based on patients' domicile was scored in accordance with the New Zealand Deprivation Index, NZDep(106) (where 1 represents a high socioeconomic status and 10 a low socioeconomic status).

6.2.2. Sample collection

Tissue samples measuring about 2 to 5 mm in diameter (mucosa and cholesteatoma) were collected from the cholesteatoma, mastoid mucosa of CSOM patients and mastoid mucosa of controls, intra-operatively. Tissue specimens were immediately placed in 500 µl of RNAlater in a sterile 2 mL screw-capped tube and in a sterile 5 mL glass vial containing Carnoy's fixative. Samples in RNAlater were transported to the laboratory on ice within 4 hours of collection, and then frozen (-20ºC). Carnoy's fixed specimens were processed for histology in the laboratory as described below.

6.2.3. Culture from swabs

A conventional microbiology swab from the cholesteatoma or mastoid mucosa was also taken at the time of tissue collection. These swabs were sent to our hospital microbiology laboratory for routine bacterial culture work and the identification of potential pathogens. In brief, the swabs were inoculated on the following culture media: Columbia Sheep Blood, Supplemented Chocolate with bacitracin, MacConkey, Colistin-Naladixic and Sabouraud Dextrose agars (Fort

Richard Laboratories Ltd). The former two were incubated at 37°C in ambient air supplemented with 5% CO_2, while the latter three were incubated at 37°C in ambient air. Swabs collected from patients with chronic infections were also plated on Brain Heart Infusion media and incubated anaerobically. Bacterial species were identified using the Vitek MS (bioMérieux) system, and antibiotic sensitivity testing was performed on the Vitek 2 (bioMérieux) or by using disc diffusion criteria as appropriate, and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines. Samples that led to the growth of a single colony or more were considered to be positive.

6.2.4. Histology and Gram staining

Tissue specimens were fixed in Carnoy's fixative for one week after collection and then transferred to 70% SDA. The samples were then processed and embedded in paraffin wax. A 5 µm section was cut onto Superfrost Plus Positively Charged Microscope Slides (Thermo Fisher Scientific, Waltham MA, USA) using a microtone. The slides were dried in an incubator at 60ºC for 1 hour and then Gram stained using standard laboratory protocols. The slides were mounted using dibutyl phthalate polystyrene xylene (Scharlau Barcelona, Spain) and covered with a glass coverslip. Gram-positive cells were visualised and imaged with an Olympus CX21LED upright microscope (Olympus Life Sciences, Waltham MA, USA). Whole tissue scanning was performed on 4 cholesteatoma specimens that contained Gram-positive cells. Scanning was conducted on the Zeiss Axio Imager z2 upright microscope (Carl Zeiss AG, Oberkochen, Germany) using Metasystems Metafer5 Vslide software.

6.2.5. DNA extraction/Sequencing

Genomic DNA was extracted from tissue specimens using the Qiagen AllPrep DNA/RNA Mini Kit as previously described(206). Bacterial 16S rRNA genes were amplified from the extracted DNA using primers specific for the V3-V4 region (S-D-Bact-0341- b-S-17 5′-TCGTCGGCAGCGTCAGATGTGTATAAG AGACAG-CCTACGGGNGGCWGCAG-3′ and S-D- Bact-0785-a-A-21 5′-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAG-GACTACHVGGGTATCTAATC C)(224, 225). Each PCR reaction contained 3 µl of template DNA (~100 ng), 0.5 U HotStar DNA polymerase (Qiagen), HotStar PCR

buffer (x1), $MgCl_2$ (2 mmol/L), equimolar concentrations of each primer and dinitrophenols (0.2 µmol/L), and PCR-grade water to a final volume of 25 µL. Samples were amplified in duplicate using the following conditions: 95ºC for 15 mins, 35 cycles of denaturing, annealing and extension (95ºC for 30 secs, 55ºC for 30 secs, 70ºC for 40 secs), and a final extension step of 3 mins at 70ºC. Negative PCR controls were included in all PCR reactions and had no detectable amplified DNA. In addition, eluate from two negative DNA extractions were subjected to bacterial PCR amplification to detect contamination from the extraction kit. All PCR amplicons were combined and purified using Agencourt AMPure magnetic beads (Beckman-Coulter, Inc. Brea, CA) as per manufacturer's guidelines. The purified products were quantified using Qubit dsDNA high sensitivity assay kits (Life Technologies, Auckland, New Zealand) and submitted to the sequencing provider at Auckland Genomics (New Zealand) for library preparation and sequencing on the Illumina MiSeq platform using 2x 300 bp paired end reads.

6.2.6. Bioinformatics

Sequence data were processed using the "DADA2" package version 1.12.1 in R statistical program version 3.6.3(226, 227). Most parameters were kept as default, except 'truncLen' was set to 250 and 200 for forward and reverse reads, respectively, and primers were removed. Quality filtered sequences that were <247 bp or >410 bp were considered non-target and removed from the dataset, and then chimeras were identified and removed. Taxonomy was assigned to amplicon sequence variants (ASVs) using the SILVA nonredundant v138 database(228). Non-target Eukaryote taxa were removed prior to the removal of ASVs with a prevalence less than 5 times in at least 5% of the samples. No rarefaction was performed as the majority of the samples would have been lost. The final dataset included 73 taxon-assigned ASVs across 19 samples. Graphs were created in R version 4.0.2. and GraphPad Prism(229, 230).

6.2.7. Droplet digital PCR

ddPCR™ was performed using the QX200 Droplet Digital PCR System and QuantaSoft Software (Bio-Rad Laboratories) on genomic DNA extracted from study samples as described previously(145). Primers selective for genes specific to *S.*

aureus, *P. aeruginosa* were optimised using pure bacterial cultures of these species and then applied to the genomic DNA of CSOM related tissue specimens and controls. A primer selective for the V1V3 hypervariable regions of the bacterial 16S rRNA gene was also applied to approximate the total bacterial load. Negative ddPCR™ controls and DNA extraction kit controls were included for each primer target and any counts were subtracted from the final sample counts. The mean value from each of the three genes was used for pairwise comparisons between cohorts (CSOM, control and cholesteatoma) followed by Tukey's *p*-value correction. *P*-value levels <0.05 are considered significant. Groups were compared using Dunn's test of multiple comparisons with the Bonferroni adjustment for multiple comparisons.

6.2.8. Multiplex PCR

A multiplex PCR reaction was used to identify the dominating taxa of GPAC in cholesteatoma tissue samples. Two genus-specific primer pairs targeting *Anaerococcus* and *Peptoniphilus* were identified from the literature(25). The genus *Anaerococcus* was further differentiated to species-level by a second multiplex PCR reaction. These included *Anaerococcus octavius*, *Anaerococcus hydrogenalis*, *Anaerococcus lactolyticus*. The sensitivity and accuracy of the primers were tested previously against 14 reference strains of GPAC species(231). All primer pairs and PCR reactions were replicated from a previous study with slight modifications(231). Positive controls for the PCR reactions were obtained from LabPLUS (Auckland, New Zealand). In brief, PCR amplification was performed using 2.5 µl genomic DNA from cholesteatoma tissue samples, 0.5 U HotStar DNA polymerase (Qiagen, Germany), HotStar PCR buffer (x1), $MgCl_2$ (2 mM), dNTPs (0.2 mM), concentration of each primer (0.25 µM), and PCR-grade water to a final volume of 25 µL. PCR amplification took place over 35 cycles as described previously(232). PCR products were analysed using a 2% agarose gel.

6.3. Results

6.3.1. Patient information

The clinical details of the participants are summarised in Table 1. There were 11 CSOM subjects whose disease extent was staged according to the joined European Academy of Otology and Neurotology and Japanese Otological Society

(EAONO/JOS) staging system (mostly stage I (n=5) and stage II (n=5))(107). One patient presented with stage IV disease. Four patients had a canal wall up (CWU) and seven a canal wall down (CWD) procedure. Based on the hospital records, most subjects were of Pasifika or Asian backgrounds. Three of eleven CSOM subjects were male, the rest female. Six subjects came from a low socioeconomic background (New Zealand Deprivation Index, NZDep 7-10)(106).The median age for CSOM subjects was 38 years (range 17- 87).

There were 10 controls, three of whom underwent cochlear implantation (CI) and seven translabyrinthine removal of a vestibular schwannoma (TL). There were significantly more (p<0.05) European subjects (n =7) in controls compared to the CSOM group (n =2). Five controls were male and five were female. Seven controls came from a low socioeconomic background (NZDep 7-10). There was no significant socioeconomic difference between the groups based on NZDep. The median age for controls was 69 years (range 32-85). Controls were significantly older than CSOM subjects (p<0.05).

***Table 1**: Demographics of participants and conventional swab results of subjects (cholesteatoma) and controls.*

	Age	Ethnicity	Sex	Side	Staging of disease	Procedure	NZDep2018	Culture
Subject 1	67	Asian	F	R	I	CWU	8	no growth
Subject 2	47	Asian	M	R	I	CWD	8	*Proprionibacterium acnes* (light)
Subject 3	28	Māori	F	R	IV	CWD	10	no growth
Subject 4	32	Asian	F	R	II	CWD	6	no growth
Subject 5	87	European	F	R	II	CWU	3	skin flora (light)
Subject 6	36	Asian	F	R	I	CWU	5	*Pseudomonas aeruginosa* (heavy)
Subject 7	38	Pasifika	M	R	II	CWD	7	*Corynebacterium jeikeium, Turicella otidis*
Subject 8	45	European	F	R	II	CWU	2	no growth
Subject 9	17	Pasifika	F	L	I	CWD	10	no growth
Subject 10	19	Pasifika	M	L	II	CWD	3	mixed bacteria, mixed anaerobes
Subject 11	43	Pasifika	F	L	I	CWD	7	no growth
Control 1	59	European	F	R		TL	3	skin flora (light)
Control 2	33	Māori	M	R		TL	10	no growth
Control 3	32	Asian	M	L		TL	7	no growth
Control 4	74	European	F	L		TL	8	no growth
Control 5	76	European	F	R		TL	7	no growth
Control 6	77	European	M	L		CI	4	no growth
Control 7	70	Pasifika	F	R		TL	9	no growth
Control 8	67	European	F	L		CI	9	no growth
Control 9	85	European	F	L		CI	3	no growth
Control 10	67	European	F	R		TL	10	no growth

M=male, F=female, R=right, L=left, Staging of disease: see text, CWU=canal wall up mastoidectomy, CWD=canal wall down mastoidectomy, TL=translabyrinthine, CI cochlear implant, NZDep=New Zealand deprivation score: see text.

6.3.2. Gram stain results

Gram-positive cocci were visible in great abundance (more than 10 cells) in the cholesteatoma samples of 5 CSOM patients with cholesteatoma (MN3, MN5, MN9, MN10, MN11), and mucosa of one CSOM patient without cholesteatoma (MN7) and no controls (Figure 1).

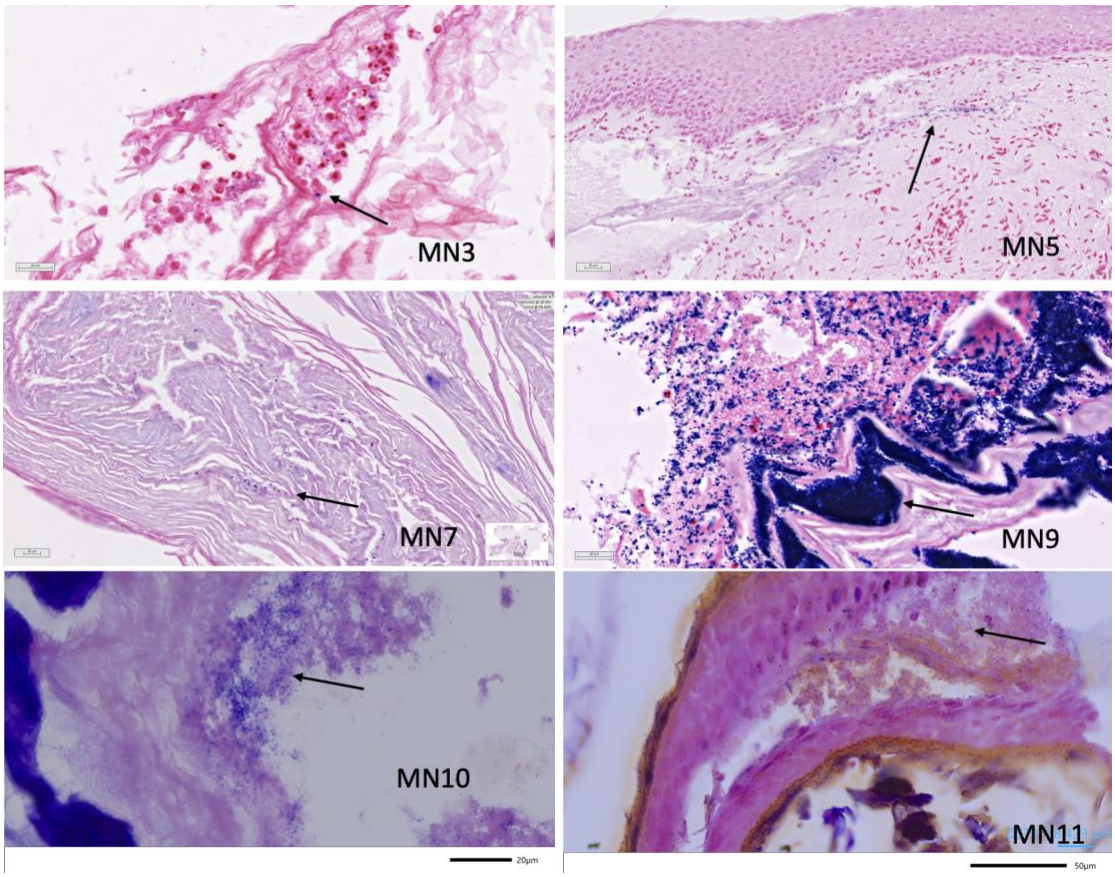


Figure 1*: Gram-positive cocci (black arrow) in cholesteatoma samples (*MN3, MN5, MN9, MN10, MN11*), and in mucosa (MN7). Scale=20 µm, apart from MN11=50 µm. MN=subject.*

6.3.3. Conventional microbiology

Using conventional hospital microbiology swab methods and reporting, only one specimen (MN6) yielded a heavy growth of *P. aeruginosa* (Table 1). Other bacteria identified in CSOM subjects were *Corynebacterium jeikeium*, *Turicella otitidis* and *Proprionibacterium acnes*, as well as skin flora, mixed bacteria and anaerobes. Two specimens were monomicrobial and two were polymicrobial. The hospital laboratory

did not identify any relevant bacteria in the remaining specimens. All controls were negative for pathogenic species.

6.3.4. Bacterial community analysis

After quality filtering, bacterial community composition in 19 samples across 11 participants (9 CSOM and 2 healthy controls) was analysed. Two DNA extraction controls were also sequenced. After quality filtration only one of these control samples had 70 reads remaining.

Many of the samples could not be rarefied due to low number of reads (<2000) (16/19, 84% of samples). Accordingly, results are reported as total number of reads. The most abundant phylum was *Firmicutes* (96.6% - 22,489 reads out of 23,884). The most abundant genera were *Anaerococcus* (22131 reads in 5 CSOM subjects) and *Peptoniphilus* (600 in 2 CSOM patients) (Figure 3 and Figure 4). Genus *Staphylococcus* was present in the amplicon sequence variant (ASV) table but at very low abundance (total of 2 reads across all samples).

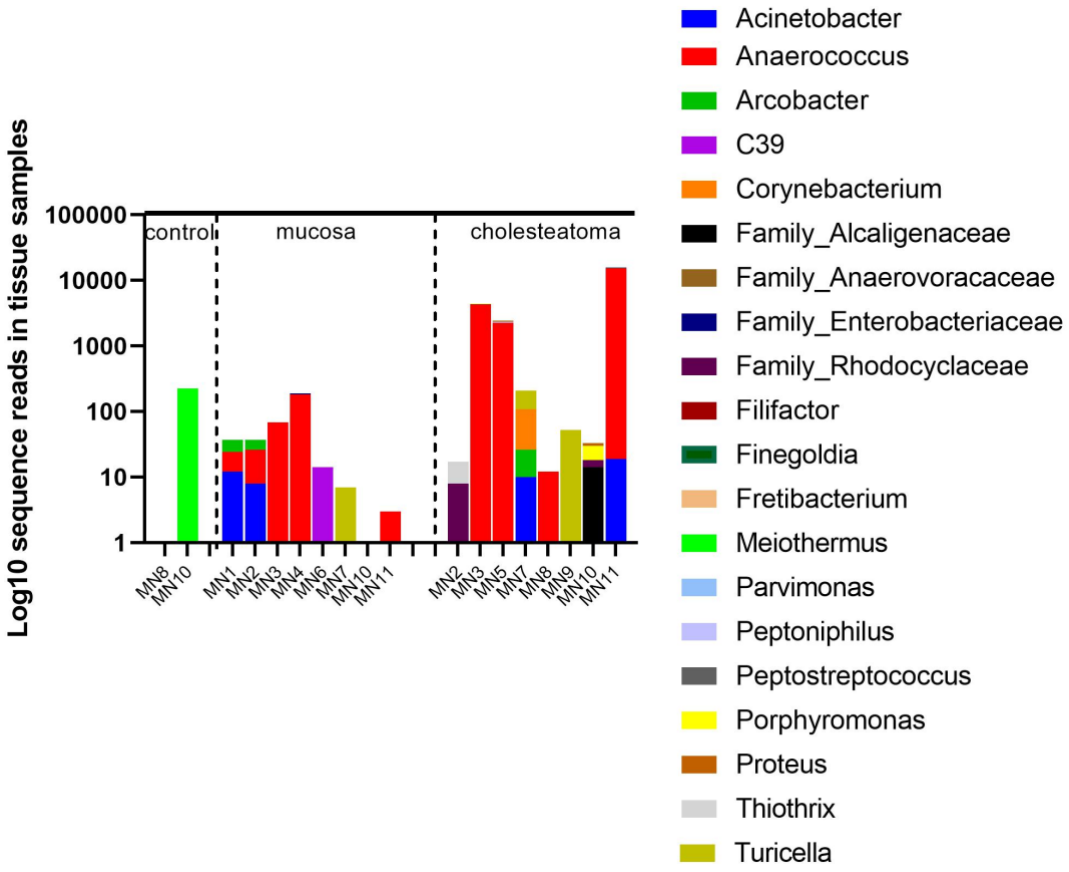


***Figure 3**: Summary of bacterial communities from tissue; mucosa and cholesteatoma*

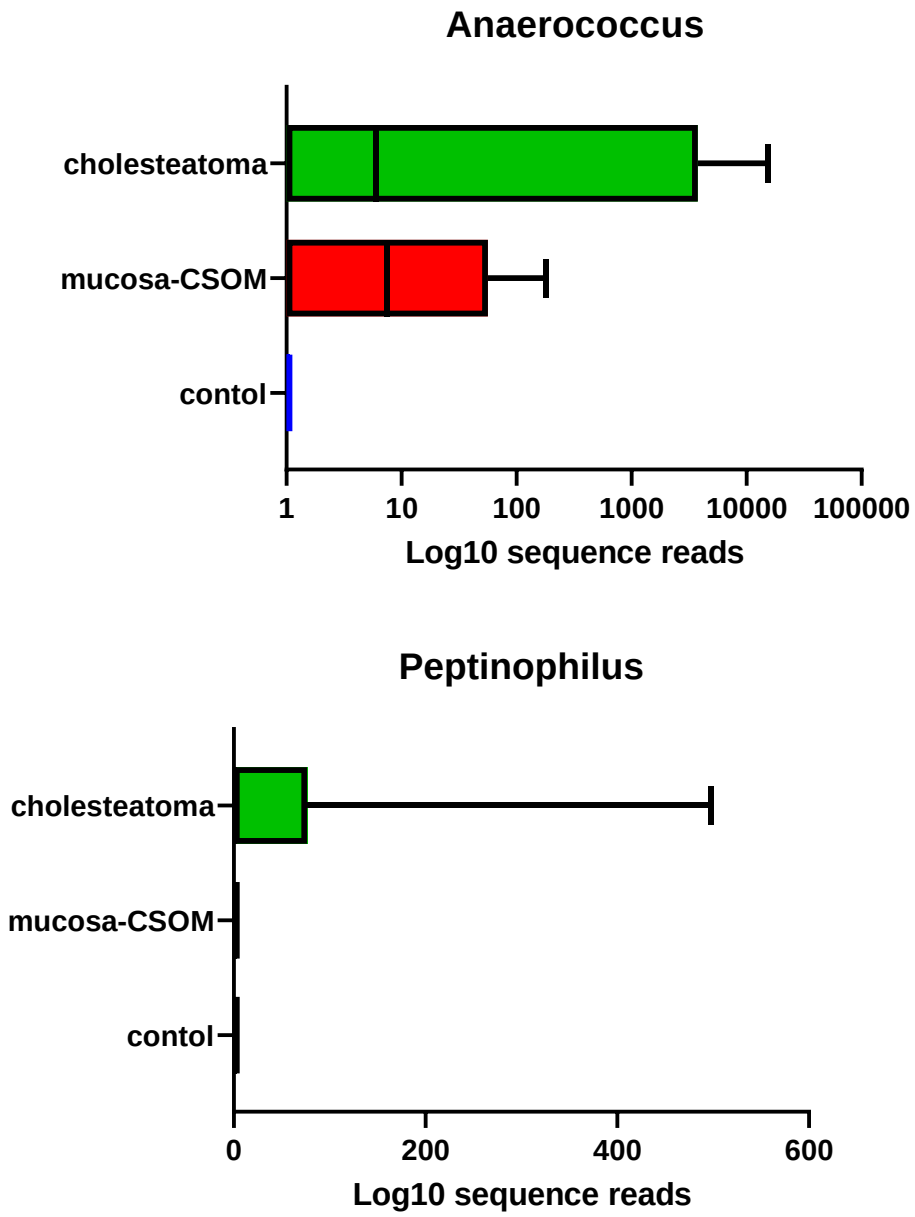


***Figure 4**: Sequence reads of genera Anaerococcus and Peptinophilus in tissue samples.*

6.3.5. Absolute abundance using ddPCR

The overall abundance of bacteria in tissue samples of CSOM patients (mucosa and cholesteatoma) was significantly higher compared to healthy controls (p<0.05) (Table 2).

***Table 2**: The average gene copies of total bacteria, genus Staphylococcus and genus Pseudomonas per tissue sample.*

	Bacterial 16S rRNA gene	*Staphylococcus*	*Pseudomonas*
CSOM mucosa	123.6 (SD 263.02)	28.3 (SD 47.8)	1.9 (SD 1.8)
CSOM cholesteatoma	30285.6 (SD 77848.8)	7.7 (SD 23.1)	88.3 (SD 209)
Controls	2.7 (SD 7.8)	1.6 (SD 11.7)	2.4 (SD 3.6)

SD- standard deviation

The abundance of clinically relevant genera *Staphylococcus* and *Pseudomonas* was also measured in the samples (Table 2). However, no significant differences were recorded between CSOM and healthy controls. In addition, no significant difference between mucosa and cholesteatoma of CSOM patients was observed. The overall gene copies for *Staphylococcus* and *Pseudomonas* reported were very low in all tissue samples (Table 2).

6.3.6. Multiplex PCR results

Based on the sequencing data, genera *Anaerococcus* and *Peptoniphilus* were targeted in the multiplex-PCR. The PCR products size for the two genera were 980 bp (*Anaerococcus*) and 510 bp (*Peptoniphilus*). Cholesteatoma from patients MN5 and MN11 had a strong PCR band for genus *Anaerococcus* (Figure 6A). Cholesteatoma from patients MN3 and MN5 had a PCR band for genus *Peptonophilus* (Figure 6A). Of the three species-specific primers tested in this study, *A. hydrogenalis* (400 bp) was identified as the dominating member of the genus *Anaerococcus* in the samples (Figures 6B).

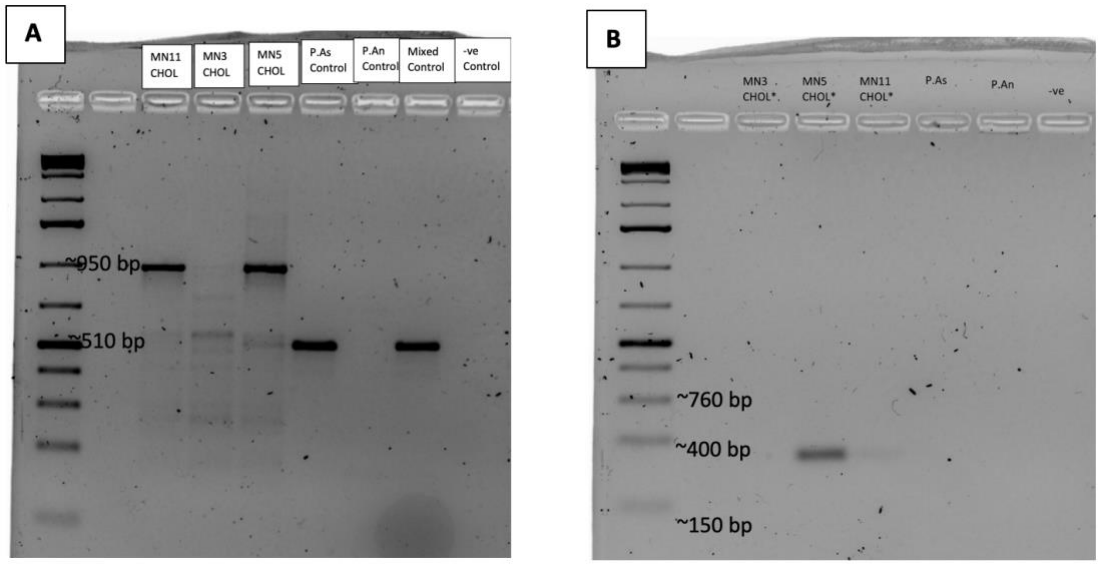


Figure 6*: A 2% agarose gel image from genus- and species-specific multiplex PCR. (A) Positive bands are visible for 3 cholesteatoma samples at ~980 bp for genus Anaerococcus and ~510 bp for genus Peptoniphilus. (B) A positive band at ~400 bp is visible for two cholesteatoma samples indicating A. hydrogenalis. Sample MN3, did not have sufficient volume or concentration of genomic DNA remaining for the species-specific multiplex PCR. A 1 Kb+ ladder was used when running the gels to identify band sizes.*

6.4. Discussion

The composition of the microbiological community observed in CSOM with cholesteatoma differed quite significantly from those that have been described in earlier studies. The likely explanation for this difference is our use of molecular as opposed to culture-based techniques. The majority of CSOM studies published to date have focused on culture results from swabs collected from ear canals or ear discharge(44–46, 52). The other explanation is the sample type examined this study. Majority of samples in CSOM literature investigate ear discharge which is prone to contamination(49, 50, 76, 233). To overcome this issue, tissue specimens were collected from the site of infection for the microbiological analyses in this study.

The main findings from this study show an absence or very low levels of *S. aureus* or *P. aeruginosa* in tissue specimens obtained from the primary site of infection in CSOM. This contrasts with majority of the papers published in this field suggesting the most dominant and prevalent organisms of CSOM to be from these two

species(44, 54, 57, 77). Differences in the capability of certain species to grow in culture media may account for much of the disparity between culture-based and molecular techniques(85). Interestingly, Gram-positive cocci, which our results suggest are anaerobic bacteria from the genera *Anaerococcus* or *Peptoniphilus*, dominate the cholesteatoma tissue bacterial community.

6.4.1. Gram-positive cocci

The identity of Gram-positive bacteria seen on our images could be assumed to be mostly *S. aureus*, given the frequency with which this bacterial species in cultured from CSOM(44–46, 52). However, considering our findings of very few reads in the sequencing data and very low counts in the ddPCR analysis of *S. aureus*, other bacteria need to be considered and may in fact be more important at the tissue level. *Alloiococcus otitidis* is another highly prevalent aerobic Gram-positive bacterium that is cultured from the outer ear canal and is considered to be part of the normal bacterial flora(169, 233). However, there was no detection of this bacteria in our sequencing data. Again, this could be due to the sample type considered in this study, which is less prone to outer ear canal contamination.

6.4.2. Gram-positive anaerobic cocci (GPAC)

Members from the genera *Anaerococcus* and *Peptoniphilus* were dominant in the bacterial communities in our specimens. They are part of a group of GPAC, that are strictly anaerobic, Gram-positive bacteria and can survive in tissue. They are common human commensal organisms, for example of the skin, oropharynx, respiratory tract (including nose), gut, and vagina(222, 234, 235).

While these anaerobes are difficult to grow using routine culture-based methods, the perceived role of GPAC, including *Anaerococcus* and *Peptoniphilus*, as pathogens in polymicrobial soft tissues infections has expanded in recent years(205, 219, 234). This is largely a result of the increased use of molecular microbiological techniques in infectious diseases. Furthermore, high levels of GPAC, especially of *Peptoniphilus*, have been shown to have a negative effect on wound healing(221, 222, 236, 237). (45,46)(15)While *Anaerococcus* has been isolated from an odontogenic brain abscess(238).

GPAC have also been implicated in systemic infections(219, 220). A review of 226 patients diagnosed with GPAC bacteraemia revealed *Anaerococcus* spp. in 43, and *A. hydrogenalis* in four patients(223). Mortality was higher in sepsis patients when bacteraemia was associated with *Anaerococcus* spp. and was commonly associated with an immunocompromised or debilitated state(223). The above observations could support GPAC's involvement in CSOM at a local (associated with cholesteatoma), regional (for example otogenic brain abscesses) and systemic level (bacteraemia and septicaemia).

The bacterial load we observed in CSOM tissue samples was high, and much higher than in healthy controls. It is plausible that the bacteria found in cholesteatoma were part of biofilms associated with keratin. The role of biofilms in the pathogenesis of CSOM has been previously suggested(34). In biofilms, pathogens co-exist in a protected polymicrobial community. In this state, pathogens are characterised by a low metabolic rate which reduces their susceptibility to both the local immune system and to antibiotics(92, 199). Bacteria can switch between the biofilm and planktonic forms, facilitating re-infection and recurrence after completion of antibiotics(34, 95, 239).

Whereas GPAC is often resistant to metronidazole, some broad-spectrum antibiotics prescribed to treat *P. aeruginosa* and *S. aureus* are effective against GPAC(240). However, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin and meropenem have high minimum inhibitory concentrations (MIC) or are resistant, and may fail to clear some GPAC infections(235, 240, 241). The sensitivity of anaerobes to antibiotics may be important when choosing antibiotics in CSOM.

This study has several limitations, most significantly that it included only a small number of patients and controls. There were differences in ethnicity and age between the CSOM patient and control groups. The small specimen size that can be collected from the middle ear meant that only low genomic DNA yields could be obtained. The extracted DNA from all specimens was completely consumed at the end of this study.

6.5. Conclusion

The molecular techniques used in this study have identified a polymicrobial community in the mucosa and cholesteatoma tissue samples from CSOM patients. Commonly reported pathogens such as *S. aureus* and *P. aeruginosa* were present, but in very low abundance. Bacteria from the GPAC group including *Anaerococcus* and *Peptoniphilus* were shown to be highly abundant in tissue specimens from CSOM patients. These bacteria can play an important role in soft tissue and systemic infections and may be pathogenic in CSOM as well. If this proves to be so, it may have implications for the choice of antibiotic therapy.

Chapter 7

General discussion

Chronic suppurative otitis media (CSOM) is one of the most common childhood diseases worldwide and represents a significant health burden to adults and children alike(1). The incidence is low in the developed world, but it has been reported to affect over 4% of New Zealand Māori children in a rural setting(242). Chapter 3 highlights the association of low socio-economic status and over-representation of minority populations in NZ. Also the risk of hearing loss as a result of the disease process and subsequent surgery was demonstrated in Chapter 3, which has previously been reported in the literature(135). While microorganisms have been implicated in CSOM, much remains unknown about the microbiology of this condition. To a large extent, the current understanding of this area is based on the culture of bacteria from swabs collected from ear discharge or the ear canal (Chapter 2). However, there are several limitations with this approach. Firstly, it is now widely acknowledged that many bacteria grow poorly if at all under standard culture conditions, so culture results provide an incomplete picture of the total microbial diversity that is present. Secondly, swabbing does not enable access to the actual site of infection (the mucosal tissue from the middle ear and mastoid, and the cholesteatoma if present), which is only accessible surgically (Chapters 4 and 6). A key aim of this thesis was to address these shortcomings, by applying culture-independent (molecular) techniques to samples obtained during surgery.

7.1. CSOM, a polymicrobial infection

A key finding of this thesis was describing the polymicrobial nature of CSOM. Chapter 4 represents one of the few publications to report on the polymicrobial nature of CSOM using sequencing techniques. Although the required technology has become more accessible in recent years, there remain very few papers investigating CSOM specifically. Chapter 4 demonstrates the heterogeneity of bacterial communities associated with CSOM and the normal middle ear, reporting novel determinations of the relative sequence abundances of these bacteria.

Subsequently, in order to better understand the microbiome at the centre of CSOM infection, we employed sequencing coupled with ddPCR and multiplex PCR to identify and determine absolute abundances of specific bacteria in cholesteatoma tissue (Chapter 6). These analyses revealed bacteria which were different to those reported using traditional culture methods.

Ultimately, infection occurs at sites of breakdown of the protective epithelial layer of the mucosa of the middle ear and mastoid. Accordingly, this thesis includes a microscopic examination of middle ear and mastoid tissue for the presence of traditionally reported bacteria (Chapter 5). Gram staining and antibody-based staining of *P. aeruginosa* and *S. aureus* were used to examine the spatial orientation of these bacteria in tissue, while cell counts confirmed a local inflammatory response. Cell counts at different sites (middle ear or mastoid), with and without cholesteatoma, were correlated with the microbiota in CSOM in order to define microbe-host interactions (Chapter 5).

It is important to discuss the findings of this thesis within the context of three CSOM sequencing-based studies that have been published. A Turkish study used next-generation sequencing on cholesteatoma samples collected intraoperatively and found that 93% of samples were polymicrobial in nature(25). Bacteria reported included members of the order *Clostridiales,* family *Staphylococcaceae, Peptoniphilaceae, Corynebacteriaceae, Fusobacteriaceae, Moraxellaceae, Porphyromonadaceae,* and genus/species *Turicella otitidis*.

In another study of patients with CSOM with and without cholesteatoma, *Firmicutes* were frequently identified on intraoperative tissue samples(27). Genera *Anaerococcus*, *Corynebacterium*, *Staphylococcus* and *Peptoniphilus* were detected in cholesteatoma. *Peptoniphilus* was dominant in patients with CSOM with cholesteatoma.

A third molecular study on patients with CSOM also confirmed the polymicrobial nature of tissue samples collected intraoperatively(26). In patients with cholesteatoma, there was increased abundance of the genera *Campylobacter, Peptococcus, Porphyromonas,* and *Prevotella*.

The polymicrobial results reported in the above studies were broadly consistent with those of Chapters 4 in this thesis, with a variety of commensal and putatively pathogenic bacteria identified. Notable bacterial genera included *Corynebacterium*, *Propionibacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Haemophilus* and *Pseudomonas*, as also reported in Chapter 4(25–27). By contrast, we found a higher relative abundance of *Alloiococcus* in cholesteatoma compared to non-cholesteatoma

and control patients, while in control patients the relative abundance of *Proprionibacterium* was higher.

One study reported lower bacterial alpha-diversity in cholesteatoma samples compared to middle ear mucosa samples, in contrast to our Chapter 4 findings of lower diversity in non-cholesteatoma patients(26). This apparent discrepancy may be related to the administration of antibiotics to the patients in this paper: quinolone antibiotics reduced the abundance of *Corynebacterium, Staphylococcus, Haemophilus* and *Enterobacter* in cholesteatoma samples, while increasing others such as *Peptococcus* and *Enterococcus*. The patients in our study described in Chapters 4 and 6 were not treated with antibiotics before surgery (apart from prophylactic antibiotics on induction of anaesthesia).

In this thesis, strict techniques were used in the recovery and identification of these bacteria (Chapter 6): specimens were collected intraoperatively through the mastoid and from the middle ear directly, avoiding contamination from the ear canal. Bacterial amplicon sequencing was then applied to the specimens to identify the overall bacterial community composition. ddPCR was used to quantify the overall bacterial load and clinically relevant bacteria (species *S. aureus* and *P. aeruginosa*).

The low abundance of *S. aureus* and *P. aeruginosa* in the tissue specimens of CSOM patients was another important finding in this thesis. However, members of the bacterial genus *Anaerococcus* and *Peptoniphilus* belonging to GPAC were identified in large relative abundance. Multiplex PCR was used to further confirm these findings and to classify the dominant bacteria to species level. In the future, GPAC need to be considered as possible pathogens in CSOM.

Limitations of the studies in this thesis are the small study populations due to funding constraints which limit the ability to generalise the above finding and apply them to the general population. Given the low number of patients presenting with CSOM in Auckland, NZ (e.g. 125 children presenting over a 10 year period, see Chapter 3), recruitment of a larger group of subjects was not feasible given the time constraints in preparing this thesis. The middle ear and mastoid specimens available for analysis are small and from collection and processing to analysis of specimens, the sequences may

reflect only a small subset of the bacterial load of the original sample. But the thesis provides a better understanding of the polymicrobial nature of CSOM.

7.2. Is there a normal middle ear microbiome?

Despite some claims that the healthy middle ear is sterile, molecular techniques have provided increasing evidence of a normal middle ear microbiome, as reported in chapters 4 and 6. The findings from Chapters 4 and 6 of this thesis were supported by another 16S rRNA gene-based study, which reported members of the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* in the normal middle ear mucosa in children and adults(27). Other studies also reported a normal middle ear microbiome, identifying nine genera (*Prevotella*, *Prevotella*, *Alloprovetella*, *Fusobacterium*, *Leptotrichia*, *Haemophilus*, *Veillonella*, *Streptococcus*, *Neisseria*) which comprised more than 90% of identified OTUs(243).

The presence of a microbiome in the normal middle ear is plausible, given the continuity of the middle ear with the nasopharyngeal microbiome via the Eustachian tube(243). Even though there may be a microbiome in the healthy middle ear, the biomass is very low (Chapters 4 and 6), and hence it can easily be missed.

Despite efforts to mitigate the risk of contamination in our specimens (Chapter 4 and 6), there is an inverse relationship between bacterial DNA concentration and specimen contamination(244). Some of the bacteria reported in Chapter 4 have been identified as background contaminant in low bacterial load specimens (244, 245). It is therefore possible that some of the very low biomass samples that have been reported positive in our studies simply reflect contamination(26, 246, 247). Considering this, extra care was taken to remove the contamination effect from the DNA extraction kits (see Chapter 6). In a recent study of a small group of cochlear implant recipients, microbial DNA could not be identified in middle ear mucosal samples(26). Since completion of the study in Chapter 4, laboratory processes have been described to standardise methods in an effort to mitigate the risk of contamination in low microbial biomass specimens, a problem commonly described across the CSOM literature(248, 249).

7.3. Potential function of the normal microbiome

7.3.1. Defence against pathogens

The commensal mucosal microbiome provides a protective shield against invading pathogens(216). This potential function of the middle ear commensal microbiome has not been investigated in the CSOM literature. But a disruption of a normal microbiome (dysbiosis, which may be caused by altered immunity or antibiotic use) may allow invasion of pathogens via the Eustachian tube or external auditory meatus, ultimately leading to CSOM. Alternatively, dysbiosis could trigger overgrowth of pathogens from the microbiome itself, as colonizing opportunistic pathogens have been identified in the normal microbiome and in Chapter 4(27, 243, 250). The concept of dysbiosis as a possible cause of CSOM has support in the literature, but has not been studied closely(251).

7.3.2. Commensals priming for mucosal immunity in CSOM

The normal microbiome could prime the immune system for the defence against future encounters with pathogens(252).

7.3.2.1. Induction and effector cells in the middle ear mucosa

Induction of immunity by pathogens usually occurs at mucosa-associated lymphoid tissue (MALT) in the gut, lung, and pharynx(253). MALT is not prominent in ear mucosa but has been described in the middle ear and Eustachian tube of temporal bones in patients with a history of otitis media, in whom induction could occur(254). In this process, memory B and T cells are produced and migrate to effector cells, which results in the expression of mucosal surface-immunoglobulin-A (S-IgA) antibodies involved in the mucosal immune response upon exposure to pathogens(255). Activation of Ig-A occurs locally at the site of exposure(256). Compared to other lymphocytes, Ig-A is 20 times more common in gut mucosa and is the main immunoglobulin active in mucosal immunity(257). There is evidence that Ig-A is the predominant immunoglobulin produced locally in the middle ear(253). In the middle ear, toll-like receptor (TLR) binding activates the innate mucosal immune system(70). Defects in TLRs have been associated with downregulation of the innate immune response and this can lead to chronic infections. In CSOM, TLR levels are

significantly lower than in normal patients(70). This could increase susceptibility to invading pathogens and the development of CSOM(70).

7.4. The role of biofilms in CSOM

It is likely that some of the bacteria identified via sequencing in Chapters 4 and 6 were part of middle ear biofilms, which have been previously described(258–260). Bacteria in biofilms are often unculturable, but can be identified by microscopic and molecular techniques.

The role of biofilms in ear infections in general and CSOM in particular has been reported(258–263). Bacteria can accumulate as heterogenous communities surrounded by a polysaccharide matrix to form biofilms. In this state, bacteria have a low metabolic rate that renders them less susceptible to antibiotics and the host's immune defences. Bacteria have the ability to cycle between the biofilm and planktonic states, and so can re-infect the host. Layers of keratin are an ideal substrate for bacterial and biofilm attachment and the chronic and recurrent nature seen in patients with CSOM may be explained by the bacterial cycle between biofilm and planktonic states(258–260).

7.5. The contribution of interstitial and intracellular bacteria to CSOM

During chronic infections, planktonic bacteria can be detected in the mucosa of middle ears. They are usually cleared by the host defences, and the host tissue returns to normal homeostasis. Some bacteria such as *Staphylococcus* are able to survive within macrophages. By exiting cells after courses of antibiotics, they can cause reinfection(201–203). Intracellular carriage of pathogens is a bacterial mechanism described to evade the host defences and could contribute to the chronicity seen in CSOM(264).

The potential significance of intracellular infection of middle ear mucosal epithelial cells as a cause of chronic infection was first raised in children with OME, a chronic ear condition related to CSOM(200).

A typical chronic mucosal inflammatory response was observed in our CSOM cases described in Chapter 5.1, as expected. Further study is required to better define the

factors that cause the persistence of the inflammatory process, but our results suggest that *Alloiococcus* and *Haemophilus* may have a role in this process. Our findings in Chapter 5.1 would suggest that in patients with CSOM genus *Alloiococcus* has a possible growth advantage, or a suppressive function on the host's immune cells, and that genus *Haemophilus* could have a negative effect on immune cell numbers in the mastoid of patients with CSOM. However, it is possible that these bacteria were contaminants or planktonic bacteria as specimens were collected using swabs. The chronic inflammatory response may actually have been due to bacteria found in tissue as reported in Chapter 6 (*Anaerococcus* and *Peptoniphilus*).

In Chapter 5.2, Gram-stain and antibody staining were used to demonstrate bacteria in tissue, below the epithelial layers in CSOM. It could not be determined with certainty if some of these bacteria were intracellular. Further study is required to determine the presence and role of intracellular bacteria in CSOM(203).

7.6. Clinical implications and future treatment approaches

7.6.1. Prevention of CSOM

For patients with CSOM who have either failed medical treatment or have a cholesteatoma, the next treatment step is mastoid surgery. Outcomes of surgical CSOM patients in a NZ paediatric hospital were reviewed in Chapter 3. An association between CSOM and low socioeconomic status has been reported by WHO(3). Chapter 3 demonstrates similar findings, with the majority of children with CSOM being of a low socioeconomic and/or minority background. It is acknowledged that addressing inequities must be considered in the prevention of CSOM.

7.6.2. The current, monomicrobial treatment approach

Much of the current understanding of the microbiology of CSOM has been based on culture-based studies, as reviewed in Chapter 2. These studies attempted to identify a primary microbe responsible for the infection, determine antibiotic sensitivities, and then use this information to treat patients with culture-guided antibiotics(44–46, 52). This treatment approach is mostly successful, as broad-spectrum antibiotics are used to cover the identified microbes and will also cover many unidentified bacteria potentially involved in the disease process. Treatment failures have been reported and

have in part been explained by the development of antibiotic resistance, for example MRSA and *P. aeruginosa* resistance to fluoroquinolones(58, 66, 99). However, despite appropriate treatment based on culture, some of these treatment failures still remain unexplained. Many of these patients undergo surgery to prevent local, regional and systemic complications, as well as mortality. (Chapter 3)

The traditional monomicrobial view when managing this disease with antibiotics remains prevalent, despite increasing evidence that CSOM is a polymicrobial infection. A better understanding of the microbiology of CSOM should reduce the number of treatment failures, and may reduce the need for extensive ear surgery in some patients. Better targeted antibiotic use can reduce the development of antibiotic resistance.

7.6.3. The polymicrobial treatment approach

High-throughput sequencing results of specimens from patients with CSOM confirmed the polymicrobial nature of CSOM (Chapter 4). These bacteria may have been in the planktonic form, in biofilms, or have survived in intracellular compartments. Treatments should aim to target bacteria in all these states.

7.6.3.1. Targeting planktonic pathogens in CSOM

Planktonic pathogenic bacteria identified by culture swabs through the ear canal have traditionally been treated with topical or systemic antibiotics. Topical antibiotics are probably superior to systemic antibiotics in achieving a dry ear(23). Conservative measures including water precautions and suction may be enough for some patients.

However, the ear canal is only a surrogate sampling site for CSOM. In Chapter 6, microbes identified by ddPCR at the primary site of infection differed from those classically reported in culture studies. The importance of anaerobes, in particular the demonstration of high absolute abundance of GPAC, and the low abundance of *S. aureus* and *P. aeruginosa*, deserve further investigation. Some of these bacteria may survive in biofilms, which may require treatment strategies other than antibiotics.

7.6.3.2. Addressing biofilms to treat CSOM

Microbes in biofilms are relatively protected from both host defences and antibiotics. Non-antibiotic therapy has therefore been suggested to treat them(263). Techniques used successfully to physically disrupt biofilm on prostheses (such as laser pressure waves, pulsed ultrasound and hydrodynamic flushing) may be contraindicated because they put the patient's middle and inner ear structures and function at risk (hearing, balance, facial nerve, taste), or may not be practical due to the limitations of anatomy(263).

In a pilot *in vitro* study, sodium 2-mercaptoethanesulfonate (MESNA) altered the properties of cholesteatoma, preventing biofilm adherence in cholesteatoma and increasing antibiotic susceptibility. The safety and *in vivo* activity of this adjuvant therapy are yet to be determined, but it is a possibly helpful approach in the management of CSOM(265).

Another strategy to break down biofilm in chronic ear disease is the use of recombinant human deoxyribonuclease (DNase). The usefulness of this agent in the treatment of cystic fibrosis led to a study in which the enzyme successfully degraded and destabilised biofilm in middle ear fluid *in vitro*, exposing bacteria to be cleared by the host defences or antimicrobials(36). This finding has not been tested or confirmed *in vivo*.

7.6.3.3. Targeting intracellular bacteria in CSOM

7.6.3.3.1. The use of systemic antibiotics against intracellular bacteria in CSOM

Although some systemically administered antibiotics can easily penetrate into human cells, intracellular bacteria have been found to persist despite antibiotic administration(264). Intracellular *S. aureus* have been observed to develop resistance in osteoblasts, rendering antibiotics ineffective(264). In addition, antibiotics may not reach therapeutic concentrations required to kill relatively resistant intracellular bacteria, which adopt a low metabolic state, in response to the harsh intracellular environment(266).

7.6.3.3.2. Antimicrobial peptides against intracellular bacteria in CSOM

Antimicrobial peptides are short peptides produced as part of the innate immune response and are designed to defend the microenvironment against pathogens. They have the ability to disrupt infected cells and may have a therapeutic use in treating intracellular bacteria, but their use middle ear disease has not been investigated. The challenge would be to apply them topically to the site of infection without disruption of unaffected host cells(267).

7.6.3.4. Novel treatments for CSOM: antisense oligonucleotides and nanoparticles

Intracellular bacteria can be targeted *in vitro* by antisense oligonucleotides, which can inhibit bacterial growth. Their delivery into the cell can be enhanced by the use of cell-penetrating proteins. Some nanoparticles have antimicrobial properties and have the ability to accumulate at the infection site, then to recognise and enter infected cells and target bacteria(266, 268). None of these substances have been trialled in CSOM.

7.7. Future research considerations

Molecular techniques will continue to provide new insights into the polymicrobial nature of CSOM. Short-read 16S rRNA gene sequencing of various hypervariable regions is the current technology of choice to identify members of the microbiome. This approach can typically identify microbes reliably to family or genus level. This technique was used in this thesis (Chapter 4). In order to reliably obtain information to species level, ddPCR and multiplex ddPCR, in addition to sequencing, were employed (Chapter 6).

Full-length 16S rRNA gene sequencing using Pacific Biosciences (PacBio) technology can identify microbes to species level, but during the time of our studies being performed, had a high error rate (5-10%) and was considerably more expensive. However, recent studies have aimed to improve the error rate and increased use will make this technology less costly and more readily available in research laboratories(269–271).

The interaction of microbes in various niches and states (planktonic, biofilm, intracellular) within anatomical regions requires more research to improve our

understanding of the pathogenesis. Future molecular studies should include an evaluation of fungi and viruses to determine their influence on the microbiome in CSOM. One molecular study postulated that CSOM is primarily a bacterial infection(251).

A call for standardised molecular techniques has been made and this should include a guide on sampling site and technique in CSOM(251). All samples in this thesis were collected intraoperatively from the primary site of infection (Chapters 4, 5, 6), to avoid contamination from the ear canal microbiome. In Chapter 6 tissue samples were examined to get a true idea of the microbiome at the tissue level.

7.8 Conclusion

In this thesis culture-independent methods were used to describe the microbiome of chronic suppurative otitis media. The inflammatory response was described by examining inflammatory cell and bacterial interactions and by staining techniques for bacteria commonly reported in CSOM. The polymicrobial nature of this infection was highlighted and compared to the normal middle ear microbiome. Intraoperative swab samples from the mastoid and middle ear of CSOM patients commonly identified *Alloiococcus*, but GPAC was commonly identified from tissue specimens, highlighting their potential importance in this condition. In CSOM intramucosal bacteria were identified which may contribute to the chronicity of this condition. An improved understanding of CSOM will hopefully lead to better treatments and outcomes for patients who present with this condition.

Appendix I

Author publication history

The following publications I contributed to as a co-author during the period of my MD.

Co-author publication:

1. McLaren H, Kim R, MacFater W, Neeff M, Jelicic T, Douglas R. 2022. Factors affecting clinic attendance for children referred with middle ear conditions. ANZ J Surg 92:3264–3267.

2. Cheung ICW, Thorne PR, Hussain S, Neeff M, Sommer JU. 2022. The relationship between obstructive sleep apnea with hearing and balance: A scoping review. Sleep Med 95:55–75.

3. Cañete OM, Purdy SC, Brown CRS, Neeff M, Thorne PR. 2021. Behavioural performance and self-report measures in children with unilateral hearing loss due to congenital aural atresia. Auris Nasus Larynx 48:65–74.

4. Hardcastle T, McKay-Davies I, Neeff M. 2020. Petrous apex pneumatisation in children: a radiological study. J Laryngology Otology 134:798–803.

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