Understanding the Risk of Emerging Bacterial Resistance from the Use of Sore Throat Over-the-Counter Topical Antibiotics

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Contents

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Thesis Summary

Within Europe, antibiotics are available in over-the-counter (OTC) topical sore throat medications. As sore throats are mainly of viral aetiology, antibiotics in these medications is poor antimicrobial stewardship. It is unknown what role OTC antibiotics (bacitracin, gramicidin, neomycin and tyrothricin) play in antimicrobial resistance. This study aims to understand whether the use of OTC antibiotics could contribute to resistance development in bacteria.

OTC antibiotics at during-use concentrations were tested against a panel of bacteria and mainly Gram-negative bacteria could resist their effects, with the exception of neomycin. After OTC exposure, clinical cross-resistance was gained to beta-lactam antibiotics (including ampicillin, cefotaxime, aztreonam and imipenem) and gentamicin and this resistance was mainly stable.

Phenotypic and genotypic changes after OTC antibiotic exposure were assessed and many changes occurred including, increased beta-lactamase activity, increased efflux activity, morphological changes, metabolic changes and mutation in membrane protein genes. It is thought that the increase in beta-lactamase activity is due to induction of AmpC, which is predominantly responsible for the clinical cross-resistance to the beta-lactam antibiotics.

Co-exposure assays were done to evaluate the impact OTC antibiotics have on aminoglycoside efficacy. It was found that gramicidin and tyrothricin both impacted the efficacy of aminoglycoside treatment, although bacitracin did not. The study concluded that gramicidin and tyrothricin depolarize the cell membrane by potassium leakage, inhibiting aminoglycoside uptake into the cell.

Along with experimental lab work, a survey was constructed to understand OTC antibiotic usage. It also sought to understand how sore throat is managed, and the knowledge of pharmacists on OTC antibiotic-containing products. Although the survey has not yet been distributed, responses from pretesting indicate that some pharmacists are unaware of OTC antibiotic-containing products.

This study highlights the development of clinical cross-resistance from exposure to OTC antibiotics, and therefore should not be used for sore throat products for patients seeking symptomatic relief.

List of Tables and Figures

List of Abbreviations

ABC - ATP-binding cassette

AK - Amikacin

AMP - Ampicillin

AMPs - Antimicrobial Peptides

AMR - Antimicrobial Resistance

ANOVA - Analysis of Variance

AST - Antimicrobial Susceptibility Testing

ATC - Anatomical Therapeutic Chemical

ATCC - American Type Culture Collection

ATM - Aztreonam

ATP - Adenosine Triphosphate

AUC - Area Under the Curve

BNF - British National Formulary

BZK - Benzalkonium Chloride

CB - Clinical Breakpoint

CCCP - Carbonyl Cyanide m-chlorophenyl Hydrazone

CFU - Colony Forming Units

CIP - Ciprofloxacin

CLSI - Clinical and Laboratory Standards Institute

CN - Gentamicin

CTX - Cefotaxime

DiOC2(3) - 3,3'-Diethyloxacarbocyanine Iodide

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic Acid

EARSS - European Antimicrobial Resistance Surveillance System

ECOFFs - Epidemiological Cut-off Values

EDTA - Ethylenediaminetetraacetic Acid

EMA - European Medicines Agency

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FDA - Food and Drug Administration

GAS - Group A *Streptococcus*

GDP - Gross Domestic Product

GDPR - General Data Protection Regulation

GI - Gastro-Intestinal

GP - General Practitioner

HS - High Sensitivity

IM - Inner membrane

InDels - Insertions and deletions

IPM - Imipenem

IU - International Units

LPS – Lipopolysaccharide

MATE - Multidrug and Toxic Compound Extrusion

MDR - Multi-Drug Resistant

MFS - Major Facilitator Superfamily

MGEs - Mobile Genetic Elements

MHA - Mueller-Hinton Agar

MHB - Mueller-Hinton Broth

MHB-F - Fastidious Mueller-Hinton Broth

MHF-A - Fastidious Mueller-Hinton Agar

MIC - Minimum Inhibitory Concentration

MRSA - Methicillin-Resistant *Staphylococcus* aureus

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NZ - No Zone

OD - Optical Density

OM – Outer membrane

OTC - Over-the-counter

PACE - Proteobacterial Antimicrobial Compound Efflux

PBP – Penicillin Binding Protein

PBS - Phosphate-buffered Saline

PHE - Public Health England

PMF - Proton Motive Force

POM - Prescription-only Medicine

PTFE - Polytetrafluoroethylene

QACs - Quaternary Ammonium Compounds

RND - Resistance-nodulation-cell Division

ROS - Reactive Oxygen Species

S/V - Surface to Volume Ratio

SD - Standard Deviation

SEM - Scanning electron Microscopy

SMR - Small Multidrug Resistance

SNPs - Single Nucleotide Polymorphisms

SXT - Trimethoprim-sulfamethoxazole

TSA - Tryptone Soya Agar

URTI - Upper Respiratory Tract Infection

USD - United States Dollars

WGS - Whole Genome Sequencing

WHO - World Health Organisation

ZOI - Zone of Inhibition

β-NAD - Beta-Nicotinamide Adenine Dinucleotide

ΔpH - Transmembrane Chemical Proton Gradient

ΔΨ - Transmembrane Electric Potential

Chapter 1: General Introduction and Project Aims

Chapter 1: General Introduction

1.1. Issue of Antibiotic resistance

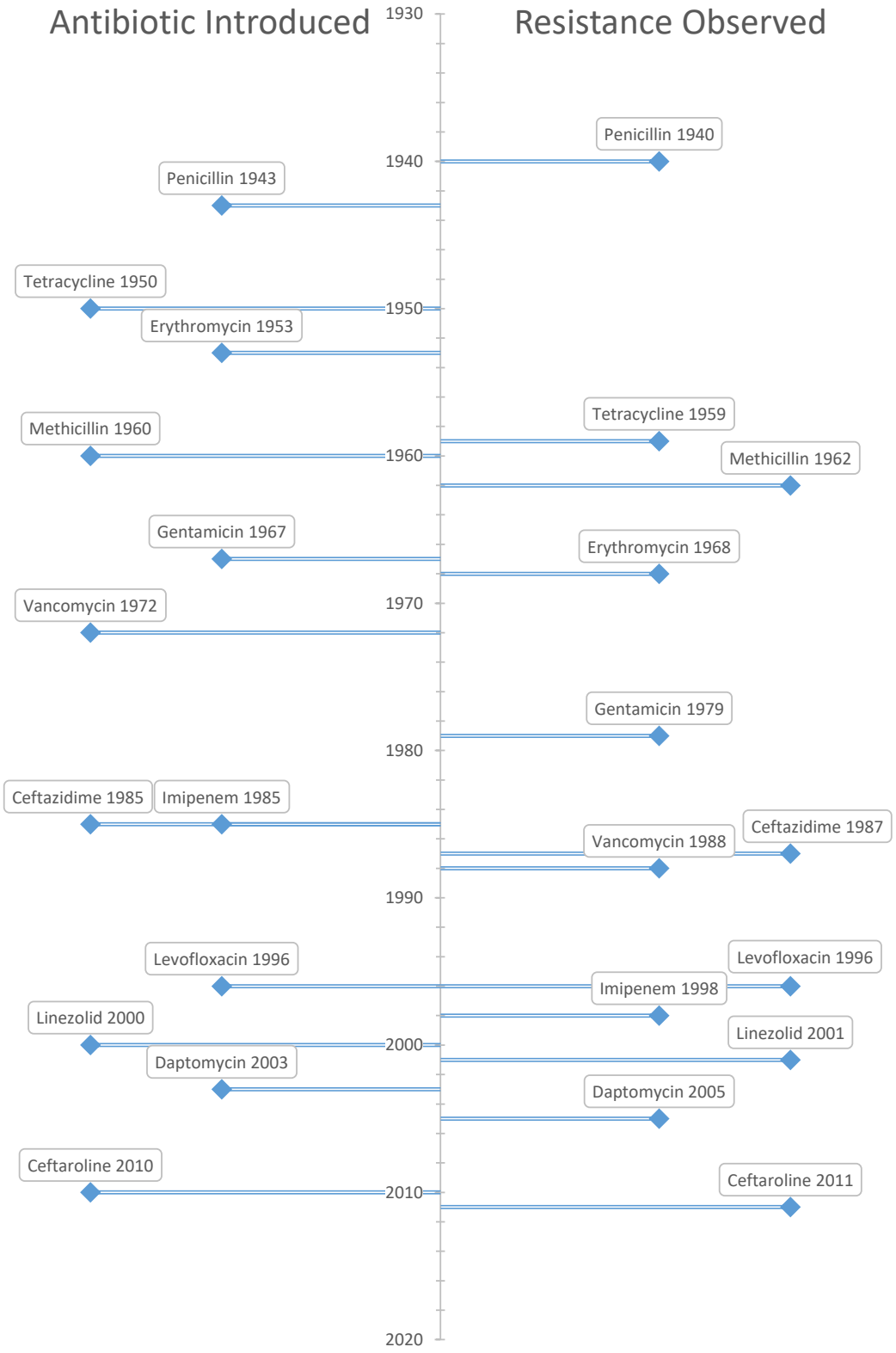
1.1.1. Rise of Antibiotic Resistance

Antibiotics are one of the most important discoveries of the twentieth century. Since their discovery (Fleming, 1929), antibiotics have been continually used to treat a wide variety of severe infections allowing the advancement of modern medicine. Treatments such as chemotherapy, invasive surgery and organ transplantation have become routine practice (Ventola, 2015).

Whilst antibiotic resistance does occur naturally and resistance genes pre-date the modern era of antibiotic use (Kashuba, *et al*., 2017), it is the rapid spread of resistance genes that is of concern. The implementation of penicillin in modern medicine has saved millions of lives around the world (Gould and Bal, 2013). However, shortly after its introduction in modern medicine, resistance to penicillin was arising (Spellberg and Gilbert, 2014). To combat this development of resistance, new antimicrobials were discovered to treat infections and ever since there has been a constant battle between resistance development and the development of new antimicrobials (Ventola, 2015; Figure 1.1).

The constant misuse and overuse of antibiotics during the 20th century gave rise to 'superbugs' which have evolved to become multi-drug resistant (MDR; Davies and Davies, 2010). This means that a bacterium is resistant to at least one antimicrobial in at least three classes of antimicrobial (Magiorakos, *et al*., 2012). This causes issues for clinicians to treat infections due to the limited treatment options available. This is evident in a group of organisms labelled the ESKAPE pathogens (*Enterococcus, faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp*.) which without the development of new antimicrobials represent a serious threat to human health (De Oliveira, *et al*., 2020).

Figure 1.1. Introduction of antibiotics into clinical practice and subsequent development of resistance. (Hayden, *et al*., 2005; Ventola, 2015).



1.1.2. Lack of New Antibiotics

Whilst the development of new antimicrobials was an effective solution to combat resistance previously, it cannot be solely relied on. Since the discovery of penicillin and its use in clinic, there has been a golden age of antibiotic discovery which ended with the lipopeptides in 1987 (Debono, *et al*., 1987). Since then, there has been a well reported discovery void in antibiotic development. This can be largely attributed to the lack of funding into antibiotic discovery. In 2017, a review estimated the cost of developing an antibiotic at approximately $1.5 billion USD (Towse, *et al*., 2017). Although, the average revenue generated from the sale of an antibiotic is estimated to be only $46 million USD (Plackett, 2020). This has caused pharmaceutical companies to focus on other types of drug development that are more profitable. For example, in 2014, the total yearly sales of patented antibiotics was about $4.7 billion USD, which is roughly the same for one top-selling cancer drug (O'Neill, 2016). A recent review found that in 1 million peer-reviewed economics articles, only 55 articles were focused on antimicrobial resistance (AMR), whilst in comparison 16,000 articles were on climate change (Roope, *et al*., 2019). This is surprising, as the devasting issue of antimicrobial resistance has been repeatedly likened to climate change (Laxminarayan, *et al*., 2013; Gelband and Laxminarayan, 2015; Harring and Krockow, 2021). This would indicate the focus of funding, especially in governing bodies, is not the development of antimicrobials and economists possibly are not aware of the full repercussions of resistance development. Further to this, from January 2010 to May 2021, only 17 new antibiotics and one related biological agent have been approved by the Food and Drug Administration (FDA) (Chahine, *et al*., 2022). This pales in comparison to the 410 newly approved anti-cancer drugs approved by the FDA between 2011 and 2020 (Wang, *et al*., 2022). This demonstrates how the rate of discovery of new antimicrobials is not in line with the rate of resistance development, and until funding structures change, other methods to combat resistance needs to be taken.

1.1.3. Current Global Burden of Antibiotic Resistance

In 2016, a landmark review by O'Neill (2016) highlighted the true effects of AMR and the predicted consequences if not addressed. This review found that, at the time, approximately 700,000 people died annually from AMR and even conceded that this number is likely to be underestimated due to poor surveillance. O'Neill went on to estimate that if AMR was not addressed, by 2050, this could result in 10 million deaths per year or one person every three seconds. The considerable loss of life estimated by 2050 would have measurable effects of the global Gross Domestic Product (GDP). This could result in a

reduction of GDP of 2% to 3.5% per year, equating to a cost of up to $100 trillion USD (O'Neill, 2014). This estimate does not take into account the effects of AMR on prophylaxis, which could cause the global economy to reduce by 7% of its GDP by 2050, equating to a cost of $210 trillion USD.

A more recent review found that the deaths directly attributable to AMR in 2019 was 1.27 million (Antimicrobial Resistance Collaborators, 2022). It was also estimated that 4.95 million deaths were associated with bacterial AMR. Furthermore, only six pathogens (*E. coli, S. aureus, K. pneumoniae, S. pneumoniae, A. baumannii* and *P. aeruginosa*) accounted for 929,000 of the 1.27 million deaths as a direct result of AMR, and 3.57 million of the 4.95 million deaths associated with AMR.

These high levels of AMR cause significant issues for clinicians. The development of resistance to first-line empirically used antibiotics in severe infections, such as fluoroquinolones and beta-lactam antibiotics (WHO, 2017a), accounts for more than 70% of deaths attributable to AMR (Antimicrobial Resistance Collaborators, 2022). A particular concern for clinicians is the development of resistance in Gram-Negative bacteria. In 2017, the WHO, released a list of priority pathogens, many of which were Gram-Negative bacteria, which have very few treatment options and therefore are in dire need of new antimicrobials (WHO, 2017b). However, the emergence of resistance is inevitable when new antimicrobials are used (Gould and Bal, 2013). Therefore, it is important to focus on the many contributing factors that drive the development of antibiotic resistance.

1.2. Combatting Antibiotic Resistance

1.2.1. Drivers of Antibiotic Resistance

Antibiotic resistance is a multi-faceted problem and therefore needs to be addressed on many different fronts (Gil-Gil, *et al*., 2019). Whilst the discovery of new antibiotics is essential to give clinicians new armaments to fight infectious diseases (Dutescu and Hillier, 2021), this effort becomes futile if resistance develops to them. The main reasons driving antibiotic resistance are i) the misuse and overuse of antibiotics, ii) lack of access to clean water, iii) lack of proper sanitation and hygiene in both humans and animals, iv) poor infection control, v) poor access to vaccines and diagnostics, vi) lack of knowledge and awareness and vii) lack of enforcement of legislation (WHO, 2021).

Whilst it has been known for some time that resistance is driven by overuse and misuse of antibiotics, the global antibiotic consumption rate has still increased by 46% from 2000 to 2018 (Browne, *et al*., 2021). This increase is driven by the unnecessary antibiotic

consumption. For example, a study found that at least 28% of prescriptions for antibiotics in the US between 2010-2015 were unnecessary since no antibiotic was needed at all (Hersh, *et al*., 2021). This indicates that there still needs to be an improvement in antibiotic stewardship and education, to ensure antibiotics are used appropriately and prudently.

1.2.2. Antibiotic Stewardship and Education

Antibiotic stewardship was first coined in 1996 and considered as the careful and responsible management of antibiotics (McGowan Jr and Gerding, 1996). There are several factors involved in antibiotic stewardship, such as selection of treatments, dosing, and duration of treatment, to minimize the impact of treatment on resistance development (Shrestha, *et al*., 2023). The principles of antibiotic stewardship also expand to the use of antibiotics in agriculture and veterinary sciences, antibiotic management in the environment and limiting the spread of disease. In all of these fields, a key theme is education. The improvement of education in both the public and healthcare professionals can help improve antibiotic stewardship. This could limit the inappropriate use of antibiotics in key areas such as the use of antibiotics in upper respiratory tract infections (URTI) and sore throat (Sangwan, *et al*., 2023).

1.3. Treatment of Sore throats

1.3.1. What is Sore throat?

Sore throat is a common condition of an upper respiratory tract infection that affects the mucosa of the throat (Kenealy, 2014). A sore throat is problematic for patients as it can make it painful to swallow. A sore throat can also be highly uncomfortable by making the throat dry and itchy. Sore throat is predominantly caused by viral infections, making approximately 80% of cases (Ebell, *et al*., 2000; Bisno, 2001; Worrall, 2007; Pelucchi, *et al*., 2012). The main viral infections linked with a sore throat are either the common cold (including rhinoviruses, parainfluenza and coronaviruses) and the flu (caused by the influenza virus; CDC 2021). The other predominant infectious cause of sore throat is bacterial infection with Group A *Streptococcus* (GAS), also known as strep throat. This bacterial infection can be very painful and uncomfortable for patients, although still considered a mild disease (CDC, 2021). Other associated symptoms with strep throat can include fever, red and swollen tonsils, white patches on tonsils, petechiae and swollen lymph nodes. Whilst a viral sore throat has associated symptoms such as cough, runny nose, hoarse voice and sometimes conjunctivitis. Although these infections are often self-

limiting and do not require medication, they often cause patients to seek treatments or at least symptomatic relief (van der Velden, 2020).

1.3.2. How are sore throats managed?

Although sore throats are mainly self-limiting and resolve themselves without medication after only a few days from the onset of symptoms (Spinks, *et al.*, 2013), they are still one of the most common reasons that patients visit primary healthcare providers (Gunnarsson, *et al*., 2020). Although home remedies such as gargling with warm salt water can help with soothing a sore throat at home, patients demand treatments from pharmacies. These treatments are aimed at relieving the pain and discomfort of a sore throat and include, ibuprofen or paracetamol, medicated lozenges containing antiseptics, anti-inflammatories and anesthetics, or the use of anesthetic sprays (Coutinho, *et al*., 2021).

However, there are still patients that demand antibiotic treatment for sore throat (Gaarslev, *et al*., 2016). This is thought to be because of the complications that can arise if GAS is not treated appropriately and can develop into acute rheumatic fever. However, by assessing sore throat adequately by using clinical score indicators such as FeverPain, Centor or McIsaac scores, patients that are at risk of having a GAS infection can be selected. Combining this with point-of-care testing, which would only be about 10-15% of sore throat cases, antibiotics can be given to those who need it, which is estimated at 3.5%-6.6% (Gunnarsson, *et al*., 2022). Although this often is not the case, as currently approximately 10% of patients consult their general practitioner (GP) with sore throat annually (Mantzourani, *et al*., 2023), with approximately 60% of sore throat consultations receiving an antibiotic (Gulliford, *et al*., 2014). This highlights the improvements that could be made in sore throat management.

1.3.3. Antibiotic stewardship in sore throat management

Acute sore throat is the leading cause of antibiotic prescription (Cohen, *et al*., 2020). With the vast amounts of antibiotics that are given out for sore throat, a disease which is often viral and therefore does not require antibiotics, this shows an evident lack of antimicrobial stewardship. In fact, a study deemed 8.8% of all systemic antibiotics in primary care in England between 2013 and 2015 inappropriate and prescribing for sore throat (23%) was the factor that contributed the most to inappropriate antibiotic prescribing (Smieszek, *et al*., 2018). Whilst there is a large proportion of antibiotics prescribed for sore throat, there are also a number of sore throat medications that contain antibiotics and are still sold legally over-the-counter in Europe (Essack, *et al*., 2019; Table 1.1). However, the

consequences of over-the-counter (OTC) sale of antibiotics for sore throat treatment are not known, but it is assumed that this would deemed as poor antibiotic stewardship.

1.4. OTC antibiotics

1.4.1. OTC antibiotics approved for Sore Throat Treatment

There are currently five different antibiotics that have the WHO anatomical therapeutic chemical (ATC) classification R02AB (WHO, 2023). This includes neomycin (R02AB01); tyrothricin (R02AB02); fusafungine (R02AB03); bacitracin (R02AB04) and gramicidin (R02AB30). This ATC classification covers antibiotics to be used in throat preparations and mouth preparations as their intended therapeutic use. These preparations are to be used to treat common minor infections of the mouth and throat.

Even though the majority of antibiotics in Europe are restricted to prescription only, there are still a number of antibiotics that are being sold legally OTC (Table 1.1; Both, *et al.*, 2015). This is not in line with the current aims of making Europe a region of best practice (European Commission, 2017). Some countries have implemented their own policies restricting the use of OTC antibiotics, for instance, France withdrawing the OTC status of any nasal or oropharynx preparations containing bacitracin, fusafungine, gramicidin or tyrothricin, for lack of evidence of therapeutic benefit, having two years previously also withdrawing the OTC status of neomycin and framycetin and sulfasuccinamide for similar reasons (WHO, 2005). It was also commented that the removal of the OTC status of these antibiotics would also prevent the emergence of antibiotic resistant strains (Both, *et al.*, 2015). This was followed by the complete withdrawal of fusafungine from the European market due to both safety concerns and lack of evidence of therapeutic benefit (EMA, 2016). Again, whilst there is minimal evidence to link fusafungine to the development of antibiotic resistance, it could not be ruled out. More recently in 2020, tyrothricin-containing lozenges were removed from the UK market and are now prescription-only medicine (POM). This is because the sale of self-care antibiotic throat lozenges was deemed as inappropriate use of antibiotics and would also send mixed messages to both patients and healthcare providers about responsible use of antibiotics (ICMRA, 2022). Despite the fact that some countries in Europe are restricting the use of these antibiotics due to the lack of evidence of benefit, safety concerns and risk of contributing to resistance, there are still many countries that continue to sell OTC antibiotics in Europe (Table 1.1).

Table 1.1. OTC antibiotic containing medications sold within Europe. The divisions of Northern, Eastern, Southern and Western Europe were based on those used in Both. *et al*. (2015). Antibiotics: T – Tyrothricin, G – Gramicidin, B – Bacitracin and N – Neomycin.

Brand	Country Sold	OTC Antibiotic	Formulation	Application	Marketed
Northern Europe					
Trachisan	Latvia, Lithuania	T	Lozenge	Sore Throat	OTC
Bacimycin	Norway	B	Ointment	Skin Infections	OTC
Bafucin	Sweden, Finland	G	Lozenge	Sore Throat	OTC
Eastern Europe					
Dorithricin	Hungary	T	Lozenge	Sore Throat	OTC
Trachisan	Bulgaria, Romania	T	Lozenge	Sore Throat	OTC
Baneocin	Bulgaria, Romania	B, N	Ointment, Powder	Skin Infections	OTC
Tyrosur	Romania	T	Gel	Skin Infections	OTC
Southern Europe					
Pulvo-47	Greece	N	Spray	Skin Infections	OTC
Trachisan	Greece, Malta	T	Lozenge	Sore Throat	OTC
Hydrotricine	Portugal	T	Lozenge	Sore Throat	OTC
Mebocaina Forte	Portugal	T	Lozenge	Sore Throat	OTC
Blastoestimulina	Spain	N	Ointment	Skin Infections	OTC/Pharmacy
Phonal	Spain	N, B	Lozenge, Spray	Surface infection of the mouth and Throat	OTC/Pharmacy
Bucometasana	Spain	T	Lozenge	Sore Throat	OTC
Cohortan	Spain	T	Ointment	Haemorrhoids	OTC
Faringotricina	Italy	T	Lozenge	Bacterial Stomatitis	OTC/Pharmacy
Golamixin	Italy	T	Spray	Bacterial Stomatitis	OTC/Pharmacy
Western Europe					
Melisana Citroen/Munt	Belgium	T	Lozenge	Sore Throat	Pharmacy
Lemocin	Belgium, Germany	T	Lozenge	Mouth and Throat Infections	OTC/Pharmacy
Dorithricin	Austria, Germany	T	Lozenge	Sore Throat	OTC/Pharmacy
Tyrosur	Germany	T	Gel	Skin Infections/Acne	OTC/Pharmacy
Micasal	Germany	T	Gel	Skin Infections/Acne	OTC/Pharmacy

1.4.2. OTC antibiotics sold in Europe

1.4.2.1. Tyrothricin

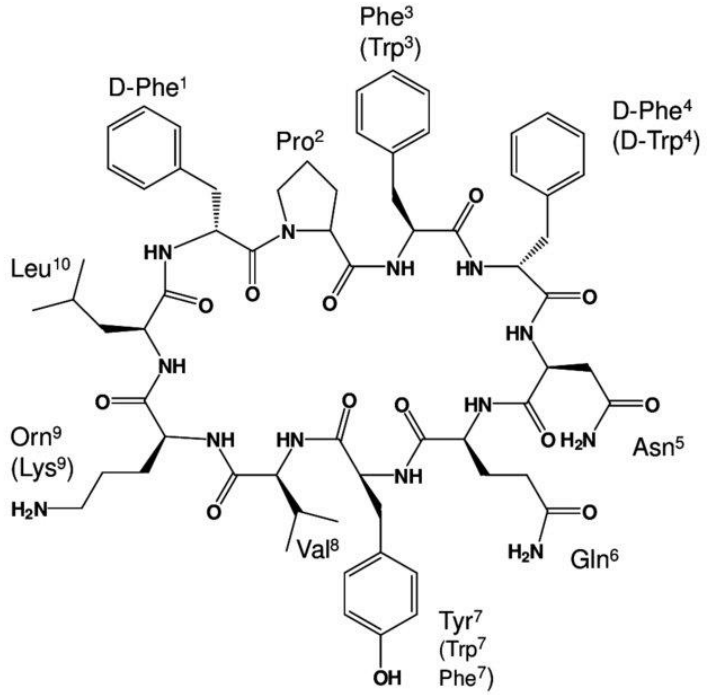
Tyrothricin is the most commonly used antibiotic in sore throat lozenges and belongs to a class of antibiotic called the antimicrobial peptides (AMPs; Table 1.1.). It is a mixture of polypeptides isolated from the soil organism *Bacillus brevis*. This mixture consists of approximately 50% - 70% tyrocidines and 25% - 50% gramicidins (Lang and Staiger, 2016). The tyrocidines are basic cyclic peptides whilst the gramicidins are neutral linear peptides (Fig. 1.2). Both groups are mixtures themselves of structurally similar peptides with minor changes to amino acid positions (Lang and Staiger, 2016). The tyrocidine component of tyrothricin has an amphiphilic nature allowing it to insert easily into bacterial membranes. The hydrophilic parts of the peptide interact with the phosphate groups of the bacterial membrane whilst the hydrophobic parts form non-selective pores within the membrane. Bacteria then leak essential intracellular components such as cations, nucleotides, amino acids and phosphates, causing a bactericidal effect (Marques, *et al.*, 2007; Pálffy, *et al.,* 2009). Tyrothricin is highly toxic to human cells due to the disruption of cell membranes and has been limited to topical treatment by oral lozenges, nasal sprays, ophthalmic solutions and skin creams.

1.4.2.2. Gramicidin

Gramicidin is a polypeptide antibiotic that was discovered in the soil dwelling organism *Brevibacillus brevis* (Dubos, 1939). Gramicidin is also an AMP that is neutral and linear and can be classed as an ionophoric antibiotic (Busath and Szabo, 1981; Fig. 1.2). Gramicidin is actually called gramicidin D which is again a mixture of structurally similar polypeptides. This is not to be confused with the cyclic gramicidin S. It has been reported to be effective at controlling various Gram-positive organisms as well as a few Gram-negative organisms (Liou, *et al.,* 2015). Gramicidin, like tyrothricin also creates pores in the bacterial cell membrane. However, these pores are smaller than the ones formed by tyrothricin and only allow the passage of monovalent cations such as potassium ions (Wallace, 1998; Wallace 2000). This causes the cell membrane to leak these vital cations and can stop biological processes within the bacteria, eventually leading to cell death. Because of the mechanism of action of gramicidin, it is highly toxic to human cells and has been limited to topical use with oral lozenges, nasal sprays, ophthalmic solutions and skin creams.

Figure 1.2. The components of tyrothricin. A) The structure of tyrocidine – a basic cyclic peptide (Rautenbach, *et al*., 2016). B) The structure of gramicidin – a neutral linear peptide. (Meikle, *et al*., 2016).

A



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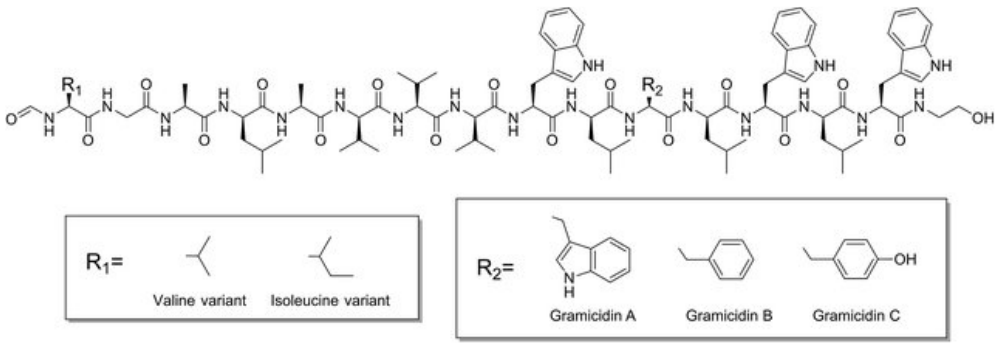


1.4.2.3. Bacitracin

Bacitracin was first isolated from *Bacillus subtilis* and is a polypeptide antibiotic that inhibits peptidoglycan synthesis. Bacitracin works by forming a complex with divalent metal ions and then binding with C55-isoprenyl pyrophosphate. This inhibits the transport of components of the bacterial cell wall through the cytoplasmic membrane, therefore stopping cell wall formation (Stone and Strominger, 1971). It is highly active against Gram-positive organisms however, activity against Gram-negative organisms is very poor (Nguyen, *et al.,* 2020). It is used topically as a skin spray or ointment, nasal spray or an oral lozenge and is poorly absorbed systematically. Although, it has been given intramuscularly in extreme cases of *Staphylococcus pneumoniae*, this is not advised due to the high chance of renal failure (Nguyen, *et al.,* 2020).

1.4.2.4. Neomycin

Neomycin is an aminoglycoside antibiotic that works by inhibition of the 30S ribosomal subunit and is often used to treat superficial infections. It has been restricted to topical and oral use due its ototoxicity and nephrotoxicity (O'Donnell, *et al*., 2015). Deafness may develop when using neomycin topically and can be irreversible after the first signs of ototoxicity even if the treatment is stopped. Neomycin is effective against both Gram-positive and Gram-negative bacteria and is often used in combination with bacitracin, polymyxin or chlorhexidine (MacDonald and Beck, 1983). Whilst its main use is for superficial skin infections either in a spray powder or topical cream, it is also used in a sore throat lozenge marketed as OTC in Spain.

1.5. Limitations of current knowledge

Whilst OTC antibiotics have been used for over 70 years, there are comparatively very few papers concerning their use in sore throat (Essack, *et al*., 2019). To firstly establish whether the use of OTC antibiotics poses a risk of resistance development, it needs to be determined how OTC antibiotics are supplied to patients and whether they are being used appropriately. Whilst there is a vast amount of information in the supply of antibiotics through prescription, information about the sale of OTC antibiotics in the community is sparse.

Previous research has claimed that OTC antibiotics, particularly tyrothricin, does not pose a risk of resistance (Stauss-Grabo, *et al*., 2014; Lang and Staiger, 2016). However more recently, the use of OTC antibiotics *in vitro* showed their potential to drive the development of bacterial resistance (Wesgate, *et al*., 2020). With the differing opinions on

the risk of resistance development from OTC antibiotics usage, further clarification is needed.

The direct effects OTC antibiotics may have on clinical antibiotics has not been explored. Whilst there are drug interactions reported with some OTC antibiotics (gramicidin, bacitracin and tyrothricin; Wishart, *et al*., 2017), none of these interactions concern other antibiotic treatments and it is therefore not known how consumption of OTC antibiotics could affect clinical treatments. For the purpose of this study, clinical treatments or clinical antibiotics are defined as antibiotics that have been prescribed to a patient.

To summarize, the current knowledge gaps are:

- Management of sore throats and supply of OTC antibiotics to patients in community pharmacies
- The effect of OTC antibiotics on bacterial resistance profiles
- Interaction of OTC antibiotics with clinical antibiotic treatments

1.6. Study Aims and Objectives

This study aims to investigate the effects of OTC antibiotic exposure on the development of antibiotic resistance and whether the continued use of OTC antibiotics could contribute to the rise of antibiotic resistance. This will be achieved firstly by understanding which organisms are most at risk of resistance development due to the use of OTC antibiotics. Then, through pre-exposure of bacteria to OTC antibiotics, assessing the changes in resistance profile to clinically relevant antibiotics. Subsequently, by elucidating the phenotypic and genotypic changes in bacteria after pre-exposure to OTC antibiotics, the mechanisms behind any changes in resistance profiles can be understood.

Further to this, the impact of OTC antibiotic use on clinical antibiotic treatment will be assessed by co-exposure. Part of the study is to understand how these antibiotics are being supplied to patients and whether sore throat is being managed appropriately within a community setting by the development of a survey. By evaluating and considering the different aspects of OTC antibiotic usage, the current usage of OTC antibiotics will be considered.

1.7. Thesis Structure

This thesis is divided into seven chapters (Figure 1.3). They are briefly described as follows:

This chapter (Chapter 1: Introduction and Project Aims) gives a brief background and context to the work described throughout the thesis. It also describes the overall aims and objectives of this thesis.

Chapter 2: Survey of Pharmacists on Treatment of sore throat; describes the processes to design and pilot a survey to further understand the management of sore throat and supply of OTC antibiotics in four countries in Europe.

Chapter 3: General Methods; outlines the general methodologies implemented in the laboratory work done throughout the whole thesis.

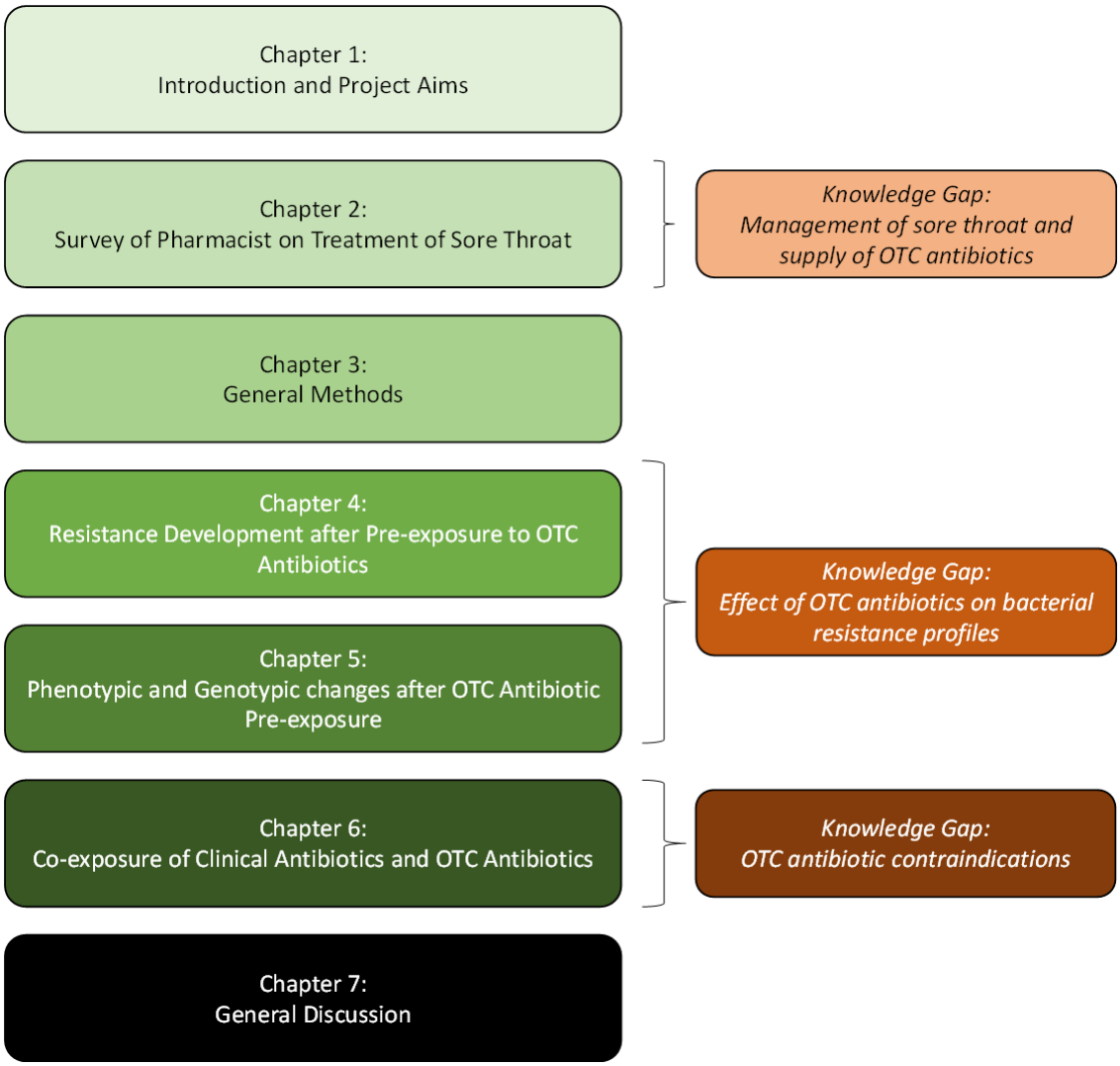
Chapter 4: Resistance development after pre-exposure to OTC antibiotics; uses the OTC antibiotics sold within Europe and discovers which organisms survive the during-use concentrations. It then goes on to explore how the resistance profile of the bacteria changes after pre-exposure to OTC antibiotics, and whether the resistance gained is stable.

Chapter 5: Phenotypic and Genotypic changes after OTC antibiotic pre-exposure; gives more information on the associated phenotypic and genotypic changes after OTC antibiotic pre-exposure in organisms that showed the development of clinical resistance in Chapter 4.

Chapter 6: Co-exposure of clinical antibiotics and OTC antibiotics; demonstrates the effects of co-exposing a bactericidal concentration of a clinical antibiotic with OTC antibiotics and the resulting ‘protection’ in bacteria. This chapter also delves to understand the mechanism behind the ‘protection’ given.

Chapter 7: General Discussion; integrate the key findings from the thesis and supplies recommendations for the future.

Figure 1.3. Overall thesis structure. The different empirical chapters cover the current limitations of knowledge outline in section 1.5.



Chapter 2: Survey for Community-Pharmacists on over-the-counter antibiotics

Chapter 2: Survey for Community-Pharmacists on OTC antibiotics

2.1. Introduction

2.1.1. Role of Community Pharmacists in Sore throat management

Sore throat is one of the main reasons patients visit primary care (Krüger, *et al*., 2021). As community pharmacists make up a key aspect of primary care, community pharmacists have a major role in the treatment and management of sore throat. Sore throat can represent a large proportion of a community pharmacists' role as approximately 10% of people present to primary care with a sore throat each year (Kenealy, 2014). There are multiple medications that are often given to help a patient manage sore throat (Table 2.1).

There are multiple tools that pharmacists can use to assist a patient on what treatments may best suit them such as the clinical scoring tools such as, FeverPAIN or Centor, which are often included in the national guidelines in the management of sore throat (Coutinho, *et al*., 2021). Along with the use of rapid antigen detection tests, these tools allow clinicians to choose the most appropriate treatments for a patient's sore throat (Llor and Bjerrum, 2014). Yet despite this information, patients still demand antibiotics despite their frequent misuse in sore throat treatments (Gaarslev, *et al*., 2016). The misuse of antibiotics has been well documented for their contribution to AMR (O'Neil, 2016). Whilst the prescribing of antibiotics in some primary care settings such as GP surgeries has been thoroughly researched (Mölter, *et al*., 2018), the use of OTC antibiotics in sore throat treatment in other primary care settings has not (Essack, *et al*., 2019). The dispensing of OTC antibiotic products to patients needs further investigation to understand their potential negative impact on AMR.

Table 2.1. The different agents commonly used to treat sore throat.

Agent Class	Mode of Action	Example used in sore throat	References
Anaesthetic	Numbing the local area	Benzocaine, Lidocaine, Benzydamine Hydrochloride	Passali, *et al*., 2022; Churbasik, *et al*., 2012; NHS, 2022
Analgesic	Pain Relief	Paracetamol, Ibuprofen, Flurbiprofen, Aspirin	Bouroubi, *et al*., 2017
Antibiotic	Fight bacterial Infections	Penicillin V, Amoxicillin, Gramicidin, Tyrothricin, Bacitracin, Neomycin, Erythromycin	Both, *et al*., 2015
Antiviral	Fight Viral Infections	Oseltamivir, zanamivir, peramivir, aciclovir	Liu, 2023
Antiseptic	Fight against microorganisms	2,4-dichlorobenzyl alcohol, amylmetacresol, Hexylresorcinol	Buchholz, *et al*., 2009
Herbal Remedy/ Home Remedy	Natural methods of sore throat relief	Honey, Saltwater gargling, Lemon, Liquorice	Story, *et al*., 2023

2.1.2. The use of OTC Antibiotic Sore Throat Treatments in Community Pharmacies in Europe

A review by Machowska and Lundborg (2019) highlighted that access to antibiotics without prescription, particularly the legal sale of OTC medicines containing antibiotics, added to the over-use and misuse of antibiotics in Europe and is an area where antibiotic stewardship can be improved. The treatment of sore throat in particular often results in the misuse of antibiotics. This is because approximately 80% of sore throat infections are viral and self-limiting, therefore not requiring antibiotics to treat (Essack, *et al*., 2019). Although some patients may consult GPs about a sore throat, patients frequently seek help from primary care (Mantzourani, *et al*., 2020). This results in community pharmacists having an important role in both the management of sore throats and also the sale of OTC sore throat antibiotics. The antibiotics currently approved for the use in OTC medicines for the treatment of sore throat in Europe are neomycin, bacitracin, tyrothricin and gramicidin (EMA, 2016; WHO, 2023). Despite these antibiotics having been used pharmaceutically for decades, there is very little information available regarding the development of resistance to them when compared to systemic clinically used antibiotics (Both *et al*., 2015). Although some studies show limited evidence of resistance development to these antibiotics (Stauss-Grabo *et al*., 2014), more recent research has suggested that the use of these antibiotics can cause cross-resistance to other clinically relevant antibiotics (Wesgate *et al*., 2020). The use of these OTC antibiotics also causes concerns regarding their safety and therapeutic benefit. Some countries have already withdrawn these antibiotics from their individual markets; for example, France in 2005 banned the use of tyrothricin, bacitracin and gramicidin, for lack of therapeutic benefit (WHO, 2005). A review by Essack, *et al*. (2019) concluded that there was a lack of evidence regarding the use of these antibiotics and that it was not possible to conduct a risk-benefit analysis of the OTC sale of these antibiotics. As with all treatments, and especially the sale of OTC medicines, a risk-benefit analysis has to be done using frameworks such as the ones described by Brass, *et al*. (2011). A key aspect of this framework is whether the OTC medicine is being misused. To further understand how these medicines are being used, it is vital to firstly understand how sore throat is managed and how these products are supplied to the patients.

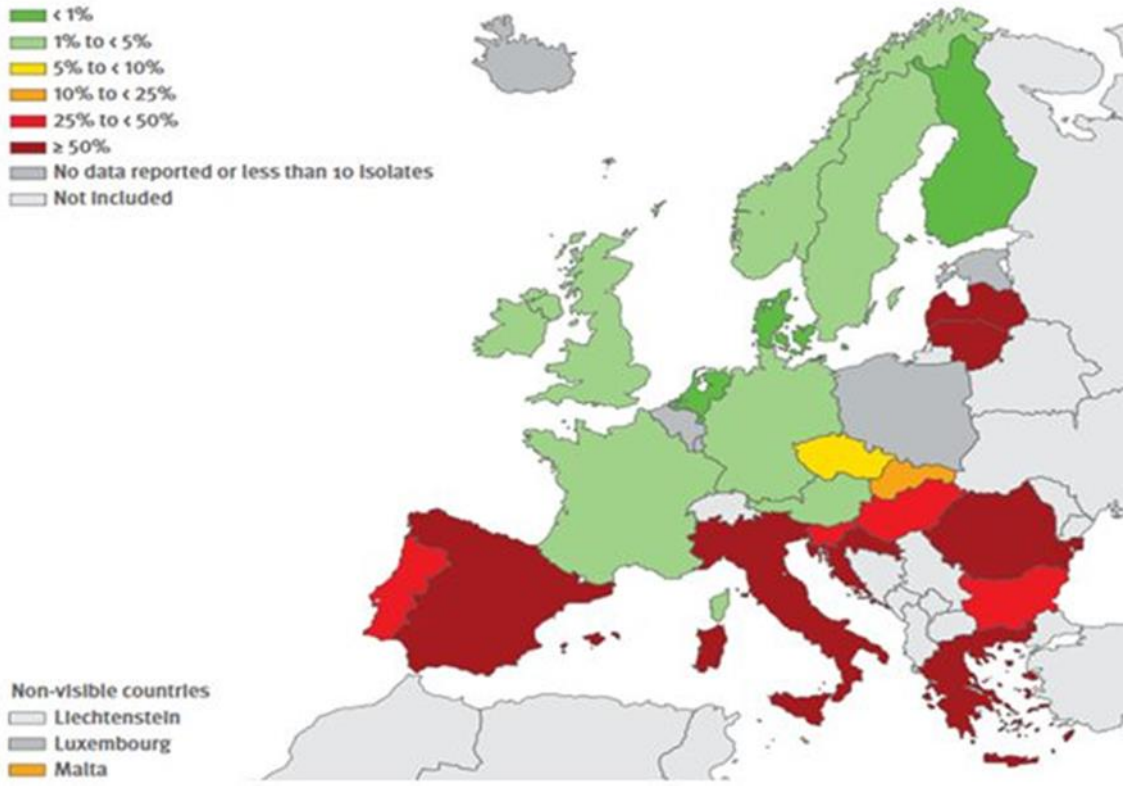
Within Europe, countries have vastly differing antibiotic consumption for systemic use (ECDC, 2021). Whilst systemic antibiotic use is carefully monitored, the usage of OTC antibiotics is largely undocumented throughout Europe. It is also unknown the reasons why some countries such as Austria, Finland, Germany and Sweden may have high OTC

antibiotic sales (Reckitt, Unpublished, 2019) despite having low systemic use of antibiotics (ECDC, 2021). All of these countries have relatively low rates of clinical infections with antibiotic resistant bacteria compared to the rest of Europe, but prudent use of antibiotics is still needed to keep these rates low. These countries have a high usage of OTC antibiotics which have been demonstrated *in vitro* to generate the emergence of antibiotic resistant bacteria (Wesgate, *et al*. 2020). It is not known the role that these OTC antibiotics may play on the rise of antibiotic resistance in these countries. The sale of OTC antibiotics is highly influenced by community pharmacists due to patients likely to seek advice from them to treat their sore throat (Both, *et al*. 2015). It is therefore important to understand the reasons for the high OTC antibiotic sales within these countries and how pharmacists within these countries can be supported to improve the stewardship of these medicines.

2.1.3. Clinical Impact of Antibiotic Resistance in Europe

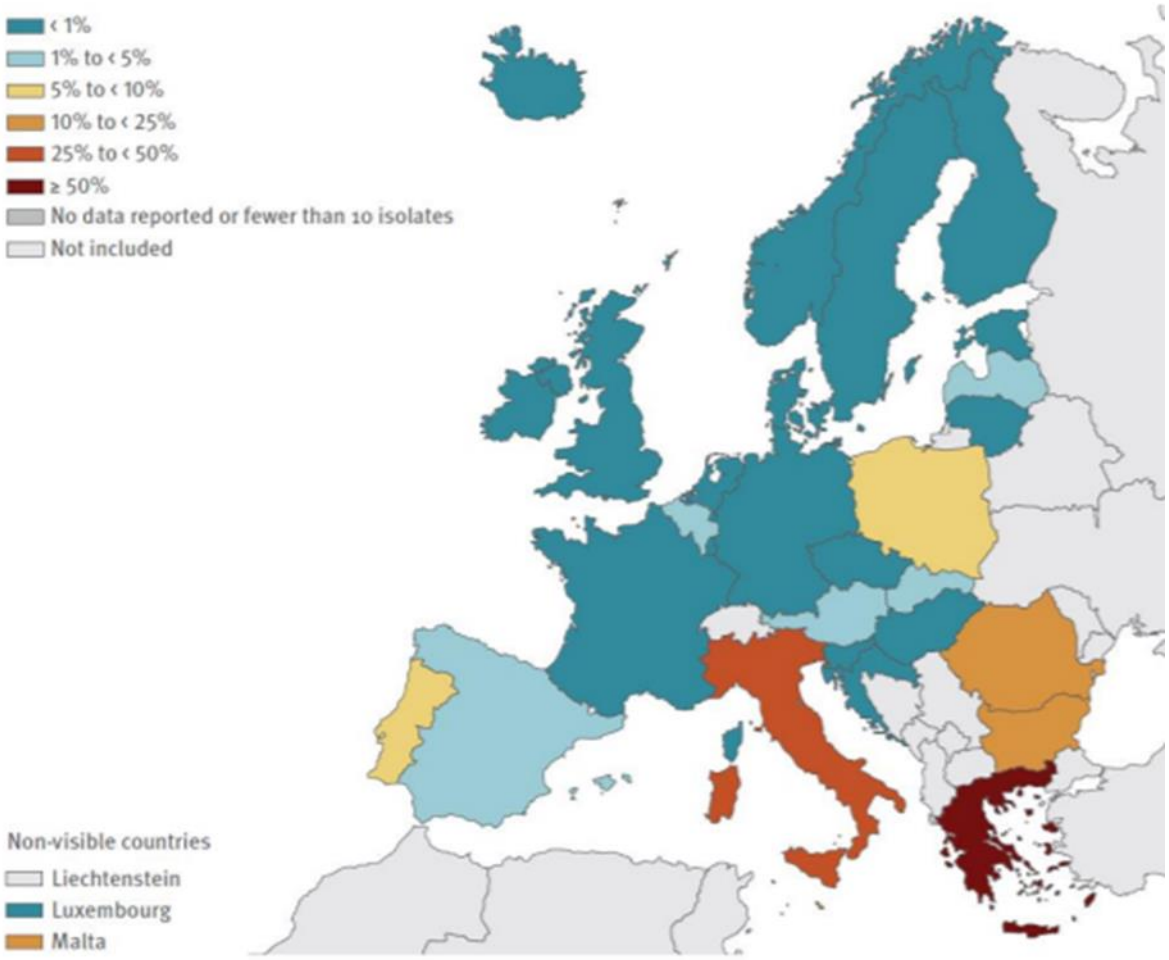
The European Antimicrobial Resistance Surveillance System (EARSS) network has seen substantial differences between Northern and Southern Europe (ECDPC, 2018; Figure 2.1 & 2.2). This is concerning as countries within these regions, such as Greece and Spain, have also demonstrated an increasing trend of antibiotic consumption which can lead to higher levels in resistance within these countries (Machowska and Lundborg, 2019).

Figure 2.1. Percentage of invasive *Acinetobacter spp*. with combined resistance to fluoroquinolones, aminoglycosides and carbapenems in 2014 (Ricciardi, 2016).



Previous reports from the EARSS network have shown significant increases of *K. pneumoniae* isolates with combined resistance of fluoroquinolones, 3rd generation cephalosporins and aminoglycosides. Other pathogens of concern include *E. coli* isolates with significant increases in resistance to 3rd generation cephalosporin resistance. This is especially worrying regarding the levels of resistance to last line antibiotics such as carbapenems or colistin (WHO, 2015). Although in Europe resistance to carbapenems remain relatively low, there has been an increasing trend of *K. pneumoniae* isolates that are carbapenem-resistant. In 2017, approximately 7.2% of *K. pneumoniae* isolates were resistant to carbapenems but this varied between 0% in the countries in Northern Europe such as Norway, to 64.7% in countries within Southern Europe such as Greece (Machowska and Lundborg, 2019; Fig. 2.2).

Figure 2.2. Percentage of invasive *Klebsiella pneumoniae* with resistance to carbapenems in 2017 (ECDC, 2018).



2.1.4. Chapter Aims

The main aim of this chapter is to understand how sore throat is managed in community pharmacies in four countries in Europe (Austria, Finland, Germany and Sweden) that are characterized by i) high sale of OTC antibiotics, ii) low usage of systemic antibiotics and iii) low rates of infections by antibiotic resistant bacteria.

The objectives of this chapter are:

- To describe how pharmacists manage patients with uncomplicated sore throat in community pharmacies and which products they supply to these patients.
- To describe pharmacists' knowledge of the mechanisms of action of OTC sore throat medications and where they get their information from.
- To describe the decision-making process for a community pharmacist to supply a patient with different types of products for sore throat.
- To describe what information community pharmacists give to patients on supplying different types of products for sore throat.
- To explore what further support would benefit pharmacists in managing patients with sore throat.

2.2. Methodology in Social Science

2.2.1. Research Design

Like any research, it is important to think about the design of the study to gain meaningful data. This is especially important in social science research as there are many different research strategies a researcher can take to complete a project. For this study, the Saunder's 'Research Onion' Model was followed (Saunders, *et al.*, 2007). This model was designed to assist those new to the field of social sciences to choose an appropriate methodology. Below detailed the different series of stages the researchers must consider.

2.2.1.1. Research Paradigm

Before designing a social study, a researcher must consider their philosophical standpoint or their research paradigm. This is because the different philosophical standpoints and approaches used in research results in different practices (Johnson and Onwuegbuzie, 2004). There are different factors that are the basis of each paradigm: ontology (the nature of reality), epistemology (how is knowledge gained), methodology (the research methods used) and methods (the tools used in social science) (Fazlioğullari, 2012). Whilst there are many branches and subsections to choose from, the four mainly considered were positivism, constructivism, critical realism and pragmatism.

A positivism philosophy is seen as the 'traditional' science standpoint (Park, *et al*., 2020). This paradigm in ontological terms says there is only one true reality. Epistemologically, positivism also says this reality is observable and measurable in and the researcher maintains an objective standpoint. Typically, this research is deductive, includes large samples and is quantitative.

In contrast to positivism is constructivism. This paradigm is of the belief that reality is complex, subjective and can be interpreted in many ways (Shannon-Baker, 2023). The gain of knowledge is constantly changing and focuses on the narratives of individuals. This paradigm often implements inductive reasoning, has small samples and uses qualitative analysis.

The critical realism paradigm can be summarized as, seeing the world as a result of the underlying structure of reality, *i.e.* what we observe may have an alternate truth (Zachariadis, 2013). As a result of this view, critical realists look for explanations behind observations. These are commonly in the form of historical analyses and studies that take place over a period of time.

Pragmatism is mainly focused on starting with a practical problem and aims to find solutions to better future practice (Baert, 2004). A pragmatist will use any of the research methods to understand the problem. A practical solution is a higher priority than which specific research methods are used. A pragmatist therefore will typically be associated with a mixed methods approach. This allows the quantitative and qualitative research to compensate for their individual weaknesses.

For this study, the pragmatism research philosophy was implemented, as the project pertains to antibiotic resistance and the supply of OTC products, in which the reality can change and needs a practical solution.

2.2.1.2. Research Approaches to theory development

The two main research approaches are either deductive or inductive. In simple terms, a deductive approach starts with a theory and measures observations to confirm or deny that theory. Whilst inductive reasoning uses the measurements of observations to develop a theory (Coccia, 2018). For this study, I have theories on the knowledge and supply of OTC antibiotics. However, this still needs to be confirmed through measurement and therefore this study will predominantly use deductive reasoning.

2.2.1.3. Methodological Choices

The methodological choice refers to whether the study will use mono or multi methods and whether it will be qualitative or quantitative (Mehrad, *et al*., 2019). As we will predominantly use a deductive research approach, a quantitative methodological choice would be best suited. Usually, the pragmatic paradigm uses mixed methods however, it was decided that a mono method would be enough to answer the research questions. Therefore, a quantitative mono method was used for this study.

2.2.1.4. Strategies within Social Science Research

There are many strategies/methodologies used in social sciences, these include: secondary research, surveys, focus groups, interviews, experimental research or observational research (SAGE, 2020). As the research approach is mono method quantitative, the strategies that would suit this approach is secondary research, surveys or experimental research. For this project, a survey was the best choice as it can gather data from a large population in a relatively short period of time. It was also decided that under a pragmatism philosophy, free-text questions could be added into a survey to give some qualitative insight into the quantitative data.

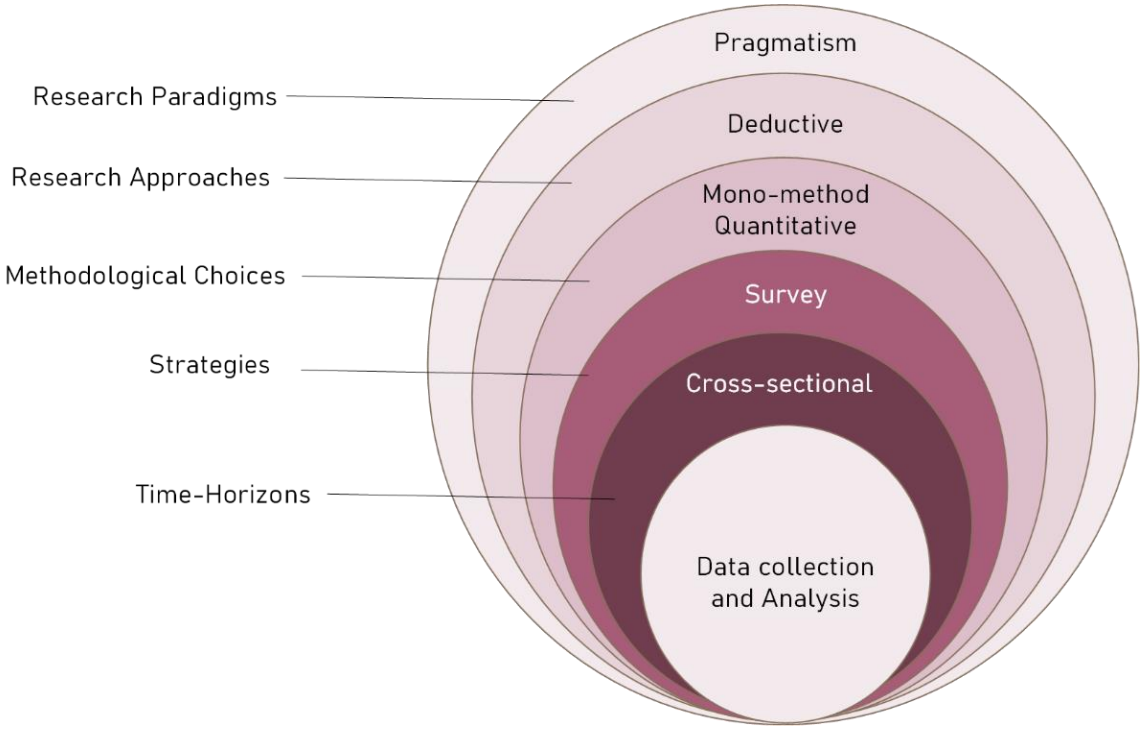
2.2.1.5. Time-Horizons

The time horizons of a study is the time frame in which the study takes place. The two main time horizons used are either a longitudinal study or a cross-sectional study (Rindfleisch, *et al.*, 2008). A longitudinal study repeatedly collects data over time and is used in research where examining the change in a population over time is important. However, a cross-sectional assesses a population at a specific time frame. This can be used to assess the differences between populations at a given time. This project will use a cross-sectional time horizon as it is looking at different populations of pharmacists in different countries.

2.2.2. Research Design Overview

The overall methodology used in the study is a cross-sectional survey. This is a mono-method quantitative study that uses a deductive research approach with a pragmatic philosophy (Figure 2.3). The development of the research study design allows the researcher to successfully and appropriately answer their research questions.

Figure 2.3. The study's 'Research Onion'. This outlines and methodology implemented in the social science study.



2.3. Survey Development

2.3.1. Construct Development

As part of the development of a survey, it is important to develop the research constructs of a survey (Agarwal, 2012). These are broad concepts that are used to define the topics addressed in the survey itself. Constructs can be developed numerous way such as through pilot interviews with the population. However, the constructs for this study were developed by the research team, including a researcher with experience in managing upper respiratory tract infection (URTI) in community pharmacies. This was mainly done by reviewing the literature within this field and through conversations with key stakeholders in the field. The stakeholders have years of experience working with community pharmacies in Europe and have a valuable insight into the OTC antibiotic market.

In total, five constructs were used in the survey:

1. Demographic data
2. How patients who come to the pharmacy are cared for and what advice do they ask for their sore throat
3. OTC products used for sore throat management
4. Common decision-making processes used when supplying a product for sore throat management
5. Further Support

These constructs represent each section of the survey (Appendix 2.1). They aim to cover the necessary themes and gather data to answer the aims and objectives.

2.3.2. Survey Sampling

The target survey population is community pharmacists working in either Austria, Finland, Germany and Sweden. Community pharmacists were specifically selected as the research relates to the supply of OTC products and therefore community pharmacists are much more likely to have conversations and interactions with patients about these products than hospital pharmacists. These countries were also chosen as they have low antibiotic consumption for systemic use (ECDC, 2021) however, from OTC antibiotics sales data (Reckitt, unpublished), these countries have high OTC antibiotic consumption.

The inclusion and exclusion criteria of the survey sample was set (Table 2.2). The sample size was calculated from how many community pharmacists are within each country. A 95% confidence interval was used with a 5% margin of error (Table 2.3). The following equation was used to calculate sample size:

$$Sample\ Size = \frac{\frac{z^2 \times p(1-p)}{e^2}}{1 + (\frac{z^2 \times p(1-p)}{e^2 N})}$$

z = z-score (1.96)

p = standard deviation

e = margin of error (percentage in decimal form)

N = population size

Table 2.2. Inclusion and Exclusion Criteria of the survey sample

Criteria Categories	Inclusion Criteria	Exclusion Criteria
Type of Pharmacist	Working as a community pharmacist	Working as a hospital pharmacist
Area of Work	Works within either Austria, Finland, Germany or Sweden	Works in countries outside of the four countries of interest
English Language ability	Ability to understand and respond in English	Pharmacists who don't understand and cannot respond in English

The main sampling strategy used was purposive sampling. This is through emailing suitable candidates for the survey through the pharmacy chains and through the Reckitt sales network in the target countries. Convenience sampling was also used to capture any additional and relevant candidates that may not be contactable by email. This is through advertisement at conferences and social media.

Table 2.3. The population of community pharmacists within each country and the ideal sample size.

Country	Approximate population of Community Pharmacists	Ideal sample size (95% Confidence Interval and 5% margin of error)
Austria	1,340	299
Finland	7,500	366
Germany	52,000	382
Sweden	5,300	359

2.3.3. Survey Dissemination

When designing the survey, it is also important to consider how the survey will be distributed. Surveys are generally either distributed through paper surveys or electronically. For this research project, it was decided that the survey would be distributed electronically as it is less expensive, less time consuming and can be widely distributed (Regmi, *et al*., 2016).

To assist with the distribution of an electronic survey, a link/QR code was made to direct participants to the participant information sheet and the survey. The distribution of the survey through an online link/QR code enables the collection of responses from a wide range of participants (Appendix 2.4). Three distribution methods for the survey were selected to ensure maximum participation:

1) Social media – The platform X (formerly Twitter) to be used to direct potential participants to the survey by clicking a link as part of the convenience sampling.

2) Conferences and sales visits – the survey to be distributed at conferences and meetings that are attended by Reckitt or the research team to direct participants to the survey by QR code as part of the convenience sampling.

3) Recruitment Emails – Recruitment emails also to be used for survey distribution (Appendix 2.3). This is through the sales networks that the funder of the research (Reckitt) has in each of the countries. For further distribution, pharmacy boards within each country and pharmacy chains to be contacted to see if they are willing to distribute the survey to maximize participation by community pharmacists.

2.3.4. Question Design

An important aspect of the survey development is the question design. This involved making sure the English in the questions were not complicated as English would presumably be a second language of most of the participants. Therefore, it is essential to make the English in the questions simple to make participants understand the questions and their responses. To get meaningful quantitative data that can be analyzed properly, closed-ended questions were mainly used. This is either in the form of directed multiple choice answers or by using tools such as Likert scales. The use of closed-ended questions is also generally easier to understand and quicker to answer. It is important that the survey does not take too long to complete as participants are more likely to complete shorter and quicker surveys (Kost and da Rosa, 2018).

2.3.5. Validity and Reliability

Validity and reliability are important considerations in survey research (Tourangeau, 2021). The measurement of validity is whether the measure of a concept is actually measuring that particular concept (Drost, 2011). There are many different ways validities can be assessed, but often face validity is firstly established (Nickerson, 2023). Face validity is to what extent the test is, on the surface, measuring what it is actually suppose to measure. For my survey, this was done by engaging with expert members of the research team and industry experts with experience of surveys that work in the target countries. For the reliability of a survey Cronbach's alpha is often used (Tavakol and Dennick, 2011). This is a test of the internal consistency or reliability within a survey. The statistic gained from Cronbach's alpha can indicate whether the responses to questions for each participant are consistent and therefore reliable. However, this was not used within the survey as the length of the survey was too short and did not allow for a proper assessment.

2.3.6. Survey pre-testing and refining

The survey was sent to three community-pharmacies within each of the four countries that work with Reckitt for the survey pre-testing. Only community pharmacists from Finland (n=2), Germany (n=1) and Sweden (n=3) responded. Respondents were asked to i) comment on the time it took to complete the survey, ii) if the English phrases used were applicable and understood in their respective country, iii) if they would change any questions or response options. The feedback recommended some changes to the wording of questions and the addition of a few options to multiple choice questions of products they would commonly use in their respective countries. The recommended wording and options from the feedback were updated in the finalized survey (Appendix 2.1). Interestingly, a respondent from Sweden said that none of the products contain an antibiotic despite saying they sell an OTC lozenge containing the antibiotic gramicidin (Appendix 2.5). Also, a respondent from Finland was aware of a product containing gramicidin but described it as a “minor antibiotic” (Appendix 2.5).

2.3.7. Study Approvals and Ethical considerations

2.3.7.1. Consent Forms and Participation

Before taking part in a survey, it is important for participants to be aware of the purpose of the study, who to contact if they have any questions, how the data will be used and managed. This done in the form of a participant information sheet (Appendix 2.2). This form appears at the start of the survey. This is followed by consent, either through using a consent form or implied consent through completion of the survey. A consent form was not used in this case as a participant could not move forward to the survey until they had read and agreed to the terms on the participant information sheet. Therefore, completion of the survey implied consent and this is made clear to the participants.

2.3.7.2. Gatekeepers

For this survey, gatekeepers are being used. Gatekeepers have a key role in the facilitation of survey distribution to potential participants. The gatekeepers used within this survey are part of the purposive sampling strategy. This initially is the Reckitt sales and regulatory networks that work with pharmacies in the target countries. The gatekeepers also have close working relationships with the pharmacy chains within each of these countries and could recruit other gatekeepers that would have access to the pharmacy chains emailing list. Alongside this, the pharmacy boards were contacted in each country to ask whether they could also be gatekeepers and distribute the survey further.

2.3.7.3. Ethical Approval

Ethical approval is needed for any research that involves human participants. For this study, ethical approval was not required in each of the target countries as the ethical approval from Cardiff University would suffice. Approval was submitted to the Cardiff University School of Pharmacy and Pharmaceutical Sciences Ethics Committee. However, approval of the study was not granted. One of the main reasons this was not granted was due to the distribution of the survey through the gatekeepers emailing list means that it would be considered as third party information. Therefore, the emailing list would have to contain only participants that have given consent to receive information and materials from third parties. Sadly, this could not be resolved in time for inclusion in the PhD thesis. However, the next sections (Section 2.4) outline the next steps that should be taken to resolve this issue.

2.4. Data collection and Analysis

2.4.1. Expanding a Pilot Survey and Questionnaire Finalization

After obtaining ethical approval, a larger pilot survey is to be run with at least five community-pharmacists from each of the target countries. The sampling used will be purposive sampling of pharmacists met during Reckitt sales visits to the pharmacies in each of those countries. This will allow to assess the distribution channels of the survey, ensuring the survey structure covers the necessary points and that the questions are suitable and clearly understood. The final feedback from the large pilot study allows the questionnaire to be finalized for the distribution of the main survey.

2.4.2. Main Survey: Data Collection and Management

2.4.2.1. Recruitment Strategy and Schedule

After the survey is finalized, a recruitment strategy is needed to get the required number of responses (Table 2.3). The survey will be active for a total of 12 weeks with the initial recruitment email containing the participant information sheet and survey link (Appendix 2.1 and 2.2). Reminder emails are then sent at the start of weeks 4, 8 and 12 to maximize responses whilst not being overbearing to the potential participants. Whilst the survey is open, the survey is to be advertised at relevant conferences and meetings attended by either the research team or the Reckitt sales network using the developed infographic that can direct potential participants to the survey (Appendix 2.4). The survey is also to be shared on the social media platform, X (formerly Twitter), by Reckitt, the pharmacy boards and chains will also be asked whether they are willing to repost the survey link.

2.4.2.2. Data collection and Maximizing Participation

To gather as many responses as possible, it is important to maximize participation. There are a few different ways that potential participation can be maximized. A key aspect is the length of the survey (Sharma, 2022). It was found that if surveys are too lengthy, participants are less likely to complete it. Therefore, to maximize the participation, the survey is kept to a time of 10 – 15 minutes which would not discourage many participants. In addition, the survey is chosen to be completed electronically, allowing its widespread dissemination. A potential complication that could arise with an electronic survey is computers within some pharmacies are locked for only specific access to necessary programs and therefore participants may not be able to complete the survey on these computers. To overcome this issue, the survey is configured so that it is also accessible on mobile devices. This can also assist with the convenience sampling through conferences and meetings through QR codes and through clicking links on social media.

2.4.2.3. Data Management

The data collected from the survey will be anonymous so there is no need to anonymize any data gathered. As there are free-text questions, there is a possibility that a respondent can put identifiable data. However, this can be anonymized through deletion. With respect to the legal basis and General Data Protection Regulation (GDPR), any data will be treated with public task as a lawful basis. This would cover the GDPR rules for the UK and these rules are also sufficient for each of the target countries. After the collection of data and the end of the survey project, the data will be permanently deleted after 5 years.

2.4.3. Data Analysis

2.4.3.1. Data Analysis

Data gathered from the survey is to be extracted into Excel and analyzed using GraphPad Prism (GraphPad Prism version 9.5.1. for Windows). Data will mainly be analyzed using descriptive statistical methods. Depending on the volume and richness of responses, analysis of free-text will be done using either thematic or content analysis (Nowell, *et al*., 2017; Kleinheksel, *et al*., 2020). For the thematic analysis, firstly, the free-text responses will be read thoroughly. Initially, codes will be created and data which relates to those codes will be highlighted. Then the codes will be collated with related data and grouped into themes. A narrative will then be written about the data with theme justification. For the content analysis, the free-text responses will be carefully read, and themes and ideas will be developed from reading the raw data. From this, a clearly defined set of coding

categories will be developed based on the themes and ideas that had been identified before. These categories will be designed into a coding schedule form and coding manual. The responses will be then coded into an Excel file, and this can allow for statistical analysis in GraphPad Prism (GraphPad Prism version 9.5.1. for Windows).

2.4.3.2. Limitations, Bias and Error Considerations

Whilst many efforts have been made to reduce limitations, bias and error, there are still some that need to be considered.

One main limitation is likely to be English is a second language for many respondents. Translation was considered, but associated costs of translating text was prohibitive. With the countries of choice, it was deemed that enough respondents would be comfortable in replying in English, since the English proficiency in these countries is high. However, a limitation to consider is that some respondents may not feel comfortable filling out a questionnaire in English or could misunderstand the questions. In addition, as the distribution of English speaking pharmacists favours an urban environment compared to more rural areas, which may be a selection bias.

Another limitation to consider is access to the survey via computer or phone. However, as these are high income countries, this is deemed to be less of an issue for this survey.

There may also be a bias through the selection of participants. This is because the recruitment email will be sent via the Reckitt sales network and therefore Reckitt has a working relationship with these pharmacists. However, the distribution through other channels such as pharmacy boards, pharmacy chains, conferences and social media can hopefully compensate for some of this bias.

2.5. Discussion

The sale of antibiotics in OTC medicines is currently not carefully monitored. Although the usage of OTC antibiotics can be estimated from the sales data of individual companies from the sore throat market (Reckitt, Unpublished, 2019), proper surveillance still needs to be implemented. There have been scientific papers suggesting that the sale of OTC antibiotics could contribute to resistance (Both, *et al*., 2015; Wesgate, *et al*., 2020), yet the supply of these antibiotics to patients is poorly understood. In fact, the sale of OTC antibiotics contradicts the antibiotic stewardship (Table 2.4) that many community pharmacists claim they are leaders in (Essack, *et al*., 2018). This survey aims to fill the knowledge gap so that we could begin to understand the reasons behind the use of OTC antibiotics. Already from

the pretesting, it was seen that pharmacists in Finland and Sweden believe that no antibiotics are sold OTC in their respective countries despite indicating they supplied sore throat lozenge products containing gramicidin. This indicates that they may not be aware that the products they are selling contain antibiotics. Further to this, antibiotics are often given with advice on taking them properly to avoid the development of resistance. This means the supply to patients may not contain the correct information on taking the medication and pharmacists may not be making patients aware that these medications contain an antibiotic.

To help resolve these issues, one aspect of the survey is to find out how community pharmacists can be further supported in the treatment of sore throat and how to effectively deliver the message to them. By improving the education and awareness of community pharmacists to OTC antibiotics and the potential impacts they may have, these medications are more likely to be used more appropriately.

Table 2.4. Core elements of Antibiotic Stewardship (GAIS, 2023).

Antibiotic Stewardship Class	Antibiotic Stewardship Element
Preventing Infection	Enhancing Infection Prevention and Control
	Controlling Source of Infection
Awareness, Education and Communication	Supporting an interdisciplinary approach
	Educating staff
Monitoring and treatment assessment	Surveillance of AMR
	Monitoring Antibiotic Consumption
	Reassessing treatment after culture results
Antibiotic consumption reduction	Using shortest duration of antibiotics based on evidence
	Only prescribing/using antibiotics when needed
	Prescribing/using appropriate antibiotics and at correct dosages

2.6. Chapter Conclusions

This chapter describes the design and process carried out to produce a robust survey on understanding how uncomplicated sore throat is managed in community pharmacies with a high OTC antibiotic usage yet low systemic antibiotic usage. Whilst the survey was not distributed failing to gain ethical approval within the lifetime of the study, there was some indication in the pretesting and development of the survey that community pharmacists may not be aware of products containing OTC antibiotics. The result of this is an inappropriate supply of these antibiotics to patients. The inappropriate use of antibiotics contributes to the development of AMR (Holloway, *et al*., 2016).

Chapter 3: General Methods and Materials

Chapter 3: General Methods and Materials

3.1. Chemicals and storage of chemicals

Unless stated otherwise, all chemicals were purchased from Fischer Sci Ltd. (Loughborough, UK). Media and diluent solutions were stored at room temperature. All antibiotic stocks and powders were stored at 4°C in the dark. Any antibiotic stocks prepared were used for a maximum of 3 months.

3.2. Media Preparation

3.2.1. Preparation of media additives

3.2.1.1. Solutions for cation-adjustment

For antibiotic testing, media needed to be cation-adjusted to ensure adequate growth and determination of MIC values. Solutions made for cation-adjustment were 10 mg/l calcium chloride dihydrate and 10 mg/l magnesium chloride hexahydrate, both made in deionized water. Each solution was filter sterilized through a 0.22 µm cellulose acetate membrane filter and was stored at 4°C and used within 3 months.

3.2.1.2. Blood preparation and β-NAD

A stock solution of 20 mg/ml beta-Nicotinamide adenine dinucleotide (β-NAD) was made in deionized water and filter sterilized using a 0.22 µm cellulose acetate membrane filter. The stock was aliquoted into 1 ml aliquots in microcentrifuge tubes and stored at -20°C and were defrosted as required.

Fresh mechanically defibrinated horse blood was used for making fastidious media. This means the blood has been mechanically agitated to promote clotting and then filtered in the pooling process (HemoStat Laboratories, 2020). The blood used contained a haemocrit value >30% as recommended in ISO 20776-1 (2020). To prepared lysed blood for fastidious broth, the fresh defibrinated horse blood was frozen at -20°C and thawed at room temperature for a minimum of three cycles. After the final thawing, an equal amount of sterile deionized water was added to the blood to ensure complete lysis of the blood, also making the solution 50% lysed blood. Lysis was confirmed by microscopy. The 50% lysed blood was clarified by centrifugation of 3,000G for 10 minutes and pelleted cell debris was removed. Further centrifugation steps were used when necessary to ensure the 50% lysed blood is clarified. Ten millimeter aliquots of the 50% lysed blood was stored at -20°C for a maximum of three months and were defrosted as required.

3.2.2. Preparation of Media

Mueller-Hinton broth (MHB) was prepared according to manufacturer's instructions. Once cooled to room temperature, the MHB was cation-adjusted using the solutions in section 3.2.1.1, to a final concentration of 20 mg/l $CaCl_2$ and 10 mg/l $MgCl_2$ as recommended in ISO 20776-1 (2020).

For the preparation of fastidious Mueller-Hinton broth (MHB-F), a final concentration of 20 mg/l β-NAD was added to MHB as prepared above. Lysed blood was defrosted and added to give a total blood concentration of 5% defibrinated blood.

Mueller-Hinton agar (MHA; Oxoid) was prepared according to manufacturer's instructions and sterilised by autoclaving for 15 minutes at 121°C/ 100 kPa (British Pharmacoepia Commission, 2008). The agar was then cooled to 55°C and then supplemented with cation solutions to give final concentrations of 20 mg/l $CaCl_2$ and 10 mg/l $MgCl_2$.

To make fastidious Mueller-Hinton agar (MHF-A), MHA was prepared as described above, then the agar was cooled to 45°C and additional supplements of β-NAD were added to give a final concentration of 20 mg/l, and mechanically defibrinated horse blood to make final concentration of 5%. To stop the agar from solidifying when the blood was added, the blood was first warmed at 37°C for 15 minutes and then added to the agar.

For the recovery and general maintenance of fridge stocks, tryptone soya agar (TSA) was used unless stated otherwise. TSA plates were bought pre-poured (E&O Laboratories) and stored at 4°C until needed.

3.2.3. pH Adjustments

Adjustments to pH was done by using 1M hydrochloric acid or 1M sodium hydroxide solutions. These were added dropwise whilst continually mixing with a magnetic stirrer until reaching to desired pH (Table 3.1). The pH was monitored continuously using a FE20 Five Easy benchtop pH meter (Mettler Toledo).

3.2.4. Sterilisation of materials

Heat stable solutions and plasticware were sterilised by autoclaving for 15 minutes at 121°C and 100 kPa using a Astell side open benchtop autoclave. Any heat-sensitive solutions were sterilised by filtration. The filter membranes used were the 0.22 µm cellulose acetate membrane filters (Sartorius, UK).

Table 3.1. Composition of buffers and Media used in this study.

Solution	**Composition**
Phosphate buffered saline (PBS), prepared from tablets (Sigma Aldrich)	137 mM NaCl; 2.7 mM KCl; 10 mM phosphate; pH 7.4
MHB	300 g/l dehydrated infusion from beef; 17.5 g/l casesin hydrolysate; 1.5 g/l starch; 20 mg/l $CaCl_2$; 10 mg/l $MgCl_2$; pH 7.3 ±0.1
MHB-F	300 g/l dehydrated infusion from beef; 17.5 g/l casesin hydrolysate; 1.5 g/l starch; 20 mg/l $CaCl_2$; 10 mg/L $MgCl_2$; 20 mg/l β-NAD; 5% lysed defibrinated blood; pH 7.3 ±0.1

3.3. Bacterial Strains and Culture Conditions

3.3.1. Bacterial strains used

The bacterial strains used for the project were bought from either, the American type culture collection (ATCC), Public Health England PHE or Fisher Scientific Ltd. (Table 3.2). These were supplied as cryovials from ATCC or PHE and as culti-loops from Fischer Scientific Ltd. and were revived following the suppliers' instructions.

3.3.2. Culture conditions used

Bacteria were either grown on plates or in liquid-culture. For bacteria grown on plates, either freezer stocks or bacterial suspensions were grown using appropriate media and incubation conditions to allow for optimal growth (Table 3.2).

Unless otherwise stated, bacteria grown in liquid-culture was done by taking 3-4 colonies from streaked plates and resuspending in 5 ml of an appropriate media (Table 3.2). Liquid cultures were incubated at an appropriate temperature 120 rpm overnight (Table 3.2). The cultures were then washed and centrifuged at 3,000G for 10 minutes at 20°C. The supernatant was discarded and the pellet resuspended in MHB or MHB-F for use in experiments.

3.3.3. Storage conditions of bacteria

Upon receipt of cultures, long-term freezer stocks were prepared. For cultures ordered from ATCC or PHE, freeze-dried pellets were revived by resuspending in 1 ml MHB or MHB-F (Table 3.2) and leaving to rehydrate for 10 minutes at room temperature. Two hundred and fifty microliters of the rehydrated pellet was added to 10 ml of MHB or MHB-F depending on the organisms needs, and was incubated at 37°C at 120 rpm for 18 hours. For cultures bought from Fisher Scientific Ltd., the culti-loops were rehydrated in 10 ml MHB or MHB-F and incubated was described previously. After incubation, 500 µl aliquots of the culture was added to cryovials along with 500 µl of 50% glycerol to give a final concentration of 25% glycerol. Cryovials were kept at -80°C for long-term storage. Before freezing, the cultures were streaked to ensure they were pure.

For short term frozen storage, cryovials were stored at -20°C for a maximum of 1 year. Working stocks of culture were maintained on TSA or MHA-F. After incubation, the plates were wrapped in parafilm to prevent desiccation and stored at 4°C for up to 2 months.

For cultures that were frozen after exposure to OTC antibiotics, bacterial cultures were washed and resuspended in MHB with 8% Dimethyl Sulfoxide (DMSO) being added as a cryoprotectant. These vials were stored at both -20°C and -80°C for short term (< 1 year) and long term (> 1 year) storage respectively

Table 3.2. Bacteria, strain number and where they were purchased from, media used and incubation conditions of test organisms.

Bacteria	**Strain Number**	**Bought from**	**Media used**	**Incubation conditions**
Staphylococcus aureus	NCTC 10788	PHE	MH	37°C, ambient air
Escherichia coli	ATCC 25922	ATCC		
Acinetobacter baumannii	ATCC 19568	ATCC		
Pseudomonas aeruginosa	ATCC 27853	Fisher Scientific Ltd.		
Enterobacter cloacae	ATCC 13047	Fisher Scientific Ltd.		
Klebsiella pneumoniae	ATCC 13883	PHE		
Streptococcus pneumoniae	ATCC 49619	Fisher Scientific Ltd.	MH-F	35°C, 5% CO_2
Streptococcus pyogenes	ATCC 19615	ATCC		
Haemophilus influenzae	ATCC 10211	Fisher Scientific Ltd.		

ATCC: American Type Culture Collection

PHE: Public Health England

3.4. Antibiotic Stock Preparation

3.4.1. Clinical Antibiotics

Clinical antibiotics used in the project were made in the recommended solvent as suggested in the ISO 20776-1 standard. The clinical antibiotics were made to a concentration of 1 mg/ml in the recommended solvent after which the stocks were filter sterilised. The clinical antibiotic stocks were stored in the dark at 4°C and were kept for a maximum of 3 months.

3.4.2. OTC antibiotics

The OTC antibiotics for testing were gramicidin, bacitracin, tyrothricin and neomycin. Gramicidin, bacitracin and tyrothricin were kindly provided in powder form by Reckitt (Slough, UK). Neomycin sulfate was purchased in powder form (Fisher Scientific Ltd., UK). All powders were stored at 4°C and in the dark.

For minimum inhibitory concentration (MIC) testing, neomycin and bacitracin powders were dissolved in water at a concentration of 5.12 mg/ml and were filter sterilised using a 0.22 µm cellulose acetate membrane filter. Gramicidin and tyrothricin powders were dissolved in methanol which when used in micro-broth dilution test gives the highest methanol concentration of 2.5% (v/v) which still allowed bacterial growth. Gramicidin and Tyrothricin stock solutions were made at a concentration of 5.12 mg/ml but were not filter sterilised using a cellulose acetate membrane filter as filtering changed the potency of the stocks when tested. All of the antibiotic stocks were tested to ensure no stocks were contaminated by diluting in phosphate-buffered saline (PBS) and plating on MHA. All stocks were stored at 4°C for a maximum of three months.

For pre-exposure and co-exposure testing, the OTC antibiotic stock solutions were made at a concentration 50 times the during-use concentration (Table 3.3).

Table 3.3. The during-use and stock concentrations of OTC antibiotics

OTC antibiotic	**During-Use concentration**	**Stock concentration**
Gramicidin	15 µg/ml	750 µg/ml
Bacitracin	5 IU/ml	250 IU/ml
Tyrothricin	200 µg/ml	10,000 µg/ml
Neomycin	250 µg/ml	12,500 µg/ml

3.5. Quantification of Bacteria

3.5.1. Serial Dilutions

Bacteria were enumerated by viable counts. This used the colony forming units (CFU) counted on agar plates and multiplying by the dilution factor of the sample to calculate the final titre. Serial dilutions were done using a 10-fold dilution series in phosphate-buffered saline (PBS; Fisher Scientific Ltd., UK). This was done by firstly adding 180 µl of PBS into 96-well plates. Then 20 µl of the sample was added to the first well and mixed thoroughly by pipetting well up and down. The 20 µl was taken from this well and added to the next well in the dilution series and this process was repeated until the required dilution.

3.5.2. Miles Misra Drop Count Method

After dilution, samples were counted using the Miles Misra drop counting method (Miles, *et al*., 1938). Three 20 µl drops for each dilution were pipetted onto an appropriate agar (Table 3.2) to allow growth of the target organisms. The three drops were then allowed to dry on the agar surface and after which plates were inverted and incubated using appropriate conditions (Table 3.2). After incubation, the colonies from each drop were counted. The average CFU per 20 µl drop was multiplied by the dilution factor to find the total viability of bacteria in the original sample.

3.5.3. Spread Plating Method

To ensure that the testing has the lowest detection limit possible, neat samples were plated by pipetting 200 µl onto the agar surface and then spreading using a disposable and sterile L-shaped spreader. Plates were incubated as described before and colonies were counted. The number of colonies was multiplied by five to give the total viability in the original sample.

3.6. Statistical Analysis

To be able to statically compare between sample groups, testing was performed in biological triplicate. The distribution o the data was seen and calculated by standard deviation (SD). Error bars on any graphs are the standard deviation values.

Statistical tests were performed using R and R studio statistical software (RStudio Team, 2020) or GraphPad Prism (GraphPad Prism version 9.5.1. for Windows). For the majority of laboratory experiments containing data with multiple means either a one, two or multi-way ANOVA (Analysis of Variance) was used. The cut-off thresholds to determine the significance of the treatment was used as $\alpha=<0.05$.

Post-hoc tests were used to determine multiple comparisons between the means of the different samples or comparing the means to the control group. The main post-hoc tests used for this was either the Tukey's or Dunnett's test which gives a comparison of all pairwise combination after one-way ANOVA or comparisons to the control group respectively.

Chapter 4: Resistance Development after Pre-exposure to OTC antibiotics

Chapter 4: Resistance development after Pre-exposure to OTC antibiotics

4.1. Introduction

4.1.1. Resistance mechanisms

Antibiotics have a wide variety of mechanisms of action, but can broadly divided into groups such as i) affecting the cell wall, ii) depolarizing the cell membrane, iii) inhibiting protein synthesis, iv) inhibiting Deoxyribonucleic acid (DNA) synthesis or v) disrupting metabolic pathways (Reygaert, 2018). With this wide array of tackling microorganisms there have been the development of an equally wide array of resistance mechanisms. Antibiotic resistance can happen through different mechanisms that can be categorised into four main groups: i) efflux pumps, ii) drug uptake limitation, iii) drug target alteration and iv) drug inactivation (Reygaert, 2018; Figure 4.1). Bacterial resistance is either intrinsically or acquired through either mutation or acquisition of mobile genetic elements (MGEs). Intrinsic resistance occurs mainly through limited drug uptake mainly in Gram-negative bacteria due to the outer membrane (Cox and Wright, 2013). Although, intrinsic resistance has also been observed through efflux pumps that are seen through an entire species of bacteria (Webber and Piddock, 2003; Hajiagha and Kafil, 2023). Acquired resistance mechanisms often make use of drug target alteration, drug inactivation and efflux pumps (Reygaert, 2018).

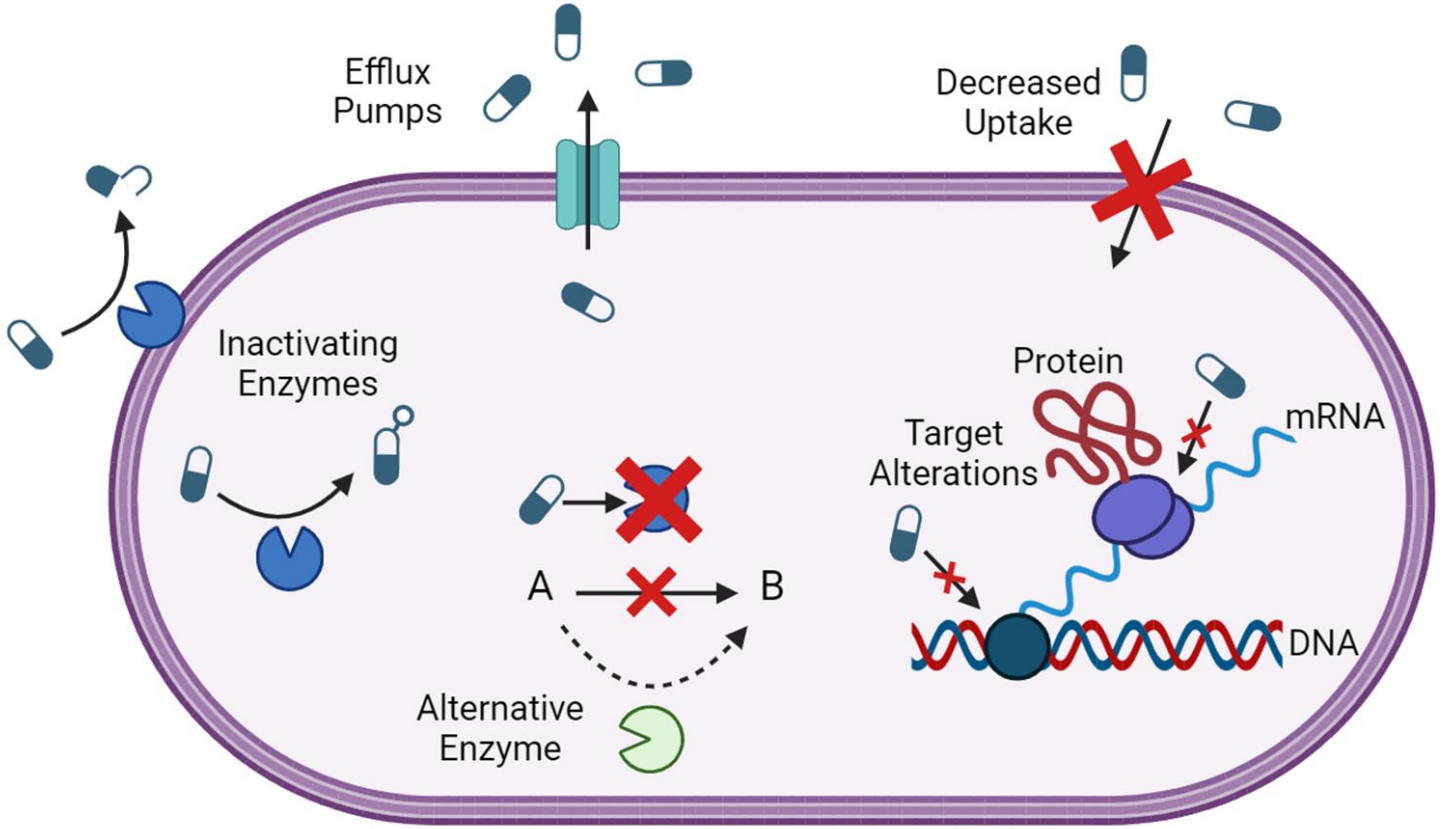


Figure 4.1. Groups of resistance mechanisms developed in bacteria. Efflux pumps, drug uptake limitation, drug target alteration and drug inactivation.

4.1.1.1. Antibiotic Efflux

The role of efflux pumps is to excrete toxic substances out of the cell (Blanco *et al*., 2016). Some efflux pumps are expressed constitutively within the bacterial cell however, they can be overexpressed when a suitable stimulus, such as antibiotics, is present (Webber and Piddock, 2003). There are six main types of efflux pump (Figure 4.2): ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), resistance-nodulation-cell division (RND), small multidrug resistance (SMR) and the proteobacterial antimicrobial compound efflux (PACE) (Hajiagha and Kafil, 2023). Most bacteria will possess different types of efflux pumps however, the level of resistance for some efflux pumps may depend on the various carbon sources that are available to the bacteria. Efflux pumps vary between Gram-positive and Gram-negative bacteria. Gram-positive generally have intrinsic chromosomally encoded efflux pumps however they have also been known to possess plasmid encoded efflux pumps. The types of efflux pump from Gram-positive bacteria are almost exclusively MFS but some can also be MATE. In contrast, Gram-negative bacteria have efflux pumps from each of the six types but the most prominent is the RND (Blair, *et al*., 2014). The PACE family of efflux pump is widespread in Gram-negative pathogens but is not well characterized yet (Hassan, *et al*., 2018).

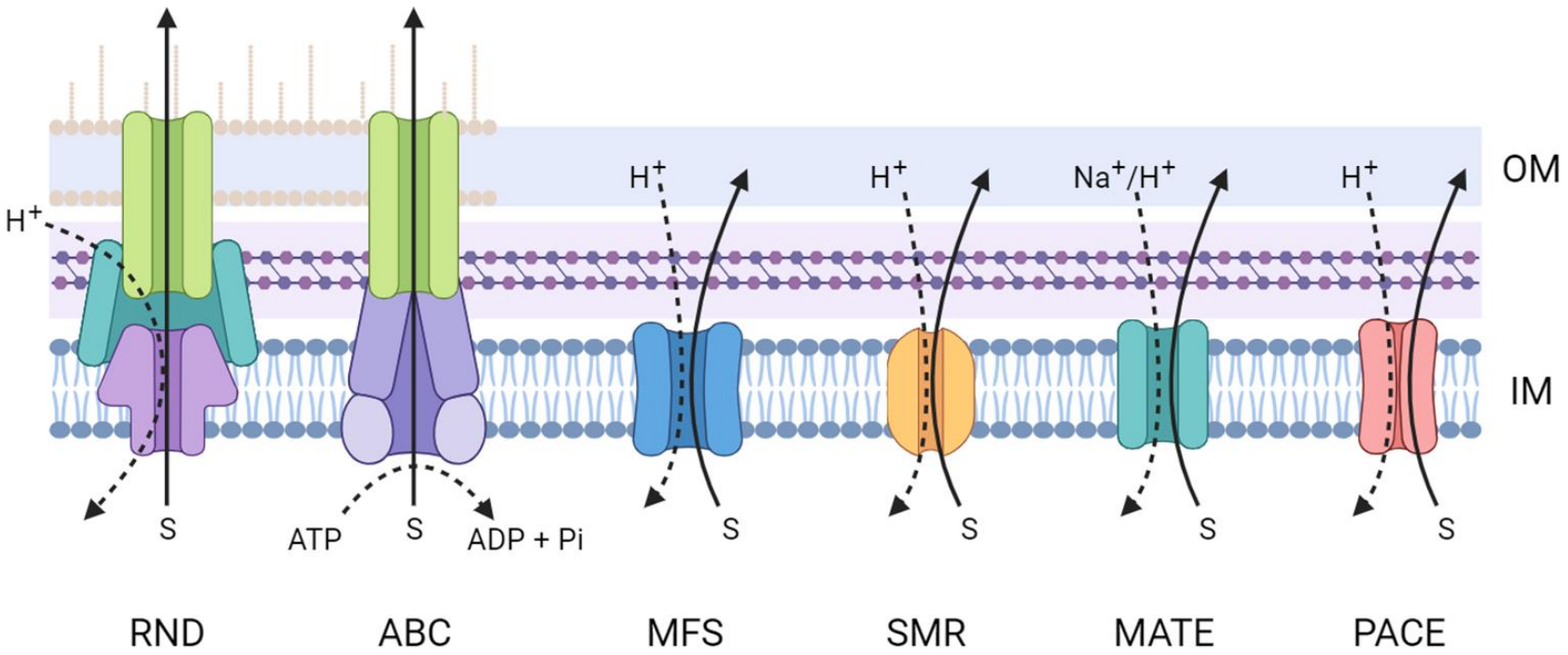


Figure 4.2. The six types of efflux pump with their location in the cell membrane and energy sources. The resistance nodulation-cell division (RND) family is only found in Gram-negative bacteria. The ATP-binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), and the multidrug and toxic compound extrusion (MATE) superfamilies are present in both Gram-positive and Gram-negative. The proteobacterial antimicrobial compound efflux (PACE) efflux pumps have not been fully characterized yet. OM – Outer membrane. IM – Inner membrane. S – Substrate. Adapted from (Hajiagha and Kafil, 2023).

4.1.1.2. Limitation of drug uptake

Limitation of drug uptake causes resistance through antibiotics not being able to reach their target site. As mentioned previously, this happens mainly to Gram-negative bacteria due to the presence of the outer membrane, but this can also happen due to changes in the bacterial porins. Another mechanism of limitation of drug uptake is a thickening of the bacterial cell walls that reduce the membrane polarity. This reduces the polarity gradients needed for certain antibiotics to enter the cell. For example, small colony variants of *S. aureus* have a vastly decreased susceptibility to aminoglycosides due to a thicker cell wall (Kahl, 2014).

4.1.1.3. Drug Target Alteration

Drug target alteration can happen in a variety of places within the bacterial cell that complement the antibiotic target site. These alterations can occur through mutations that select for a decreased or complete inhibition of binding affinity of the antibiotic to the target (Beceiro, *et al*., 2013). These mutations can also be passed to other bacteria through horizontal gene transfer. A key example of this is the *mecA* gene which encodes for a methicillin-resistant penicillin binding protein (PBP). This is the key gene that has been globally disseminated and causative of methicillin-resistant *Staphylococcus aureus* (MRSA) (Wielders *et al*., 2002).

4.1.1.4. Drug Inactivation

Drug inactivation occurs in two main ways: drug degradation and chemical addition. A prolific example of drug inactivation by drug degradation is the beta-lactamases. These enzymes were acquired by bacteria to negate the effects of penicillin by cutting the beta-lactam ring in the antibiotic structure that was essential for blocking cell wall synthesis (Bush and Bradford, 2016). Drug inactivation by chemical addition occurs mainly through the addition of an acyl group, phosphoryl group or adenyl group by the bacterial transferases. The most widely utilised transfer addition is acylation which can inhibit the action of aminoglycosides, chloramphenicol, streptogramins and fluoroquinolones (Blair, *et al*., 2015; Ramirez and Tolmasky, 2010; Robicsek, *et al*., 2006; Schwarz, *et al*. 2004).

4.1.2. Development of Resistance

Antibiotic resistance has been present for thousands of years however, it is only the overuse and misuse of them that causes issues to arise in modern medicine (Ventola, 2015). Whilst some bacteria are innately resistant to antibiotics, such as Gram-negative bacteria and benzyl penicillin (Breijyeh, *et al*., 2020), it is the acquisition of resistance that is of primary concern. This acquisition of resistance means that a bacterium that was once susceptible to an antibiotic can now survive its exposure (Munita and Arias, 2016). Antibiotic resistance can be acquired through mutations in the bacterium's own genome or through horizontal transfer of MGEs such as plasmids (van Hoek, *et al*., 2011). Horizontal transfer allows the gene to mobilise away from a single clonal strain and become widespread (Bengtsson-Palme, *et al*., 2018). Different factors can play a role in the mobilisation of genes, but this can be induced by environmental stresses such as heavy metals, biocides, and antibiotics (Pal, *et al*., 2015). When these genes are integrated into other bacteria, they often come at a fitness cost however, the continued environmental selection pressure can help establish the gene within bacteria (Bjorkman and Andersson, 2000). The spread of resistance genes often occurs in complex microcosms such as the gut microbiome (Broaders, *et al*., 2013).

Mutation can also be a source of resistance development. Mutations naturally occur in bacteria during replication. Most mutations are deleterious to the cell, however, with environmental stresses such as antibiotic treatment, some mutations may confer resistance and select for the survival of these bacterial mutants (Reygaert, 2018).

4.1.3. Testing for Antibiotic Resistance

It is important to monitor the development and spread of resistant bacteria and particularly resistance genes (Zhou, *et al*., 2021). This is to inform clinicians about the antibiotic resistance within local areas as it can affect empirical treatment planning (Kapoor, *et al*., 2017). It is also important to monitor the spread of resistance to determine antibiotic regulation and prescribing guidelines on both a national and international basis. Traditionally, the MIC of antibiotics have been determined by various antimicrobial susceptibility testing (AST) such as microbroth-dilution, Etest strips or clinical resistance profiles being determined by the Kirby-Bauer disk diffusion method (Khan, *et al*., 2019). These standard techniques are used commonly in most countries around the world with minor variations. The Clinical and Laboratory Standards Institute (CLSI) are the most commonly used guidelines globally. However, in Europe, most countries follow the guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

These guidelines use epidemiological cut-off values (ECOFFs) to determine whether an organism would be resistant or not and whether treatment would therefore be successful or not. It is important to know what guidelines to follow as the MIC breakpoint values in EUCAST guidelines are generally higher than the CLSI guidelines and therefore could change the recommended clinical treatments (Khan, *et al*., 2019).

4.1.4. Clinical Impact of Resistance

Antibiotic resistance is a huge issue for clinicians due to increasing number of bacterial infections that are clinically resistant to various antibiotics and as such are very difficult to treat (Laxinarayan, 2013). This means that patients are often hospitalised for longer and are at higher risk or getting a hospital-acquired infection (Rosman, *et al*., 2015). Not only is antibiotic resistance an issue but also cross-resistance which can occur from certain resistance mechanisms such as efflux pumps and decreased uptake of antibiotics (Figures 4.1 & 4.2). Whilst cross-resistance often occurs within the same antibiotic classes due to the high similarity in mechanism of action, it can also occur between different antibiotic classes (Périchon and Courvalin, 2009).

It has already been demonstrated how the use of OTC antibiotics, can cause cross-resistance to clinical antibiotics *in vitro* (Wesgate, *et al*., 2020). It is not known however, how these antibiotics can affect resistance development *in vivo.* It is also unknown how these OTC antibiotics could be affecting environments such as the gut microbiome. If the gut microbiome is disrupted, patients can be vulnerable to opportunistic infections following the suppression of commensal organisms (Bengtsson-Palme, *et al*., 2018). Therefore, we need to consider the use of OTC antibiotics and the long-term complications for systemic and topical antibiotic treatments.

4.1.5. Chapter Aims

The aim of this chapter is to determine which bacteria can survive the during-use concentration of OTC antibiotics that would be present in saliva when used for the treatment of sore throat and what cross-resistances may form to clinical systemic antibiotics. This will be achieved through pre-exposing bacteria to a during-use concentration of OTC antibiotic that would be realistically found in saliva during lozenge dissolution. The cross-resistances to clinical systemic antibiotics will then be determined using standard protocols. The stability of any resistances formed will be assessed through passing the bacteria multiple times and reassessing the resistance profile.

4.1.6. Principle of Experiments and Rationale

Previous literature has shown that neomycin is effective against both Gram-positive and Gram-negative bacteria and therefore is likely to be effective and may not allow the bacteria to grow and therefore develop resistances. Bacitracin, gramicidin and tyrothricin however are highly effective against Gram-positive bacteria but have little to no activity against Gram-negative bacteria. Multiple papers have claimed that resistance does not develop to these antibiotics as they do not have a defined target in the bacteria but rather randomly insert themselves into bacterial membranes or cell walls (Stauss-Grabo, *et al*., 2014; Lang and Staiger, 2016). Whilst many say these OTC antibiotics are to treat Gram-positive bacteria and not Gram-negative bacteria, it still needs to be considered what effects they may have on Gram-negative bacteria. Previously researchers have claimed that because most sore-throat infections are caused by Gram-positive bacteria, they will not come into contact with Gram-negative bacteria, however, these antibiotics are likely to travel further down the gastrointestinal tract where they can come into contact with a wider variety of Gram-negative bacteria. It has also already been previously shown that bacterial exposure to these antibiotics could lead to cross-resistance, although it is not known which classes of antibiotics are most at risk and the stability of resistance.

4.2. Methods

4.2.1. OTC Antibiotic Pre-exposure

The panel of test bacteria (Chapter 3; Section 3.3) were exposed to during-use concentrations of the different OTC antibiotics. The during-use concentration was determined by using the largest amount of OTC antibiotic in any one product (sore throat lozenge) within Europe and the documented average amount of saliva products dissolve in. When an antibiotic lozenge is used, it is instructed to be slowly dissolved in the mouth which should take approximately 30 minutes (Maheshwari, *et al*., 2013). The average person produces between 0.5 l and 1.5 l per day of saliva, therefore an approximate amount of saliva produced in 30 minutes is 20 ml (Iorgulescu, 2009). Considering the amounts of the OTC antibiotics used based on sore throat products that contain the highest amount of each antibiotic in Europe, and the amount of saliva (20 ml), the during use OTC antibiotic concentration can be estimated (Table 4.1). However, it is worth noting that these products are been used in a dynamic environment and therefore the concentration of the products will be further diluted as they pass through the gastrointestinal tract.

Table 4.1. The during-use concentrations the OTC antibiotics in saliva.

OTC Antibiotic	**Bacitracin**	**Neomycin**	**Gramicidin**	**Tyrothricin**
Lozenge amount	100 IU	5 mg	0.3 mg	4 mg
Saliva concentration during lozenge dissolution (During-Use concentration)	5 IU/ml	250 µg/ml	15 µg/ml	200 µg/ml

Initial exposure was done by firstly picking 3-4 bacterial colonies to inoculate 5 ml of an appropriate broth (Chapter 3, Table 3.2) in triplicate and incubating at 37°C under constant agitation at 150 rpm. After incubation, bacteria were washed by centrifuging at 3,000 G for 10 minutes at ambient temperature and resuspending the pellet in 5 ml phosphate-buffered saline (PBS). Each culture was then adjusted to an OD_{625} of 0.08 – 0.15 to give a bacterial concentration of approximately 1 x 10^8 CFU/ml.

The OTC antibiotic exposures were made by diluting the antibiotic exposure stocks, as described earlier, and diluting 1 in 10 with an appropriate media (Chapter 3, Table 3.2), for

the bacteria tested to give the during-use concentration (Table 4.1). This was further diluted with media to give exposure concentrations of 90%, 75%, 50%, 25%, 5% and 1% in respect to the saliva concentration during lozenge dissolution (Table 4.1) to mimic the product dilution through the gastrointestinal product. To bijoux bottles, 1 ml of the OTC antibiotic exposure concentrations in an appropriate media (Chapter 3, Section 3.3.2) was added in triplicate and 5 µl of the adjusted bacterial culture was added to give the final bacterial concentration of approximately 5.5 x 10^5 CFU/ml. The bottles were then incubated 18 h ± 2 h at 37°C under agitation at 150 rpm with the lids slightly loosened to maximise air exchanges. After incubation, the bottles were checked for turbidity and any those that presented no turbidity were incubated further for a maximum of seven days; turbidity was checked daily. Any cultures that had gone turbid were decanted into 1.5 ml microcentrifuge tubes and were washed three times by centrifuging at 10,000 G for 1 minute and resuspending the pellet in 1 ml PBS. The culture was tested by micro-broth dilution and disk diffusion.

4.2.2. EUCAST Disk Diffusion

The method was based on the EUCAST disk diffusion test (EUCAST, 2019). All bacteria were streaked onto an appropriate media (Chapter 3, Section 3.3) and incubated overnight at 37°C in ambient air for non-fastidious bacteria and at 35°C in 5% CO_2 for fastidious ones. After incubation, 3-4 colonies were picked using a 10 µl loop and resuspended in 5 ml of a suitable culture broth. All bacteria were then incubated overnight shaking at 150 rpm at 37°C. After incubation, the bacteria were washed in PBS. Each culture was then diluted to OD_{625} 0.08-0.15. Bacteria in each culture was enumerated by serially diluting in PBS and drop counting (Miles, *et al.*, 1938) on a suitable agar to ensure the correct bacterial concentration was used to create the bacterial lawn. The plates were incubated at 37°C in ambient air for 16-18 h for MH plates and 35°C in 5% CO_2 for 16-18h for MH-F plates.

To inoculate the antibiotic test plate, a bacterial lawn was streaked on a suitable media by streaking across the whole plate in three directions using a cotton swab. When making the bacterial lawn, the swabs used for Gram-negative organisms were squeezed against the side of the tubes as to not over inoculate the plates, this was not necessary for Gram-positive organisms. Two agar plates were streaked for each test and tests were performed in biological triplicate. Plates were streaked within 15 minutes of OD_{625} adjustment.

Four antibiotic disks per plate (Table 4.2) were placed onto the agar surface at set distances using a disk dispenser to allow clear reading of the zones of inhibition. Once the disks were

placed on the agar surface, a sterile pair of tweezers was used to ensure that the disk made full contact with the agar. The disks were dispensed within 15 minutes of the plates being streaked with the bacterial inoculum. MH plates were incubated at 37°C and MH-F plates at 35°C at 5% CO_2 for 18 ± 2 hours.

After incubation, zones of inhibition around antibiotic disks were measured to the nearest millimeter using calipers. The MH plates were read from the back of the plates using a dark background and illuminated by reflective light. The MH-F plates were read from the top of the plates with the lid removed using reflective light. When reading the MH-F plates, care taken to read only the inhibited growth and not the haemolysis. Results were then interpreted using the EUCAST clinical breakpoint tables (EUCAST, 2023).

Table 4.2. The antibiotic disks and classes used for testing cross-resistance. *Enterobacterales* include *E. coli*, *E. cloacae* and *K. pneumoniae*.

Bacteria	Antibiotic Disk	Antibiotic class	Content (μg)	Code
S. aureus	Benzylpenicillin	Penicillins	1 unit	**P**
	Cefoxitin	Cephalosporins	30	**FOX**
	Ciprofloxacin	Fluoroquinolones	5	**CIP**
	Gentamicin	Aminoglycosides	10	**CN**
	Erythromycin	Macrolides	15	**E**
	Tetracycline	Tetracyclines	30	**TE**
	Rifampicin	Rifampicin	5	**RD**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**
P. aeruginosa	Piperacillin (with tazobactam)	Penicillins	30 (6)	**TZP**
	Ceftazidime	Cephalosporins	10	**CAZ**
	Imipenem	Carbapenems	10	**IPM**
	Ciprofloxacin	Fluoroquinolones	5	**CIP**
	Amikacin	Aminoglycosides	30	**AK**
	Aztreonam	Monobactams	30	**ATM**
	Tobramycin	Aminoglycosides	10	**TOB**
	Meropenem	Carbapenems	10	**MEM**
A. baumannii	Imipenem	Carbapenems	10	**IPM**
	Meropenem	Carbapenems	10	**MEM**
	Ciprofloxacin	Fluoroquinolones	5	**CIP**
	Levofloxacin	Fluoroquinolones	5	**LEV**
	Amikacin	Aminoglycosides	30	**AK**
	Gentamicin	Aminoglycosides	10	**CN**
	Tobramycin	Aminoglycosides	10	**TOB**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**

Table 4.2 - continued

Enterobacterales	Ampicillin	Penicillins	10	**AMP**
	Cefotaxime	Cephalosporins	5	**CTX**
	Imipenem	Carbapenems	10	**IPM**
	Ciprofloxacin	Fluoroquinolones	5	**CIP**
	Gentamicin	Aminoglycosides	10	**CN**
	Aztreonam	Monobactams	30	**ATM**
	Chloramphenicol	Chloramphenicol	30	**C**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**
H. influenzae	Piperacillin (with tazobactam)	Penicillins	30 (6)	**TZP**
	Cefotaxime	Cephalosporins	5	**CTX**
	Imipenem	Carbapenems	10	**IPM**
	Ciprofloxacin	Fluoroquinolones	5	**CIP**
	Tetracycline	Tetracyclines	30	**TE**
	Chloramphenicol	Chloramphenicol	30	**C**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**
	Rifampicin	Rifampicin	5	**RD**
S. pyogenes	Benzylpenicillin	Penicillins	1 unit	**P**
	Levofloxacin	Fluoroquinolones	5	**LEV**
	Vancomycin	Glycopeptides	5	**V**
	Erythromycin	Macrolides	15	**E**
	Tetracycline	Tetracyclines	30	**TE**
	Chloramphenicol	Chloramphenicol	30	**C**
	Rifampicin	Rifampicin	5	**RD**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**

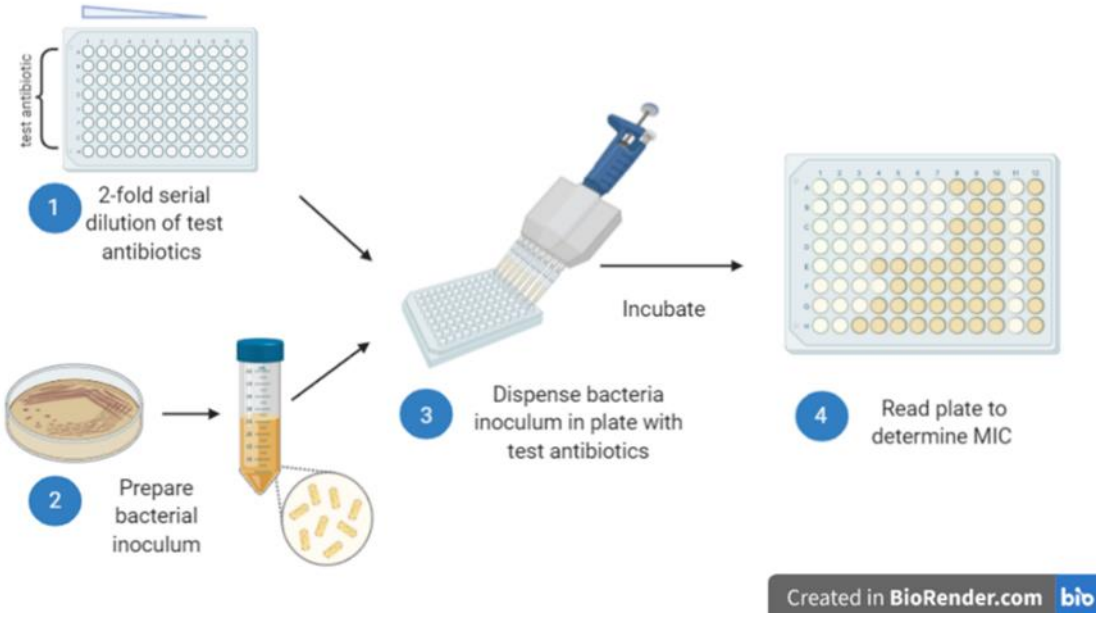
Table 4.2 - continued

S. pneumoniae	Ampicillin	Penicillins	2	**AMP**
	Levofloxacin	Fluoroquinolones	5	**LEV**
	Vancomycin	Glycopeptides	5	**V**
	Erythromycin	Macrolides	15	**E**
	Tetracycline	Tetracyclines	30	**TE**
	Chloramphenicol	Chloramphenicol	30	**C**
	Rifampicin	Rifampicin	5	**RD**
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	1.25 – 23.75	**SXT**

4.2.3. MIC – Broth Microdilution

The microbroth dilution test method was based on the ISO 20776 – 1 standard protocol (ISO, 2020). All cultures were initially streaked onto MHA or MHA-F plates to check for purity. 3-4 colonies were picked from overnight culture plates and added to 5 ml MHB or MHB-F, which were incubated overnight at 37°C under constant agitation at 150 rpm. After incubation, the culture was centrifuged at 3,000 G for 10 minutes at ambient temperature. The supernatant was then discarded, and the pellet was resuspended in 5 ml fresh MHB. The OD_{625} was adjusted to 0.08 – 0.15 which equates to approximately 1 x 10^8 CFU/ml. The inoculum was then diluted 100-fold to achieve a starting inoculum of approx. 1 x 10^6 CFU/ml.

MIC test was performed in 96 well plates (Figure 4.3). The MIC plates were filled with diluent and the antibiotic was diluted using the VIAFLO pipette (Integra, Zizers, Switzerland) and VIAFLO ASSIST pipetting assistant (Integra, Zizers, Switzerland) to allow high-throughput testing. Columns 1-12 were filled with 50 µl of the recommended diluent as suggested in the ISO 20776-1 standard, Annex B (Appendix 4.1). For testing OTC antibiotics, sterile distilled water was used as a diluent. The antibiotic storage stock solution (Chapter 3, Section 3.4) was diluted 1 in 10 with the appropriate diluent to make the testing stock solution for MIC testing. Fifty microlitres of the testing stock solution was then added to column 1 and was serially diluted by pipetting 50 µl up and down three times until column 10 after which the volume left in the pipette tip was discarded. This gave a test concentration range of 128 µg/ml to 0.25 µg/ml for all antibiotics tested. Fifty microlitres of the bacterial inoculum (approximately 1 x 10^6 CFU/ml) was added to all wells except column 11 which had 50 µl of MHB or MHB-F added as a negative control which gave a final bacterial concentration of 5 x 10^5 CFU/ml. Each bacterial strain was tested in at least triplicate. The plates were then incubated statically at 37°C for 18 ± 2 hours. The plates were shaken gently before reading and the MICs were then taken as the lowest concentration that had no visible turbidity in the well by naked eye.



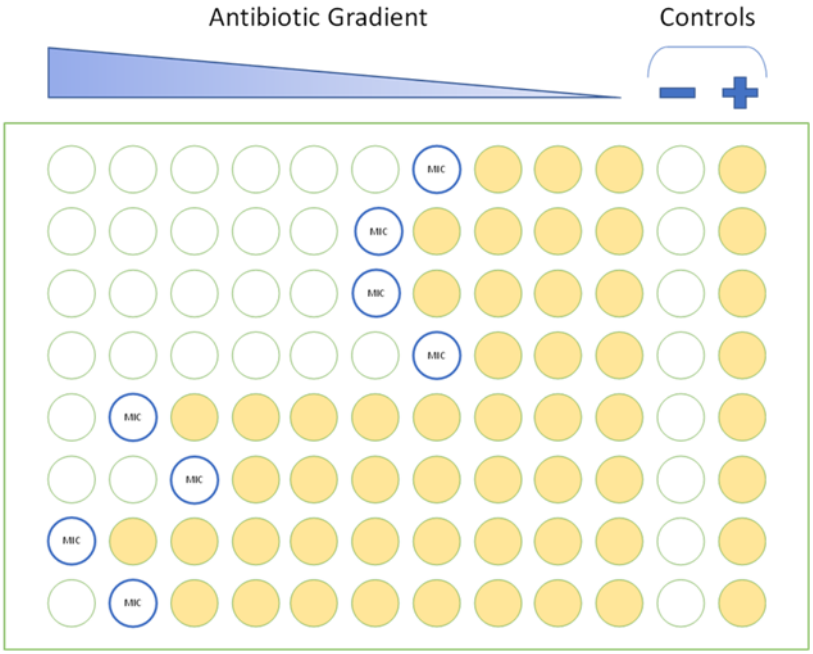


Figure 4.3. MIC microbroth dilution test. The figure shows the set up and the layout of the 96-well plates. The yellow wells indicate growth and the blank wells, no growth. The MIC was taken as the lowest concentration with no growth observed. Columns 1-10 have a decreasing concentration of antibiotic and contain 5 x 105 CFU/ml of bacteria in MHB. The total volume in each well is 100 µl. Columns 11 and 12 are negative and positive growth controls, respectively.

4.2.4. Passaging Bacteria

After initial pre-exposure to OTC antibiotics, turbidity check following additional incubation or not, washed cultures (Section 4.2.1) were also passaged further by taking 100 µl of the washed culture and diluting in 900 µl of PBS to give a bacterial concentration of approximately 1 x 10^8 CFU/ml. Bacterial antibiotic susceptibility was tested by disk diffusion (Section 4.2.2) at passages 1, 5 and 10. The remaining culture was centrifuged once more and resuspended in a freezing solution of 1 ml PBS containing 8% DMSO. The cultures were then frozen at -80°C for phenotypic and genetic characterization later in the project.

4.2.5. Statistical Analyses

All experiments were conducted in biological triplicate. Statistical significance for the disk diffusion data was determined by Two-way ANOVA followed by Šídák's multiple comparisons test on the *S. aureus* data and a Tukey's multiple comparisons test on the other organisms using GraphPad Prism (GraphPad Prism version 9.5.1. for Windows).

4.3. Results

4.3.1. Survival of bacteria after OTC Antibiotic Pre-exposure

The survival of bacteria after exposure to 100-1 % during-use OTC antibiotics concentrations for 24 hours are presented in Table 4.3. After incubation of up to a maximum of 7 days, no additional test bacteria were able to grow in 100% of the during-use concentrations. Only the cultures that were able to survive 100% of the during-use concentrations after 24 hours were chosen for further testing as other antibiotic and bacteria combinations would not realistically be able to survive the concentrations of these antibiotics found in saliva and therefore would be at less risk of emerging bacterial resistance.

Table 4.3. Maximum OTC antibiotic concentrations allowing bacterial growth after 24 hours exposure. The during-use concentration for each antibiotic (100%) is as follows: Neomycin - 250 µg/ml; Tyrothricin – 200 µg/ml; Bacitracin 5 IU/ml; Gramicidin 15 µg/ml. The green boxes indicates that the bacteria were able to grow in the during-use concentration of the OTC antibiotic and were taken forward for further testing. The yellow boxes indicates that bacteria were able to grow in a diluted concentration of OTC antibiotic. The orange boxes indicate that no growth was observed even in the lowest concentration tested.

Bacteria	Highest percentage of the during-use concentrations of OTC antibiotics that showed visible growth after 24 hours (%)			
	Neomycin	Tyrothricin	Bacitracin	Gramicidin
S. aureus	NG	NG	25	100
A. baumannii	1	25	100	100
P. aeruginosa	25	100	100	100
E. coli	NG	100	100	100
E. cloacae	NG	100	100	100
K. pneumoniae	NG	100	100	100
H. influenzae	NG	25	100	100
S. pyogenes	NG	NG	NG	NG
S. pneumoniae	NG	NG	NG	NG

NG = No growth seen in any concentrations.

4.3.2. Disk diffusion results after OTC Antibiotic Pre-exposure

Diameters of the disk diffusion testing were measured in accordance with the EUCAST standard protocol. These diameters were interpreted for their clinical significance using the clinical breakpoint tables (EUCAST, 2023). The clinical breakpoints are separated into three groups: clinically sensitive, intermediate and clinically resistant.

Along with clinically significant changes, statistical significance in zone of inhibition (ZOI) was assessed. This was to demonstrate that some ZOI, whilst not having clinically significant changes, have statistically significant decreases. This demonstrates in increased tolerance of the bacteria to the clinical antibiotic.

Staphylococcus aureus was only able to survive in the during-use concentration of gramicidin (15 µg/ml; Table 4.3) and therefore only the development of cross-resistance after gramicidin exposure was tested. The antibiotics used for determining cross-resistance were taken from the antibiotics used in clinic or had known defined resistance criteria (Table 4.2; EUCAST, 2023). The baseline susceptibility showed that *S. aureus* was susceptible to all antibiotics except ciprofloxacin for which an intermediate resistance was observed. After gramicidin exposure, 1/3 replicate was clinically resistant to ciprofloxacin and 1/3 replicate was clinically resistant to tetracycline (Table 4.4). Even with 1/3 replicate showing clinical resistance, there was not a significant difference in zone of inhibition between the baseline and gramicidin pre-exposed cultures in ciprofloxacin ($p=0.1156$) or tetracycline ($p=0.3951$).

However, after gramicidin pre-exposure 2/3 replicates developed clinical trimethoprim-sulfamethoxazole resistance, but overall there was a significant reduction in zone of inhibition ($p=0.0025$) between the baseline and after gramicidin pre-exposure.

Table 4.4. *S. aureus* disk diffusion results**.** Cross-resistance of *S. aureus* to clinically relevant antibiotics after pre-exposure to gramicidin (15 µg/ml). Green (●): zone of inhibition corresponding to clinical susceptibility; Yellow (●): zone of inhibition corresponding to intermediate susceptibility; Red (●): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)	
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure
S. aureus	P (1 unit)	26	26	1	35	37
				2	37	33
				3	36	34
	FOX (30 µg)	22	22	1	29	33
				2	30	30
				3	31	28
	CIP (5 µg)	50	21	1	27	27
				2	28	23
				3	28	NZ
	CN (10 µg)	18	18	1	20	20
				2	22	20
				3	23	19
	E (15 µg)	21	21	1	25	27
				2	26	24
				3	27	23
	TE (30 µg)	22	22	1	28	16
				2	30	24
				3	32	25
	RD (5 µg)	26	26	1	30	26
				2	32	26
				3	32	28
	SXT (1.25 - 23.75 µg)	17	14	1	24	NZ
				2	25	23
				3	26	NZ

P – Benzylpenicillin; FOX – Cefoxitin; CIP – Ciprofloxacin; CN – Gentamicin; E – Erythromycin; TE – Tetracycline; RD – Rifampicin; SXT – Trimethoprim-sulfamethoxazole.

Acinetobacter baumannii was able to survive in the during-use concentration of both gramicidin (15 µg/ml) and bacitracin (5 IU/ml; Table 3). The baseline susceptibility profile showed that *A. baumannii* had innate clinical resistance to trimethoprim-sulfamethoxazole and intermediate clinical resistance to ciprofloxacin (Table 4.5). After gramicidin (15 µg/ml) pre-exposure, 1/3 replicate became clinically resistant to amikacin and 2/3 replicates became intermediately resistant to imipenem. (Table 4.5). After bacitracin (5 IU/ml) pre-exposure, all three replicates had clinical resistance to amikacin, 1/3 isolate showed clinical resistance to tobramycin and 1/3 replicate showed intermediate clinical resistance to imipenem and levofloxacin (Table 4.5). After gramicidin or bacitracin pre-exposure, *A. baumannii* remained clinically susceptible to meropenem or gentamicin.

Whilst there were only a few instances of culture becoming clinically resistant to an antibiotic, there were many that had statistically significant reductions in zone of inhibition. After either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) pre-exposure, there were statistically significant reductions in zone of inhibition for imipenem, meropenem, ciprofloxacin, levofloxacin and amikacin ($p<0.0001$). There was no significant change in zone in inhibition for gentamicin, tobramycin or sulfamethoxazole.

Table 4.5. *A. baumannii* disk diffusion results**.** Cross-resistance of *A. baumannii* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml) and bacitracin (5 IU/ml). Green (●): zone of inhibition corresponding to clinical susceptibility; Yellow (●): zone of inhibition corresponding to intermediate susceptibility; Red (●): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)		
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure
A. baumannii	IPM (10 µg)	24	21	1	34	27	22
				2	31	23	25
				3	34	23	25
	MEM (10 µg)	21	15	1	34	24	21
				2	30	24	23
				3	32	23	21
	CIP (5 µg)	50	21	1	31	23	22
				2	28	22	22
				3	31	22	22
	LEV (5 µg)	23	20	1	32	25	21
				2	31	23	23
				3	32	23	24
	AK (30 µg)	19	19	1	23	19	18
				2	25	20	16
				3	23	18	18
	CN (10 µg)	17	17	1	19	17	18
				2	19	17	17
				3	20	18	18
	TOB (10 µg)	17	17	1	18	18	17
				2	18	17	16
				3	19	17	18
	SXT (1.25 - 23.75 µg)	14	11	1	NZ	NZ	NZ
				2	NZ	NZ	NZ
				3	NZ	NZ	NZ

IPM – Imipenem; MEM – Meropenem; CIP – Ciprofloxacin; LEV – Levofloxacin; AK – Amikacin; CN – Gentamicin; TOB – Tobramycin; SXT – Trimethoprim-sulfamethoxazole.

Pseudomonas aeruginosa was able to survive the during-use concentrations of gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml; Table 4.3). The baseline susceptibility profile of *P. aeruginosa* showed that there was intermediate susceptibility to piperacillin/tazobactam, ceftazidime, imipenem, ciprofloxacin and aztreonam. The baseline profile also showed that there was clinical susceptibility to amikacin, tobramycin and meropenem (Table 4.6). After gramicidin (15 µg/ml) pre-exposure, 2/3 replicates became clinically resistant to imipenem and 1/3 replicate gained resistance to ciprofloxacin (Table 4.6). One out of three replicates also was clinically susceptibility to meropenem but would be clinically resistant to treatment in meningitis infections as the breakpoints for meningitis are different according the EUCAST clinical breakpoint tables (EUCAST, 2023). After bacitracin (5 IU/ml) pre-exposure, 2/3 replicates became clinically resistant to ciprofloxacin. After tyrothricin (200 µg/ml) pre-exposure, 1/3 replicate became clinically resistant to piperacillin/tazobactam (Table 4.6).

After pre-exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml), there was a statistically significant reduction in meropenem zone of inhibition ($p<0.05$; Table 4.6).

Table 4.6. *P. aeruginosa* disk diffusion results. Cross-resistance of *P. aeruginosa* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml). Green (🟢): zone of inhibition corresponding to clinical susceptibility; Yellow (🟡): zone of inhibition corresponding to intermediate susceptibility; Red (🔴): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).(*) – Clinical breakpoint for meningitis infections.

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)			
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure	After Tyrothricin (200 µg/ml) exposure
P. aeruginosa	TZP (30[6] µg)	50	18	1	26	23	22	25
				2	26	22	22	22
				3	26	24	22	NZ
	CAZ (10 µg)	50	17	1	26	18	21	24
				2	25	22	22	23
				3	25	25	22	22
	IPM (10 µg)	50	20	1	23	18	22	22
				2	24	17	23	21
				3	23	21	22	21
	CIP (5 µg)	50	26	1	32	27	25	28
				2	30	24	25	26
				3	30	28	26	26
	AK (30 µg)	15	15	1	24	21	20	21
				2	25	20	20	21
				3	24	21	19	20
	ATM (30 µg)	50	18	1	27	25	23	25
				2	26	24	23	25
				3	26	26	22	25
	TOB (10 µg)	18	18	1	23	21	20	22
				2	23	20	20	21
				3	22	22	20	21
	MEM (10 µg)	20	14 (*20)	1	30	22	26	23
				2	34	18*	26	24
				3	29	28	20	26

TZP – Piperacillin/Tazobactam; CAZ – Ceftazidime; IPM – Imipenem; CIP – Ciprofloxacin; AK – Amikacin; ATM – Aztreonam; TOB – Tobramycin; MEM – Meropenem.

Escherichia coli also grew in the during-use concentrations of gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml)(Table 4.3). The baseline susceptibility profile showed that *E. coli* was clinically susceptible to all the antibiotics tested in the panel (Table 4.7). After gramicidin (15 µg/ml) pre-exposure, 1/3 replicate became clinically resistant to cefotaxime and gentamicin and 2/3 replicates gained intermediate susceptibility to ciprofloxacin (Table 4.7). After bacitracin (5 IU/ml) pre-exposure, all replicates gained clinical resistance to ampicillin and 1/3 replicate gained clinical resistance to gentamicin and aztreonam. After tyrothricin (200 µg/ml) pre-exposure, again all three replicates gained clinical resistance to ampicillin and 2/3 isolates gained resistance to gentamicin.

There was a significant reduction in zone of inhibition of the ampicillin disk after bacitracin (5 IU/ml; $p=0.0014$) or tyrothricin (200 µg/ml; $p<0.0001$) pre-exposure. There was also a significant reduction in zone of inhibition of cefotaxime after gramicidin (15 µg/ml) pre-exposure ($p=0.0125$). Finally, there was a significant in aztreonam zone of inhibition after bacitracin (5 IU/ml) pre-exposure ($p=0.0204$) showing an increased resistance to these clinical antibiotics after OTC exposure. There were no other significant changes in zone of inhibition from the baseline susceptibility profile.

Table 4.7. *E. coli* disk diffusion results. Cross-resistance of *E. coli* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml). Green (🟢): zone of inhibition corresponding to clinical susceptibility; Yellow (🟡): zone of inhibition corresponding to intermediate susceptibility; Red (🟠): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).(*) – Clinical breakpoint for meningitis infections.

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)			
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure	After Tyrothricin (200 µg/ml) exposure
E. coli	AMP (10 µg)	14	14	1	20	15	NZ	NZ
				2	20	14	NZ	NZ
				3	20	14	11	NZ
	CTX (5 µg)	20	17 (*20)	1	29	24	26	27
				2	30	24	27	25
				3	29	NZ	27	26
	IPM (10 µg)	22	19	1	30	25	24	24
				2	31	25	27	22
				3	30	26	27	25
	CIP (5 µg)	25	22	1	34	24	30	29
				2	34	24	30	30
				3	35	29	31	26
	CN (10 µg)	17	17	1	18	17	17	15
				2	18	19	18	17
				3	18	NZ	NZ	NZ
	ATM (30 µg)	26	21	1	31	28	29	29
				2	32	28	NZ	29
				3	32	30	29	28
	C (30 µg)	17	17	1	22	25	26	20
				2	23	23	27	25
				3	25	24	23	23
	SXT (1.25 - 23.75 µg)	14	11	1	24	25	25	22
				2	25	25	22	25
				3	26	25	24	23

AMP – Ampicillin; CTX – Cefotaxime; IPM – Imipenem; CIP – Ciprofloxacin; CN – Gentamicin; ATM – Aztreonam; C – Chloramphenicol; SXT – Trimethoprim-sulfamethoxazole.

Enterobacter cloacae survived the during-use concentrations of gramicidin (15µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml)(Table 4.3). The baseline susceptibility profile of *E. cloacae* showed that it was clinically resistant to ampicillin (Table 4.8). There were 2/3 *E. cloacae* replicates that were intermediately susceptible to cefotaxime. There was also 1/3 replicates that had intermediate susceptibility to imipenem and 2/3 replicates that had intermediate susceptibility to aztreonam whilst the last replicate was clinically resistant to that antibiotic. After gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) pre-exposure, 3/3 replicates gained clinical resistance to cefotaxime, gentamicin and aztreonam. After exposure to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) all 3 replicates also gained intermediate susceptibility to imipenem. After tyrothricin (200 µg/ml) pre-exposure however, 2/3 replicates became clinically resistant to imipenem whilst the last replicate showed intermediate susceptibility to that antibiotic. After gramicidin (15 µg/ml) pre-exposure, 1/3 replicate became clinically resistant to ciprofloxacin and 1/3 replicate became clinically resistant to chloramphenicol. 2/3 replicate also gained intermediate susceptibility to ciprofloxacin and 1/3 replicate gained intermediate susceptibility to trimethoprim-sulfamethoxazole. After bacitracin (5 IU/ml) pre-exposure, 2/3 replicates also gained intermediate susceptibility to trimethoprim-sulfamethoxazole. After tyrothricin (200 µg/ml) pre-exposure, 1/3 replicate gained intermediate susceptibility to ciprofloxacin whereas all three replicates became intermediately susceptible to trimethoprim-sulfamethoxazole.

There was a significant reduction in the zone of inhibition of imipenem after gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) pre-exposure ($p<0.0001$). There was a significant reduction in zone of inhibition of ciprofloxacin after gramicidin (15 µg/ml) pre-exposure ($p<0.0001$) and of bacitracin (5 IU/ml) pre-exposure ($p=0.0473$). There was also a significant reduction in gentamicin zone of inhibition after gramicidin (15 µg/ml) pre-exposure ($p=0.0166$). There were significant reductions in zone of inhibition of aztreonam after gramicidin (15 µg/ml); $p=0.0078$), bacitracin (5 IU/ml; $p=0.0338$) or tyrothricin (200 µg/ml; $p=0.0239$) pre-exposure. There were no other statistically significant changes in zone of inhibition.

Table 4.8. *E. cloacae* disk diffusion results. Cross-resistance of *E. cloacae* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml). Green (●): zone of inhibition corresponding to clinical susceptibility; Yellow (●): zone of inhibition corresponding to intermediate susceptibility; Red (●): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).(*) – Clinical breakpoint for meningitis infections.

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)			
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure	After Tyrothricin (200 µg/ml) exposure
E. cloacae	AMP (10 µg)	14	14	1	NZ	NZ	NZ	NZ
				2	NZ	NZ	NZ	NZ
				3	NZ	NZ	NZ	NZ
	CTX (5 µg)	20	17 (*20)	1	18*	NZ	NZ	NZ
				2	19*	NZ	NZ	NZ
				3	20	NZ	NZ	NZ
	IPM (10 µg)	22	19	1	23	20	20	18
				2	22	20	19	19
				3	20	19	20	18
	CIP (5 µg)	25	22	1	31	24	25	24
				2	32	NZ	26	25
				3	32	24	26	26
	CN (10 µg)	17	17	1	18	NZ	15	16
				2	18	16	15	14
				3	18	15	16	16
	ATM (30 µg)	26	21	1	24	16	17	18
				2	20	15	15	16
				3	25	13	16	13
	C (30 µg)	17	17	1	21	18	18	18
				2	21	18	19	19
				3	21	15	19	19
	SXT (1.25 - 23.75 µg)	14	11	1	15	13	13	13
				2	16	14	13	13
				3	15	14	14	13

AMP – Ampicillin; CTX – Cefotaxime; IPM – Imipenem; CIP – Ciprofloxacin; CN – Gentamicin; ATM – Aztreonam; C – Chloramphenicol; SXT – Trimethoprim-sulfamethoxazole.

Klebsiella pneumoniae grew in the during-use concentrations of gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml)(Table 4.3). The baseline susceptibility profile showed that *K. pneumoniae* is clinically resistant to ampicillin, but susceptible to all the other antibiotics tested (Table 4.9).

After gramicidin (15 µg/ml) pre-exposure, *K. pneumoniae* only developed clinical resistance in 1/3 replicate to chloramphenicol but there were no other changes in clinical susceptibility. After bacitracin (5 IU/ml) pre-exposure, again 1/3 replicate gained clinical resistance to chloramphenicol but all three replicates gained intermediate susceptibility to imipenem (Table 4.9). After tyrothricin (200 µg/ml) pre-exposure, all three replicates gained clinical resistance to cefotaxime. 2/3 replicates gained clinical resistance to gentamicin and 1/3 replicate gained clinical resistance to chloramphenicol. There was also 1/3 replicate that gained intermediate susceptibility to aztreonam after tyrothricin (200 µg/ml) pre-exposure (Table 4.9).

There was a statistically significant reduction in cefotaxime zone of inhibition after bacitracin (5 IU/ml) pre-exposure ($p=0.0004$) and tyrothricin (200 µg/ml) pre-exposure ($p<0.0001$). There was also a significant reduction in imipenem zone of inhibition after bacitracin (5 IU/ml) pre-exposure ($p<0.0001$). After gramicidin (15 µg/ml) and bacitracin (5 IU/ml) pre-exposure, there were significant reductions in zone of inhibition of ciprofloxacin ($p=0.0039$ & $p=0.0019$). Only pre-exposure to tyrothricin (200 µg/ml) resulted in a statistically significant reduction of zone of inhibition ($p=0.0019$). There was also a reduction in zone of inhibition of aztreonam after bacitracin (5 IU/ml; $p=0.0080$) or tyrothricin (200 µg/ml; $p=0.0009$) pre-exposure. There were also significant reductions in the zone of inhibition of chloramphenicol after gramicidin (15 µg/ml; $p<0.0001$), bacitracin (5 IU/ml; $p<0.0001$) or tyrothricin (200 µg/ml; $p=0.0004$) pre-exposure.

Table 4.9. *K. pneumoniae* disk diffusion results. Cross-resistance of *K. pneumoniae* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml), bacitracin (5 IU/ml) and tyrothricin (200 µg/ml). Green (🟢): zone of inhibition corresponding to clinical susceptibility; Yellow (🟡): zone of inhibition corresponding to clinical susceptibility; Red (🟠): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).(*) – Clinical breakpoint for meningitis infections.

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)			
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure	After Tyrothricin (200 µg/ml) exposure
K. pneumoniae	AMP (10 µg)	14	14	1	NZ	NZ	NZ	NZ
				2	NZ	NZ	NZ	NZ
				3	NZ	NZ	NZ	NZ
	CTX (5 µg)	20	17 (*20)	1	29	24	22	NZ
				2	28	24	21	NZ
				3	26	25	22	NZ
	IPM (10 µg)	22	19	1	29	25	21	24
				2	28	23	19	24
				3	26	24	20	26
	CIP (5 µg)	25	22	1	33	27	25	29
				2	31	26	26	29
				3	30	26	27	27
	CN (10 µg)	17	17	1	21	21	17	14
				2	20	20	17	15
				3	21	19	17	17
	ATM (30 µg)	26	21	1	32	29	26	25
				2	31	29	27	27
				3	33	28	29	27
	C (30 µg)	17	17	1	23	22	21	20
				2	25	17	19	20
				3	24	12	12	14
	SXT (1.25 - 23.75 µg)	14	11	1	21	23	22	22
				2	22	23	16	20
				3	22	22	21	22

AMP – Ampicillin; CTX – Cefotaxime; IPM – Imipenem; CIP – Ciprofloxacin; CN – Gentamicin; ATM – Aztreonam; C – Chloramphenicol; SXT – Trimethoprim-sulfamethoxazole.

Haemophilus influenzae survived the during-use concentrations of both gramicidin (15 µg/ml) and bacitracin (5 IU/ml)(Table 4.3). The baseline susceptibility profile showed that it was susceptible to all the antibiotics tested in the panel (Table 4.10).

There were very few changes in clinical resistance after exposure to either of the OTC antibiotics. After gramicidin (15 µg/ml) pre-exposure, 1/3 replicate gained clinical resistance to ciprofloxacin and 1/3 replicate also gained intermediate susceptibility to trimethoprim-sulfamethoxazole. There were no changes in clinical susceptibility after bacitracin (5 IU/ml) pre-exposure (Table 4.10).

There was a statistically significant reduction in the zone of inhibition of imipenem ($p=0.0026$) and chloramphenicol ($p=0.0460$) after bacitracin (5 IU/ml) pre-exposure). The zone of inhibition for ciprofloxacin was also significantly reduced after gramicidin (15 µg/ml) pre-exposure ($p=0.0007$). There were no other significant changes in zone of inhibition after OTC antibiotic pre-exposure.

Table 4.10. *H. influenzae* disk diffusion results. Cross-resistance of *H. influenzae* to clinically relevant antibiotics after exposure to gramicidin (15 µg/ml) and bacitracin (5 IU/ml). Green (●): zone of inhibition corresponding to clinical susceptibility; Yellow (●): zone of inhibition corresponding to intermediate susceptibility; Red (●): zone of inhibition corresponding to clinical resistance. NZ = No zone. CB – Clinical Breakpoint. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).(*) – Clinical breakpoint for meningitis infections.

Bacteria	Antibiotic Disk	CB (mm)		Replicate	Zone of inhibition (mm)		
		S ≥	R <		Baseline	After Gramicidin (15 µg/ml) exposure	After Bacitracin (5 IU/ml) exposure
H. influenzae	TZP (30 – 6 µg)	27	27	1	33	30	29
				2	32	31	27
				3	33	31	30
	CTX (5 µg)	27	27	1	35	33	31
				2	33	33	30
				3	34	33	28
	IPM (10 µg)	20	20	1	34	32	27
				2	34	31	26
				3	30	31	22
	CIP (5 µg)	30	30	1	37	33	35
				2	33	15	33
				3	38	34	31
	TE (30 µg)	25	25	1	30	30	30
				2	30	29	29
				3	32	30	33
	C (30 µg)	28	28	1	37	30	33
				2	35	31	29
				3	34	33	28
	SXT (1.25 – 23.75 µg)	23	20	1	29	25	26
				2	29	22	25
				3	28	24	25
	RD (5 µg)	18	18	1	32	28	29
				2	31	27	27
				3	31	25	30

TZP – Piperacillin/Tazobactam; CTX – Cefotaxime; IPM – Imipenem; CIP – Ciprofloxacin; TE – Tetracycline; C – Chloramphenicol; SXT – Trimethoprim-sulfamethoxazole; RD – Rifampicin.

4.3.3. MICs of cross-resistance results after OTC pre-exposure

The clinical antibiotics for which clinically significant changes in susceptibility were recorded, were tested by microbroth-dilution (Table 4.11). Unfortunately, aztreonam and trimethoprim-sulfamethoxazole were not able to be tested due to interruptions in either manufacturing or shipping during the COVID-19 pandemic. During MIC testing by microbroth-dilution, there were fewer changes in clinical significance than during disk-diffusion testing (Table 4.12). No changes could be observed in clinical susceptibility to ciprofloxacin for *P. aeruginosa* as the concentration range tested was above the clinical breakpoints. To determine whether bacitracin affects ciprofloxacin resistance in micro-broth dilution testing, it would require re-testing at a lower concentration. After pre-exposure of *E. coli* to gramicidin (15 µg/ml), there was a clinically significant change in ciprofloxacin resistance. After tyrothricin (200 µg/ml) pre-exposure, *E. coli* cultures became clinically resistant to gentamicin (Table 4.11). *Klebsiella pneumoniae* gained clinical intermediate susceptibility to imipenem after bacitracin (5 IU/ml) pre-exposure and gained clinical resistance to gentamicin after tyrothricin (200 µg/ml) pre-exposure. *Enterobacter cloacae* was already clinically resistant according to the micro-broth dilution testing and remained resistant after pre-exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). However, there was clinical change for imipenem susceptibility from susceptible to intermediately susceptible after exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) (Table 4.11).

Table 4.11. MICs cross-resistances developed after OTC antibiotic exposure**.** The standard deviations are shown in brackets. Green (🟢): MIC corresponding to clinical susceptibility; Yellow (🟡): MIC corresponding to intermediate susceptibility; Orange (🟠): MIC corresponding to clinical resistance. Clinical interpretation according to EUCAST breakpoints (EUCAST, 2023).

Bacteria	Antibiotic	Minimum Inhibitory Concentration (µg/ml) (±SD)			
		Baseline	Gramicidin	Bacitracin	Tyrothricin
S. aureus	SXT	NT	NT		
A. baumannii	IPM	≤0.25(±0.00)	0.46(±0.29)		
	AK	2.00 (±0.00)		3.33 (±1.15)	
P. aeruginosa	IPM	0.75(±0.27)	1.17(±0.41)		
	CIP	≤0.25(±0.00)*		≤0.25(±0.00)*	
E. coli	CIP	≤0.25(±0.00)	0.46(±0.29)		
	AMP	4.00(±0.00)		6.67 (±2.31)	7.33(±1.15)
	CN	1.33(±0.58)			3.00(±1.00)
K. pneumoniae	IPM	1.00(±0.00)		2.50(±1.22)	
	CTX	0.25(±0.00)			3.33(±1.03)
	CN	0.58(±0.14)			1.08(±0.38)
E. cloacae	CTX	4.33(±0.58)	6.00(±2.00)	4.00(±0.00)	6.67(±2.31)
	IPM	0.67(±0.26)	2.17(±0.98)	2.33(±0.82)	3.67(±0.82)
	CN	0.92(±0.14)	1.67(±0.58)	2.00(±0.00)	2.00(±1.00)
	ATM	NT	NT	NT	NT
	CIP	≤0.25(±0.00)	≤0.25(±0.00)		
	SXT	NT		NT	NT

NT = Not tested. (*) = Unable to determine clinical significance. SXT: Trimethoprim-sulfamethoxazole; IPM: Imipenem; AK: Amikacin; CIP: Ciprofloxacin; AMP: Ampicillin; CN: Gentamicin; CTX: Cefotaxime; ATM: Aztreonam.

4.3.4. Summary of clinical changes in cross-resistance after initial OTC antibiotic pre-exposure

There were distinct differences in susceptibility profile when testing by a disk-diffusion method or by a micro-broth-dilution method (Table 4.12). Disk-diffusion testing showed many more changes in clinical susceptibility profiles when compared to micro-broth dilution testing. However, there are instances where the comparison between to two methods was not possible as sourcing the antibiotic powders for both trimethoprim-sulfamethoxazole and aztreonam during the pandemic meant that testing was unable to be carried out. There were many resistances to beta-lactam antibiotics such as ampicillin, imipenem, aztreonam and cefotaxime which changes could be seen by disk-diffusion whereas these changes were not apparent by microbroth dilution testing (Table 4.12).

Table 4.12. Summary of changes in clinical susceptibility with Disk-diffusion or Micro-broth dilution testing**.** The antibiotics entered in the table are ones that showed clinical changes in susceptibility according to EUCAST clinical breakpoints (2023). Where the box contains a (-), there were no clinical changes in clinical susceptibility. Where boxes are grey (), these combinations of organisms and OTC antibiotic were not tested. Antibiotic in bold showed agreement between the two protocols.

Bacteria	Gramicidin (15 µg/ml)		Bacitracin (5 IU/ml)		Tyrothricin (200 µg/ml)	
	Disk Diffusion	Micro-broth Dilution	Disk Diffusion	Micro-broth Dilution	Disk Diffusion	Micro-broth Dilution
S. aureus	SXT	-				
A. baumannii	IPM	-	AK	-		
P. aeruginosa	IPM	-	CIP	-		
E. coli	**CIP**	**CIP**	AMP	-	AMP, **CN**	**CN**
E. cloacae	CTX, **IPM**, CIP, CN, ATM	**IPM**	CTX, **IPM**, CN, ATM, SXT	**IPM**	CTX, **IPM**, CN, ATM, SXT	**IPM**
K. pneumoniae			**IPM**	**IPM**	**CTX**, CN	**CTX**

SXT: Trimethoprim-sulfamethoxazole; IPM: Imipenem; AK: Amikacin; CIP: Ciprofloxacin; AMP: Ampicillin; CN: Gentamicin; CTX: Cefotaxime; ATM: Aztreonam.

4.3.5. Stability of cross-resistance results after 1, 5 and 10 passages

The stability of resistance was measured by re-testing the susceptibility profiles by disk-diffusion testing after passaging in an appropriate broth. Disk-diffusion was the preferred method of choice as it was more-high through-put than MIC determination and it was better for testing beta-lactamase resistances (ISO,2020). In addition, all the antibiotics could be sourced from manufacturers. Stability was done by repeatedly passaging the culture with or without antibiotic after initial exposure and re-testing at passage 1, 5 and 10. Cultures were deemed as stable if they maintained resistance after initial exposure and kept their change in clinical susceptibility from the baseline without any further antibiotic exposure in at least two out of the three replicates tested.

Resistance to trimethoprim after exposure to gramicidin (15 µg/ml) in *S. aureus* was unstable after only one passage (Table 4.13). *Acinetobacter baumannii* had a stable resistance for up to 10 passages to amikacin after bacitracin (5 IU/ml) pre-exposure. However, resistance to imipenem was unstable after only one passage.

Resistance in *P. aeruginosa* to imipenem after gramicidin (15 µg/ml) pre-exposure was stable after 10 passages. Resistance to ciprofloxacin was not stable after 5 passages post-bacitracin (5 IU/ml) exposure (Table 4.13).

Resistance in *E. coli* to ampicillin was stable after bacitracin (5 IU/ml) and tyrothricin (200 µg/ml) pre-exposure after 10 passages. Gentamicin resistance was stable after tyrothricin (200 µg/ml) pre-exposure after 10 passages. However, ciprofloxacin resistance after gramicidin (15 µg/ml) pre-exposure was unstable after one passage (Table 4.13).

Klebsiella pneumoniae had stable resistance for 10 passages to both cefotaxime and gentamicin after tyrothricin (200 µg/ml) pre-exposure. Resistance to imipenem was only stable up to 5 passages after bacitracin (5 IU/ml) pre-exposure.

Enterobacter cloacae showed the most number of stable resistances (Table 4.13). Resistance to both cefotaxime and gentamicin was stable after 10 passages after pre-exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). Resistance to imipenem was also stable for 10 passages after pre-exposure to gramicidin (15 µg/ml) or bacitracin (5 IU/ml) and was stable after five passages after pre-exposure to tyrothricin (200 µg/ml). Resistance to aztreonam was stable for 10 passages after gramicidin (15 µg/ml) pre-exposure, five passages after tyrothricin (200 µg/ml) pre-exposure and only one passage after bacitracin (5 IU/ml) pre-exposure. Ciprofloxacin was unstable after one passage after gramicidin (15 µg/ml) pre-exposure, and trimethoprim resistance was unstable after one passage after either bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) pre-exposure (Table 4.13).

Table 4.13. Stability of resistance after passaging once, 5 times and 10 times without antibiotic. Stable resistance was determined if the passages kept their clinical resistance following testing. Stable: Green (●); Unstable: Red (●). Combinations not tested: grey (●).

Bacteria	Antibiotic	Gramicidin			Bacitracin			Tyrothricin		
		P1	P5	P10	P1	P5	P10	P1	P5	P10
S. aureus	SXT	Unstable	Unstable	Unstable						
A. baumannii	IPM	Unstable	Unstable	Unstable						
	AK				Stable	Stable	Stable			
P. aeruginosa	IPM	Stable	Stable	Stable						
	CIP				Stable	Unstable	Unstable			
E. coli	CIP	Unstable	Unstable	Unstable						
	AMP				Stable	Stable	Stable	Stable	Stable	Stable
	CN							Stable	Stable	Stable
K. pneumoniae	IPM				Stable	Stable	Unstable			
	CTX							Stable	Stable	Stable
	CN							Stable	Stable	Stable
E. cloacae	CTX	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
	IPM	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Unstable
	CN	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable
	ATM	Stable	Stable	Stable	Stable	Unstable	Unstable	Stable	Stable	Unstable
	CIP	Unstable	Unstable	Unstable						
	SXT				Unstable	Unstable	Unstable	Unstable	Unstable	Unstable

4.4. Discussion

4.4.1. OTC Antibiotic Fate

The during use concentrations of the OTC antibiotics (Table 4.2) were chosen from the highest amounts found within products sold in Europe. Of note, in the case of bacitracin, it is often used as bacitracin zinc. This not only has a different solubility to bacitracin but also a different biological activity. Therefore, although there were products that contained a higher amount of bacitracin zinc, the product with the highest amount of pure bacitracin was chosen for adjusting test concentrations (Table 4.2).

The test concentrations chosen were also based on the average amount of saliva produced per day. This can vary from 0.5 l – 1.5 l although there are also outliers within that population due to changes in salivation when eating or drinking (Iorgulescu, 2009). The amount of saliva produced for a single person can vary during the day so the concentrations found in saliva would vary depending on when they take a lozenge. The instructions are to let a lozenge slowly dissolve in your mouth which should take approximately 30 minutes, although this could be quicker for some people (Maheshwari, *et al.*, 2013). If people eat or drink after taking the lozenge the concentrations of the active ingredients would also drastically decrease. All these factors make it difficult to accurately adjust antibiotic concentrations that would be found within lozenges. Twenty mL of saliva was chosen as a starting concentration as this would represent the majority of people and further dilutions were chosen to reflect consumption of food or drink.

One further consideration is that OTC antibiotics found in sore-throat medications will travel further down the gastro-intestinal (GI) tract and potentially put pressure on the gut microbiome (Langdon, *et al*., 2016). It has already been demonstrated that antibiotics consumed orally can have detrimental effects on the gut microbiome. Antibiotic-led eradication of some species in the gut microbiome can lead to opportunistic infections such a *Clostridioides difficile* associated colitis (Demols, *et al*., 1996). Recent literature has linked the health and biodiversity of the gut microbiome population to other disease such as Parkinson's, obesity and dietary intolerances (Bosch, 2020). Exposure to oral antibiotics should be considered as there could be wider implications. The literature on the effect of OTC antibiotics on the gut microbiome is very limited. Dubos suggested in 1939, when it was first discovered, that tyrothricin precipitates out of solution at pH 4.5 and also becomes insoluble when digested with proteolytic enzymes although this did not appear to affect its bactericidal power (Dubos, 1939). Due to the pH changes and proteolytic enzymes

that the OTC antibiotics would encounter whilst in the stomach, it is likely that tyrothricin and perhaps gramicidin would become insoluble but maintain some activity (Dubos, 1939). It has been demonstrated that gramicidin is not inactivated by the stomach (Henderson, 1946). However, the study also stated that the large quantities of Gram-negative organisms in the intestines could quench the antibiotic activity (Henderson, 1946). This is likely due to gramicidin binding to the outer membranes of Gram-negative bacteria. Although, Henderson (1946) stated that under experimental conditions and with adequate amounts of gramicidin, there was still an effect on the Gram-positive flora in the microbiome. Overall, there could be an effect of these antibiotics on the microbiome but additional testing would be required for confirmation.

Further evidence of these antibiotics affecting the microbiome as was in a letter to an editor (Demols, *et al*., 1996). This letter by Demols, *et al*., (1996) described a clinical case of a 26-year old male who had been hospitalised with *C. difficile* associated colitis. The patient had no immunosuppression, had not travelled recently and had no other relevant medical history. The only medication he had been taking was Tyro-drops, a lozenge for local pharyngitis relief containing 1 mg of tyrothricin. The authors concluded that the tyrothricin is the likely etiological cause of the colitis and that clinicians should be asking patients with *C. difficile* colitis whether they have taken medications for oral pharyngitis relief. They also state that the occurrence of tyrothricin-induced pseudomembranous diarrhoea is likely to be underestimated.

Whilst there is evidence that some of these OTC antibiotics could be affecting the gut microbiome (Demols, *et al*., 1996), a review stated that tyrothricin is destroyed in the gastro-intestinal tract (Lang and Staiger, 2016). However this was not referenced in the review. Whilst it is unknown what effects OTC antibiotic consumption has on the gut microbiome, it has already been shown that gramicidin is not inactivated by the stomach (Henderson, 1946) and it has been more recently questioned whether tyrothricin could disturb the bacterial flora (Palm, *et al*., 2018). More research is needed to determine what effects OTC antibiotics has on gut microbiome diversity and the development of cross-resistance genes.

4.4.2. Cross-resistance Development

Of the bacterial panel tested, it was mainly Gram-negative bacteria that could survive the during-use concentration of OTC antibiotics (Table 4.3). However, none of the bacteria tested could survive exposure to neomycin even at realistically low concentrations (Table 4.3). Gram-negative bacteria are already resistant to these OTC antibiotics but the presence of the antibiotics in the environment can still exert a pressure on the bacteria (Larsson and Flach, 2022). When bacteria are put under pressure, this can cause the activation of resistance genes or changes in bacterial phenotype that can lead to cross-resistance (Tumah, 2009; Poole, 2012 Maillard, 2018; Adkin*, et al*., 2022). The main cross-resistance that emerged after pre-exposure to either gramicidin, bacitracin or tyrothricin was to beta-lactams and aminoglycosides (Table 4.12). Interestingly, when testing the resistance profiles via disk diffusion testing compared to micro-broth dilution testing, there were different clinical interpretations of resistance (Table 4.12).

When testing the MIC by broth micro-dilution, there were no statistically significant changes in MIC ($p>0.05$), although some replicates of the MICs went above a clinically significant threshold. This is because the values are close to the clinical breakpoint. This could be due to the 2-fold dilution of the antibiotic and therefore causing a high variance for low numbers. It is also worth noting that, although it appears there is not much of a change in MIC after 18h ±2h incubation, when the bacteria were incubated for longer (48 h) the exposed bacteria could grow in higher concentrations (results not shown). It is possible that after OTC exposure, bacterial growth rate decreases affecting turbidity and thus determination of MIC after 18 ± 2 h, whilst longer growth time would enable growth in higher bacterial concentrations and higher turbidity. This may then be an issue with MIC via broth microdilution testing. However, when comparing this to a disk diffusion method, the change in antibiotic concentration through the agar is much more gradual and therefore results interpretation is clearer even with small changes in the ZOI. The use of a test based on broth micro-dilution may therefore demonstrate that an antibiotic is effective based on a set incubation time although this may not be the case, overall affecting clinical interpretation.

4.4.3. Clinical Relevance of Resistance

Cross-resistance developed as a result of exposure to the OTC antibiotics may be of clinical concern. Here identified cross-resistance were mainly observed in Gram-negative bacteria and to aminoglycoside and beta-lactam antibiotics. Gentamicin is mainly used to treat Gram-negative bacilli including *E. coli*, *E. cloacae* and *K. pneumoniae* (Krause, *et al*., 2016) notably to treat serious bacteraemia, complicated urinary tract infections and endocarditis (Gonzalez and Spencer, 1998).

We observed cross-resistance to many beta-lactam including ampicillin, imipenem, aztreonam and cefotaxime (Table 4.12). Ampicillin is a widely used antibiotic and is especially important in certain clinical settings such as dentistry, where structurally similar antibiotics such as amoxicillin are commonly used (Roda, 2007). Penicillins, particularly amoxicillin, are is especially relevant to sore throat medicines as they can be used to treat respiratory tract infections (Zoorob, *et al.,* 2012).

Resistance to the 3rd generation cephalosporin, cefotaxime, was also seen in *E. cloacae* and *K. pneumoniae*. Cephalosporins are broad-spectrum antibiotics and use to treat both community- and hospital-acquired pneumonia (Lupia, *et al.*, 2020). Resistance development to this class of antibiotics can make lower-respiratory tract infections difficult to treat. Cefotaxime is also used for treating meningitis, especially in emergency treatments with patients allergic to benzylpenicillin (BNF, 2023) and failure of this emergency treatment due to resistance could be life-threatening.

Resistance to aztreonam, a monocylic beta-lactam antibiotic, was observed in *E. cloacae* and it was stable after multiple passages (Table 4.13). Aztreonam is an effective antibiotic for treating aerobic Gram-negative infections (BNF, 2023). With the rise of antibiotic resistance such as carbapenem resistance, aztreonam is being reconsidered for a wider therapeutical use as they are stable against Ambler class B metallo-beta-lactamases (Ramsey and MacGowan, 2016). Although it is currently mainly used for meningitis, it can be used for lower-respiratory tract infections (Brewer and Hellinger, 1991).

Possibly some of the most concerning resistance development observed in our study, is the gain of clinical resistance to imipenem. This is a carbapenem antibiotic which are broad-spectrum and are resistant to hydrolysis by most beta-lactamases. Due to these features, carbapenems are considered as ‘last-line’ antibiotics and are used to treat patients with antibiotic resistant bacteria. The emergence of multi-drug resistant bacteria is already a

threat to the effectiveness of this class of antibiotics (Queenan and Bush, 2007; Indrajith, *et al*., 2021).

OTC antibiotics are used in the treatment of sore-throats, and as such it is likely that bacteria causing these infections will come into contact with these antibiotics if a patient is taking them. Cross-resistance developed to the various antibiotic classes as a result of OTC antibiotic exposure could cause difficulties in treating upper and lower respiratory tract infections.

4.4.4. Stability of Resistance

The development of resistance is of concern but the fact that most of the antibiotic resistance observed were stable is more concerning as this could indicate a genetic change (Munita and Arias, 2016). Antibiotic clinical susceptibility changes have arisen due to the environmental stresses exerted following bacterial exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). External stresses can change multiple phenotypic characteristics including resistance phenotypes in bacteria (Foster, 2005).

Exposure to OTC antibiotics could be evoking stress responses in bacterial that can lead to these resistance phenotypes (Poole, 2012). Bacterial stress response is highly regulated and under conditions of stress, such as antibiotic exposure, gene regulation can change. Gene regulation is often activated by an energy stress which can modulate genes such as the *rsbU* genes (Abee and Wouters, 1999). The mechanisms of action of gramicidin, bacitracin and tyrothricin are likely to cause energy stresses, as the disruption of the cell membrane can cause disruptions in ATP production. Once stress genes have been activated this can cause a change in the regulation of stress polymerases such as RpoS (Schellhorn, 2020). These polymerases allow bacteria to cope with a large variety of environmental stresses as they control many other genes involved in stress response (Dawan and Ahn, 2022). These are involved in a range of phenotypic characteristics such as cell morphology, cell division or metabolism. The activation of these stress polymerases also has other evolutionary advantages such as mistranslation (Samhita, *et al*., 2020). This adds errors in the bacterial genome and can lead to beneficial mutations. These mutations could allow the bacteria to overcome environmental stresses such as antibiotic exposure (Schmutzer and Wagner, 2023).

The stability of the resistances could indicate that the mutational changes create a benefit for the bacteria but does not result in a dramatic fitness-cost for the bacteria. Whilst these mutations could just be changing the regulation of already present resistance genes, a concern could be the development of novel resistance genes in bacteria, but this would need to still be investigated in future research (Hawkey, 1998).

4.5. Chapter Conclusions

Of the four OTC antibiotics sold throughout Europe, only neomycin was highly effective even at low realistic concentrations. Of the bacteria tested, mainly Gram-negative could survived the during-use concentrations of gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). The key antibiotics of concern with resistance development following OTC antibiotic pre-exposure are gentamicin and beta-lactam antibiotics including penicillins, cephalosporins, carbapenems and monobactams. Clinical resistance developing in Gram-negative bacteria such as *Enterobacterales*, *Acinetobacter* and *Pseudomonas* spp. is of serious concern. Most antibiotic clinical resistances were stable, especially in *Enterobacterales* (particularly *E. cloacae*). Stable resistances could result in the spread of resistance with implication for treatment.

Chapter 5: Phenotypic and Genotypic Changes after OTC Antibiotic Exposure

Chapter 5: Phenotypic changes after Pre-exposure to OTC antibiotics

5.1. Introduction

5.1.1. Bacterial Stress Responses to Antibiotics

5.1.1.1. Overview of bacterial stress response

There are many environmental factors that bacteria have to overcome to be able to survive including; nutrient starvation, oxidative stress, membrane damage, temperature or ribosome disruption. These stresses can be caused by competition with other organisms in the environment, heavy metal ions or antimicrobials (Poole, 2012). These factors can cause stress in bacteria that elicit a wide variety of highly regulated responses that have evolved over time to protect bacteria (Dawan and Ahn, 2022).

5.1.1.2. Gene regulation in stress response

Gene regulation in the bacterial stress response is highly regulated. The classical example of bacterial stress response is the stringent response which occurs due to amino acid deprivation (Boutte and Crosson, 2013). The limited nutrient availability causes an increase in alarmones, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Irving, 2021). These alarmones control multiple functions within the cell such as physiology and metabolism (Irving, 2021). The alarmones also activate alternative sigma factors such as RpoS and RpoH. Both of these sigma factors can then compete with the vegetative sigma factor, RpoD (Foster, 2005). The different sigma factors then cause a change in transcription rate and which specific set of genes are transcribed (Paget, 2015). This causes a higher rate of mutations to be introduced into bacterial DNA and can lead to bacteria to evolve and adapt to the environment, such as the development of resistance to antibiotics (Woodford and Ellington, 2007).

5.1.1.3. The role of stress response in antibiotic resistance

Antibiotics within the environment can cause stresses in bacteria. As they have different mechanisms of action, antibiotics can cause a diverse range of stresses in bacteria (Munita, 2016). It is the adaptive response to these stresses that can cause bacteria to not only survive specific antibiotics but also cross-resistances to other antibiotics (Milisav, *et al*., 2012). This generally occurs when there are responses that are designed to cover a broad range of antimicrobial compounds such as changes to the cell membrane, efflux pumps or changes into the bacterial growth state, such as biofilm formation (Poole, 2012). Whilst

these responses are important in bacteria initially tolerating the exposure to antibiotics, it can also promote the emergence of novel resistance genes and therefore persistence of resistance in the bacterial populations (Alnahhas and Dunlop, 2023). This is due to the genetic changes that occur when bacteria are stressed and activate alternative polymerases that increases the rate of mutations in the bacterial DNA sequence. This can lead to advantageous mutations enabling antibiotic exposure survival (Loh, *et al*., 2010). This allows novel resistance gene mutations to develop and eventually spread.

5.1.2. Phenotypic changes associated with Antibiotic Resistance

5.1.2.1. Role of Growth in Antibiotic Resistance

Bacterial growth is made of four different phases: the lag phase, the exponential phase, the stationary phase and the death phase (Wang, *et al*., 2015). Bacterial growth is often important for the mechanism of action of many antibiotics (Stokes, *et al*., 2019). It has been proven many times that antibiotics preferentially kill bacteria that are replicating (Brauner, *et al*., 2016). Therefore, a common mechanism bacteria use to tolerate antibiotic pressure is the reduction in growth rate. This not only reduces the activity of antibiotics in bacteria but also has the effect of lowering metabolism and therefore the conservation of energy within the cell (Bertrant, 2019). Previously, it has been demonstrated that during growth of the bacterial population, extension of the lag phase is not only important in protecting bacteria but can also promote the regrowth of the bacterial population after removal of the antibiotic stress (Li , 2016). Growth rate can also be affected in bacteria by the activation of antibiotic resistance genes by causing a fitness cost of nutrients and thus lowering the growth rate (Melnyk, *et al*., 2015).

5.1.2.2. Role of Efflux in Antibiotic Resistance

Efflux is extremely important for the survival of bacteria when faced with toxic substances. Efflux pumps allow bacteria to regulate their internal environment and remove any substances that could be toxic to the bacterial cell (Soto, 2013). There are different types of efflux pump that can force out different antibiotics and, in many cases a single efflux pump has multiple substrates (Chapter 4; Figure 4.2). Efflux pumps play a pivotal role in antibiotic resistance as they are one of the key mechanisms that bacteria can express to adapt to a new environment quickly (Ebbensgaard, *et al*., 2020). This is because the selective pressure exerted on bacteria by compounds such as biocides, heavy metals or antibiotics, can cause an over expression of efflux pumps which allow bacteria to tolerate otherwise lethal concentrations of these compounds (Blanco, *et al*., 2016). Whilst initial over expression of

efflux pumps does not necessarily mean an increase in MIC (Piddock, 2006), the tolerance or low-level of resistance in bacteria can progress to full resistance to an antibiotic. This is because although treatments are often given at concentrations well above the MIC of the antibiotics, the concentrations *in vivo* vary and the over-expression of efflux in some environmental niches may allow bacteria to survive treatment (Andersson, *et al*., 2020). This survival also creates the opportunity for mutations to occur in the bacteria and a resistant population can then flourish under the selective pressure of any further antibiotic treatment (Emara, *et al*., 2023). Therefore, it is important to understand whether treatments such as OTC antibiotics cause an over-expression of efflux in bacteria as this can allow populations of bacteria to tolerate other clinically used antibiotics and possibly develop full resistance to them.

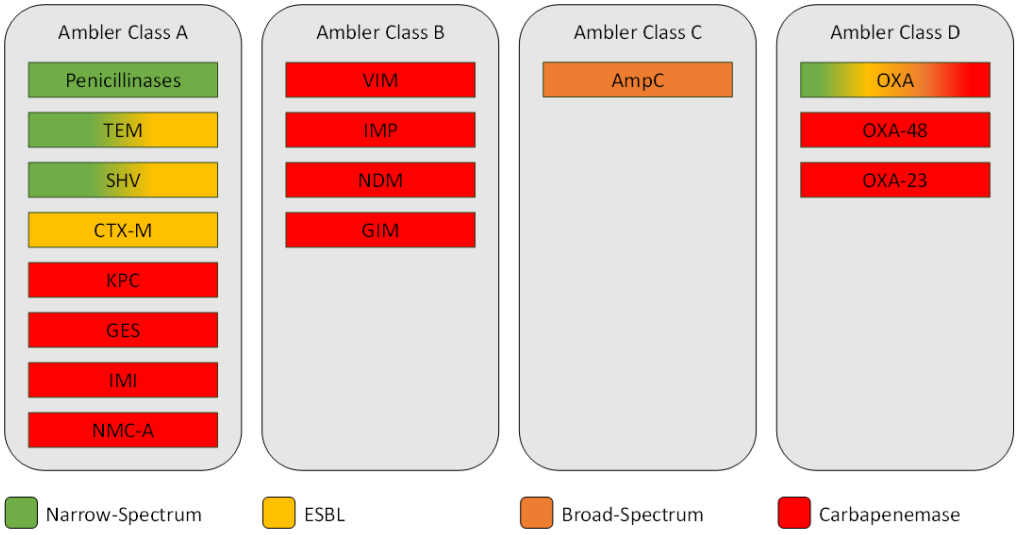
5.1.2.3. Virulence changes in Antibiotic Resistance

Bacterial virulence is the ability of bacteria to be able to enter and damage a host organism (Webb and Kahler, 2008). Virulence is dictated by a multitude of genes known as virulence factors (Sharma, *et al*., 2017). The relationship between virulence and antibiotic resistance is extremely complicated and was previously thought that the development of antibiotic resistance came at a fitness cost to bacteria and therefore have lowered virulence *in vivo* (Andersson and Levin, 1999). However, more recent studies have shown that the acquisition of resistance genes does not necessarily pose a fitness cost to the bacteria and therefore lowered virulence, but is actually often increasing virulence (Beceiro, *et al*., 2013). This is thought to be due to the complex nature of antibiotic resistance and virulence gene regulation. Both antibiotic resistance and virulence are highly regulated in bacteria and often the regulation pathways for both are intertwined (Schroeder, *et al*., 2017). Therefore, the expression of antibiotic resistance genes often influences the expression of virulence genes and *vice versa*. This is particularly observed when either resistance and virulence genes occur on the same plasmid, or when bacteria are in a biofilm state and communicate through either quorum-sensing or two-component systems (Schroeder, *et al.*, 2017). As both antibiotic resistance and virulence evolve concurrently, we must also aim to stop the spread of increased virulence in bacterial populations. Thus, it is important to understand how OTC antibiotics may be affecting virulence in bacteria as this could be both spreading any antibiotic resistance genes and also assist in the development of increased virulence.

5.1.2.4. Enzyme Regulation and Antibiotic Resistance

A common mechanism of antibiotic resistance used is antibiotic inactivation (Reygaert, 2018). This is done by enzymes either cleaving or modifying the antibiotic and making it become inactive (Wright, 2005; Ramirez and Tolmasky, 2010; Wilson, 2013). There are four broad classes of enzymes that inactivate antibiotics: hydrolases, transferases, redox enzymes and lysases (Wright, 2005). The most famous example of antibiotic inactivation by enzymes are the hydrolases that cleave the beta-lactam ring, beta-lactamases. This group of enzymes can disrupt the biological activity of antibiotics such as penicillins, cephalosporins, monobactams or carbapenems, (Poole, 2004). There are four different classifications of beta-lactamases depending on their mechanism of action (Noster 2021; Figure 5.1).

Figure 5.1. Ambler Classification of Beta-lactamases in *Enterobacterales*. Class A, C and D are serine active site hydrolases whereas Class B uses metal zinc as an active center. (Figure adapted from Noster 2021.)



Worryingly, beta-lactamases can confer broad-spectrum resistance to other beta-lactam antibiotics and are rapidly spreading globally (Hammerum, *et al*., 2010; Bush and Bradford, 2020). More recently, there has been a rise in carbapenemases that can inactivate some of our most important clinical antibiotics, the carbapenems (Halat and Moubareck, 2020). Whilst many of these beta-lactamases are constitutively expressed at low levels, over-expression may be induced by damage to the bacterial cell wall (Harris and Ferguson, 2012). This often occurs as a result of bacteria coming into contact with beta-lactam antibiotics.

5.1.2.5. Morphological Changes in Antibiotic Resistance

During antibiotic stress, bacteria can change their morphology to assist in the adaptation to the stress (Monahan, *et al*., 2014), although the role of cell morphology in antibiotic resistance is still poorly understood. It is currently thought that bacteria may change their cell morphology as an attempt to decrease the intracellular concentrations of antibiotics to a sub-lethal level (Banerjee, *et al*., 2021). This can be achieved by changes to the cell surface to volume ratio (S/V). For reducing intracellular concentrations of antibiotics, it is best for the bacteria to reduce the S/V. This is also commonly seen with an over-expression of efflux pumps and the reduction in porin expression (Ojkic, *et al*., 2022). However, for those agents which are membrane active, the best morphological change is an increase in S/V. This is because for membrane bound antibiotics, an increase in surface area of the cell would effectively decrease the antibiotic surface density (Ojkic, *et al.*, 2022). This could prevent lysis in bacteria and allow the bacteria to tolerate the antibiotic treatment. Whilst morphology can play a role in antibiotic resistance, it is often not the only phenotypic change and often works synergistically with porin expression and efflux activity. It is not known what effects the exposure of OTC antibiotics may have on the change of bacterial morphology although as they are membrane active agents, they may cause increases in S/V.

5.1.3. Evolution of Bacterial Resistance

Antibiotic resistance is a natural phenomenon with a plethora of antibiotic genes being found a 10,000-year-old microbial community found in the permafrost (Kashuba *et al*., 2017). Although this does occur naturally, the rate at which antibiotic resistance is spreading in the modern-day era of medicine is unnatural. This is due to the copious amounts of antibiotics that are used by humans and creates a selective pressure for bacteria that have resistance genes to survive and flourish (Tenover, 2006). The concern is not only the spread of resistance, but the spread of multi-drug resistance which could culminate in infections being untreatable by the current antibiotics at our disposal (Boucher, 2009).

Antibiotic resistance can be acquired through mutations in the bacterium's own genome or can be through horizontal transfer by MGEs such as plasmids (Munita and Arias, 2016). For the spread of novel resistances, and known resistances, horizontal transfer allows the gene to mobilise away from a single clonal strain and become widespread (Bengtsson-Palme, *et al.*, 2018). Different factors can play a role in the mobilisation of genes, but this can be induced by environmental stresses such as heavy metals, biocides, and antibiotics (Pal *et*

al., 2015). When these genes are integrated into other bacteria, they often come at a fitness cost however, the continued selection pressure within the bacteria's environment can help establish the gene within the host (Bjorkman and Andersson, 2000).

5.1.4. Chapter Aims

The main aim of this chapter is to understand the phenotypic and genotypic changes associated with pre-exposure to OTC antibiotics. This will be achieved by analysing changes seen in bacteria after OTC antibiotic pre-exposure such as growth rate, efflux pump activity, virulence, beta-lactamase activity, changes in morphology, metabolomic changes or mutational changes. Phenotypic and genotypic changes will be explored in Gram-negative ESKAPE pathogens, particularly *Enterobacter cloacae.* This is due to these organisms showing the greatest changes in clinical cross-resistance after OTC antibiotic exposure (Chapter 4).

5.1.5. Principle of Experiments and Rationale

There are many phenotypic and genotypic changes that can occur in bacteria after exposure to environmental stresses such as antibiotics. By measuring phenotypic changes such as growth rate, efflux pump activity, virulence, enzyme activity, morphology or metabolomic changes, we can begin to understand how resistance occurs and how the bacteria are adapting due to pre-exposure of OTC antibiotics. This is important to understand these changes and the role they play in antibiotic resistance. It is also important to understand genetic changes that can occur due to pre-exposure of OTC antibiotics in bacteria and whether this could result in evolutionary changes that lead to the emergence of antibiotic resistance.

5.2. Methods

5.2.1. Growth rate testing

5.2.1.1. Culture preparation

Cultures were pre-exposed to OTC antibiotics as described in Chapter 4, Section 4.2.1. The cultures were then washed by centrifuging the bacteria at 3,000 xG for 10 minutes at 20°C. Supernatant was removed and pellet was resuspended in 5 ml of MHB. The washed cultures were then adjusted with MHB to an OD_{625} of 0.08 – 0.15 which equates to a bacterial inoculum of approximately 1 x 10^8 CFU/ml. The cultures were diluted 100-fold in MHB to 1 x 10^6 CFU/ml. Bacterial viability in culture were enumerated as previously (Chapter 3, Section 3.4) to check the correct concentration.

5.2.1.2. Plate inoculation and Plate reader set-up

To each of the wells in a flat-bottomed 96-well plate, 50 µl of sterile distilled water was added. Then 50 µl of each bacterial inoculum was added to the corresponding wells to give a final bacterial concentration of 5 x 10^5 CFU/ml. Control wells containing 50 µl of sterile distilled water and 50 µl MHB were used as a control for contamination and background absorbance. The plate reader was set to a temperature of 37°C. An absorbance of 600nm was read every 15 minutes for a total of 48 hours with orbital shaking of 4 mm for 3 seconds being done before every reading.

5.2.1.3. Data analysis

Growth curves were analysed using the 'Growthcurver' package in R (Sprouffske and Wagner, 2016). The growth curves were firstly plotted in Excel. The growth curves were then modelled and analysed by 'Growthcurver' by using the command 'SummarizeGrowthByPlate'. The growth curves were blanked using the default settings and the data was trimmed to 20 hours to ensure the growth curves were comparable. After fitting a logistic curve to the growth curve data, the resulting output data was analysed using a two-way ANOVA and a Dunnett's multiple comparisons test in GraphPad Prism (GraphPad Prism version 9.5.1. for Windows).

5.2.2. Efflux pump activity assay

5.2.2.1. Bacterial culture preparation

Bacterial cultures were prepared by firstly washing the bacteria by centrifuging at 3,000 xG for 10 minutes at room temperature, discarding the supernatant and resuspending the pellet in 5 ml PBS. The cultures were then adjusted in 5 ml fresh MHB to an OD_{600} of 0.2. The cultures were incubated for 3 hours shaking at 150 rpm at 37°C until they reached mid-log phase (OD_{600} of 0.6 – 0.8). After incubation, bacteria were washed again by centrifuging the culture at 3,000 xG for 10 minutes at room temperature and the supernatant was discarded. The pellet was then resuspended in 10 ml PBS to give a final culture density of approximately OD_{600} of 0.4. Bacterial cultures of *A. baumannii*, *E. coli*, *E. cloacae* and *K. pneumoniae* were pre-exposed to OTC antibiotics as described previously (Chapter 4, Section 4.2.1).

5.2.2.2. Preparation of Stock solutions and Controls

A positive control of boiled cells was used. This was done by taking 1 ml of prepared suspension (~5 x 10^8 CFU/ml) and adding to a microcentrifuge tube and placing in a dry bath at 95°C for 10 min. A PBS only control was used to measure background fluorescence.

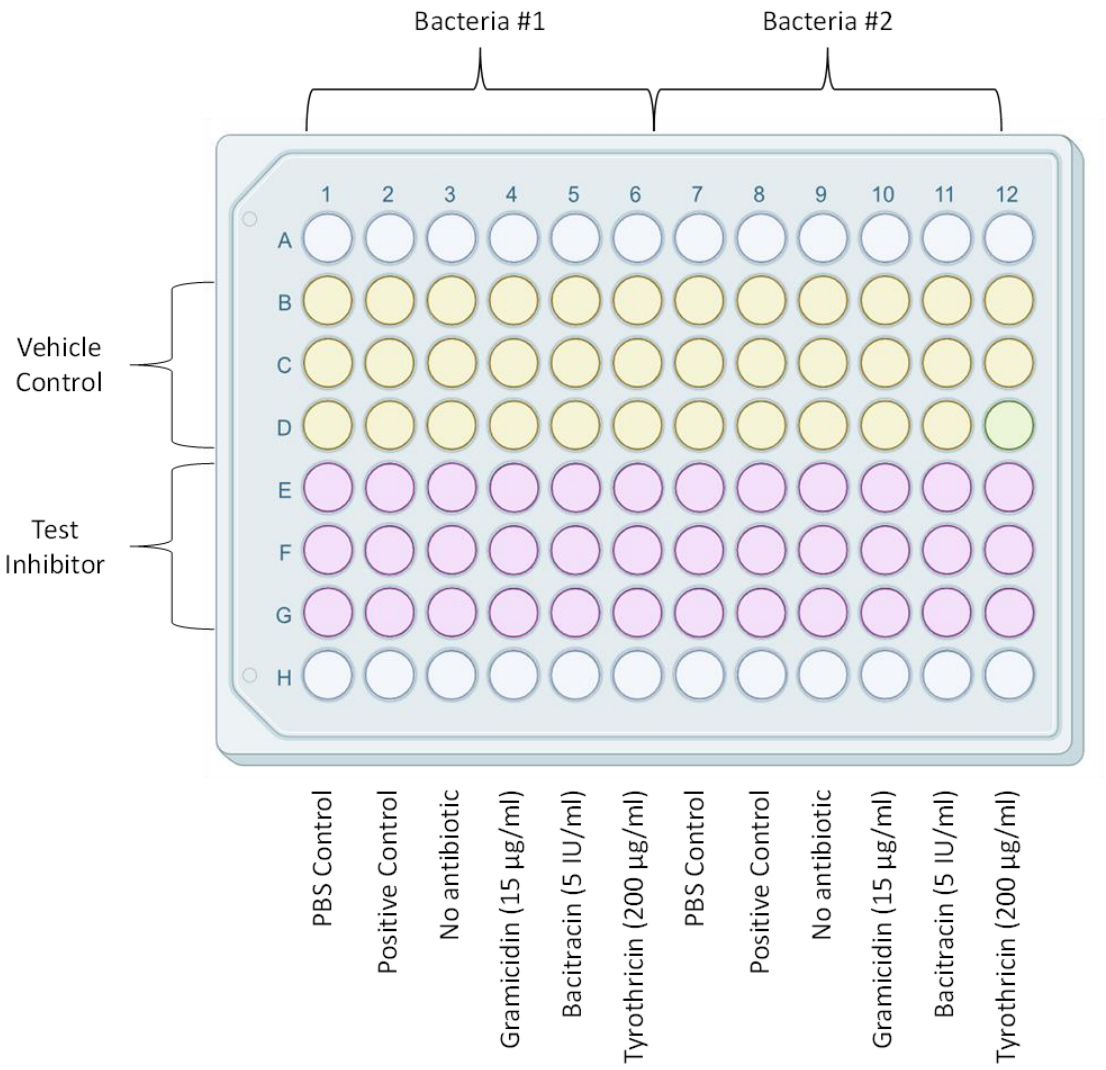
The test uses ethidium bromide as a measurement for efflux activity. A 10 mg/ml ethidium bromide stock solution was made in water. Two microlitres of ethidium bromide stock was added to 998 µl of water to result in a final solution of 0.02 mg/ml equating to a concentration of 0.005 mg per well. An efflux pump inhibitor, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used to stop proton-led efflux pump activity. A stock solution of 1 mg/ml CCCP was made in DMSO. Two hundred microlitres of this stock was added to 1,800 µl of PBS to make the test inhibitor solution of 0.1 mg/ml. A vehicle control of DMSO added to PBS was also used.

5.2.2.3. Test Method and Plate reader parameters

Before starting the test, an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) was set to a temperature of 37°C. The vehicle control and test inhibitor (CCCP) were also added to injector bottles A and B respectively. Both injectors were primed with 700 µl of their respective solutions. To a black opaque 96-well plate, 100 µl of the PBS control, positive control (boiled cells) and test suspensions were added in triplicate for both the vehicle control and test inhibitor (Figure 5.2). Fifty microlitres of the ethidium bromide solution was then added to each well. The plate was then immediately added to the plate reader and read for 10 minutes with a reading being taken every 2.5 minutes at 530_{ex} 590_{em} with

shaking being carried out before each reading. After the first 10 minutes, 50 µl was injected into wells from solution A or B with shaking being done after injection. The fluorescence was read for a further 50 minutes.

Figure 5.2. Plate layout for Efflux pump assays.



5.2.2.4. Data analysis

After acquiring the fluorescence absorbance readings, the data was plotted in MS Excel to ensure there were no anomalies. The background fluorescence was then removed using the mean of the background fluorescence control. The data was then trimmed to include only those readings after injection, and the change in fluorescence after injection was plotted by adjusting the data relative to the first reading after injection in each well. The area under the curve (AUC) of each condition was calculated in GraphPad Prism (GraphPad

Prism version 9.5.1. for Windows). The mean of the net area and standard error of mean for each condition per bacteria was analysed by two-way ANOVA and a Dunnett's Post-hoc test was used to compare each treatment to the untreated control.

5.2.3. Virulence testing with *Galleria mellonella*

5.2.3.1. Culture preparation

Bacterial cultures of *A. baumannii*, *E. coli*, *E. cloacae* and *K. pneumoniae* were pre-exposed to OTC antibiotics as previously described (Chapter 4, Section 4.2.1). The cultures were then centrifuged at 3,000 xG for 10 minutes and 20°C. The pellets were then washed in PBS three times and the final pellet was suspended in 5 ml PBS. After washing, the bacterial cultures were adjusted to OD_{625} at a value of 0.08-0.15, corresponding to a bacterial titre of approximately 1 x 10^8 CFU/ml. The bacteria were then diluted in ten-fold dilutions in PBS to reach the desired injection titre. The viable counts were done as described previously (Chapter 3, Section 3.4) to ensure the correct tire of the bacterial inoculum.

5.2.3.2. Maintenance and injection of Galleria mellonella

Galleria mellonella larvae were used as an infection model to estimate bacterial virulence (Wand, *et al*., 2011). *Galleria mellonella* larvae (TruLarv, Biosystems, UK) were raised on an antibiotic free medium. The larvae were kept in the dark at 4°C for a maximum of one week before testing to avoid the development of larvae into pupae. Before injecting, the *Galleria* were separated into groups of 10 individuals into 90 mm diameter sterile petri dishes, with a cellulose filter paper, and allowed to reach room temperature before injection. Each group of 10 was used for one biological replicate of bacterial culture.

Galleria mellonella larvae were injected in the last left proleg using a 50 µl Hamilton GASTIGHT syringe with a Polytetrafluoroethylene (PTFE) luer lock (26202, Hamilton, Nevada, USA) and a 25 gauge needle (300600, Becton Dickinson, New Jersey, USA). The needle was changed every 5 injections to maintain sharpness and therefore minimize trauma to the larvae. The larvae were injected with 10 µl of bacterial cultures containing approximately 1 x 10^5 CFU. Before the first injection, the syringe was rinsed three times in 70% (v/v) ethanol and three times in sterile distilled water. The syringe was also rinsed this way in between infections with different bacterial conditions. Controls of untreated larvae, stabbed larvae, larvae injected with PBS and a positive control of DMSO were used. This was done to ensure that the larvae did not die due to natural causes, the trauma of stabbing or injection and that the injected material entered the larvae.

5.2.3.3. Galleria mellonella incubation and monitoring

After injection, the *Galleria mellonella* larvae were incubated at 37°C and the survival of the larvae in each petri dish was monitored every 24 hours for a total of 7 days. Larvae that had died usually become darker in colour but this is not always the case. To ensure the larvae were dead, they were gently stimulated using a sterile pipette tip and if they did not move, they were considered as dead. Any dead larvae were removed from the petri dish as to not further contaminate any other larvae in the petri dish.

5.2.3.4. Statistical analysis

All experiments were performed in technical replicates of 10 larvae and in biological triplicate of three bacterial cultures. The mean and standard deviation for the survival of *Galleria mellonella* larvae of each bacterial condition was determined using GraphPad Prism (GraphPad Prism version 9.5.1. for Windows). Statistical analysis was done by two-way ANOVA with a Dunnett's Post-Hoc test.

5.2.4. Beta-lactamase production

5.2.4.1. Pre-test set-up

Beta-lactamase activity was measured using nitrocefin which gives a colorimetric output when cleaved by beta-lactamases (ab197008, Abcam, Cambridge, UK). *Enterobacter cloacae* was pre-exposed to OTC antibiotics as previously described (Chapter 4, Section 4.2.1). Before performing the assay, the positive control from the kit (ab197008) was reconstituted using 20 µl of beta-lactamase buffer (ab197008).

5.2.4.2. Standard curve preparation

Firstly, a standard curve was prepared using hydrolysed nitrocefin. This was done by adding 8 µl of nitrocefin (Supplied from the Kit, ab197008) to 16 µl of beta-lactamase hydrolysis buffer (Supplied from the Kit, ab197008) and 56 µl of DMSO in a microcentrifuge tube. This mixture was incubated in a dry bath at 60°C for 30 minutes. The reaction was then cooled to room temperature and centrifuged briefly. Using the 2 nM hydrolysed Nitrocefin stock solution, a standard curve was prepared to give an end concentration of 0, 4, 8, 12, 16, 20 nmol/well. Each dilution of the standard curve was done in duplicates (2 x 100µl).

5.2.4.3. Sample Preparation

Microcentrifuge tubes were pre-weighed and labelled. One milliliter of the bacterial samples (~1 x 10^9) that were either pre-exposed to OTC antibiotic or not, were added into microcentrifuge tubes and were centrifuged at 10,000 xG for 10 minutes. The supernatant was carefully removed and the tubes were reweighed to determine the wet weight of the

bacterial pellet. The pellet was resuspended in five microliters of beta-lactamase buffer (supplied from kit, ab197008) per mg of pellet weight. The samples were then sonicated in a sonicating water bath at (55 HZ) for 5 minutes and then kept on ice for a further 5 minutes. Any insoluble material was collected by centrifugation at 16,000 xG at 4°C for 20 minutes. The supernatant was collected and added to a fresh microcentrifuge tube and kept on ice for testing.

5.2.4.4. Assay Procedure

Before starting, all reagents and materials were equilibrated to room temperature. A master mix of 48 µl of beta-lactamase assay buffer (supplied from kit, ab197008) and 2 µl nitrocefin (supplied from kit, ab197008) was made per reaction plus extra to ensure accurate pipetting. The standard curve was added to the plate by adding 100 µl of the standard curve solutions (Section 1.2.4.2.) to the plate in duplicate. A positive control was also added by adding 48 µl of beta-lactamase assay buffer to the positive control wells and 2 µl of the positive control enzyme (supplied from kit, ab197008). To each of the sample wells, 25 µl of the samples and 25 µl of the beta-lactamase assay buffer were added. To each of the sample wells and the positive control wells, 50 µl of the master mix was added quickly and immediately read on the microplate reader at 490 nm. The plate reader was set then to read the sample every 2.5 minutes at 490 nm in a kinetic mode for 60 minutes at room temperature protected from light, with linear shaking of 3 mm for 3 seconds before each reading.

5.2.4.5. Calculation of Beta-lactamase Activity and Statistical Analysis

For the calculation of the beta-lactamase activity of the pre-exposed cultures, firstly the standard curve needed calculating. For this, the average absorbance of the standard curve wells was plotted against the nmol concentration of hydrolysed nitrocefin per well. A linear trendline was calculated and the trendline equation was used to find the amount of hydrolysed nitrocefin. The activity of beta-lactamase (B) is the ΔOD_{490nm} = absorbance at T2 – absorbance at T1; and was calculated using the following equation:

$$B = \frac{(Corrected\ absorbance - [y\ intercept])}{Slope}$$

After finding the total activity of the beta-lactamase (B), the activity was then found using the following equation:

$$BL\ Activity = \left(\frac{B}{(T2 - T1)\ \times\ V}\right)\ \times\ D$$

B = Total beta-lactamase activity

T1 = Time point 1

T2 = Time point 2

V = Volume (µl)

D = Dilution Factor

This give the activity in mU/µl and could be then calibrated to the amount of nitrocefin hydrolysed per mg of protein. The 1 unit definition of beta-lactamase activity is the amount of enzyme that generates 1 µmol of hydrolysed nitrocefin per minute at pH 7.0 at 25°C.

The beta-lactamase activity was calculated over 10, 20, 30, 40, 50 and 60 minutes. The statistical differences in beta-lactamase activity were assessed by two-way ANOVA and using a Dunnett's post-Hoc multiple comparisons test in GraphPad Prism (GraphPad Prism version 9.5.1. for Windows).

5.2.5. Scanning electron Microscopy (SEM)

5.2.5.1. SEM sample preparation

Samples were pre-treated with OTC antibiotics as previously described and no OTC antibiotic treatment was used as a negative control. After treatment, 1 ml of each sample was transferred into sterile microcentrifuge tubes. These were centrifuged at 10,000 xG for 1 minute and the supernatant was discarded. The pellet was then resuspended in 1 mL PBS by pipetting gently up and down to ensure the suspension was homogenous. The sample was washed a further three times using the same procedure. After washing, the sample was centrifuged again at 10,000 xG for 1 minute and the supernatant was discarded. The pellet was then resuspended in a fixative solution (2% glutaraldehyde in PBS, pH7.4). The samples were then incubated at room temperature for two hours to fix the cells. After fixing, the samples were centrifuged again at 10,000 xG for 1 minute, and the supernatant was discarded. The pellet was then resuspended in PBS and incubated at room temperature for 3 minutes. After which, the samples were then sequentially dehydrated using an increasing concentration of ethanol (50%, 70%, 80%, 90%, 95%, 100%) by

centrifuging, resuspending and incubating at room temperature for 3 minutes. After sample dehydration, a 10-fold dilution was done to ensure a proper dispersion of cells for SEM, so single cells could be imaged. After diluting, samples are prone to aggregation, therefore they were thoroughly vortexed and immediately added to a 0.22 µm polycarbonate filter membranes on a three-way manifold system attached to a vacuum pump. The vacuum pump was immediately stopped after the sample passed through the membrane as to not over dry the sample which could damage the bacteria. The membranes were then placed in individual sterile vented petri dishes and placed in a bell jar with desiccant overnight.

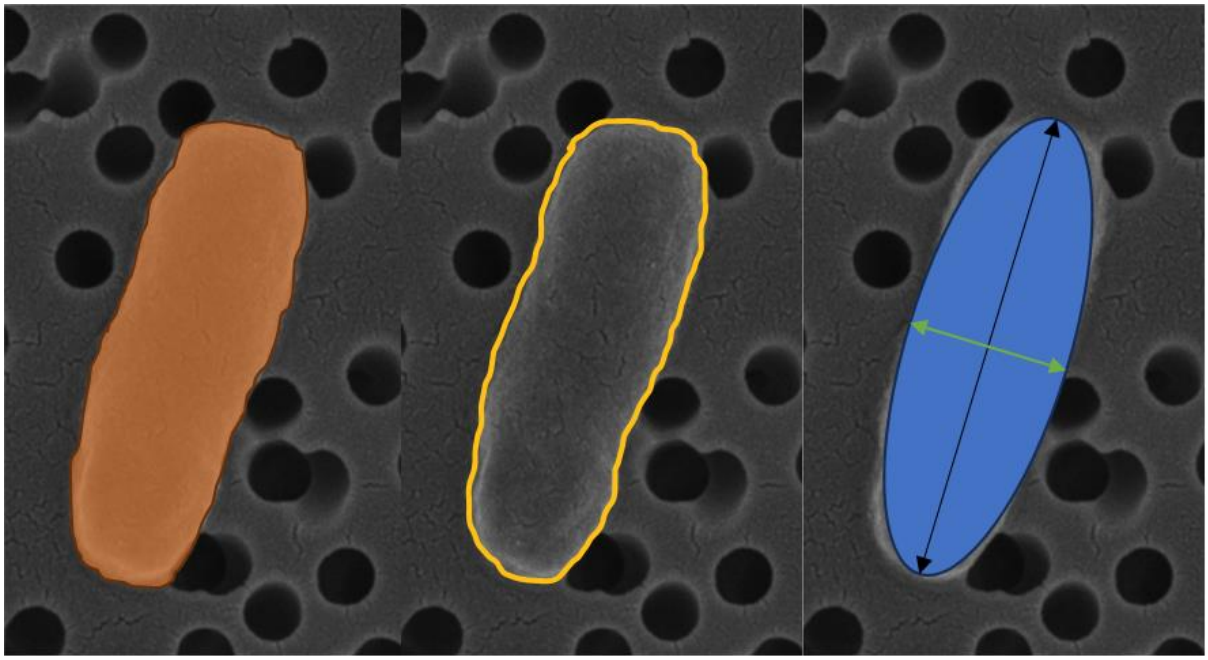
5.2.5.2. Image acquisition

To prepare the samples for SEM imaging, small squares were cut from the filter membranes and were mounted onto 12.5 mm stainless steel stubs using Leit adhesive carbon tabs. The samples were then coated with 20 nm AuPd sputter coater (SC500, Biorad, UK). SEM images were acquired using a beam energy of 5 kV using an in-lens detector on a Sigma HD Field Emission Gun Scanning Electron microscope (Carl Zeiss Ltd., Cambridge, UK) at a 5-7 mm working distance.

5.2.5.3. Cell analysis

The cells from the SEM images were analysed using Fiji, a redistribution package of ImageJ (Schindelin, *et al*., 2012). To analyse the cells, the scale was firstly set using the scale bar from the SEM image. The image was then put in 8-bit format and a threshold was set to have a dark background and highlight the cells in white. Any 'holes' within the cells were filled using the binary setting, 'Fill holes' or by filling in the 'holes' in the cells manually. After this, the wand tracing tool was used to highlight the perimeter of the cell. During cell analysis, multiple parameters were used to assess the cell shape (Figure 5.3). The results from the parameters measured were then analysed using one-way ANOVA with a Dunnett's Post-Hoc test in GraphPad Prism (GraphPad Prism version 9.5.1. for Windows).

Figure 5.3. The structural parameters assessed in the Fiji software from SEM images. The parameters measured were: area (orange), perimeter (yellow), the major axis (black arrow), the minor axis (green arrow), aspect ratio and roundness.



Parameter	How it is measured
Area	Area of the selection, *i.e*. area of the bacteria (orange)
Perimeter	The length of the outside boundary of the selection (yellow)
Major Axis	The major axis of the 'Fit Ellipse' (blue)
Minor Axis	The minor axis of the 'Fit Ellipse' (blue)
Aspect Ratio	Major axis/minor axis
Roundness	4 x area/π x Major axis^2

5.2.6. DNA extraction and quantification

5.2.6.1. DNA Extraction

DNA was extracted using the PureLink® Genomic DNA Kit (K-1820-01; Invitrogen). All reagents were prepared as instructed in the kit.

Cultures were pre-exposed to OTC antibiotics as before and then washed in PBS. The first step of the DNA extraction is to prepare the bacterial lysate. To prepare the lysate, one millilitre of washed culture was transferred to a microcentrifuge tube. This was pelleted and the supernatant was removed. The pellet was then resuspended in 180 µl of PureLink® Genomic Digestion Buffer. Twenty microlitres of Proteinase K (20 mg/ml) was added to assist lysing the cells. This was mixed briefly by vortexing. The tubes were then incubated at 55°C in a heat block for 4 hours with a brief vortex every 30 minutes. After incubation, 20 µl of RNAse A (20 mg/ml; in 50 mM Tris-HCl, pH 8.0, 10 mM Ethylenediaminetetraacetic acid [EDTA]) was added to the lysate, vortexed and incubated at room temperature for 2 minutes. To this, 200 µl of PureLink® Genomic Lysis/Binding Buffer was added and mixed well by vortexing. Two hundred microlitres of ethanol (98%) was then added to the lysate and mixed by vortexing for 5 seconds.

After lysate preparation, the DNA was extracted by binding to a spin column. The prepared lysate was added to the PureLink® Spin Column in a collection tube. The column in the collection tube was centrifuged at 10,000 xG for 1 minute at room temperature. After centrifugation, the collection tube was discarded and the PureLink® Spin Column was placed into a clean collection tube.

After binding the DNA to the column, the DNA was then washed by adding 500 µl of Wash Buffer 1, supplied with the kit and prepared with ethanol. The column was then centrifuged at 10,000 xG for 1 minute at room temperature. The collection tube was discarded and the PureLink® Spin Column was placed into a new collection tube. The DNA was washed a second time with 500 µl of Wash Buffer 2, supplied with the kit and prepared with ethanol. The column was centrifuged at 17,000 xG for 3 minutes at room temperature. The collection tube was discarded and the spin column was placed in a sterile 1.5 ml microcentrifuge tube.

After washing, the DNA was eluted from the spin column by adding 50 µl of PureLink® Genomic Elution Buffer to the column an incubated at room temperature for 1 minute. The column was centrifuged at 17,000 xG for 1 minute after which, the column was removed and discarded. The purified DNA was stored at -20°C until needed.

5.2.6.2. DNA Quantification

DNA quantification was done using the Qubit® 4 Fluorometer using a Qubit® dsDNA HS(High Sensitivity) Assay Kit (Q32851; ThermoFisher). All reagents were brought to room temperature before conducting assays.

Before the assay, a Qubit® working solution was prepared by diluting the Qubit® dsDNA HS reagent 1:200 in Qubit® dsDNA HS Buffer in a sterile 50 ml conical centrifuge tube. The amount of working solution required was calculated by using the number of samples tested plus two standards plus one for excess with each tube requiring a total volume of 200 µl.

The standards were prepared by adding 190 µl of working solution to two 0.5 ml thin-walled Qubit® assay tubes (Q32856; ThermoFisher). To the other tubes for the test samples, 198 µl of working solution was added. Ten microlitres of each Qubit® standard was added to the appropriate tubes and vortexed for 3 seconds. To each of the test samples, 2 µl of DNA extract was added and vortexed for 3 seconds. All tubes were then incubated for 2 minutes at room temperature. The fluorometer was then calibrated using the standards supplied with the kit. After this, 2 µl sample quantity was selected and each of the samples were read with the output being in ng/µl. For any samples which the reading was too high (>600 ng/ml), the sample was diluted in PureLink® Genomic Buffer and retested.

5.2.7. DNA sequencing and Bioinformatic Analysis

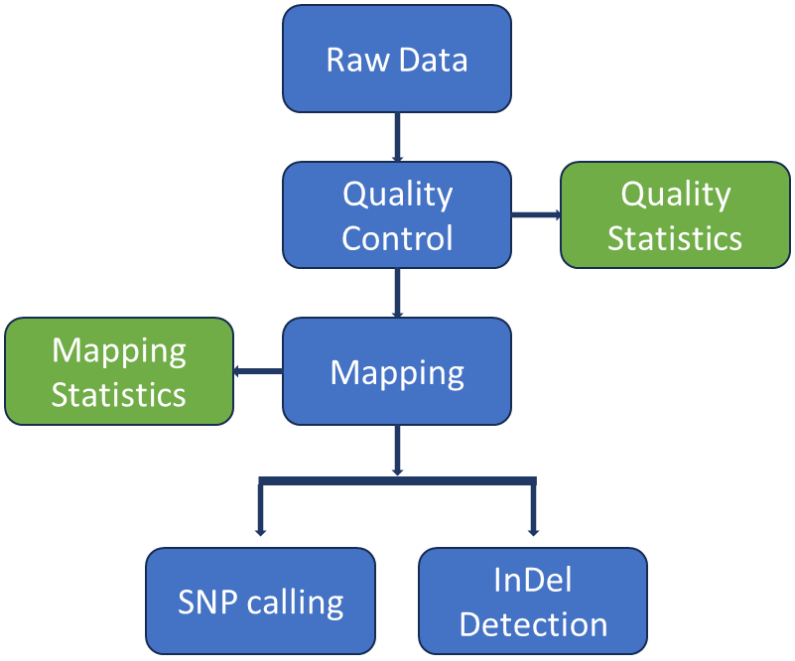
5.2.7.1. DNA sequencing

Purified DNA samples were sent to Novogene for sequencing. Whole genome sequencing (WGS) was done using an Illumina HiSeq™ Sequencing platform. The library preparation consisted of randomly fragmenting the DNA into 350 bp fragment sizes. The resulting fragments were then end repaired, A-tailed and ligated with an Illumina adapter. These were then PCR amplified, size selected and purified. The DNA was then sequenced using high-throughput sequencing and was then analysed using a bioinformatic workflow.

5.2.7.2. Bioinformatic analysis and workflow

The bioinformatics workflow was done to analyse and visualise the data (Figure 5.4).

Figure 5.4. Bioinformatic workflow. The boxes in blue are processes undertaken on the data. Boxes in green are the outputs from the data.



The raw data from the Illumina sequencing was assessed firstly for quality of reads by error rate and Qphred scores by the Casava software version 1.8. The reads were then mapped to a reference genome, CP001918 (www.ncbi.nlm.nih.gov/nuccore/CP001918), using the BWA (Burrows-Wheeler Aligner; Li and Durbin, 2009) with parameters 'mem -t 4 -k 32 -M'. The genomic analysis toolkit (GATK; McKenna, *et al*., 2010) was used to call Single nucleotide polymorphisms (SNPs) and Insertions and deletions (InDels) from BAM files and ANNOVAR (Wang, *et al*., 2010) was used to annotate the variants.

5.2.8. Metabolomic testing

5.2.8.1. Reagent preparation

The metabolomic testing was done using an Omnilog plate reader (Hayward, CA, USA). Procedure used was optimized for use on Gram-Negative bacteria. The IF-0a plate inoculating fluid was firstly prepared by using 125 ml of IF-0a GN/GP Base inoculating fluid (1.2x; Biolog) and adding 25 ml of sterile distilled water. The IF-0a+dye mix was also made by using 125 ml of IF-0a GN/GP Base inoculating fluid (1.2x; Biolog) and adding 1.8ml of Biolog Redox Dye A mix (100x; Biolog) and 23.2 ml of sterile distilled water. The IF-10a+dye mix was made by using 125 ml of IF-10a GN Base inoculating fluid (1.2x; Biolog) and adding 1.5 ml of Biolog Redox Dye A mix (100x; Biolog) and 23.5 ml of sterile distilled water. A solution of 2M sodium succinate/200 µM ferric citrate (100x; Sigma) was also prepared in water and filter sterilised by 0.22 µm nitrocellulose filter.

5.2.8.2. Culture preparation

Four cultures were grown overnight at 37°C, shaking at 120 RPM in 10 ml MHB+CA with either no antibiotic, gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). After incubation, cultures were centrifuged at 4,000 G. The cultures were then washed once in PBS and the final pellet was resuspended and adjusted in IF-0a plate inoculating fluid. The culture was transferred in 20 x 150 sterile capped test tubes (E+K Scientific) with final volume of the cultures being 16 ml with a 42% transmittance value when read on the Biolog Turbidimeter (Biolog). To separate 25 ml tubes, 20 ml of the IF-0a+dye mix was added along with 4 ml of adjusted cultures to give a (1:5 dilution) 85% T cell suspension.

5.2.8.3. Plate Inoculation and Incubation

The plates tested were the PM1, PM5 and PM9 (Table 5.1). These test for changes in growth with different carbon sources, nutrient supplements and osmolytes, respectively. For inoculation of the PM1 plate (Biolog), 11 ml of 85% T cell suspension was transferred to a sterile reservoir. The plates were then inoculated with 100 µl of cell suspension per well. For the PM5 plate (Biolog), 12 ml of the 85% T cell suspension was supplemented with 120 µM of the 2M sodium succinate/ ferric citrate 200 µM (100x) solution. This was then transferred into a sterile reservoir and 100 µl of was added to each well in the PM5 plates. For the inoculation of the PM9 plates, 150 µl of the 85% T cell suspension was added to 15 ml of the IF-10a+dye mix (1:100 dilution). This was transferred into a sterile reservoir and 100 µl was added into each well.

Table 5.1. Biolog Plates used in metabolomic testing.

Plate	**Name**	**Metabolomic Focus**
PM1	Carbon Sources	Carbohydrate utilization, energy production and sugar metabolism
PM5	Nutrient Supplements	Assessing the changes in various biosynthetic pathways in the cell and secondary metabolite metabolism
PM9	Osmolytes	Looking at the bacterial response to different osmotic stresses

After inoculation, the plates were added into the OmniLog plate reader (Biolog). The plates were incubated at 37°C and were read for 48 hours. The positions and the type of plate was logged using the OmniLog software.

5.2.8.4. Data analysis

Data analysis was done using the 'opm' package in R Studio (RStudio Team, 2020; Vaas, *et al*., 2013). The 'opm', 'opmdata' and 'opmextra' library packages were loaded to read the opm files from the Biolog reader. Metadata was added by installing and loading the library 'openair' and merging the metadata with the kinetic output data. XY plots were firstly done to ensure there were no faults in the kinetic readings. Heatmaps were generated on the basis of aggregated curve parameters by installing and loading the libraries; 'tidyr', 'reshape2', 'remotes', 'gplots' and 'grofit'. The four growth parameters were aggregated using a spline fit algorithm. Heatmaps were then generated from the subset data, AUC. Confidence interval plots were generated for each well in each plate using the AUC subset data from the aggregated data. Similarly, radial plot was made using the AUC subset data from the aggregated data.

5.3. Results

5.3.1. Changes in growth rate after OTC antibiotic exposure and under OTC antibiotic pressure

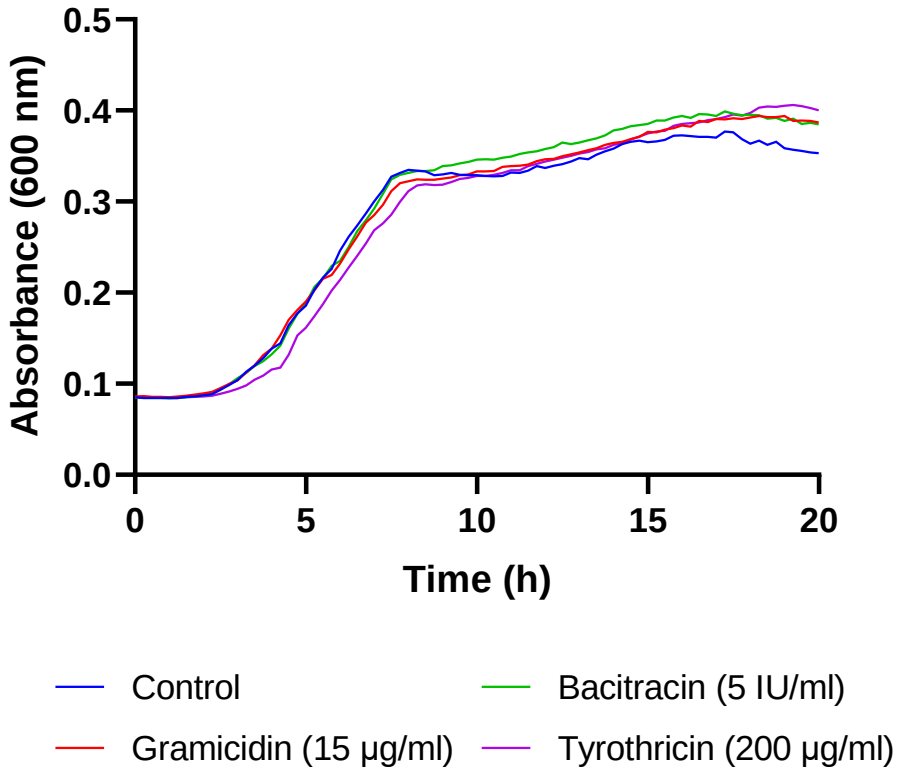
The results output from the GrowthCurver program look at different parameters of the bacterial growth curves. The 'k' parameter measures the carrying capacity, which is the maximum possible population size in a particular environment. The 'r' parameter measures the intrinsic growth rate of the population.

When looking at the *E. coli* growth curves after OTC antibiotic exposure there were some significant differences (Figure 5.5, Table 5.2). The growth curve parameters were assessed in 'Growthcurver' and the parameters were statistically analysed. There were significant increases in the 'r' parameter after pre-exposure to either gramicidin ($p=0.0233$) or tyrothricin ($p=0.0095$). The 'r' parameter is the growth rate of the population if there were no restrictions imposed on the population size. There were also significant increases in the 't_gen' parameter, which is the fastest generation time of the growth curve, after pre-exposure to either gramicidin ($p=0.0071$) or tyrothricin ($p=0.0013$). There was also a significant increase in the 't_mid' parameter, which is when the population density reaches half of the carrying capacity of the population (K), after pre-exposure to gramicidin ($p<0.0001$) bacitracin ($p=0.0001$) or tyrothricin ($p<0.0001$).

Table 5.2. Growth curve parameters from the GrowthCurver output of *E. coli* either not exposed or pre-exposed to OTC antibiotics. The cells highlighted green are statistically significant from the control ($p<0.05$).

Parameter	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
k	0.271 (±0.010)	0.287 (±0.014)	0.295 (±0.019)	0.293 (±0.010)
r	0.889 (±0.079)	0.663 (±0.010)	0.740 (±0.060)	0.636 (±0.027)
t_mid	5.570 (±0.029)	5.989 (±0.109)	5.939 (±0.227)	6.596 (±0.135)
t_gen	0.784 (±0.068)	1.045 (±0.016)	0.940 (±0.078)	1.092 (±0.047)
auc_e	3.889 (±0.161)	3.979 (±0.166)	4.115 (±0.202)	3.879 (±0.142)

Figure 5.5. Growth curves of the raw absorbance data of *E. coli* ATCC 25922 after 24 hours pre-exposure with OTC antibiotics. Bacteria were either pre-exposed to gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control of no antibiotic pre-exposure was used as a comparison for growth.

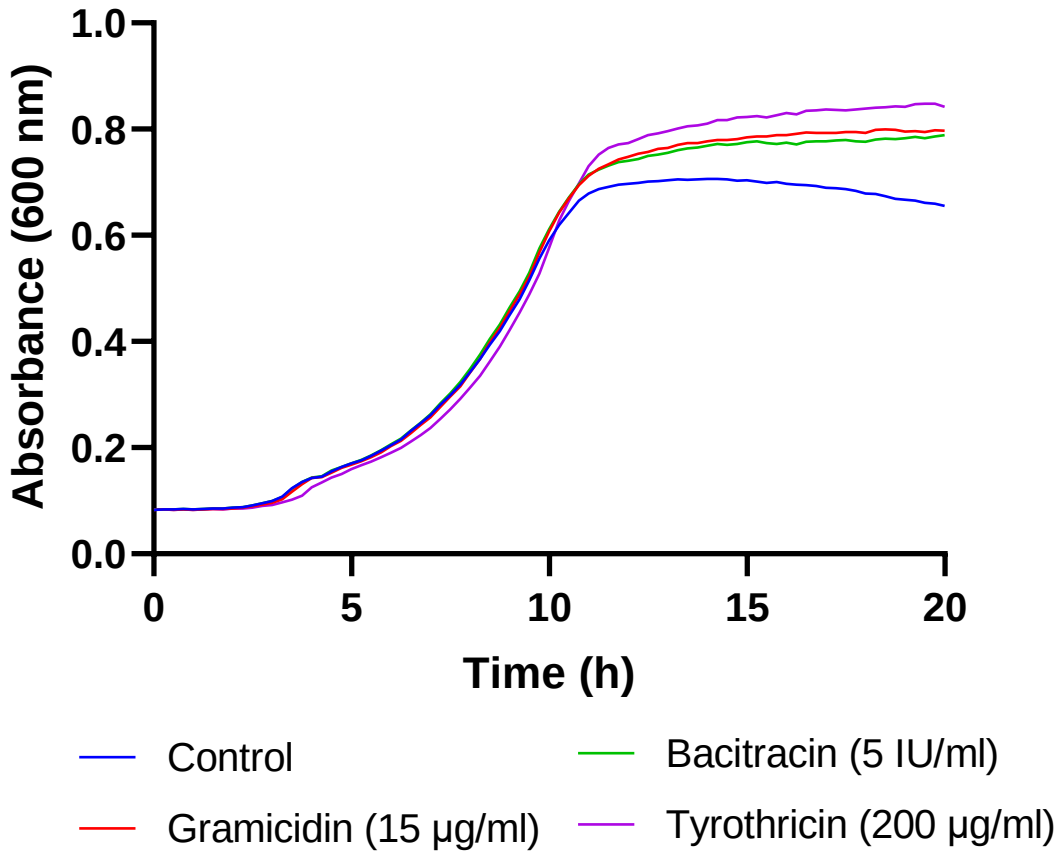


Exposure or pre-exposure of *E. cloacae* to gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) did not decrease bacterial population (Figure 5.6). Looking at growth curve parameters obtained with 'Growthcurver' (Table 5.3), pre-exposure caused a significant increase in the 't_mid' parameter after tyrothricin pre-exposure (p=0.0146). There were no significant changes in either the growth rate 'r' or the maximal growth rate 't_gen'. There were, however, significant increases in the area under the curve of the experimental data (auc_e) after pre-exposure to either gramicidin (p=0.0241), bacitracin (p=0.0448) or tyrothricin (p= 0.0043) due to the lack of population decline, or death phase, in the pre-exposed cultures (Figure 5.6). The elongated stationary phase in the pre-exposed cultures can indicate adaptation to stress (Jaishankar and Srivastava, 2017) but this would require further testing to confirm.

Table 5.3. Growth curve parameters from the GrowthCurver output of *E. cloacae* either not exposed or pre-exposed to OTC antibiotics. The cells highlighted green are statistically significant from the control ($p<0.05$)

Parameter	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
k	0.617 (±0.054)	0.719 (±0.084)	0.706 (±0.106)	0.761 (±0.070)
r	0.752 (±0.036)	0.681 (±0.071)	0.687 (±0.088)	0.698 (±0.051)
t_mid	8.172 (±0.185)	8.651 (±0.325)	8.529 (±0.385)	9.053 (±0.188)
t_gen	0.924 (±0.044)	1.026 (±0.104)	1.019 (±0.125)	0.997 (±0.072)
auc_e	7.359 (±0.526)	8.182 (±0.688)	8.107 (±0.919)	8.370 (±0.610)

Figure 5.6. Growth curves of the raw absorbance data of *E. cloacae* ATCC 13047 after 24 hours pre-exposure with OTC antibiotics. Bacteria were either pre-exposed to; gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control of no antibiotic pre-exposure was used as a comparison for growth.

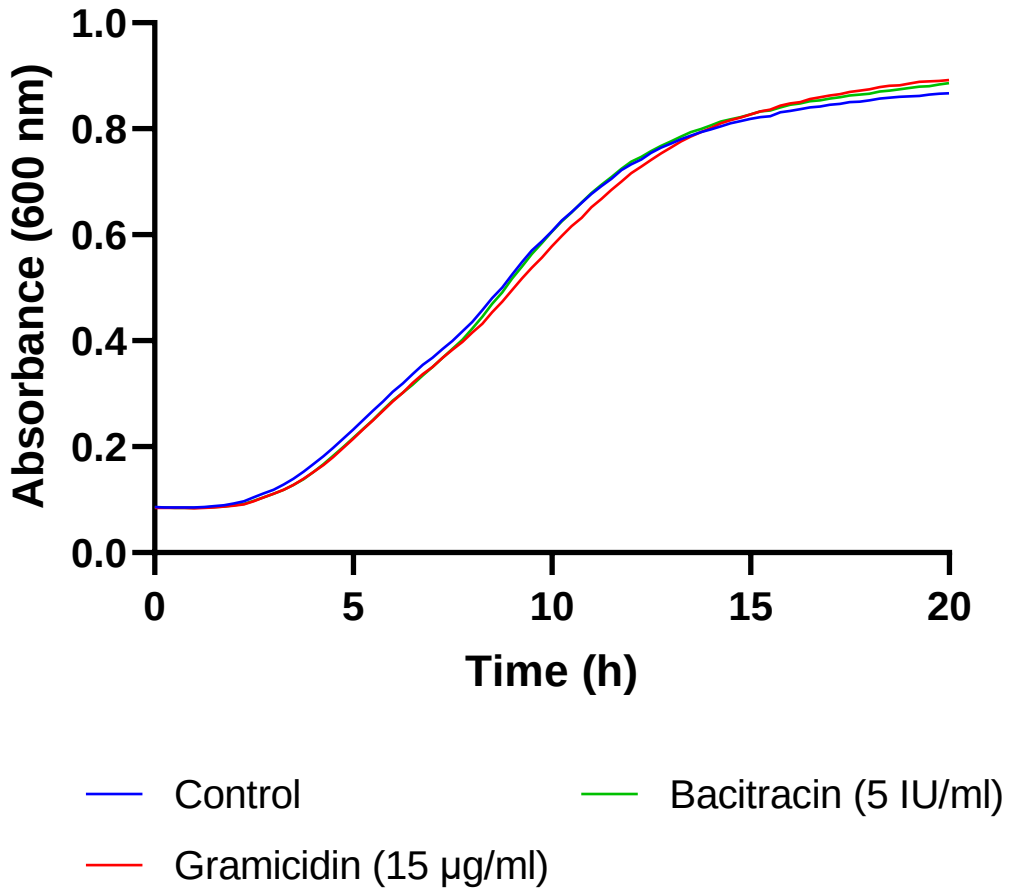


The growth curves of *A. baumannii* after either pre-exposure to gramicidin (15 µg/ml) or bacitracin (5 IU/ml), showed one significant change compared to the antibiotic-free control (Figure 5.7, Table 5.4). Neither the growth rate 'r' or the maximal growth growth rate 't_gen' had any significant change. There were no significant changes in the area under the curve ('auc_e') either. The only significant change was the 't_mid' value that was significantly different after gramicidin pre-exposure (p=0.0107).

Table 5.4. Growth curve parameters from the GrowthCurver output of *A. baumannii* either not exposed or pre-exposed to OTC antibiotics. The cells highlighted green are statistically significant from the control (p<0.05)

Parameter	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)
k	0.774 (±0.031)	0.803 (±0.040)	0.789 (±0.076)
r	0.463 (±0.036)	0.439 (±0.048)	0.474 (±0.041)
t_mid	8.377 (±0.297)	8.896 (±0.444)	8.583 (±0.433)
t_gen	1.503 (±0.111)	1.591 (±0.167)	1.468 (±0.127)
auc_e	8.902 (±0.117)	8.816 (±0.095)	8.904 (±0.520)

Figure 5.7. Growth curves of the raw absorbance data of *A. baumannii* ATCC 19568 after 24 hours pre-exposure with OTC antibiotics. Bacteria were either pre-exposed to; gramicidin (15 µg/ml) or bacitracin (5 IU/ml). A negative control of no antibiotic pre-exposure was used as a comparison for growth.

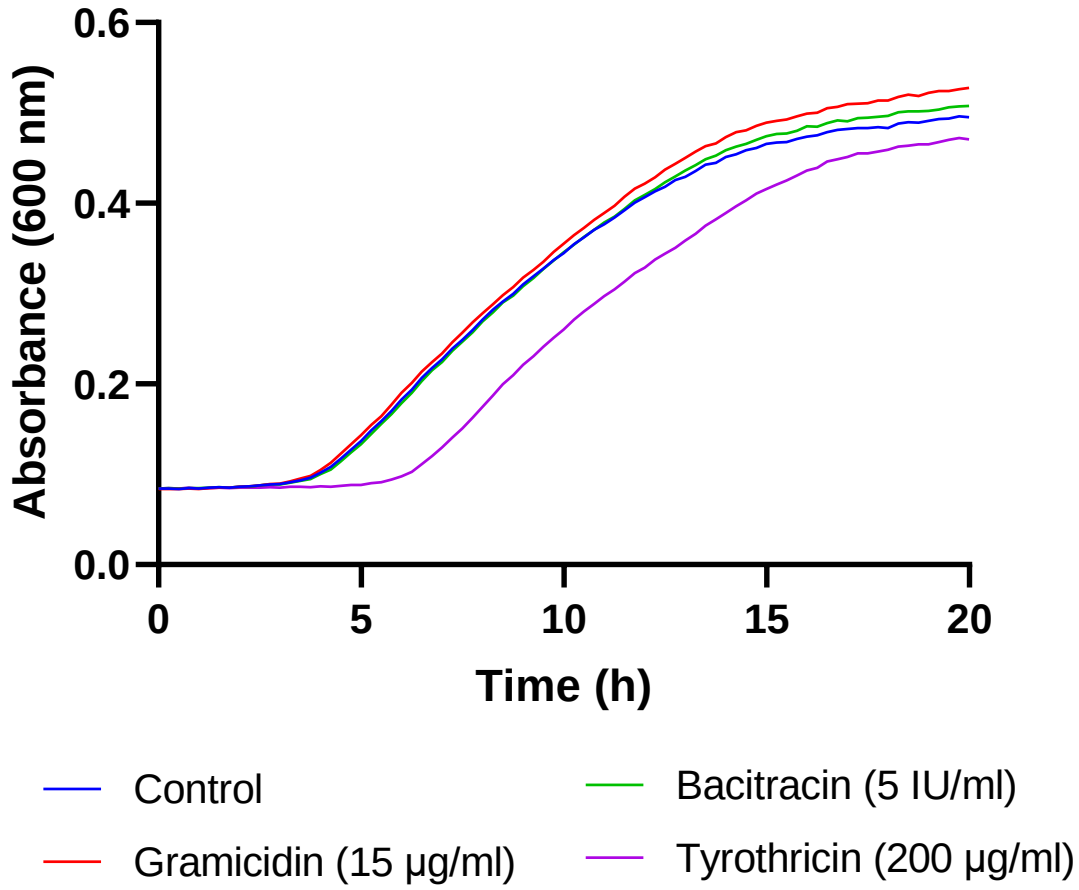


The growth curves for *K. pneumoniae* show that there is a clear increase in the lag phase after pre-exposure to tyrothricin (200 µg/ml; Figure 5.8). After obtaining the growth curve parameters from 'Growthcurver' (Table 5.5), there was a significant increase in the 't_mid' parameter after tyrothricin pre-exposure ($p<0.0001$). This correspondingly also caused a significant increase in the area under the curve ($p<0.0001$) showing there was an overall decrease in growth. Although there were these changes, there were no significant changes in either the growth rate 'r' or the maximal growth rate 't_gen'.

Table 5.5. Growth curve parameters from the GrowthCurver output of *K. pneumoniae* either not exposed or pre-exposed to OTC antibiotics. The cells highlighted green are statistically significant from the control ($p<0.05$)

Parameter	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
k	0.403 (±0.057)	0.435 (±0.064)	0.415 (±0.053)	0.382 (±0.031)
r	0.477 (±0.037)	0.454 (±0.038)	0.468 (±0.027)	0.483 (±0.033)
t_mid	8.607 (±0.388)	8.784 (±0.543)	8.802 (±0.419)	10.667 (±0.331)
t_gen	1.460 (±0.108)	1.534 (±0.127)	1.485 (±0.084)	1.439 (±0.097)
auc_e	4.510 (±0.499)	4.789 (±0.495)	4.567 (±0.421)	3.515 (±0.181)

Figure 5.8. Growth curves of the raw absorbance data of *K. pneumoniae* ATCC 13883 after 24 hours pre-exposure with OTC antibiotics. Bacteria were either pre-exposed to; gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control of no antibiotic pre-exposure was used as a comparison for growth.

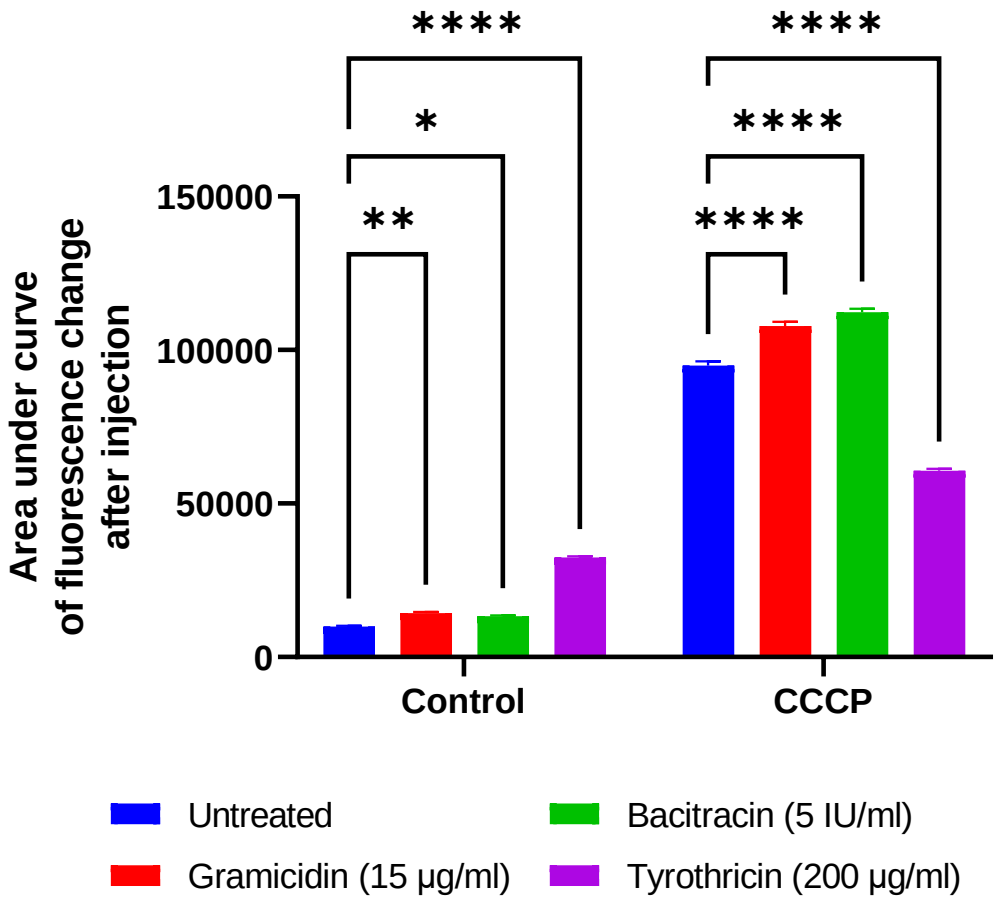


5.3.2. Efflux pump activity

When measuring efflux activity, the accumulation of ethidium bromide within cells inversely correlated to the amount of efflux activity; a higher efflux activity is associated with a lower fluorescence.

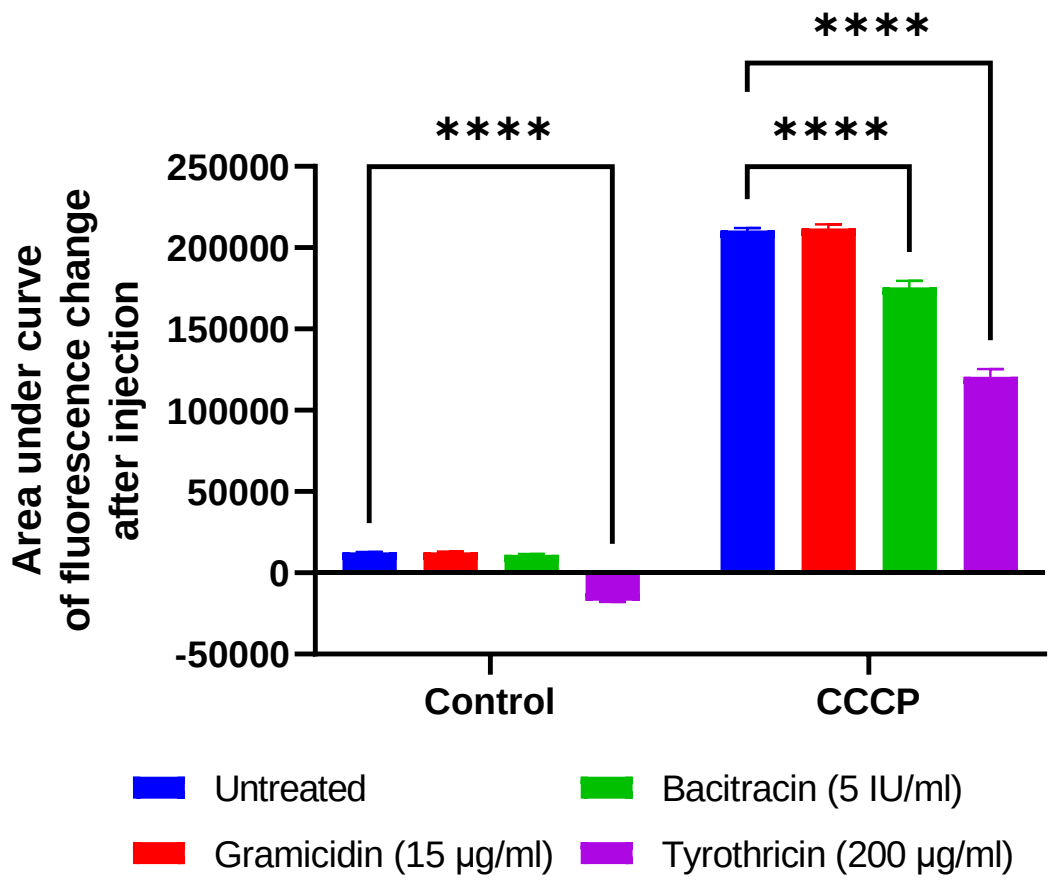
There was a significant increase in AUC of fluorescence in *E. coli* that were pre-exposed to either gramicidin (15 µg/ml; p=0.0040), bacitracin (5 IU/ml; p=0.0242) or tyrothricin (200 µg/ml; p<0.0001) and not treated with the efflux pump inhibitor CCCP (Figure 5.9). When the cultures were treated with CCCP, there was a significant increase in AUC for *E. coli* pre-exposed to gramicidin (15 µg/ml; p<0.0001) or bacitracin (5 IU/ml; p<0.0001) when compared to the untreated cultures. However, there was significant decrease in cultures pre-exposed to tyrothricin (200 µg/ml; p<0.0001).

Figure 5.9. The AUC of measured fluorescence change after injection of either the vehicle control or test inhibitor (0.025 mg/ml CCCP) in *E. coli* cultures either untreated or OTC antibiotic pre-exposed. ns – not significant (p > 0.05), * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001, **** - p ≤ 0.0001.



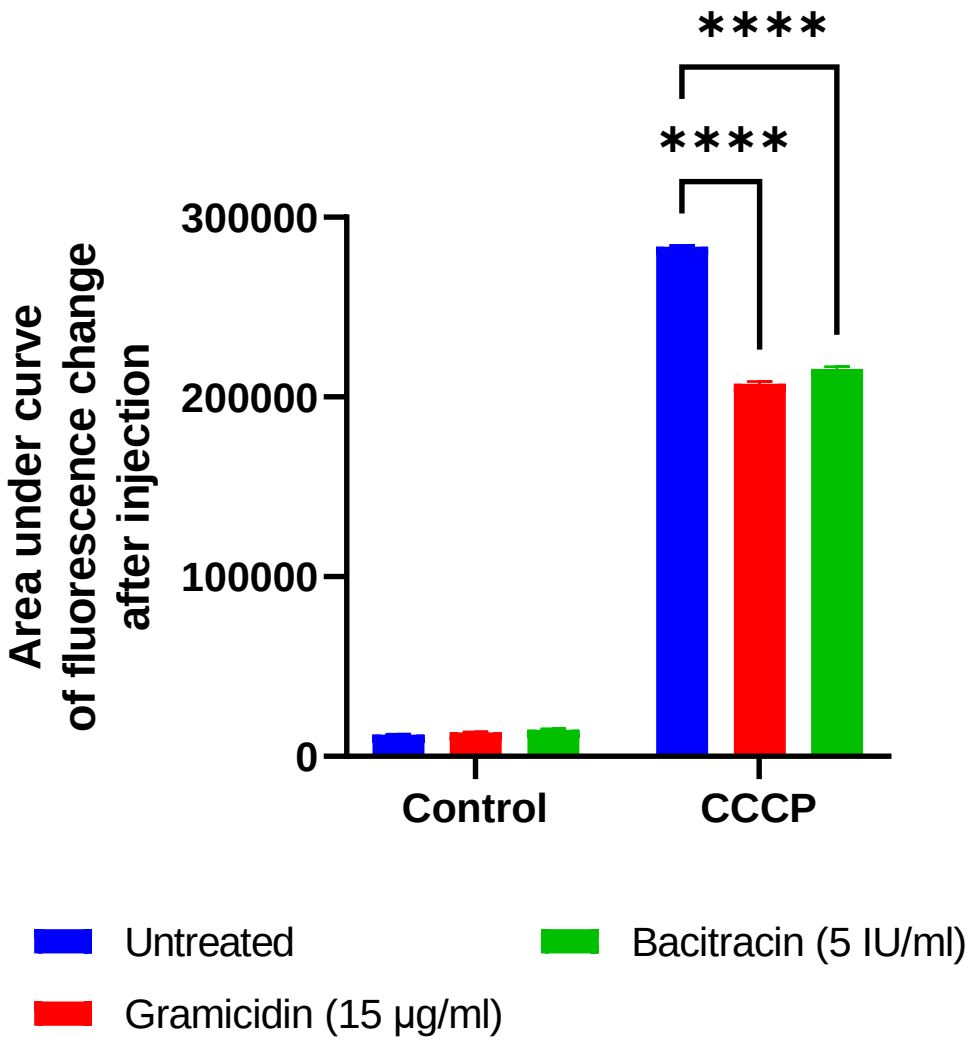
There were no significant changes in the AUC of *E. cloacae* cultures pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) and that were not treated with CCCP (Figure 5.10). However, bacteria pre-exposed to tyrothricin (200 µg/ml) had a significant decrease in AUC when not treated with the efflux pump inhibitor ($p<0.0001$) compared to the untreated cultures. When cultures were treated with CCCP (0.025 mg/ml), there were no significant changes in AUC of *E. cloacae* culture pre-exposed to gramicidin (15 µg/ml; $p=0.9687$). However, bacteria pre-exposed to either bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) had significant decreases in AUC ($p<0.0001$; Figure 5.10) compared to the untreated cultures.

Figure 5.10. The AUC of measured fluorescence change after injection of either the vehicle control or test inhibitor (0.025 mg/ml CCCP) in *E. cloacae* cultures either untreated or OTC antibiotic pre-exposed. ns – not significant ($p > 0.05$), * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.



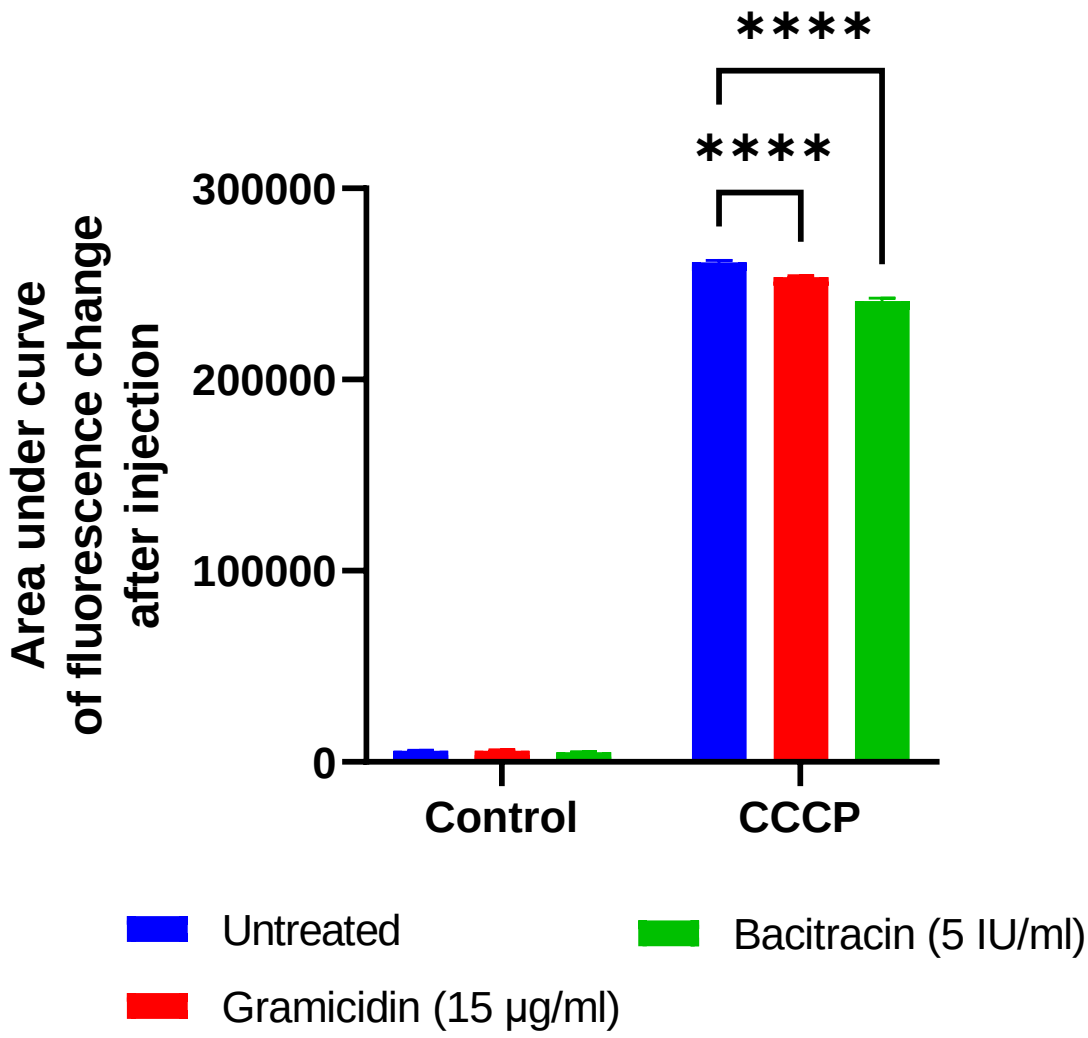
There were no significant changes in the AUC of *A. baumannii* cultures pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) and that were not treated with CCCP (Figure 5.11). However, when bacteria were treated with the efflux pump inhibitor, there were significant decreases in AUC of *A. baumannii* cultures that were pre-exposed to either gramicidin (15 µg/ml; $p<0.0001$) or bacitracin (5 IU/ml; $p<0.0001$) when compared to the untreated cultures.

Figure 5.11. The AUC of measured fluorescence change after injection of either the vehicle control or test inhibitor (0.025 mg/ml CCCP) in *A. baumannii* cultures either untreated or OTC antibiotic pre-exposed. ns – not significant ($p > 0.05$), * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.



No significant changes in the AUC were observed in *K. pneumoniae* cultures pre-exposed to either gramicidin (15 µg/ml; p=0.9799) or bacitracin (5 IU/ml; p=0.7910) and that were not treated with CCCP (Figure 5.12). However, there was a significant decrease in the AUC in bacteria pre-exposed to either gramicidin (15 µg/ml; p<0.0001) or bacitracin (5 IU/ml; p<0.0001) in the presence of CCCP (0.025 mg/ml) when compared to the untreated cultures.

Figure 5.12. The AUC of measured fluorescence change after injection of either the vehicle control or test inhibitor (0.025 mg/ml CCCP) in *K. pneumoniae* cultures either untreated or OTC antibiotic pre-exposed. ns – not significant (p > 0.05), * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001, **** - p ≤ 0.0001.



The efflux assay showed that *E. coli* cultures that were pre-exposed to any of the OTC antibiotics, and without the presence of an efflux pump inhibitor, showed a significantly higher fluorescence. This indicates that more ethidium bromide was able to enter and accumulate in cells rather than being pumped out by efflux pumps. The lowered efflux activity could be due to the OTC antibiotics effecting the pumps by lowering the cell membrane potential and therefore affecting ATP production (Benarroch and Asally, 2020). However, when the efflux pump inhibitor (CCCP) was added there was significantly more ethidium bromide accumulating in cells after gramicidin (15 µg/ml) or bacitracin (5 IU/ml) pre-exposure, but significantly less after tyrothricin (200 µg/ml) pre-exposure.

In *E. cloacae* cultures that were pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml), there was no change in ethidium bromide accumulation when compared to the control and without the presence of an efflux pump inhibitor. This would indicate that there was no change in efflux activity. However, when the *E. cloacae* cultures were pre-exposed to tyrothricin, there was a decrease in the accumulation of ethidium bromide in cells when compared to the control. When the efflux pump inhibitor was used however, there was no significant difference in the uptake of ethidium bromide in the cultures after gramicidin (15 µg/ml) pre-exposure, although there were significant decreases in ethidium bromide accumulation after either bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) pre-exposure.

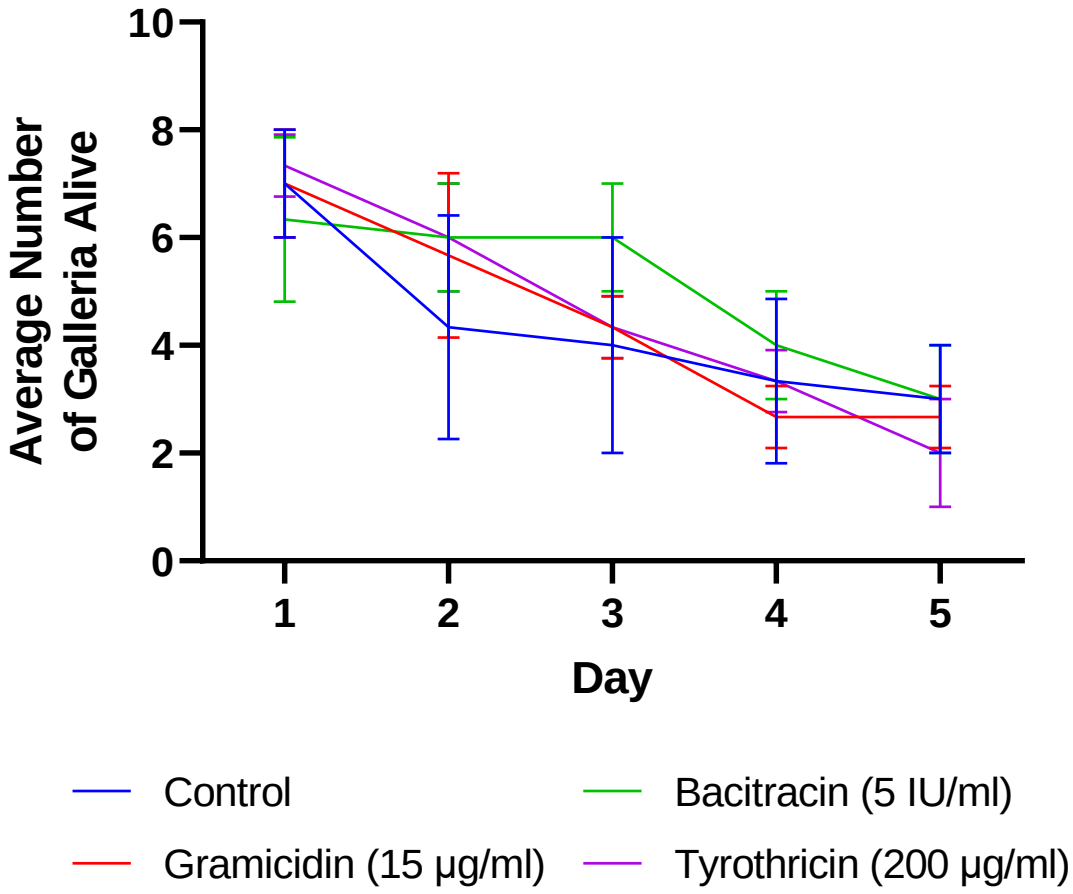
In *A. baumannii*, cultures that were pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) and without an efflux pump inhibitor, showed that there were no significant differences in ethidium bromide accumulation indicting no change in efflux activity. Although in the presence of an efflux pump inhibitor, culture that were pre-exposed to OTC antibiotics had less accumulation of ethidium bromide.

Similarly, *K. pneumoniae* cultures that were pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml) had no significant differences in ethidium bromide accumulation indicating no change in efflux activity. Although, when in the presence of an efflux pump inhibitor, there was a significantly decreased uptake of ethidium bromide in culture that were pre-exposed to either gramicidin (15µg/ml) or bacitracin (5 IU/ml).

5.3.3. Changes in Virulence after OTC antibiotic exposure

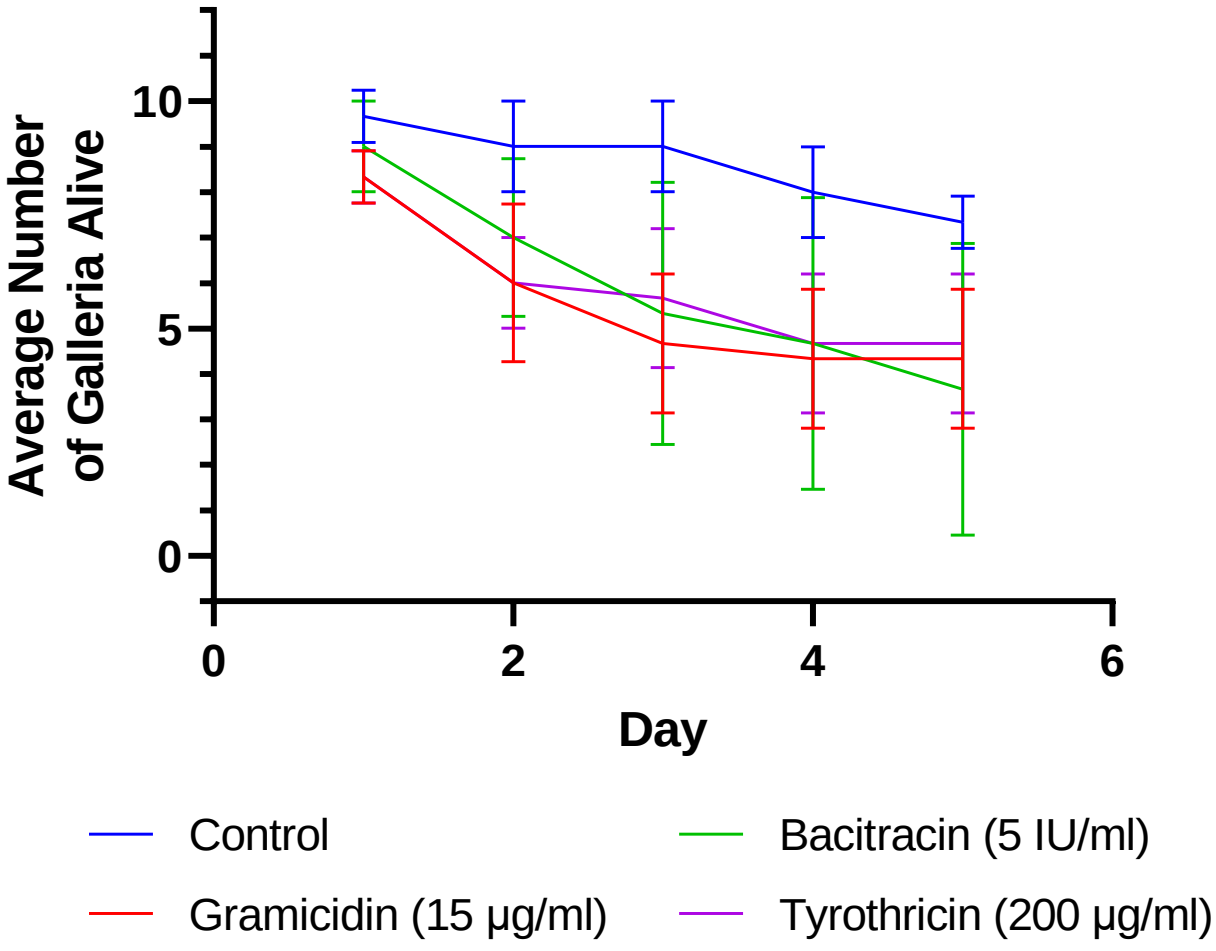
After injection of *E. coli* that was pre-exposed to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml), there were no significant changes in the survival of *Galleria mellonella* on days 1 to 5 when compared to the control ($p>0.05$). (Figure 5.13).

Figure 5.13. Survival of *Galleria mellonella* after *E. coli* injection. *Escherichia coli* was pre-exposed to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control consisted of untreated *E. coli*. The mean number of *Galleria* alive was plotted with the error bars representing the SD.



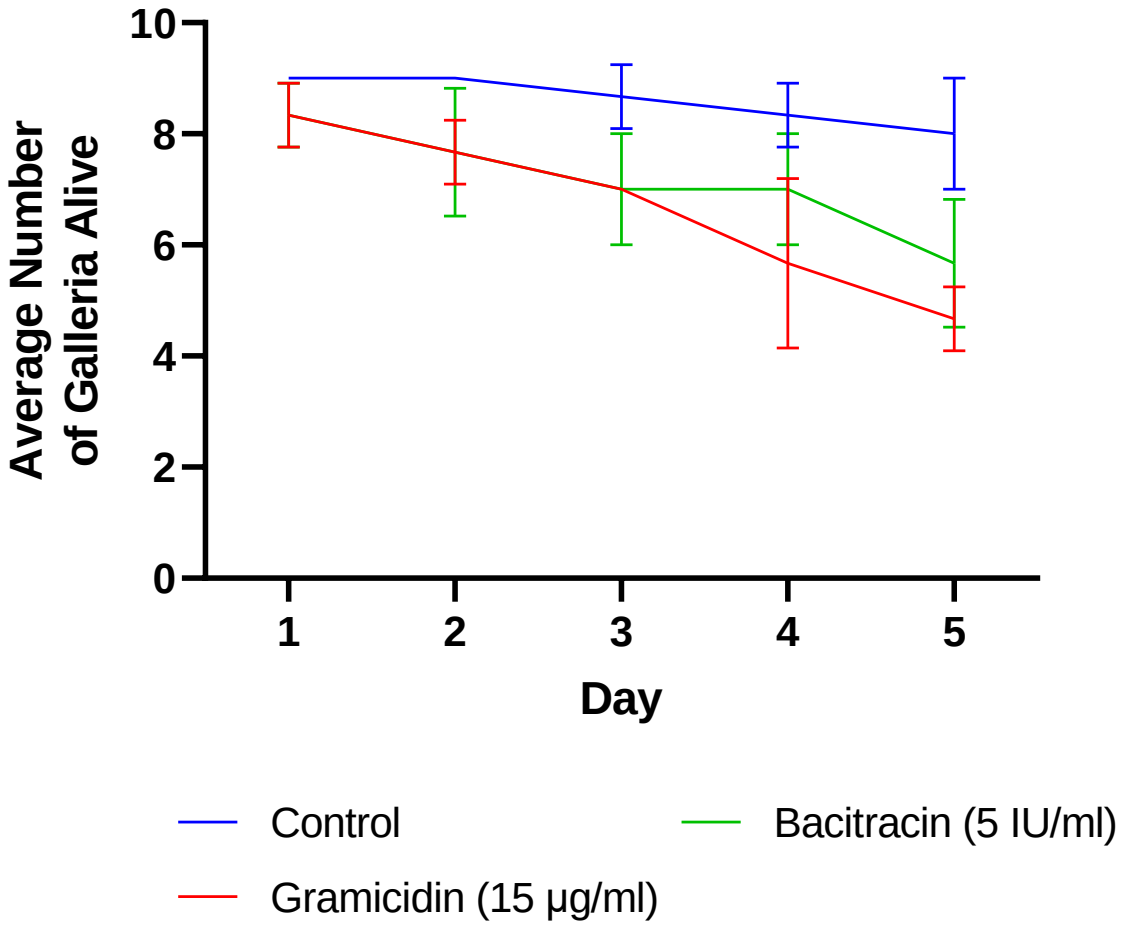
After injection of *E. cloacae,* there was no significant difference in survival after day 1 (Figure 5.14). However, after day 2 there was a significant reduction in survival of those *Galleria mellonella* injected with *E. cloacae* pre-exposed to tyrothricin (p=0.0476). On day 3, there was only a significant difference in the *Galleria mellonella* survival between the control and injection with *E. cloacae* pre-exposed to gramicidin (p=0.0433). There were then no further significant differences (p>0.05) from the control on either day 4 or day 5.

Figure 5.14. Survival of *Galleria mellonella* after *E. cloacae* injection. *Enterobacter cloacae* was pre-exposed to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control consisted of untreated *E. cloacae*. The mean number of *Galleria* alive was plotted with the error bars representing the SD.



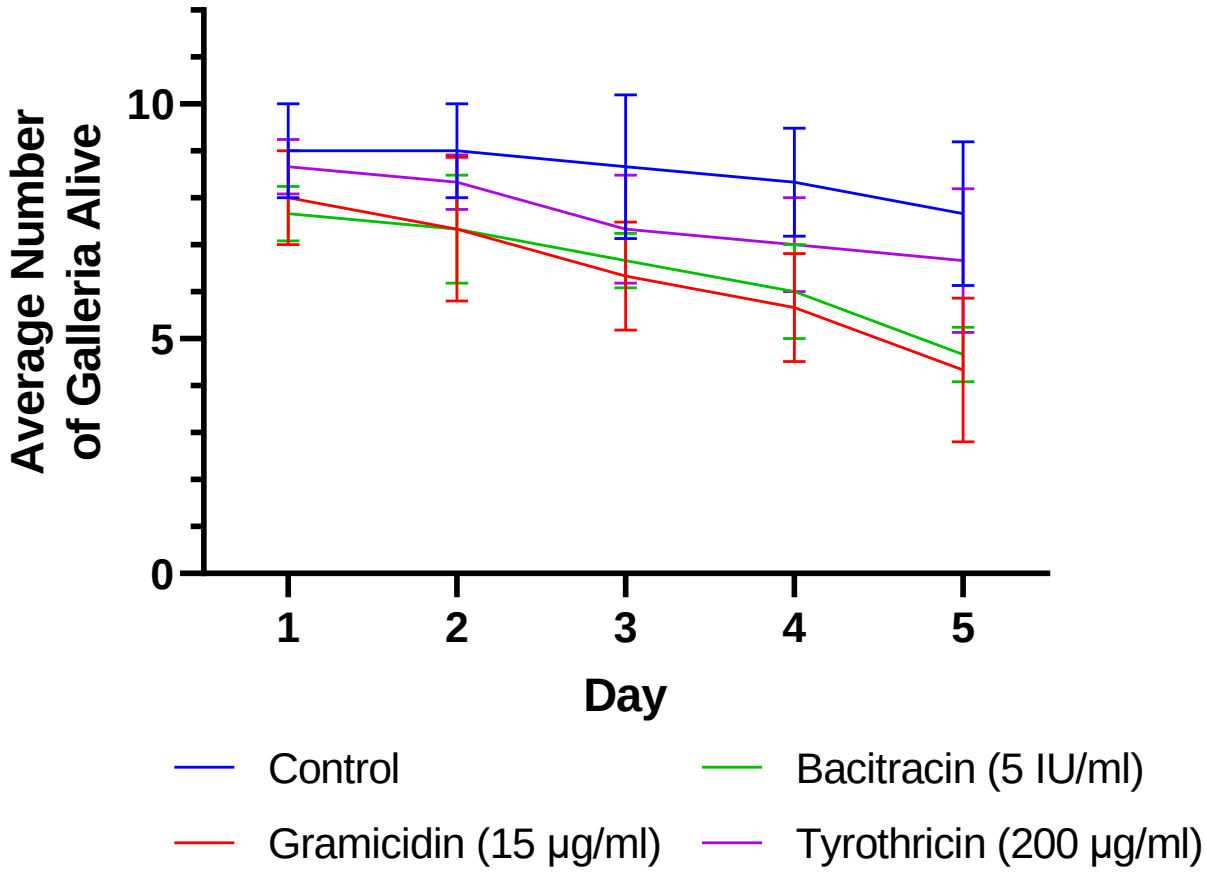
After injection of *A. baumannii*, there was no significant difference in the survival of *Galleria mellonella* after day 1 or day 2 (Figure 5.15). However, after three days, there was a significant difference in survival after both gramicidin pre-exposure (p=0.0336) and bacitracin pre-exposure (p=0.0336) when compared to the control (Figure 5.15). Four days post-injection, there was no significant difference (p>0.05) from the control after bacitracin pre-exposure, although there was a significant difference (p=0.0008) after gramicidin pre-exposure. After five days there was a significant difference in survival after gramicidin pre-exposure (p<0.0001) and bacitracin pre-exposure (p=0.0028).

Figure 5.15. Survival of *Galleria mellonella* after *A. baumannii* injection. *Acinetobacter baumannii* was pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml). A negative control consisted of untreated of *A. baumannii.* The mean number of *Galleria* alive was plotted with the error bars representing the SD.



After injection of *K. pneumoniae,* there were no significant differences (p>0.05) in survival of *Galleria mellonella* after day 1 or day 2 (Figure 5.16). On day 3 however, there was a significant difference in larvae survival between the control and *K. pneumoniae* pre-exposed to gramicidin (p=0.0383). On day four there was a significant difference in survival after both gramicidin pre-exposure (p=0.0156) and bacitracin pre-exposure (p=0.0383). There were also significant differences in survival on day 5 in cultures that were pre-exposed to gramicidin (p=0.0021) or bacitracin (p=0.0059)

Figure 5.16. Survival of *Galleria mellonella* after *K. pneumoniae* injection. *Klebsiella pneumoniae* was pre-exposed to either gramicidin (15 µg/ml) or bacitracin (5 IU/ml). A negative control consisted of untreated *K. pneumoniae*. The mean number of *Galleria* alive was plotted with the error bars representing the SD.



When looking at *E. coli* culture that were pre-exposed to any of the OTC antibiotics, there were no significant changes in the survival of the *G. mellonella* from the infection control. This would indicate that the OTC antibiotics did not affect virulence in *E. coli*.

However, in *G. mellonella* injected with *E. cloacae*, there were significant changes in survival with bacteria pre-exposed to tyrothricin (200 μg/ml) on day 2, and with bacteria pre-exposed to gramicidin (15 μg/ml) on day 3. This would indicate that gramicidin or tyrothricin may cause a slight increase in virulence in *E. cloacae*, although further repeats may be needed to confirm.

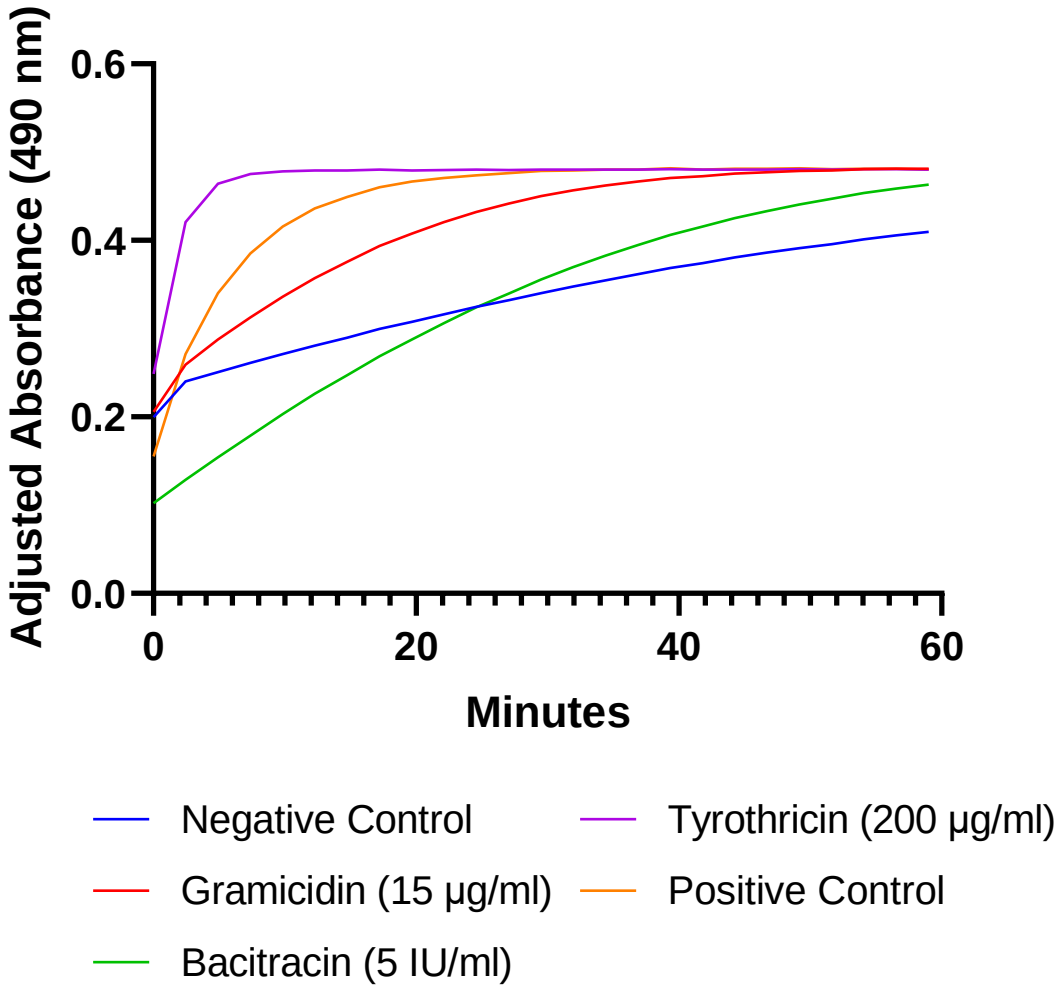
There were no differences in survival of *G. mellonella* after two days following *A. baumannii* infection, although after three days there was a significant difference in survival of *G. mellonella* that were injected with bacteria pre-exposed to either gramicidin (15 μg/ml) or bacitracin (5 IU/ml). Although on day 4, it was only gramicidin that had significantly less survival than the control. But at day 5, both of the culture that were pre-exposed to OTC antibiotic had a lower survival. This shows that both gramicidin and bacitracin had an increased virulence in *A. baumannii*.

In *G. mellonella* that were infected with *K. pneumoniae*, there were no differences in survival after 2 days. After 3 days however, there was a significant decrease in survival with bacteria pre-exposed to gramicidin (15 μg/ml), and on days 4 and 5 there were significant decreases in survival with bacteria pre-exposed to either gramicidin (15 μg/ml) or bacitracin (5 IU/ml). This again shows that cultures after gramicidin or bacitracin pre-exposure appear to have an increased virulence

5.3.4. Changes in Beta-lactamase activity after OTC antibiotic exposure

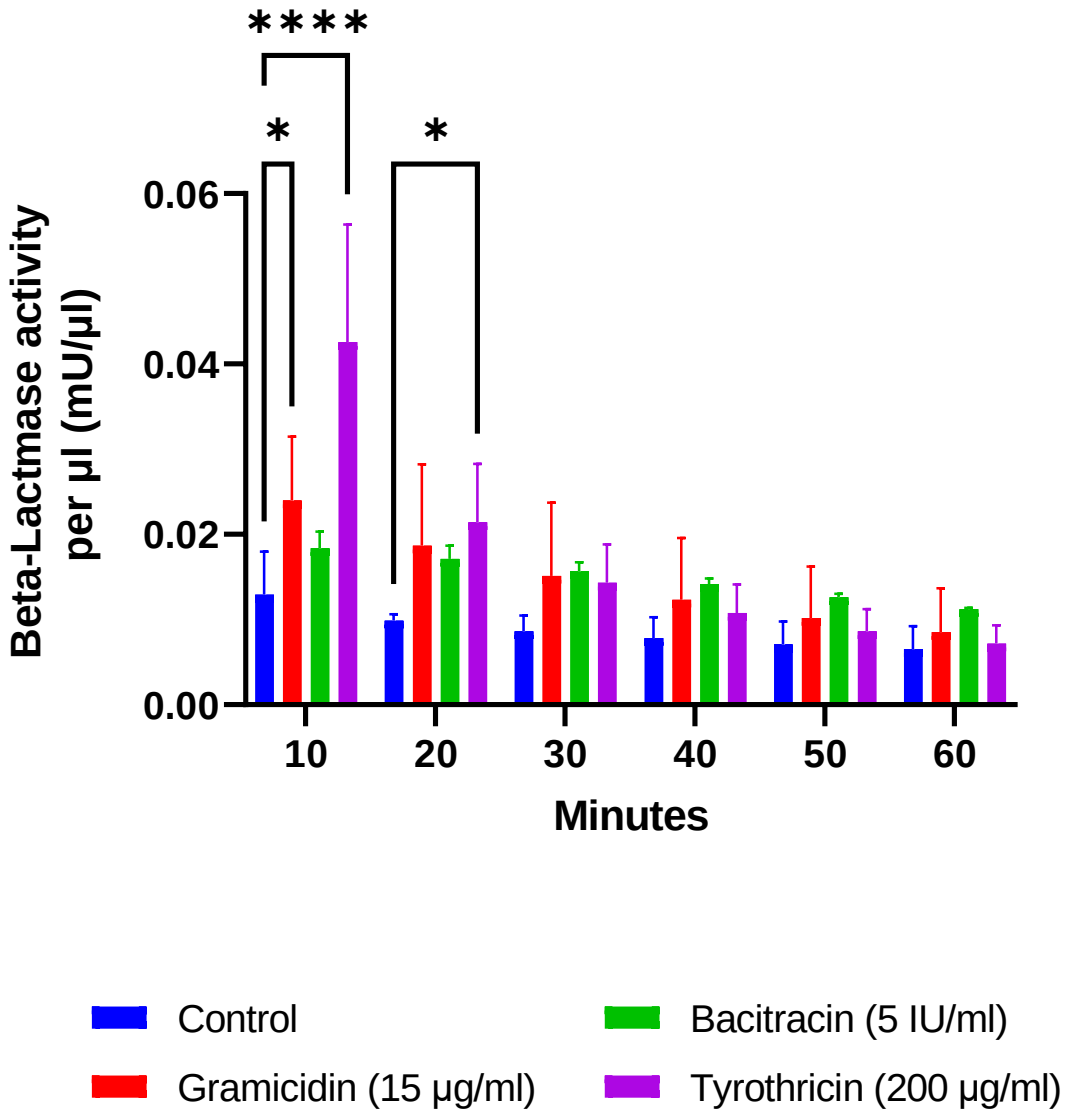
By looking at the adjusted raw data curves, the mean absorbance of the *E. cloacae* pre-exposed to any of the OTC antibiotics appears to increase at a faster rate than the mean absorbance of not treated bacteria (negative control) (Figure 5.17).

Figure 5.17. The mean adjusted raw absorbance data from the beta-lactamase activity of *E. cloacae* either with no antibiotic pre-exposure (negative control), pre-exposure to gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). Also included is the positive control included in the kit (ab197008).



When looking at the beta-lactamase activity per microliter, there was a significant increase in beta-lactamase activity over approximately 10 minutes in both the cultures that were pre-exposed to gramicidin ($p=0.0380$) and tyrothricin ($p<0.0001$). There was only a significant change in beta-lactamase activity over approximately 20 minutes in the cultures that were pre-exposed to tyrothricin ($p=0.0283$; Figure 5.18).

Figure 5.18. The beta-lactamase activity per microliter of *E. cloacae* culture that have either been pre-exposed to gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). A negative control of an *E. cloacae* culture that has not been pre-exposed to any antibiotics was used. ns – not significant ($p > 0.05$), * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.



5.3.5. Changes in bacterial structure and morphology after OTC antibiotic exposure

The parameters analysed from the SEM image analysis were area, perimeter, major axis, minor axis, aspect ratio and roundness (Figure 5.3 and Table 5.6)

There were no statistical differences seen in bacteria that were pre-exposed to either gramicidin (15 µg/ml; p=0.8352), bacitracin (5 IU/ml; p=0.7834) or tyrothricin (200 µg/ml; p=0.9648) when analysing the area of the bacteria (Figure 5.19; A).

There were also no differences seen in the perimeter of the bacteria (Figure 5.19; B) when exposed to either gramicidin (15 µg/ml; p=0.9992), bacitracin (5 IU/ml; p=0.4238) or tyrothricin (200 µg/ml; p=0.5173).

There were also no statistically different changes in the major axis of the 'Fit of Ellipse' (Figure 5.19; C) when treated with either gramicidin (15 µg/ml; p=0.9971), bacitracin (5 IU/ml; p=0.6534) or tyrothricin (200 µg/ml; p=0.1962).

When looking at the minor axis of the 'Fit of Ellipse' (Figure 5.19; D), there were no statistically significant changes when pre-exposed with gramicidin (15 µg/ml; p=0.1663) or tyrothricin (200 µg/ml; p=0.0507). However, there was a significant change when pre-exposed to bacitracin (5 IU/ml; p=0.0039).

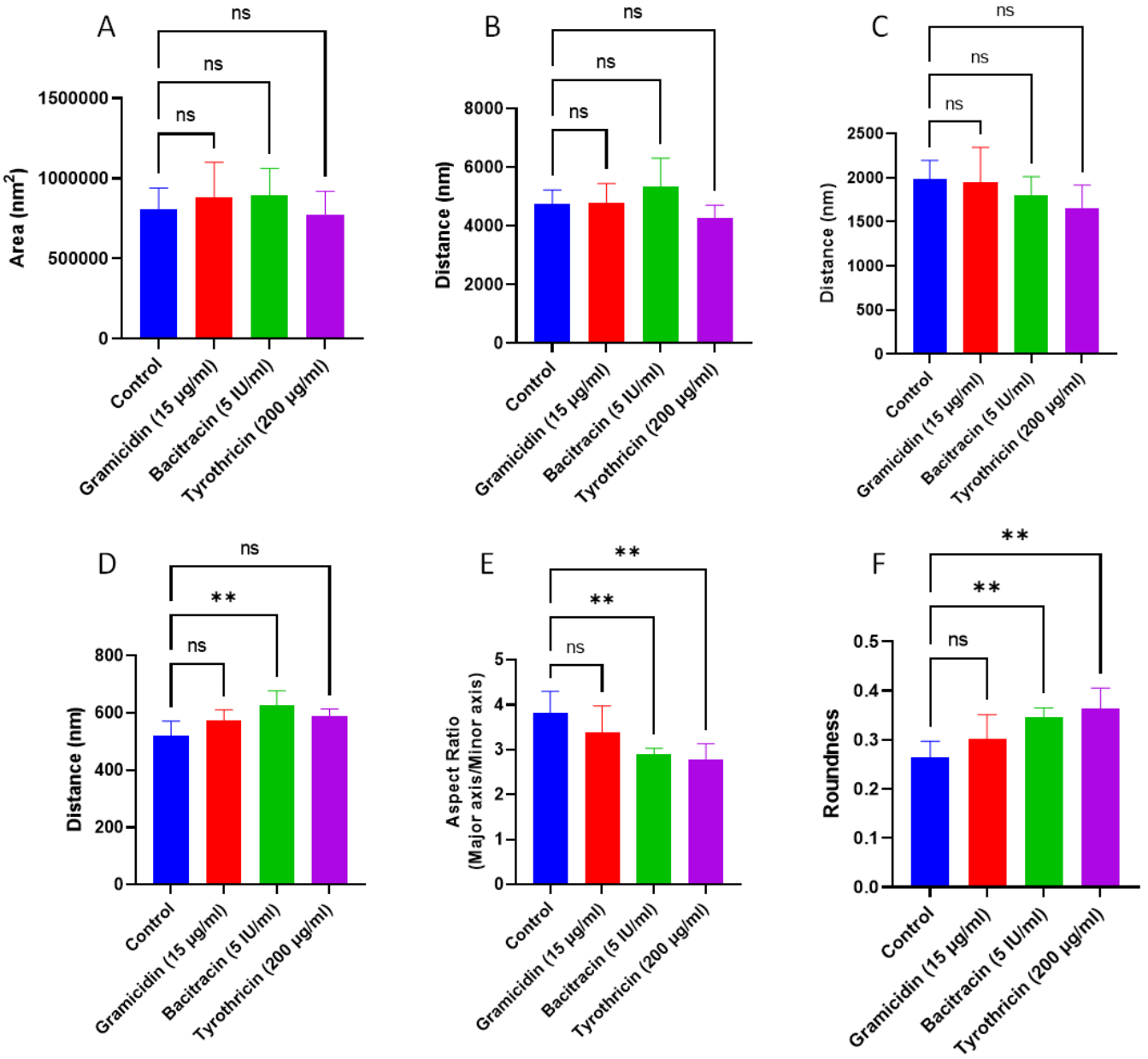
The aspect ratio (Figure 5.19; E) shows there were no significant differences after pre-exposure with gramicidin (15 µg/ml; p=0.2824). However, there were significant differences after pre-exposure to bacitracin (5 IU/ml; p=0.0076) or tyrothricin (200 µg/ml; p=0.0036).

There were no statistically significant differences when looking at the roundness of cells (Figure 5.19; F) when pre-exposed to gramicidin (15 µg/ml; p=0.3172). However, there were significant changes when pre-exposed with bacitracin (5 IU/ml; p=0.0089) or tyrothricin (200 µg/ml; p=0.0022).

Table 5.6. Structural parameters from the SEM image analysis of *E. cloacae* either not exposed or pre-exposed to OTC antibiotics. Mean (±SD). The cells highlighted green are statistically significant from the control ($p<0.05$)

Parameter	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
Area	810660(±129365)	883800(±216512)	893212(±168804)	770454(±150278)
Perimeter	4758(±463.5)	4802(±642.4)	5324(±984.8)	4258(±443.0)
Major	1979(±216.6)	1951(±393.9)	1808(±204.6)	1654(±262.8)
Minor	520.3(±51.29)	573.0(±38.28)	624.7(±52.39)	590.5(±23.08)
Aspect Ratio	3.825(±0.4789)	3.395(±0.5823)	2.888(±0.1410)	2.791(±0.3434)
Roundness	0.2648(±0.03231)	0.3014(±0.04952)	0.3468(±0.01796)	0.3628(±0.04285)

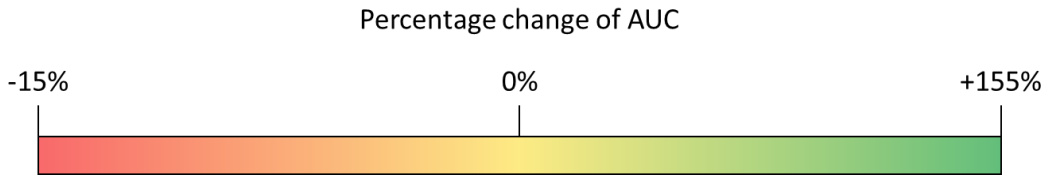
Figure 5.19. The SEM cell analysis shows comparison between; Area (A), Perimeter (B), Major axis (C), Minor axis (D), Aspect Ratio (E) and Roundness (F). ns – not significant ($p > 0.05$), * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.



5.3.6. Metabolomic Changes after OTC Antibiotic Pre-exposure

The plates for the metabolomic testing were chosen to assess the effects of OTC antibiotic pre-exposure on carbon source metabolism, biosynthetic pathways and the bacterial response to osmotic stresses (Table 5.7). Due to the large volume of data acquired from the metabolomic testing, only the key data are presented. Full data in Appendices 5.3 – 5.14.

Table 5.7. Key metabolomic changes in *E. cloacae* were chosen and they were classified by their role in bacteria. The percentage change in AUC from the control groups are presented and coloured by a gradient using red for downregulated growth, yellow for no change and green for upregulated growth.



Role in bacteria	Metabolite	Percentage change in AUC from the control after OTC antibiotic pre-exposure (%)		
		Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
Stress Protectant	D-trehalose	8.35	1.85	2.46
	D-trehalose + 6% NaCl	-7.59	6.36	14.98
Oxidative Stress	Glutathione	7.14	-0.52	6.00
	Glutathione + 6% NaCl	31.78	9.63	7.76
	Menadione	3.77	1.83	2.85
NADH/NADPH production	b-Nicotinamide Adenine Dinucleotide	0.85	1.18	1.00
Osmotic Stress	Ectoine + 6% NaCl	8.78	23.42	28.44
Low level penicillin protection	L-Glutamine	-4.73	-0.48	-13.62
Biofilm formation	Spermine	1.73	1.69	1.35
	Spermidine	1.55	0.78	-2.41
	Putrescine	1.62	1.43	-5.26
Fructose-Mannose Pathway	L-Mannose	8.75	0.38	8.58
	L-Fucose	35.54	153.51	36.06
	L-Fructose	5.89	-0.81	3.33
Amino Acid and Nucleotide Metabolism	L-Isoleucine + L-Valine	6.18	1.35	1.61
	Thymidine	8.21	1.18	2.20
	Uracil	5.38	2.28	3.12
	b-Alanine	2.33	1.35	1.60
	Adenine	9.97	1.83	2.75

There were many changes in the metabolome when *E. cloacae* was pre-exposed to OTC antibiotics. As these results were done in single replicate, statistical analysis was not possible but the changes observed can still give an idea of what metabolomic changes can in the cells after OTC exposure.

After OTC antibiotic pre-exposure, there was an up regulation in trehalose metabolism (Table 5.7). This could be indicating the bacteria are under stress as trehalose can be used as a stress protectant (Arguelles, 2000).

There was also an upregulation in the presence of glutathione (Table 5.7). This could be due to glutathione being important protecting bacteria from both oxidative and osmotic stress (Masip, *et al*., 2006). This could explain the upregulated growth in the presence of menadione due to bacterial adaptation to oxidative stress (Rowe, *et al*., 2020). The upregulation in b-Nicotinamide Adenine Dinucleotide can cause a higher production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Spaans, *et al*., 2015) which can combat reactive oxygen species (ROS).

When analysing the growth of bacteria under different osmotic stresses, bacteria that were pre-exposed to any of the OTC antibiotics grew better in the presence of ectoine (Table 5.7). Ectoine helps organisms tolerate osmotic stress (Richter, *et al*., 2019). These stresses arise from either the concentration of ions extracellularly or intracellularly and stopping biological processes. The OTC antibiotics may have been synergistic with ectoine as they disrupt the membranes and ion gradients across them.

It was found that there was a downregulation in glutamine metabolism (Table 5.7). This could be a result of the bacteria increasing the intracellular concentration of glutamine which can confer tolerance to penicillin (El Khoury, *et al*., 2017).

There was also an upregulation of spermine after pre-exposure to all OTC antibiotics, which plays a key role in growth and biofilm development (Michael, 2018). There was also an upregulation in both spermidine and putrescine after gramicidin (15 µg/ml) and bacitracin (5 IU/ml) pre-exposure, but not tyrothricin (200 µg/ml) pre-exposure (Table 5.7). These compounds both play a role in biofilm formation (Thongbhubate, *et al*., 2021). Therefore, biofilm formation may be affected after OTC pre-exposure, especially gramicidin (15 µg/ml) and bacitracin (5 IU/ml) pre-exposure. However, this would need to be determined through further testing as there are many factors which affect biofilm formation.

There also appears to be an upregulation in the fructose mannose pathway (KEGG, 2000), particularly in cultures pre-exposed to either gramicidin (15 µg/ml) or to tyrothricin (200 µg/ml) as the upregulation in L-Mannose, L-Fucose, and L-Fructose would suggest (Table 5.7). This is not unusual in bacteria to use different sugars as carbon sources (Muchaamba, *et al*., 2019) because the pre-exposure to OTC antibiotics could have caused this upregulation in metabolism due to inducing a nutrient stress.

When looking at changes in the bacterial biosynthetic pathways, there was an upregulation of the amino acid metabolism and nucleotide metabolism with the upregulation of L-Isoleucine + L-Valine, Thymidine, Uracil, b-Alanine and Uracil (Table 5.7). Both the amino acid and the nucleotide metabolism would indicate oxidative stress in bacteria (Sharma and Curtis, 2022). Especially changes in nucleotide metabolism are a well-conserved mechanism implemented by bacteria to handle a diverse range of stresses (Fitzsimmons, *et al*., 2018).

5.3.7. Mutational changes after OTC Antibiotic Pre-exposure

5.3.7.1. Single Nucleotide Polymorphisms

There were several mutational changes observed after OTC antibiotic pre-exposure. There were many SNPs found in the DNA after OTC antibiotic exposure (Table 5.8). In the control (no OTC antibiotic pre-exposure), there were a number of mutations that were different from the reference genome (CP001918) and were also not found in any of the bacteria pre-exposed to OTC antibiotics. These SNP mutations were found predominantly in the *merA* gene but also in the *merP* gene. These genes are part of the *mer* operon which is involved in resistance to mercury (Boyd and Barkay, 2012). There were also mutations in genes involved in the *mer* operon after pre-exposure to gramicidin (15 µg/ml) such as *merA* and *merD*. There were also mutations found in the *merA* gene after bacitracin (5 IU/ml) and tyrothricin (200 µg/ml) pre-exposure. There were SNPs found in *arsC* after bacitracin (5 IU/ml) pre-exposure and in *arsB* after tyrothricin (200 µg/ml) pre-exposure. These genes are involved in the *ars* operon and involved in the reduction and detoxification of arsenate (Ben Fekih, *et al*., 2018). There were multiple mutations in the *cusA* gene after pre-exposure to bacitracin (5 IU/ml; Table 5.8). This gene is part of a copper and silver efflux pump system that is part of the RND protein superfamily (Franke, *et al*., 2003). After bacitracin (5 IU/ml) pre-exposure, there was also a mutation in the *murE* gene. This gene encodes for the UDP-N-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase which is involved in cell wall biosynthesis (El Zoeiby, *et al*., 2003). After gramicidin (15 µg/ml) pre-exposure, there was a mutation in the *pilV* gene which is involved in the production of type IV pili. There were mutations found in five other genes that are not characterized (Table 5.8). There were some consistent mutations found in the gene labelled ECL_01016 and ECL_04767 after pre-exposure to any of the OTC antibiotics (Table 5.8). There was also a mutation in the gene labelled ECL_04873 after bacitracin (5 IU/ml) pre-exposure. In addition, after tyrothricin (200 µg/ml) pre-exposure, there were mutations in the gene labelled ECL_03819 and multiple mutations in the genes labelled ECL_04900.

5.3.7.2. InDels

There were no insertions or deletions in bacteria that were not pre-exposed to any OTC antibiotic or to bacitracin (5 IU/ml). There were six insertions and one deletion in the *pilV* gene that is involved in the production of a type IV pili after gramicidin (15 µg/ml) pre-exposure (Table 5.9). There were also two insertions and two deletions in intronic code after gramicidin (15 µg/ml) pre-exposure. After tyrothricin pre-exposure (200 µg/ml), there was a deletion in the ADF60336.1 gene, which encodes for a transposase. There were also two insertions in the ADF64424.1 gene which has unknown function.

Table 5.8. Single Nucleotide Polymorphisms (SNP) after pre-exposure to OTC antibiotics.

Gene	SNP Mutations after pre-exposure			
	Control	Gramicidin (15 µg/ml)	Bacitracin (5 IU/ml)	Tyrothricin (200 µg/ml)
arsB				T2913517C, C2913520T, T2913541A, C2913547T, G2913562T, G2913568A, G2913583A
arsC			G2914951A, C2914960A, C2914963G, G2915002T, G2915014A	
cusA			G4995925C, G4995934A, A4995955G, G4995961A, A4995964G, C4995970A, G4995985C, T4995997C, C4996000A, T4996003C, G4996007A, C4996009A, G4996012A, G4996015T, G4996021T, T4996027A, T4996032C, C4996042G, G4996048A, C4996051T, T4996060C, T4996078C, C4996093G, C4996095T, A4996099G, T4996114C, C4996117A, T4996126A, G4996129C, A4996132G, G4996133A, T4996141C, G4996147A, T4996150C, G4996162T, T4996168C, T4996186C, A4996189G,	

Table 5.8 - continued

ECL_01016		**G1036083A, C1036101T, T1036146C**	**G1036083A, C1036101T, T1036146C**	**G1036083A, C1036101T**
ECL_03819				T3907712G
ECL_04767		G4877630T	G4877630T	G4877630T
ECL_04873			G4983129A, A4983130T	
ECL_04900				T5006057A, G5006063A, T5006066C, A5006067T, C5006069A, G5006094T, T5006096C, A5006107G, C5006108T, T5006129C, C5006132T, T5006138A
merA	T3910490C, A3910494C, T3910497C, T3910505C, C3910511T, T3910514C, C3910515A, T3910526C, C3910538G, G3910541A, C3910542T, C3910547A, G3910556A, C3910676G, C3910691T, C3910706T, C3910715T, A3910718G, C3910724G, A3910725G, T3910727C, G3910736A, A3910745C, A3910766G	C3909796A, A3909812G, C3910304G, C3910391T, G3910397A, C3910400G	C3910391T, C3910400G	C3910304G, C3910311A, G3910313A, C3910316G, T3910385G, C3910391T, G3910397A, C3910400G

Table 5.8 - continued

merD		**C3911364T, T3911367C, A3911369G, A3911370T, G3911374T, C3911376T, C3911392T**		
merP	G3909124A			
murE			C883250T	
pilV		T440274G		

Table 5.9. Insertion and Deletion mutations after OTC antibiotic pre-exposure.

Gene	InDel Mutations after OTC exposure					
	Gramicidin (15 µg/ml)			Tyrothricin (200 µg/ml)		
	Position	Reference	Alternative	Position	Reference	Alternative
pilV	440255	AG	A			
	440257	CCT	C			
	440263	GTCCTACA	G			
	440271	G	GGA			
	440278	T	TG			
	440280	GTTCA	G			
	440285	GTCTGTA	G			
Intronic	5006337	TGTCACGAC	T			
	5006347	A	ATATATT			
	5006352	GATTT	G			
	5006359	C	CA			
ADF60336.1				764476	CT	C
ADF64424.1				5006074	C	CA
				5006075	T	TCGTCATCGTCATCGTCA

5.4. Discussion

5.4.1. Role of growth rate in antibiotic resistance

The effect of OTC antibiotics on growth rate showed that in *E. coli*, both gramicidin and tyrothricin had an overall increased growth rate and faster generation time. All of the OTC antibiotics caused a significant increase in the t_mid suggesting an extended lag phase. This was similarly the case in *E. cloacae* and *K. pneumoniae* after tyrothricin (200 µg/ml) pre-exposure. There was also an extension of lag phase in *A. baumannii* after gramicidin (15 µg/ml) pre-exposure. An extension of lag phase may be caused by the bacteria adapting to a new environment (Rolfe, *et al*., 2012). This also creates an advantage in bacteria to resist antibiotics; an extension of lag phase is the first change in bacteria as a response to antibiotic stress (Fridman, *et al*., 2014). This is because often antibiotics are more effective in actively growing cells and therefore an extension of lag phase can cause antibiotics to tolerate high concentrations of antibiotics and subsequently can promote the evolution of antibiotic resistance. Therefore, pre-exposure to any of the OTC antibiotics in *E. coli* or pre-exposure to tyrothricin (200 µg/ml) in *E. cloacae* could be causing bacteria to be able to tolerate other antibiotics for longer periods of time allowing the possibility of resistance development.

5.4.2. Efflux pump activity in antibiotic resistance

Many of the efflux experiments showed that there were no changes in efflux after pre-exposure to OTC antibiotics, except in *E. cloacae* after tyrothricin (200 µg/ml) pre-exposure. However, many of the experiments showed in the presence of CCCP, there was a lower accumulation of ethidium bromide. Ethidium bromide is taken into the cell via porins (Martins, *et al*., 2013). There are various pathways that bacteria have, to reduce the intracellular concentrations of toxic compounds (Anes, *et al*., 2015), and therefore the lower accumulation of ethidium bromide may be due to other reasons than efflux such as modulation to the cell membrane composition (Delcour, 2009) or expression of porins (Masi and Pagès, 2013). Other effects of the CCCP on the cell could be the cause of the lowered accumulation of ethidium bromide as CCCP is a proton motive force (PMF) inhibitor and will affect cell functions such as metabolism, cell membrane potential and efflux (Strahl and Hamoen, 2010). Therefore, further experimentation would be needed to determine the cause of this lowered accumulation as it could be due to other changes in the cell such as, porin expression, membrane potential or membrane composition changes.

5.4.3. Antibiotic Resistance and Virulence

Virulence in bacteria is also a concern for clinicians as an increase and spread of virulence factors can results in more infection (Peterson, 1996). This combined with antibiotic resistance, makes treating patients very difficult. Previously, it was thought that bacteria that were antibiotic resistant, had a decreased virulence due to the fitness cost of the resistance (Beceiro, *et al*., 2013). However, due to the nature of the virulence and resistance gene regulation, the two aspects are often combined and increase in resistance could also mean increase in virulence (Schroeder, *et al*., 2017). Increased virulence could be linked to changes in outer membrane structures such as fimbriae, through enhanced adhesion (Jonson, *et al*., 2005). Here, an increase in virulence following OTC antibiotic pre-exposure was observed on occasion, although, differences in survival of *Galleria mellonella* over time was not always consistent, possibly because of the small set (910 larvae) used. However, further experimentation would be needed to confirm this.

5.4.4. Change in bacterial morphology and its role in antibiotic resistance

The role of bacterial morphology is still poorly understood but it can play a role in the development of antibiotic resistance (Ojkic, *et al*., 2022). The results show that after pre-exposure to any of the OTC antibiotics there were no significant changes in the area, perimeter or major axis (length) of the cells. However, there were significant increases in the width of the cells after bacitracin (5 IU/ml) pre-exposure indicating the cells were ‘fatter’. There were also significant decreases in the aspect ratio of the cells after either bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). The aspect ratio is the major axis of the cell/ minor axis of the cell. Therefore, a decrease in the aspect ratio shows the cells are becoming ‘shorter’ and ‘fatter’. There was also a significant increase in the roundness of cells after bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) pre-exposure. All these data mean the cells were decreasing the S/V. Our research hypothesis however expected an increase in their S/V ratio, as the OTC antibiotics are membrane active, according to Ojkic, *et al*. (2022). There are examples of resistance by a decrease in S/V to membrane active agents such as chlorhexidine (Tattawasart, *et al*., 2000). There was also no significant change in cell morphology after gramicidin (15 µg/ml) pre-exposure when compared to the untreated control. However, differences in cell size due to growth, add to variability within the data. Increasing the number replicates could determine further distinctions between the cell morphologies after treatments. Further experiments would also be required to assess why the cells would decrease their S/V.

5.4.5. Beta-lactamase Activity and the Impact of Clinical Resistance

E. cloacae ATCC 13047 is known to carry 11 beta-lactamase genes (Ren, *et al*., 2010). Possibly the most notable of these beta-lactamase genes is the AmpC gene. This is a broad spectrum Ambler Class C beta-lactamase and can confer resistance to, broad-spectrum cephalosporins such as cefotaxime, ceftazidime and ceftriaxone (Harris and Ferguson, 2012). When induced at high enough concentrations, it can also give rise to monobactam resistance (Jacoby, 2009). However, AmpC normally is only expressed at clinically insignificant levels and therefore does not give the resistant phenotype. But when AmpC is upregulated by mutations in the promotor region, clinical resistance can be observed (Corvec, *et al*., 2007). AmpC can also be upregulated by changes in transcription that occurs when there is disruption in the bacterial cell-wall recycling processes. This is called inducible expression and often occurs in AmpC when the bacteria come into contact with beta-lactam antibiotics (Tamma, *et al*., 2019), although it is possible that other membrane damaging compounds such as membrane active biocide have the same affect.

When *E. cloacae* was pre-exposed to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) there was a higher average beta-lactamase activity. This is significant in the cultures pre-exposed to either gramicidin (15 µg/ml) or tyrothricin (200 µg/ml) per microliter in the reaction wells (Figure 5.18). This higher activity could be due to induced AmpC expression and could explain the resistance to cefotaxime and aztreonam seen in Chapter 4. To confirm this, qPCR needs to be done on the *ampC* gene is culture after OTC antibiotic exposure.

5.4.6. Changes in the Metabolome and the Associated Phenotypic Changes

There were many changes in the metabolome when *E. cloacae* was pre-exposed to OTC antibiotics. As these results were done in single replicate, statistical analysis was not possible, but the changes observed can still give an idea of what metabolomic changes can in the cells after OTC exposure.

Many of the changes seen in the metabolomic data indicate that the OTC antibiotics caused various stresses which in turn changed the bacterial metabolome. A common theme occurring was the upregulation in metabolism to deal with oxidative stress (Table 5.7). This could be expected as many bactericidal antibiotics can generate varying levels of reactive oxygen species with contribute to the killing of the bacterial cell (Dwyer, *et al*., 2014).

In the case of the OTC antibiotics, it has been proposed that gramicidin can cause perturbations in the ion gradients across the cell membrane which causes the oxidative

stress (David and Rajasekaran, 2015). This oxidative stress can then cause DNA damage leading to mutation. It can also result in the upregulation of NADPH to regenerate glutathione to detoxify ROS. The disruption in ion gradients can also cause an osmotic stress and therefore explain the upregulation in ectoine to help bacteria cope with this (Table 5.7). Upregulation in amino acid and nucleotide metabolism is often seen in bacteria that are under oxidative stress (Sharma and Curtis, 2022).

There are various pathways in which bacteria may upregulate the production of NADPH. The main pathways are: the oxidative pentose phosphate pathway, the Entner-Doudoroff pathway, and the isocitrate dehydrogenase step of the tricarboxylic acid cycle (Spaans, *et al*., 2015). The fructose mannose pathway does not generate NADPH which is used for anabolic redox reactions, but generates NADH which is used for oxidative reactions (Harold, 1986). Therefore, it is unlikely this plays a role in protection from oxidative stress, but rather generates energy for other processes such as efflux, damage repair or enzyme production.

There was interestingly a downregulation in glutamine metabolism (Table 5.7). This has been previously seen in *S. pneumoniae* and caused the bacteria to accumulate intracellular glutamine (El Khoury, *et al*., 2017). This gave a low-level of resistance to penicillin and could be related to the beta-lactamase activity (Section 5.3.4) and penicillin resistance seen in Chapter 4, but would need further testing to confirm this.

Finally, there were changes in metabolism related to biofilm formation. Whilst pre-exposure to gramicidin (15 µg/ml) and bacitracin (5 IU/ml) upregulated the genes, tyrothricin (200 µg/ml) mainly downregulated them. It is unknown what effects this will have on development of biofilm but would warrant further investigation as this is a mechanism bacteria use to protect themselves from antibiotics (Singh, *et al*., 2017).

5.4.7. Mutational Changes after OTC antibiotic exposure

There were multiple mutations in *E. cloacae* after exposure to OTC antibiotics. Notably, there were changes in the *mer* operon. This operon is involved in resistance to mercury and the central enzyme involved in mercury resistance in the *merA* enzyme which catalyses the reduction of mercury Hg(II) making it Hg(0) and volatile which diffuses away from cells (Wagner-Döbler, 2003). Although this can contribute to heavy metal resistance, the *merA* gene has not been conclusively linked to antibiotic resistance (Martani, *et al*., 2022). Similarly, there were mutations found in an operon that is related to metal or metalloid resistance, the *ars* operon, which confers resistance to arsenate (Ben Fekih, et al., 2018).

Although these genes have not been linked with antibiotic resistance, there have been numerous links between tolerance to heavy metal and the development of antibiotic resistance (Edet, *et al*., 2023). One such instance is *cusA* for which there were many mutations after bacitracin (5 IU/ml) pre-exposure. The *cus* operon encodes for a RND efflux pump which confers resistance to copper and silver ions, Cu(I) and Ag(I) (Gudipaty, *et al*., 2012). This has been linked to resistance to tigecycline, a glycylcycline antibiotic, that is used to treat serious bacterial wound and digestive infections (Townsend, *et al*., 2007). This change could be important as the fate of OTC antibiotics after a patient using them would be in the digestive tract.

There were also mutations found in the *murE* gene after bacitracin (5 IU/ml) pre-exposure. This gene plays a role in the biosynthesis of the bacterial cell wall (De Lencastre, *et al*., 1999). When disrupted however, it can cause an accumulation of UDP-MurNAc dipeptides (Gardete, *et al*., 2004), which are later converted into UDP-MurNAc pentapeptides (Taguchi, Kahne and Walker, 2019). Interestingly, these peptides are suppressors of the *ampR* gene which regulates the expression of *ampC* (Vadlamani, *et al*., 2015). If *murE* production is compromised, 1,6-anhydro-MurNAc-peptides in the cytosol will displace UDP-MurNAc-pentapeptide and activate the *ampC* gene (Vadlamani, *et al*., 2015). This could explain the heightened beta-lactamase activity (Section 5.3.4). however, further experiments would be needed to validate this.

Mutations were also found in the *pilV* gene (Table 5.8 & Table 5.9). This gene is involved in the formation of a type IV pilus (Alm and Mattick, 1995). Pili are responsible for adhesion bacteria to host cells and therefore play a crucial role in infection (Psonis and Thanassi, 2019). Although, they are also involved in twitching motility (Mattick, 2002). Pili are predominantly made proteins called major pilins but also are made of minor pilins, such as *pilV*, which are essential for the formation and function of a pilus (Jacobsen, *et al*., 2020). Further experiments such as motility assays and biofilm formation assays could be used to determine the phenotypic effects associated with the mutations in *pilV*.

There were also several mutations found in uncharacterized genes and it would be difficult to hypothesize what effects these mutations are having on the bacteria. One common theme amongst the known mutations however is that they are all membrane proteins. This could indicate that *E. cloacae* has changes to its membrane after exposure to OTC antibiotics that could result in the phenotypic changes seen. Most notably the gain of cross-resistance to clinical antibiotics.

5.5. Chapter Conclusions

There were many phenotypic changes is bacteria after pre-exposure to OTC antibiotics. It was seen that OTC antibiotics could cause extensions in lag phase of bacterial growth, allowing for bacteria to adapt and tolerate different stresses. The combination of increase in efflux pump activity, change in morphology, the metabolome and genetic changes to membrane proteins could cause the cross-resistance seen in Chapter 4. The most notable phenotypic change was the increase in beta-lactamase activity. This is likely due to the chromosomally encoded *ampC* gene that is present in *E. cloacae*. This when overexpressed can cause resistance to 3rdgeneration cephalosporins and monobactams. This expression can occur as a result of membrane damage and the accumulation of 1,6-anhydro-MurNAc-peptides which displace UDP-MurNAc-pentapeptide. This accumulation activates *ampR* which activates *ampC* which gives the resistant phenotype (Harris, 2015). The reasons behind this may need further investigation however, mutations in genes such as *murE* could result in this effect. Therefore, treatment of OTC antibiotics could be inducing beta-lactamase expression thus creating resistant populations. Long-term, this could cause issues for clinicians as beta-lactams are the most widely used antibiotics in clinics (Bush and Bradford, 2016).

Chapter 6: Co-exposure of Clinical Antibiotics and OTC Antibiotics

Chapter 6: Co-exposure of Clinical Antibiotics and OTC antibiotics

6.1. Introduction

6.1.1. How antibiotics enter bacterial cells

There are many different antibiotic classes that affect different bacterial cell targets. Many antibiotics need to get through bacterial structures to reach their target, for example aminoglycosides which target the 30S ribosomal subunit within the bacterial cytoplasm (Kotra, *et al.*, 2000). Antibiotics can accumulate within cells by either diffusion through porins, diffusion through the bilayer or self-uptake (Kapoor, *et al.*, 2017). As antibiotics need to accumulate in the cell to reach an effective concentration, there are mechanisms that prevent their accumulation leading to clinical resistance. This is either through decreasing the uptake of antibiotics or by increasing their efflux. The main mechanism utilized naturally by bacteria is the decreased uptake of antibiotics by reducing the permeability of bacterial membranes (Reygaert, 2018). This is especially seen in Gram-negative bacteria due to the presence of the outer membrane which can stop antibiotics from reaching their target. The outer membrane is an effective barrier against the entry of lipophilic molecules (Prajapati, *et al.*, 2021). However, small hydrophilic molecules can enter into cells via porins, proteins that form pores that span the membrane (Nichols, 2017).

Antibiotic resistance can occur due to the decrease in number of porin channels, or porin channel size and hence cause resistance to antibiotics such as beta-lactams, tetracycline, chloramphenicol or fluoroquinolones (Nikaido, 2003). Although, it is not just the number of porins that span the membrane that can lead to resistance, but also decreased antibiotic uptake by changes in the bacterial membrane potential. The bacterial membrane potential is the transmembrane electric potential ($\Delta\Psi$). Along with the transmembrane chemical proton gradient (ΔpH), the membrane potential is a key factor in the PMF and energy production in the cell (Strahl and Hamoen, 2010). The membrane potential gradient is especially important in aminoglycoside activity (Bruni and Kralj, 2020). Although the exact mechanism of aminoglycoside uptake into the bacterial cell is still contested, it is agreed that a decrease in bacterial cell membrane potential can reduce the uptake of aminoglycosides and thus lead to a decrease in their antimicrobial efficacy (Ezraty, *et al.*, 2013; Damper and Epstein, 1981). Chemicals that could depolarize bacterial cell

membranes should be considered during antibiotic treatments especially during aminoglycoside treatments.

6.1.2. Antibiotic Antagonism

Antibiotic therapies can be administered using two antibiotics to utilize their synergistic effects. This allows for more efficient treatments, and therefore clearance of the infection at lower concentrations (Cottarel and Wierzbowski, 2007; Tängdén, 2014; Coates, *et al*., 2020). Conversely, antibiotic antagonism can lead to treatment failure which in turn could result in the development of antibiotic resistance. Furthermore, antibiotic antagonism could compromise the treatment of life-threatening infections such as the treatment of pneumococcal meningitis, where combined treatment with chlortetracycline and penicillin compared to penicillin alone resulted in an increase in mortality from 30% to 79% (Cates, *et al*., 1951; Garrod, 1972).

The antagonistic relationship between bacteriostatic and bactericidal antibiotics has been previously described (Johansen, *et al*., 2000). Ocampo and colleagues demonstrated the that a bacteriostatic antibiotic used in combination with a bactericidal antibiotic, resulted in the bacteriostatic antibiotic interfering with the bactericidal antibiotic's activity (Ocampo, *et al*., 2014). It was hypothesized that this was a result of the bactericidal antibiotics being most potent on cells that are actively dividing. Whilst the bacteriostatic antibiotics affected the bacterial cell division. This effect on cell division reduced the activity of the bactericidal antibiotics. It is therefore important to consider antibiotics or any other antimicrobial compounds that can interfere with cell division and antagonize bactericidal antibiotics.

Antagonism has not just been seen with antibiotics but also with biocides such as benzalkonium chloride (BZK) (Short, *et al*., 2021). Sub-inhibitory concentrations of BZK were shown to antagonize aminoglycoside activity and promote the emergence of resistant mutants. Short and colleagues (2021) concluded that the reason for antagonism was the dissipation of the bacterial cell membrane potential which aminoglycosides require to enter bacterial cells.

It is not currently known whether OTC antibiotics such as gramicidin, tyrothricin or bacitracin interact with any other antibiotics. However, it is known that gramicidin (also a major component of tyrothricin) depolarizes mammalian cell plasma membranes by disrupting the ion gradient (Shin, *et al*., 2013). As for bacitracin, its interaction with cell membrane potential has not been investigated.

6.1.3. Chapter Aims

The aim of this chapter is to explore whether OTC antibiotics could interfere with clinical treatments. This will be achieved by co-exposing OTC antibiotics at a during-use concentration with clinical antibiotics at a bactericidal concentration. The mechanism behind any interference will be explored using membrane potential and potassium leakage assays.

6.1.4. Principle of Experiments and Rationale

Previous literature has shown that compounds such as biocides can interfere with clinical antibiotic treatments such as aminoglycosides. This happens at sub-MIC levels of certain biocides such as BZK depolarizing the bacterial cell membranes which aminoglycosides require to enter cells and kill bacteria. Cell membrane potential is determined by the concentration of mainly potassium ions either side of the membrane. Antibiotics such as gramicidin, form ionophores and therefore form pores in bacterial membranes which allow the passage of monovalent cations such as sodium and potassium. As gramicidin is a large component of tyrothricin, it is likely that tyrothricin would have the same effect. As for bacitracin, it inhibits cell-wall biosynthesis, although it is unknown what effect it will have on cell membrane potential. However, as many of the OTC antibiotics, such as gramicidin, bacitracin or tyrothricin, affect bacterial cell membrane or cell wall (Stone and Strominger, 1971; Busath and Szabo, 1981; Wallace, 1998; Wallace 2000; Lang and Staiger, 2016), they may interfere with the uptake of clinical antibiotics (Short, *et al*., 2021).

6.2. Methods

6.2.1. Bacterial Strains and Culture Conditions

The strains used is this chapter were *A. baumannii* ATCC 19568, *E. coli* ATCC 25922, *E. cloacae* ATCC 13047 and *K. pneumoniae* ATCC 13883. The bacteria used in this testing were selected from the bacteria that showed changes in resistance after pre-exposure to OTC antibiotics (Chapter 4). These organisms are also part of the ESKAPE pathogens that cause significant clinical challenges (Mulani, *et al*., 2019). Bacteria were grown on TSA for routine culture maintenance or in MHB for antibiotic experiments (Chapter 3, Table 3.2).

6.2.2. Antibiotics Selected and Preparation of Antibiotic Stocks

Clinical antibiotics were selected based on the aminoglycoside cross-resistance development seen after pre-exposure (Chapter 4; Table 4.12).

Table 6.1. Co-exposure combinations tested.

Organism	OTC Antibiotic	Clinical Antibiotic
A. baumannii	Gramicidin, Bacitracin	Amikacin, Gentamicin, Tobramycin
E. coli	Gramicidin, Tyrothricin, Bacitracin	Gentamicin
E. cloacae	Gramicidin, Tyrothricin, Bacitracin	Gentamicin
K. pneumoniae	Gramicidin, Tyrothricin, Bacitracin	Gentamicin

Clinical antibiotic stocks were prepared in sterile distilled water to a concentration of 1 mg/ml and sterilized through a 0.22 µm membrane filter. Bacitracin stock was prepared at a concentration of 250 IU/ml in sterile distilled water and as filter sterilized as before. As Gramicidin and Tyrothricin are poorly soluble in water, the stocks were dissolved in methanol at concentrations of 0.75 mg/ml and 10 mg/ml respectively.

6.2.3. Measurement of Minimum Inhibitory Concentration (MIC)

Aminoglycoside MIC were determined using a standard microbroth-dilution test (ISO, 2020) (Chapter 4, Section 4.2.3). Aminoglycoside stock solution was serially diluted 2-fold in sterile deionized water to give a final concentration range of 0.25 - 128 µg/ml. Bacterial test inocula were prepared in MHB to give a final concentration of 5 x 10^5 CFU/ml.

6.2.4. Co-exposure Assays

The co-exposure assay was based on the time to kill assay from Short *et al*. (2021). Overnight liquid bacterial cultures (5 ml) were pelleted by centrifugation at 3,000G for 10 minutes and resuspended in 5 ml of PBS. One hundred microlitres of washed culture was then added to 5 ml MHB and incubated at 37°C, shaking at 120 RPM for 2 hours. After incubation, the aminoglycosides were added to the bacterial test suspension; the concentration used depended upon the aminoglycoside and the bacterial species (Table 6.2).

Table 6.2. Concentration of aminoglycosides used for each co-exposure assay. This corresponded to twice the MIC.

Organism	**Antibiotic**	**MIC (µg/ml)**	**Final Concentration (µg/ml)**
A. baumannii	Amikacin	1	2
	Gentamicin	1	2
	Tobramycin	1	2
E. cloacae	Gentamicin	2	4
E. coli	Gentamicin	2	4
K. pneumoniae	Gentamicin	1	2

One hundred microlitres of the OTC antibiotic stocks were also added to the test suspension to give a final concentration of 15 µg/ml for gramicidin, 200 µg/ml for tyrothricin and 5 IU/ml for bacitracin. These concentrations are based on the during-use concentrations of the antibiotics found in sore throat lozenges sold in Europe and were calculated taken into consideration antibiotic concentration in the lozenges and the dilution by an average man's saliva when taken by mouth, which means a lozenge in dissolved in approximately 20 ml (Iorgulescu, 2009; Maheshwari *et al*., 2013). Negative controls contained only aminoglycoside and positive control consisted of aminoglycosides co-exposed with BZK (4 µg/ml). After the addition of antibiotics, bacterial concentration was immediately evaluated by taking 20 µl from the test suspension and drop counting on TSA using the Miles and Misra method following serial dilution in PBS. Test suspensions

were then incubated at 37°C under shaking at 120 RPM for 3 hours. After incubation, bacterial concentration was enumerated as described above. All TSA plates were incubated at 37°C overnight. Colonies in each drop were counted and the average was taken. The final colony forming units per millilitre was calculated and $\log_{10}$ reduction at 3 hours was compared with the controls.

6.2.5. Membrane Potential

To measure the change in membrane potential, the BacLight™ bacterial membrane potential kit was used (B34950; ThermoFisher Scientific, Massachusetts, USA). *A. baumannii* only was used to measure the effects of OTC antibiotics on bacterial membrane potential since Enterobacteriaceae did not interact with the fluorescent dye used (data not shown). Five ml overnight cultures of *A. baumannii* was pelleted by centrifugation at 3,000 G for 10 min and resuspended in 5 ml MHB. The bacterial suspension was adjusted to a cell density of approximately 10^8 CFU/ml in MHB. One hundred µl of gramicidin (750 µg/ml) and bacitracin (250 IU/ml) stocks were added to 5 ml of adjusted cell suspension to give a final concentration of 15 µg/ml and 5 IU/ml respectively. A negative control (polarized cells) consisting of no antibiotic treatment was used. Suspensions were incubated for 3 hours at 37°C under shaking at 120 RPM. After incubation, cultures were diluted 100-fold in PBS to give an approximate cell density of 10^6 CFU/ml. One ml of suspension was aliquoted to a flow cytometry tube from the antibiotic exposed and untreated tubes. Two additional flow cytometry tubes were aliquoted from the untreated sample for the unstained and positive (depolarized) controls. To the positive control tube, 10 µl of 500 µM CCCP was added and mixed for 10 seconds to give a final concentration of 5 µM CCCP. To each flow cytometry tube, except the unstained control, 10 µl of 3 mM DiOC2(3) (3,3'-diethyloxacarbocyanine iodide) was added and incubated for 30 min at room temperature before analysing by flow cytometry. Samples were analysed using a BD LSR Fortessa flow cytometer. The channels used for detecting the green and red fluorescence were the FITC-A and PE-Texas Red-A channels respectively. The forward and side scatter of the flow cytometer was adjusted using the unstained cells to determine the gating for the bacterial population. In total, each sample had 10,000 events recorded. The mean red and green fluorescence was recorded and compared between samples to determine the change in membrane potential.

6.2.6. Potassium Leakage

A. baumannii was grown overnight in 5 ml MHB at 37°C, under shaking at 120 RPM. The culture was then washed three times in deionised sterile water by centrifuging at 3,000 G. One hundred µl of triple-washed culture was added to 5 ml of deionised sterile water to give a final concentration of approximately 2 x 10^7 CFU/ml. One hundred µl of gramicidin and bacitracin were then added to give final concentrations of 15 µg/ml and 5 IU/ml, respectively. Negative controls consisting of water or methanol, and a positive control consisting of cell treated with 4 µg/ml BZK were used. Samples were incubated statically at room temperature for 3 hours. After incubation, the cultures were filtered into sterile tubes through a 0.22 µm nitrocellulose filter membrane. The potassium in each cell-free sample was measured using inductively coupled plasma spectrometry (ICP) and concentrations were compared between samples to determine leaked potassium.

6.2.7. Statistical Analysis

All experiments were conducted in biological triplicate. Statistical significance was determined by one-way ANOVA followed by a Tukey Post-hoc test on the data using GraphPad Prism (GraphPad Prism version 9.5.1. for Windows). Time-kill experimental data was log-transformed before analysis.

6.3. Results

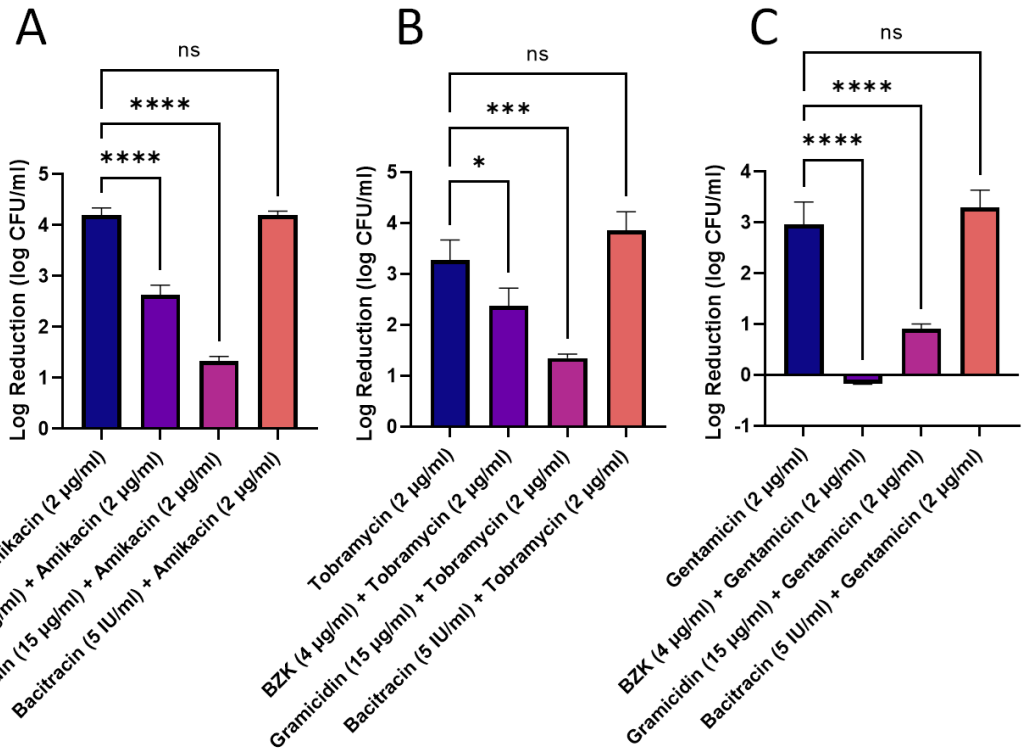
6.3.1. MIC Results

Aminoglycoside MIC for the different test bacteria are reported in Table 6.2.

6.3.2. OTC antibiotics protecting *A. baumannii* against aminoglycoside activity

We confirmed that amikacin (2 µg/ml) was strongly bactericidal (4.20 ± 0.14 log_{10} reduction in CFU/ml) against *A. baumannii* after 3 hours exposure (Figure 6.1A). But when co-exposed with either BZK (4 µg/ml) or gramicidin (15 µg/ml), bactericidal activity of amikacin significantly ($p < 0.0001$) decreased to 2.63 ± 0.19 log_{10} CFU/ml with BZK and 1.33 ± 0.09 log_{10} CFU/ml with gramicidin (Figure 6.1A). Similar results were observed with tobramycin (3.27 ± 0.40 log_{10} CFU/ml reduction alone; Figure 6.1B) and gentamicin (2.96 ± 0.44 log_{10} CFU/ml reduction alone; Figure 6.1C). There was a significant reduction in their bactericidal efficacy when exposed to BZK (2.37 ± 0.35 log_{10} CFU/ml reduction; $p=0.0401$) for gramicidin (1.35 ± 0.08 log_{10} CFU/ml reduction ($p=0.0004$) for tobramycin (Figure 6.1B). There was also significant decreases in bactericidal efficacy when co-exposed to BZK (-0.17 ± 0.01 log_{10} CFU/ml reduction; $p<0.0001$) or gramicidin (0.91 ± 0.09 log_{10} CFU/ml reduction ($p<0.0001$) for gentamicin (Figure 6.1C). In contrast, bacitracin (5 IU/ml) did not reduce the bactericidal efficacy of any of the aminoglycosides (Figure 6.1).

Figure 6.1. Bactericidal efficacy of aminoglycoside co-exposed OTC antibiotics in *A. baumannii*. (A) *A. baumannii* treated with amikacin (2 µg/ml) for 3 hours. There was a significant difference in $\log_{10}$ reduction when co-exposed with BZK (4 µg/ml; p<0.0001) or gramicidin (15 µg/ml; p<0.0001). When co-exposed to bacitracin (5 IU/ml) the difference in bactericidal efficacy to amikacin alone was not significant (p>0.9999). (B) *A. baumannii* treated with tobramycin (2 µg/ml) for 3 hours. Tobramycin efficacy was significantly reduced when co-exposed to either BZK (4 µg/ml; p=0.0401) or gramicidin (15 µg/ml; p=0.0004) but not with bacitracin (5 IU/ml; p=0.2085). (C) *A. baumannii* treated with gentamicin (2 µg/ml) for 3 hours. Gentamicin bactericidal efficacy was significantly decreased when exposed to BZK (4 µg/ml; p<0.0001) or gramicidin (15 µg/ml; p<0.0001) but not with bacitracin (5 IU/ml; p=0.5136). ns – not significant (p > 0.05), * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001, **** - p ≤ 0.0001.



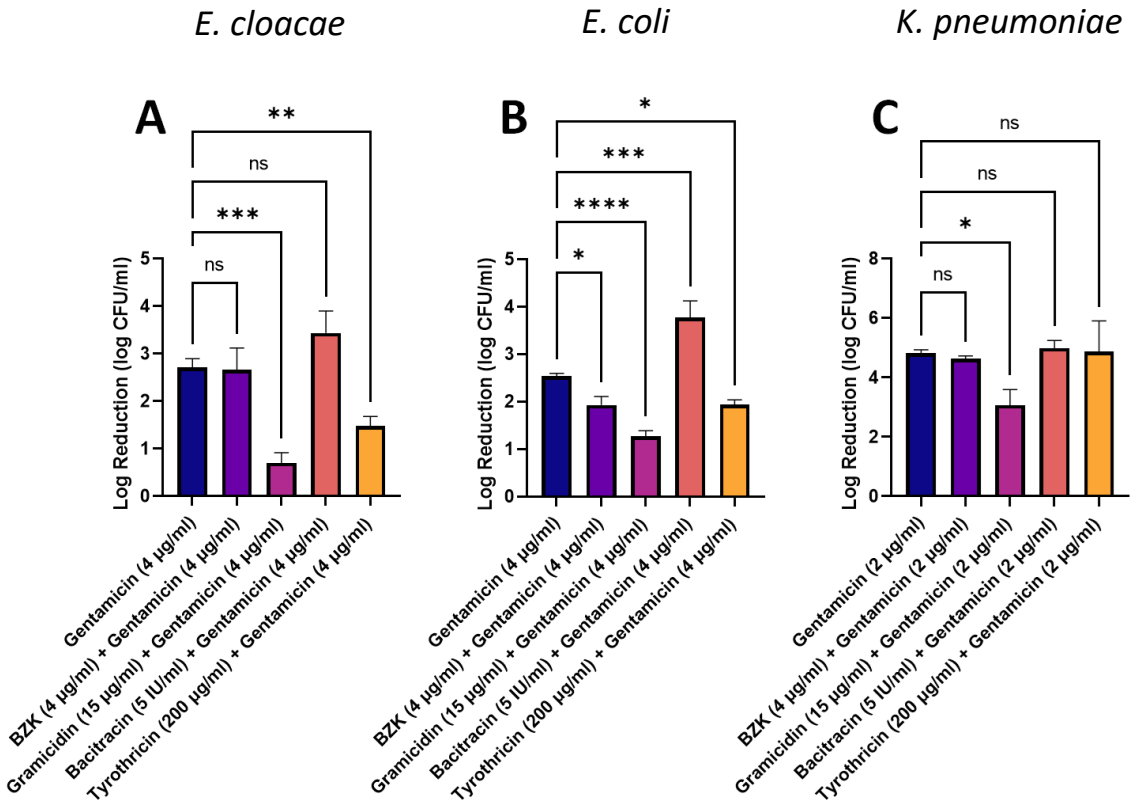
6.3.3. OTC antibiotics protecting ESKAPE *Enterobacteriaceae* against gentamicin activity

Gentamicin at the concentrations tested (Table 6.2) was confirmed to be bactericidal after 3 hours exposure against *E. cloacae* (2.71 ± 0.19 log_{10} CFU/ml reduction), *E. coli* (2.53 ± 0.06 log_{10} CFU/ml reduction) and *K. pneumoniae* (4.82 ± 0.11 log_{10} CFU/ml reduction)(Figure 6.2). BZK (4 µg/ml) did not affect the bactericidal efficacy of gentamicin (2.67 ± 0.46 log_{10} CFU/ml reduction; p=0.9998) in *E. cloacae* (Figure 6.2A). Gentamicin co-exposure with gramicidin (15 µg/ml) or tyrothricin (200 µg/ml) significantly reduced the efficacy of the aminoglycoside, 0.70 ± 0.22 log_{10} CFU/ml reduction with gramicidin (p=0.0002) and 1.47 ± 0.20 log_{10} CFU/ml reduction with tyrothricin (p=0.0074)(Figure 6.2A).

In *E. coli*, co-exposure to BZK (4 µg/ml), gramicidin (15 µg/ml) or tyrothricin (200 µg/ml) negatively impacted the efficacy of gentamicin (1.93 ± 0.18 log_{10} CFU/ml reduction (p=0.0271) with BZK; 1.26 ± 0.13 log_{10} CFU/ml reduction (p<0.0001) with gramicidin; 1.47 ± 0.20 log_{10} CFU/ml reduction (p=0.0225; Figure 6.2B) with tyrothricin. In contrast, the combination of gentamicin with bacitracin significantly contributed to an increased bactericidal efficacy of the aminoglycoside (3.77 ± 0.35 log_{10} reduction; p=0.0001)(Figure 6.2B).

In *K. pneumoniae* co-exposure data were different (Figure 6.2C). Only the combination of gentamicin (2 µg/ml) with gramicidin (15 µg/ml) significantly decreased the efficacy of the aminoglycoside (3.07 ± 0.53 log_{10} reduction; p= 0.0172)(Figure 6.2C). Co-exposure of gentamicin with BZK (4 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) did not results in statistically significant changes in bactericidal activity (p=0.9913, p=0.9947, p>0.9999, respectively).

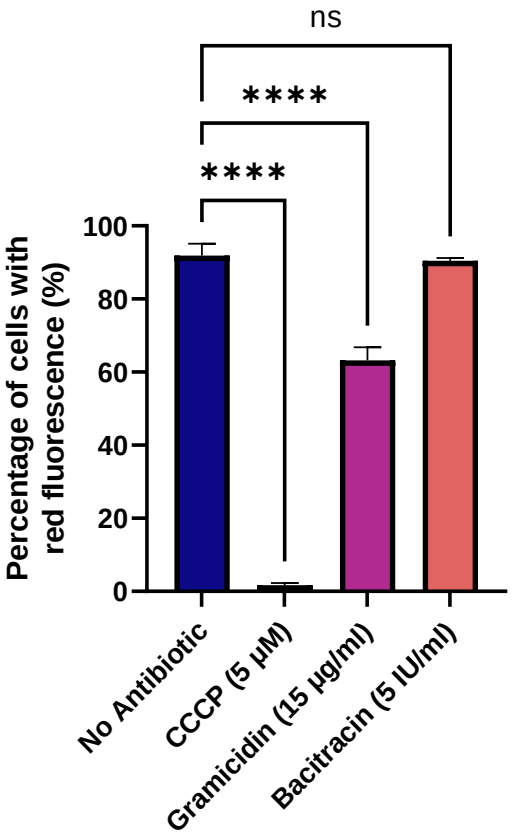
Figure 6.2. Bactericidal efficacy of aminoglycoside co-exposed OTC antibiotics in *Enterobacteriaceae*. (A) *E. cloacae* treated with gentamicin for 3 hours. Co-exposed to gramicidin or tyrothricin significantly decreased gentamicin efficacy when compared to gentamicin treatment alone (p=0.0002 & p=0.0074 respectively). (B) *E. coli* cultures treated with gentamicin for 3 hours. There was a significant decrease in gentamicin efficacy when cultures were co-exposed to BZK (p=0.0217), gramicidin (p<0.0001) or tyrothricin (p=0.0225). (C) *K. pneumoniae* treated with gentamicin for 3 hours. Co-exposure to gramicidin significantly decreased efficacy when compared to gentamicin alone (p=0.0172). ns – not significant (p > 0.05), * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001, **** - p ≤ 0.0001.



6.3.4. OTC antibiotics abolishing membrane potential

There was a change in cell membrane potential in *A. baumannii* suspensions exposed to gramicidin (15 µg/ml) but not the cultures exposed to bacitracin (5 IU/ml) after 3 hours exposure. When bacteria were not exposed to an antibiotic, their cell membrane potential remained intact with an average of 91.90% ± 3.20% of cells exhibiting red fluorescence (Figure 6.3). After exposure to CCCP (5 µM) for 10 seconds, *A. baumannii* cell membrane potential was supressed with treated cells having an average red fluorescence of 1.66% ± 0.65% ($p<0.0001$). Cell membrane potential was also lowered in cultures exposed to gramicidin (15 µg/ml) with the average red fluorescence being 63.20% ± 3.62% ($p<0.0001$). In contrast, cells' exposure to bacitracin (5 IU/ml) did not significantly decrease the mean red fluorescence (90.47% ± 0.70%; $p=0.8890$).

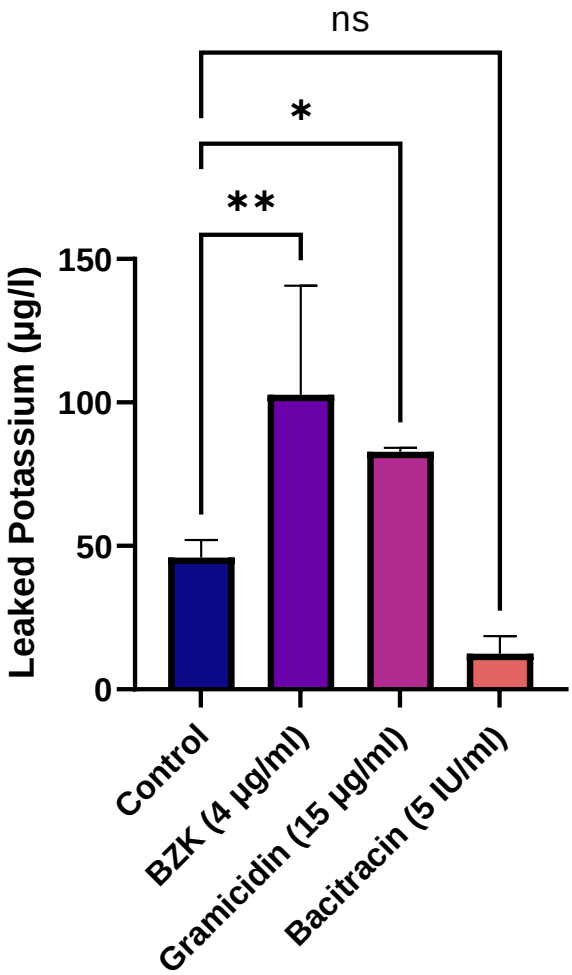
Figure 6.3. *A. baumannii* membrane potential following exposure to OTC antibiotics. CCCP (5 µM) was used as a positive control for membrane depolarisation ns – not significant ($p > 0.05$), * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.



6.3.5. OTC antibiotics causing potassium leakage in bacteria

When bacteria were not exposed to antibiotics the potassium concentration in solution was 45.95 ± 6.16 µg/l (Figure 6.4). Exposure of *A. baumannii* to BZK (control) and gramicidin (15 µg/ml) led to a significant leakage in potassium with BZK (102.70 ± 38.02 µg/l; p=0.0074) and with gramicidin (82.83 ± 1.30 µg/l; p=0.0493). Bacitracin (5 IU/ml) did not lead to the potassium leakage in *A. baumannii* (12.43 ± 6.09 µg/l; p=0.0684).

Figure 6.4. Potassium concentration in solution following exposure to OTC antibiotics in *A. baumannii*. ns – not significant (p > 0.05), * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001, **** - p ≤ 0.0001.



6.4. Discussion

6.4.1. Contraindications of OTC medicines and clinical treatments

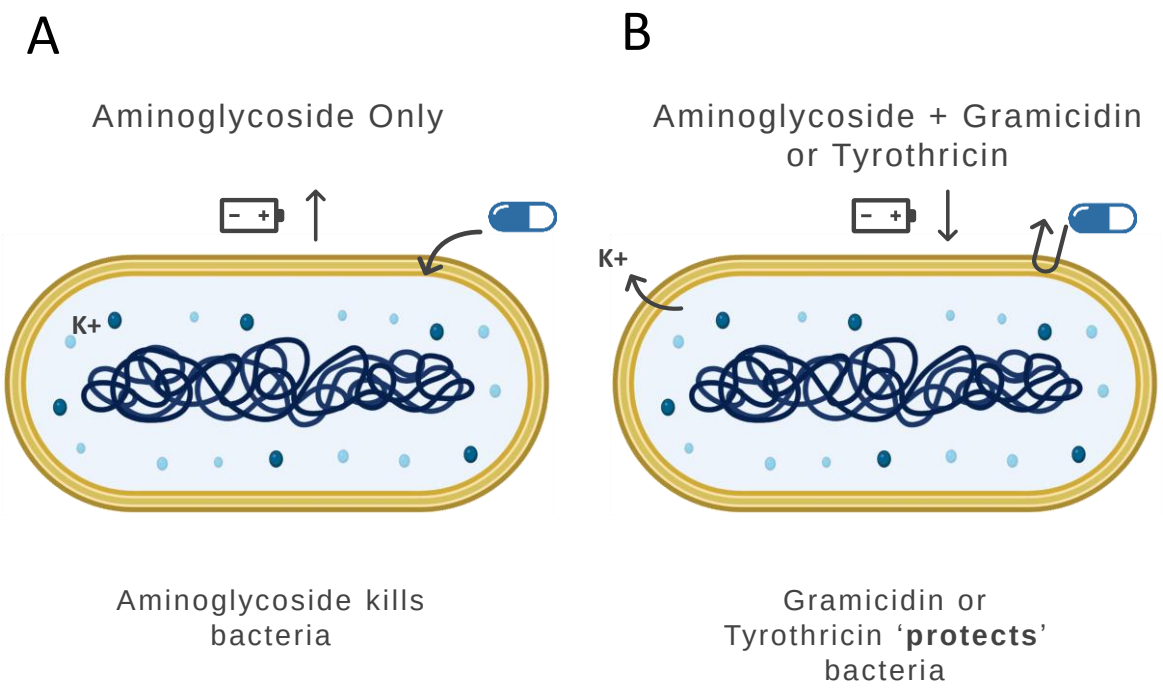
Aminoglycosides inhibit protein synthesis by binding to the 30S ribosome and therefore need to enter bacterial cells to be effective (Krause, *et al*., 2016). Aminoglycoside activity is dependent on the bacterial cell membrane potential as this potential is required for the aminoglycosides to enter into cells (Taber, *et al*., 1987). The cell membrane potential is often determined by the concentration of intracellular ions, in particular potassium ions (Stautz, *et al*., 2021). Our results showed that in bacterial cells that have only been treated with aminoglycosides, the intracellular concentrations of potassium ions remain high (Figure 6.4) and therefore the cell membrane potential remains polarized (Figure 6.3) allowing aminoglycosides to enter cells and exert their bactericidal effect (Figure 6.5A). However, cells co-exposure with gramicidin led to potassium leakage (Figure 6.4) and membrane depolarization (Figure 6.3) preventing aminoglycosides to enter bacterial cells, and subsequently decreased the aminoglycoside bactericidal efficacy (Figure 6.1), demonstrating a 'protection' effect (Figure 6.5B).

Gramicidin acts as an ionophore and therefore has similar effects to compounds such as indole (Chimerel, *et al*., 2012). Gramicidin forms channels across the cell membrane and disrupts the ionic homeostasis resulting in membrane depolarization and leakage of monovalent cations (Meikle, *et al*., 2016).

The explanation for the decreased aminoglycoside efficacy when co-exposed to tyrothricin (Figures 6.1 & 6.2) is likely to be similar to gramicidin. Tyrothricin is partly made from gramicidin (approximately 25-50%) so is expected to have a similar mechanism of action, but it also contains another antimicrobial peptide, tyrocidine. The mechanism of action of tyrocidine is currently unproven but is thought to work by binding of the bacterial cell membrane and embedding within it forming pores (Marques, *et al*., 2007; Pálffy, *et al*., 2009).

In contrast, bacitracin's mechanism of action is to inhibit the formation of the bacterial cell wall. This is done by bacitracin forming a complex with part of the bacterial cell wall, C55-isoprenyl pyrophosphate (Stone and Strominger, 1971) with no impact on cell membrane potential. This would explain co-exposure to bacitracin has no impact on aminoglycoside efficacy (Figures 6.1 & 6.2).

Figure 6.5. Mechanism of protection during co-exposure with gramicidin or tyrothricin. (A) When bacteria are exposed to only aminoglycosides, the intracellular potassium concentration is high and the cells is polarized. This allows aminoglycosides to enter into bacteria and kill them. (B) When co-exposed with either gramicidin or tyrothricin, the OTC antibiotics disrupts the bacterial membranes. This cause intracellular potassium to leak out and depolarizes cells. Aminoglycosides cannot enter into depolarized cells and therefore the gramicidin or tyrothricin will 'protect' the bacteria from aminoglycoside activity.



The use of OTC antibiotics has been questioned for many years due to safety concerns and lack of therapeutic benefit with some countries banning their use (WHO, 2005). Our study indicates that OTC antibiotics such as gramicidin and tyrothricin have the potential to antagonize aminoglycoside activity. Aminoglycosides are crucial treatments used by clinicians for sometimes life-threatening infections (Serio, *et al*., 2018), but it remains to be shown that OTC antibiotics and aminoglycosides can be present at the same time against bacterial pathogens in a patient. Aminoglycosides are poorly absorbed orally and are therefore often administered by injection (BNF, 2023). However, there are instances where aminoglycosides are given orally such as neomycin in the treatment of hepatic coma or pre-operative sterilisation or paromomycin in the treatment of parasitic infections. Not only are aminoglycosides given orally, but also by inhalation; such as the use of tobramycin in cystic fibrosis patients or liposomal amikacin in the treatment of *Mycobacterium avium complex*

(BNF, 2023). In both administrations of aminoglycosides, it is possible that bacteria can come into contact with OTC antibiotics and could interfere with clinical aminoglycoside treatments. However, as the OTC antibiotics in these formulations are AMPs, they are usually active even at very low concentrations and therefore it is not known for how long these antibiotics exert their effect on bacteria. Nevertheless, the results showed a potential impact of OTC antibiotics decreasing the efficacy of a class of systemic antibiotic and provided an explanation as to the mechanism of antibiotic antagonism.

It has been suggested that OTC antibiotics do not directly cause the development of AMR (Stauss-Grabo, *et al*., 2014) because they are AMPs, without a defined target site and strong bactericidal action. These OTC antibiotics have a strong bactericidal activity against Gram-positive bacteria and are considered to have a low or no activity against Gram-negative bacteria. A more recent study showed that these OTC antibiotics can elicit responses that generate not resistance to the OTC antibiotics themselves but cross-resistance to clinical antibiotics (Wesgate, *et al*., 2020). Here, it is shown that gramicidin and tyrothricin can decrease significantly the efficacy of aminoglycosides in Gram-negative bacteria.

6.4.2. Reflection and expansion to other antibiotics

The study findings indicate that OTC antibiotics can interfere with aminoglycoside treatments. However, there are other classes of clinically used antibiotics that OTC antibiotics could possibly also interfere with. It also needs to be considered in which environments the protect mechanism from OTC antibiotic usage, and therefore decrease in clinical antibiotic efficacy, would occur. Whilst here it was demonstrated that the during-use concentrations during sore-throat medication dissolution was enough to depolarize the bacterial cell membrane, OTC antibiotic concentrations will vary depending on their usage. These OTC antibiotics are not just found in sore throat medications but also topical creams for skin infections, where the during-use concentrations are much higher as they encounter less dilution of the antibiotic when compared to a sore throat product (Table 6.3). It is unknown whether the concentrations in these environments would have same protection mechanism and therefore would require further investigation.

Table 6.3. Topical products that are available OTC in Europe and are used in applications other than sore throat. The divisions of Northern, Eastern, Southern and Western Europe were based on those used in Both. *et al.,* (2015). Antibiotics: T – Tyrothricin, G – Gramicidin, B – Bacitracin and N – Neomycin. (Table adapted from Chapter 1; Table 1.1).

Brand	Country Sold	OTC Antibiotic	Application	Marketed	Antibiotic Strength/g
Northern Europe					
Bacimycin	Norway	B	Skin Infections	OTC	500 IU
Eastern Europe					
Baneocin	Bulgaria, Romania	B, N	Skin Infections	OTC	250 IU/ 5000 IU
Tyrosur	Romania	T	Skin Infections	OTC	1 mg
Southern Europe					
Pulvo-47	Greece	N	Skin Infections	OTC	2.23 mg
Blastoestimulina	Spain	N	Skin Infections	OTC/Pharmacy	3.5 mg
Cohortan	Spain	T	Rectal Ointment	OTC	1 mg
Western Europe					
Tyrosur	Germany	T	Skin Infections/Acne	OTC/Pharmacy	1 mg
Micasal	Germany	T	Skin Infections/Acne	OTC/Pharmacy	1 mg

As for antibiotics that OTC co-exposure could be of concern, polymyxins would be worth investigating. Polymyxins, much like aminoglycosides, having self-promoted uptake into bacteria which may be disrupted by changes in the cell membrane potential (Chopra, 1988). Alongside polymyxins, co-exposure with OTC antibiotics could also affect treatments, particularly bactericidal antibiotics that work intracellularly if they are at a concentration that disrupts the cell membrane potential. Other antibiotics such as tetracycline may also be affected. Tetracycline enters bacteria through a carrier-mediated transport which is energy dependent (Chopra and Roberts, 2001). As the cell membrane potential plays a pivotal role in energy production within bacteria (Strahl and Hamoen, 2010), the change in membrane potential could also affect the tetracycline uptake. However, further testing would be required to confirm the antagonism of OTC antibiotics to other clinical antibiotics.

6.5. Chapter Conclusions

Aminoglycosides can be used to treat external infections such as in the eyes, wounds or ears (Short, *et al*., 2021). Whilst it is unlikely that these antibiotics will come into contact with aminoglycosides when contained in sore throat medications, OTC antibiotics are used in other types of medications such as topical skin preparations (Both, *et al*., 2015). When a patient is undergoing aminoglycoside treatment for external infections such as wound, eye or ear infections, it needs to be considered whether the patients has used or is currently using an OTC antibiotic as this could cause treatment to fail. When antibiotic treatments fail, it could result in the development of AMR.

Chapter 7: General Discussion and Conclusion

Chapter 7: General Discussion and Conclusion

7.1. Summary of Project Findings

This thesis sought to understand whether there is a risk of the use of OTC topical antibiotics in sore throat treatments to the emergence of bacterial resistance. The primary objectives of this thesis were to establish which organisms were most at risk of resistance development and the impact on clinical treatments. As part of understanding their impact in treating sore throat in a community setting, the supply of OTC antibiotics in sore treatments was also investigated. This was primarily done to assess whether these medications are being given appropriately to patients and to understand the current management of sore throat. The knowledge gap covered, the objectives and the major findings of this study are summarized below (Table 7.1).

Table 7.1. Summary of project findings.

Knowledge Gap	Objective	Major Findings
Management of sore throat and supply of OTC antibiotics	Understand how sore throat is managed in community pharmacies in four European countries	A survey was developed but not fully distributed. Pre-testing of the survey indicates that community pharmacies may not be aware of OTC products containing antibiotics which results in an inappropriate supply to patients.
Effect of OTC antibiotics on bacterial resistance profiles	To highlight which bacteria are at risk of developing resistance	None of the bacteria tested could survive even in a realistically low during use concentration of neomycin. Of the other antibiotics tested, it was mainly Gram-negative bacteria that could survive the during-use concentrations of gramicidin, bacitracin and tyrothricin.

Table 7.1 - continued

	Determining the changes in resistance profiles to clinical antibiotics and whether these changes are stable	The main changes in clinical resistance following OTC antibiotic exposure were to either gentamicin or to beta-lactam antibiotics. Most of these changes were stable in *Enterobacterales.*
	Exploring the phenotypic and genotypic changes after pre-exposure to OTC antibiotics	There were extensions to lag phase allowing bacteria to adapt to stresses. There was also an increase in efflux pump activity, change in morphology, increase in beta-lactamase activity, changes in the metabolome and genetic changes to membrane proteins after OTC antibiotic exposure.
OTC antibiotic contraindications	Explore whether the use of OTC antibiotics could interfere with clinical antibiotic treatments	Gramicidin could protect *A. baumannii* from aminoglycosides whereas bacitracin did not. In the *Enterobacterales*, both gramicidin and tyrothricin could protect from the bactericidal action of gentamicin.
	Understand the mechanism behind the interference in treatment	The mechanism behind the protection is due to the leakage of potassium which causes a depolarization of the bacterial cell membrane.

7.2. Mechanisms of Resistance from the use of OTC antibiotics

There were various changes that occurred in the bacteria after exposure to either gramicidin, bacitracin or tyrothricin (Figure 7.1; Table 7.2). The pre-exposure of either gramicidin, bacitracin and tyrothricin gave rise to resistance to beta-lactam antibiotics (ampicillin, cefotaxime, aztreonam, imipenem) and gentamicin (Chapter 4). The mechanisms behind this resistance were discussed and detailed in Chapter 5. The reasoning behind the beta-lactam resistance seen is thought mainly to be due to the increased beta-lactamase activity (Figure 7.1; ⑤). In *E. cloacae*, the increased activity of the AmpC beta-lactamase can give rise to the resistance of third-generation cephalosporins (cefotaxime) and monobactams (aztreonam) (Tamma, *et al*., 2019), but it does not entirely explain resistance to imipenem as imipenem resists cleavage by AmpC. However, it has been previously seen that AmpC production combined with reduction of imipenem influx via porin mutation can cause this resistance phenotype (van Boxtel, *et al*., 2016), although this would require further investigation to confirm the change in porins.

As for the gentamicin resistance seen after pre-exposure to either gramicidin, bacitracin or tyrothricin, the mechanism could be a result as a combination of the changes seen in the cell (Figure 7.1). There are various changes that can occur in a cell that can lead to resistance to aminoglycosides such as gentamicin (Reygaert, 2018): increase in efflux (Figure 7.1; ⑥), and changes in morphology that causes the cells to decrease their S/V ratio (Figure 7.1; ⑦), which effectively dilutes the intracellular concentration of compounds in the bacterial cytosol (Ojkic, *et al*., 2022). Other changes can include modifications in the cell membrane, changes in the membrane potential (Figure 7.1; ③) and over-expression of modifying enzymes (Reygaert, 2018). These changes can cause resistance by either limiting the uptake of aminoglycosides, or in the case of modifying enzymes, inactivating the antibiotic. Within this work, there was an increase in efflux which could be contributing to the aminoglycoside resistance (Chapter 5, Section 5.3.2). There were some changes seen in the bacterial cell morphology where there was a decrease in S/V ratio. This was unexpected as membrane targeting compound are expected to increase the S/V (Ojkic, *et al*., 2022). However, this may increase cross-resistance to gentamicin as it has an intracellular target. Therefore, the decrease in S/V may cause the intracellular concentration of gentamicin to be effectively diluted. There were also changes in the metabolome and mutations in the genes of membrane proteins (Figure 7.1; ⑧ and ⑨) which could decrease the uptake of aminoglycosides into the cell. These changes in metabolome may cause a change in the bacterial cell membrane potential which can

reduce the uptake of aminoglycosides into the cell (Webster and Shepherd, 2023). Mutations in membrane proteins can also affect the uptake of aminoglycosides and decrease the permeability into the cell (Garneau-Tsodikova and Labby, 2016). Whilst there is evidence of some changes to membrane proteins within this study (Chapter 5, Section 5.3.7), it is unclear whether these have an effect on aminoglycoside uptake.

The protection mechanism seen during the co-exposure testing in Chapter 6 was due to a decrease in the uptake of aminoglycosides into the cell. This was predominantly seen in gramicidin and tyrothricin as they both caused leakage of potassium ions (Figure 7.1; ②). This perturbation in ion homeostasis disrupts the bacterial cell membrane potential (Figure 7.1; ③). This is essential for aminoglycosides to enter into bacterial cells and work (Bruni and Kralj, 2020), without it, the aminoglycosides are inhibited from entering into cells (Figure 7.1; ④).

Figure 7.1. The effects of OTC antibiotic (gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml) exposure on Gram-negative bacteria tested. ①- OTC antibiotic stress, ②- potassium leakage, ③ - membrane potential depolarization, ④ - inhibition of aminoglycoside entry, ⑤- upregulation of beta-lactamase activity, ⑥ - increased efflux pump activity, ⑦ - changes in morphology, ⑧ - changes in metabolism, ⑨ - DNA mutation in membrane protein genes, ⑩ - resistance to gentamicin and beta-lactam antibiotics. The effects seen for each antibiotic are summarized in Table X.

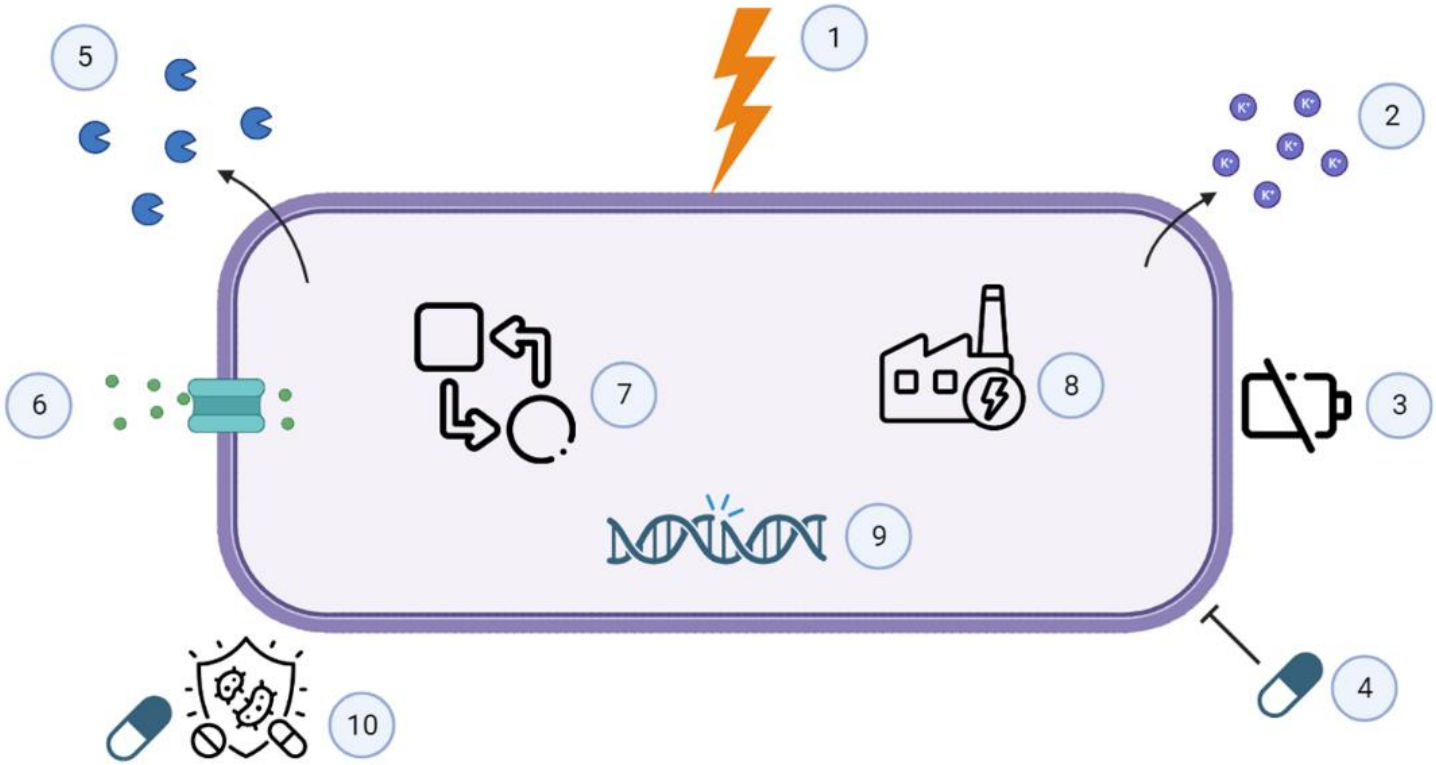


Table 7.2. The effects after exposure to either gramicidin (15 µg/ml), bacitracin (5 IU/ml) or tyrothricin (200 µg/ml). (✓) green: Effect seen after exposure to the OTC antibiotic. (✗); red: Effect not seen after exposure to the OTC antibiotic

Effects seen within bacterial cells	**After Gramicidin (15 µg/ml) exposure**	**After Bacitracin (5 IU/ml) exposure**	**After Tyrothricin (200 µg/ml) exposure**
OTC antibiotic stress	✓	✓	✓
Potassium leakage	✓	✗	✓
Membrane potential depolarization	✓	✗	✓
Inhibition of aminoglycoside entry	✓	✗	✓
Upregulation of beta-lactamase activity	✓	✓	✓
Increased efflux pump activity	✗	✗	✓
Change in morphology	✗	✓	✓
Change in metabolism	✓	✓	✓
DNA mutation in membrane protein genes	✓	✓	✓
Resistance to gentamicin and beta-lactamase	✓	✓	✓

7.3. Considerations for the Usage of Agents that cause Membrane Damage

The resistance that develops from the exposure to gramicidin, bacitracin and tyrothricin all resulted from the damage to the bacterial membrane. There are many other agents that can cause membrane damage to bacteria (Maillard and Pascoe, 2023). It could therefore be a concern that these agents, such as biocides, can cause similar resistances in these bacteria. These may include disinfectants or antiseptics such as phenolics, Quaternary Ammonium Compounds (QACs), biguanides, organic acids, alcohols and iodine (Maillard and Pascoe, 2023). Other commonly used agents such as non-ionic, cationic, anionic and amphoteric surfactants could be included in this, as they also interact with the bacterial membranes (Sharma, *et al*., 2022). Disinfectants, antiseptics and surfactants are used in a wide range of applications including healthcare settings, food industries, cosmetic industries, hygiene products, veterinary practice and farming (Maillard, 2007; Condell, *et al*., 2012; Pereira and Tagkopoulos, 2019; Jones and Joshi, 2021; Sharma, *et al*., 2022). Therefore, it is a concern whether the usage of these compounds can be creating pools of resistant isolates in these environments. This an issue as infections from these environments can be difficult to treat and spread antibiotic resistance (Larsson and Flach, 2021). There has been concern on whether the use of biocides can contribute to the development of antimicrobial resistance, particularly after the widespread usage of biocides during the COVID-19 pandemic (Chen, *et al*., 2021). It has already been demonstrated that exposure to commonly used biocides such as triclosan, chlorhexidine or BZK have resulted in the development of cross-resistance to some antibiotics (Wesgate, *et al*., 2016; Wesgate, *et al*., 2020; Maillard, 2022; Pena, *et al*., 2023). Some of the cross-resistances seen in these studies from exposure to biocides are similar to those that developed from exposure to OTC antibiotics (Chapter 4). The mechanisms behind these resistance have been previously investigated (Poole, 2002; Gilbert and McBain, 2003; Tumah, 2009; Maillard, 2018; Adkin, *et al*., 2022). As the main stress exerted by disinfectants, antiseptics and surfactants are mainly on the cell membranes, they have commonalities in the resistance mechanisms in bacteria with the effects seen in this study. Although we currently focus on antimicrobial stewardship to reduce the development of resistance and use antibiotics appropriately, it could also be time to introduce stewardship of other membrane active compounds such as disinfectants, antiseptics and surfactants.

7.4. The Role of Pharmacists in the Improvement of Antibiotic Stewardship

With increasing AMR development, there is more emphasis on the importance of antimicrobial stewardship (Essack, *et al*., 2018). Pharmacists in particular play a key role in antimicrobial stewardship, as they are often in contact with patients and can influence treatment options (Both, *et al*., 2015). This is particularly important in the case of ailments such as sore throat, where the misuse of antibiotics is high (Gaarslev, et al., 2016). As many patients come to pharmacists for sore treatments, they can supply the patient with treatments for symptomatic relief that patients are often actually demanding (van Driel, *et al*., 2006). Alongside this, the pharmacists can use diagnostic tools and scoring indicators to not only choose an appropriate treatment but also the educate patients can antibiotic misuse (Coutinho, *et al*., 2021). From the pre-testing conducted within this work, it was clear that some pharmacists were unaware of the OTC antibiotics used within sore throat medications (Chapter 2). However, to get a clearer picture of the awareness of these products and how sore throat is managed, the full survey would still need to be completed. Although, if pharmacists are unaware of the products they are supplying to patients, they will not be supplying them appropriately, which is poor antimicrobial stewardship. Therefore, education of these products to pharmacists is key to improving the antimicrobial stewardship.

7.5. Future Work

The work carried out within this study assessed many of the previously unknown effects that exposure to OTC antibiotics has on bacteria. However, further research should be conducted to build upon this work and give further understanding of the mechanisms behind the resistances seen. As many of these mechanisms was linked to damage of the bacterial cell membrane, I would recommend the future work to expand to the risk of emerging resistance from other membrane active compounds such as disinfectants, antiseptics and surfactants. As for understanding the effects of OTC antibiotics, I would recommend that the changes on membrane composition should be assessed, in particular, porin production within bacteria. Any changes to the LPS should also be considered, as this can occur in bacteria after exposure to antimicrobial peptides (Bahar and Ren, 2013). I would also recommend to add to the work on beta-lactamase activity and assess whether the higher activity is linked to upregulated expression.

Finally, a key piece of future work is to complete the survey that was developed in Chapter 2. This would give crucial information on how sore throats are managed, the use of OTC antibiotic-containing sore throat products and the awareness of them containing an antibiotic.

7.6. Conclusions and Recommendations

On completion of the survey, a report of the data will be sent to the pharmacy regulatory boards within each country. This will highlight the need for education of the pharmacists within each of these countries. With better education on these products, more appropriate treatments will be supplied to patients which is an improvement of antimicrobial stewardship. Moreover, manufacturers should be clear that their products contain an antibiotic.

Within this study there was also evidence on how the use of OTC antibiotics, particularly gramicidin, bacitracin and tyrothricin, can cause the development of clinical cross-resistance. As the development of resistance is a major risk factor of the usage of these antibiotics, they should not have OTC status and should be provided as prescription only, limiting potential misuse of these products.

Overall, this work does not support the use of OTC antibiotics in sore throat products aiming to provide symptomatic relief.

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Appendices

Appendix 2.1. The finalized survey to be sent for further piloting

Understanding how patients with an uncomplicated sore throat are managed in community pharmacies in Europe.

Introduction

This is a survey aimed at community pharmacists in a range of European countries, focused on understanding how patients with uncomplicated sore throat are managed in the pharmacy. For the purposes of this survey, we will be referring to uncomplicated sore throat simply as “sore throat”. We define this as a sore throat that is not accompanied by high temperature (fever), not linked to extreme difficulties in swallowing liquids, and in general when patients are not presenting with any systemic symptoms.

Your answers will help us to map pharmacists’ contributions towards caring for patients with sore throat, how they are being supported to fulfil this role, and understand any needs for further support. It is important that any developments are shaped by you, so any further support is appropriate, useful, and tailored to you.

All answers are anonymous and will be kept strictly confidential. The survey is completely voluntary and you can stop at any time.

Thank you for considering taking part in our survey. Please read the information below and continue to the next page, if you are happy to participate.

[Insert Scroll box containing Participant information sheet]

Section A

This sections aims to collect some general information about you, the place where you work, and the number of patients with sore throat in your pharmacy.

In which country is the pharmacy you work in based?

- Austria
- Finland
- Germany
- Sweden

How old are you?

- Under 30 years
- 30-39 years
- 40-49 years
- 50-59 years
- 60 years or over
- Prefer not to say

Where do you currently work?

- An independent/single pharmacy that you own
- An independent/single pharmacy that someone else owns
- A corporate owned pharmacy (also known as chain pharmacy) that has up to 10 pharmacies
- A corporate owned pharmacy (also known as chain pharmacy) that has more than 10 pharmacies

On an average day, how many patients visit your pharmacy for minor ailments (in general, not just sore throat)?

- Under 5
- 5-9
- 10-14
- 15-19
- 20-24
- 25-29
- 30 or over

How many of these ailments relate to sore throat?

- Under 5
- 5-9
- 10-14
- 15-19
- 20-24
- 25-29
- 30 or over

Free text: Is there anything else you would like to tell us in relation to this section?

Section B

This section aims to understand how you care for patients who come to the pharmacy you work in and ask for advice for their sore throat.

How effective do you think the following therapeutic agents and/or interventions are to manage sore throat symptoms?

	Not at all effective	Slightly effective	Moderately effective	Very effective	Extremely effective
Anaesthetics					
Antibiotics					
Anti-inflammatories/analgesics					
Antivirals					
Home remedies (e.g. herbal tea, steam inhalation etc.)					
Topical antiseptics					

How have you found out information about how effective the different agents and/or interventions are for managing sore throat symptoms? Please select all that apply.

- Session(s) included in your pharmacy degree
- Sessions(s) included in postgraduate degree(s) you have completed
- One-off sessions in conferences, seminars, or other continuing professional development activities
- Resources provided by your employer
- Marketing information from the companies that are supplying these products
- Your own searches for resources available in print/online
- Other

If you selected Other, please specify:

Free text: Is there anything else you would like to tell us in relation to this section?

Section C

This section aims to understand in more detail the landscape of the specific products that are available to sell for sore throat management.

What type of products are available to buy for managing sore throat symptoms in the country that you are working? Please select all that apply.

- Gargles
- Lozenges
- Sprays
- Tablets/capsules
- Other

If you selected Other, please specify:

What specific products come to mind when deciding how to treat a patient's sore throat in the pharmacy? Please select all that apply.

- Lemocin
- Dorithricin
- Trachisan
- Bafucin
- Strepsils
- Strefen
- Difflam
- Zyx
- Septabene
- Other

If you selected Other, please specify:

Of the products you selected above, which ones do you think contain an antibiotic? Please select all that apply.

- Lemocin
- Dorithricin
- Trachisan
- Bafucin
- Strepsils
- Strefen
- Difflam
- Zyx
- Septabene
- None of them
- Other

If you selected Other, please specify:

Please tell us how you have found out which of these products contain an antibiotic. Please select all that apply.

- Marketing materials that are available with the product packages
- Marketing information provided by supplier companies' representatives

- Resources provided by your employer
- Your own searches for resources available in print/online
- Not applicable
- Other

If you selected Other, please specify:

Free text: Is there anything else you would like to tell us in relation to this section?

Section D

This section aims to explore common decision-making processes when community pharmacists supply a product for sore throat management.

When a patient asks for advice on managing their sore throat symptoms, which of the below do you routinely complete **before deciding** whether to supply a product? Please select all that apply.

- Brief patient history, mainly led by patient-volunteered information
- Brief patient history, including duration and nature of symptoms
- Detailed patient history, including duration and nature of symptoms, pharmacological and non-pharmacological patient information
- Clinical scoring tools (e.g. FeverPAIN/CENTOR)
- Diagnostic point of care tests (e.g. throat swabs for screening against Streptococcus A)
- Other

If you selected Other, please specify:

Once you **have decided** to supply a product, which of the below do you routinely complete? Please select all that apply.

- Advise patients on how to use the product
- If the product contains an antibiotic, advise patients on specific antibiotic-related factors (e.g. do not share left-over product with friends/family, do not re-use next time you have the same symptoms without speaking to you or another pharmacist first etc.)
- If the product doesn't contain an antibiotic, explain reasons to the patients
- Refer patient to the leaflets included in each package
- Advise patients to return to the pharmacy for further support if symptoms don't improve within 3-5 days
- Advise patients to return to the pharmacy or seek medical attention for further support if symptoms don't disappear after 7 days
- Advise patients to seek medical attention if symptoms get worse (e.g. high temperature/fever, extreme difficulties in swallowing water, other systemic effects)
- Other

If you selected Other, please specify:

How have you found what information to share with patients, depending on the product that you are supplying? Please select all that apply.

- Session(s) included in your pharmacy degree
- Sessions(s) included in postgraduate degree(s) you have completed
- One-off sessions in conferences, seminars, or other continuing professional development activities
- Marketing information from the companies that are supplying these products
- Resources provided by your employer
- Your own searches for resources available in print/online
- Other

If you selected Other, please specify:

Free text: Is there anything else you would like to tell us in relation to this section?

Section E

This section aims to understand what, if any, further support mechanisms would increase your confidence in managing patients with sore throat symptoms in the pharmacy.

How do you feel about the importance of the following for community pharmacists?

	Not at all important	Slightly important	Moderately important	Very important	Extremely important
Additional education on types of products that are available for managing sore throat symptoms and their effectiveness					
Additional education on common side-effects of different products					
Diagnostic point of care tests to help decide on whether a product containing antibiotics needs to be supplied					
Additional education on reasons why					

antibiotics are not the best choice for all patients with sore throat symptoms					

If you have stated that additional education on any of the areas above is important, what format would you like this to be provided as? Please rank in order of preference.

	1st	2nd	3rd	4th	5th
Online live webinars/sessions					
Face-to-face seminars/conferences					
Online self-directed reading					
Printed materials sent to your pharmacy					
Online resources that you can print at your convenience					

If you would like additional information provided to you in a way not stated in the list, please let us know.

Free text: Is there anything else you would like to tell us in relation to this section?

Final Page

Thank you for taking your time to fill in this questionnaire. Your responses are greatly appreciated!

Appendix 2.2. Participant Information Sheet

Project Title: Understanding how patients who present at community pharmacies with a sore throat are managed

We would like to invite you to take part in our study. Before deciding to take part, it is important to understand why the research is being done and what it involves for you. Please read the following information carefully and ask if anything is not clear or you would like further information. Thank you for reading this.

1. **About the research**

Uncomplicated sore throat is one of the most common ailments that community pharmacists face. This is often a self-limiting disease however the urge of patients to seek treatment and symptomatic relief can cause patients to take medications unnecessarily. To treat sore throat, patients often use over-the-counter medicines bought from community-pharmacies. It is therefore important to know the role that community pharmacists have in the management and the sale of these over-the-counter medicines. Your answers will help us map these contributions to the management of sore throat and what further support can be given to community pharmacists.

We are recruiting community-pharmacists who work in Austria, Finland, Germany or Sweden, as it is currently unknown how sore throats are managed in community pharmacies and what products are involved in the management of sore throat.

2. **Who will conduct the research?**

Data collection and analysis are being undertaken by Andrew Robertson (a PhD research student at Cardiff University) and Dr. Efi Mantzourani (a member of staff at Cardiff University).

3. **Why have I been chosen to take part?**

As a community pharmacist working in either Germany, Austria, Sweden or Finland, you have first-hand experience with managing patients who complain of sore throat. Your answers will be valuable to us, to help map pharmacists' contributions towards caring for patients with sore throat, how pharmacists are being supported in this role and understand any needs for further support.

4. **What would I be asked to do if I took part?**

For this research, data will be collected using an online survey. Completion of the survey is entirely voluntary, and you may withdraw at any point.

The online survey should take around 10-15 minutes to complete.

5. **What are the possible benefits of me taking part?**

There are no direct advantages or benefits of your participation, although the information gathered from this research will be invaluable in understanding how sore throat is managed and what further support can be given to community pharmacists.

6. What are the risks and disadvantages of me taking part?

There are no foreseeable risks in taking part in the study . The only disadvantage is that the completion of the survey takes approximately 10-15 minutes of your time.

7. What will happen to the results from the study?

The results from the study will be analysed and written as part of a PhD thesis. It is expected that the results will also be used for an academic publication, and shared with regulatory bodies in countries of interest and industrial partners.

8. What happens if I do not want to take part or if I change my mind?

It is up to you to decide whether or not to take part. If you do decide to take please tick the box at the end of this page saying "I confirm I have read the information above and agree to take part in the survey".

9. What information will you collect about me?

We would like to collect demographic information at the beginning of the survey (country of work and age). If you would like to leave this blank, you are welcome to do so. If you are not happy with any of the questions in the survey, you can leave them blank.

10. If I decided to take part, will my participation be kept confidential?

The survey responses are completely anonymous and there is no way of linking any of the information back to you. If any of your responses in free-text questions identify you, any personal information will be managed in accordance with data protection legislation. Please see 'What will happen to my Personal Data?' (below) for further information.

11. What will happen to my Personal Data?

For this research we are not looking to collect any data that is classed as personal according to the General Data Protection Regulation (GDPR). However, if you decide to add any personal data (such as name, email address or contact details) through answers in the free-text questions of the survey, this will be processed by GDPR using public task as a lawful basis. Any answers containing personal will be anonymized as soon as possible by the research team using generalization to ensure the data is no longer identifiable.

If you contact us with a query regarding the survey, we will answer any questions and delete the email promptly.

Cardiff University is the data controller and is committed to respecting and protecting your personal data in accordance with your expectations and Data Protection legislation.

Further information about Data Protection, including:

- your rights
- the legal basis under which Cardiff University processes your personal data for research
- Cardiff University's Data Protection Policy
- how to contact the Cardiff University Data Protection Officer
- how to contact the Information Commissioner's Office

may be found at https://www.cardiff.ac.uk/public-information/policies-and-procedures/data-protection.

12. What if I have a complaint?

If you have any concerns or complaints during this research project, please contact a member of the research team (details below) who will address the issue. If you remain unhappy and wish to complain formally, you can do this by contacting the Director of Research, Cardiff School of Pharmacy and Pharmaceutical Sciences, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3NB, phrmyresoffice@cardiff.ac.uk.

13. Who has reviewed the study?

This project has been reviewed by the Cardiff University Research Ethics Committee and has been given a favourable ethical opinion for conduct. The approval number is: [Insert here]

Contact Details:

At any point during the study if you have queries or concerns, please email one of the research team.

Andrew Robertson – RobertsonAA@cardiff.ac.uk

Dr. Efi Mantzourani – MantzouraniE1@cardiff.ac.uk

Appendix 2.3. Participant Recruitment Email

Subject: Are you a community pharmacist in Germany, Austria, Sweden or Finland? Help us to help you!

Dear Colleague,

My name is Andy and I'm a PhD student at Cardiff University. I am working on understanding how patients with uncomplicated sore throat are managed in the pharmacy. We need your help so we can map pharmacists' contributions towards caring for patients with sore throat, how they are being supported to fulfil this role, and understand any needs for further support – only you can help us with this as you are the people who have first-hand experience.

This survey is aimed at community pharmacists who work in Austria, Finland, Germany or Sweden. All answers are anonymous and will be kept strictly confidential. The deadline for submitting responses is [insert date]

You can take part in the survey by clicking the link below. The first page will give you more information to the background of the research, and details about how your information will be used. If you would like any more information before deciding whether to take part in the study, please do not hesitate to contact me at RobertsonAA@cardiff.ac.uk.

[Insert survey link (containing participant information sheet)]

Thank you for supporting research for community pharmacies and helping us to help you!

Best Regards,

Andy Robertson (on behalf of the research team)

Appendix 2.4. Social media and Conference Infographic

We are looking for community pharmacists to share their experiences of supporting patients with uncomplicated sore throat, how they are being supported to fulfil this role, and understand any needs for further support

The Project

To understand how patients with sore throat are managed in community pharmacies and what further support is needed

Who do we need?

We need community pharmacists who work in Austria, Finland, Germany or Sweden to take part

How do I take part?

To take part please click the link above or scan the QR code and complete the survey. This should only take 10-15 minutes of your time

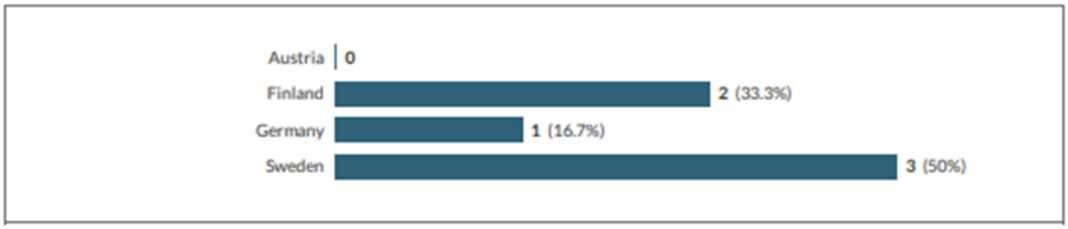
Have your say!

QR Code

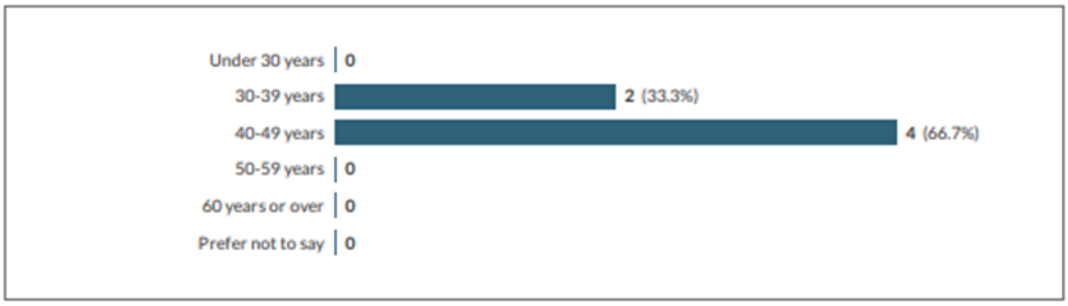
If you have any questions, contact Andrew Robertson
RobertsonAA@cardiff.ac.uk

Appendix 2.5. Survey suggested changes and free-text responses

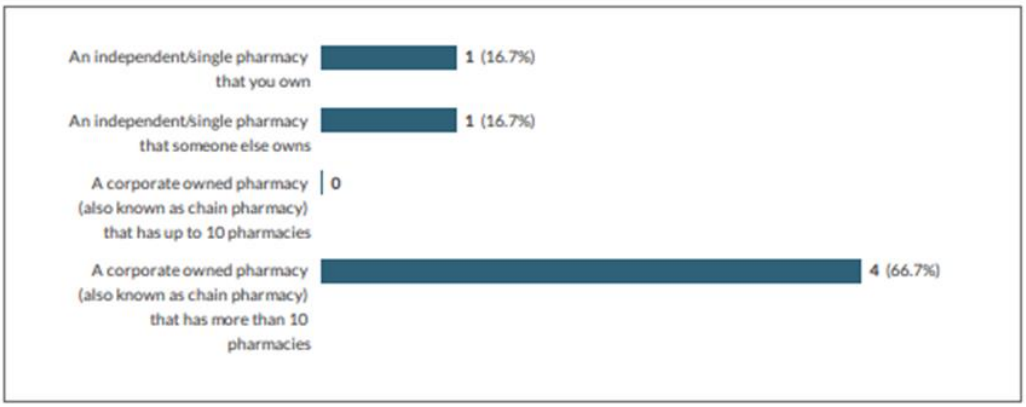
1 In which country is the pharmacy you work in based?



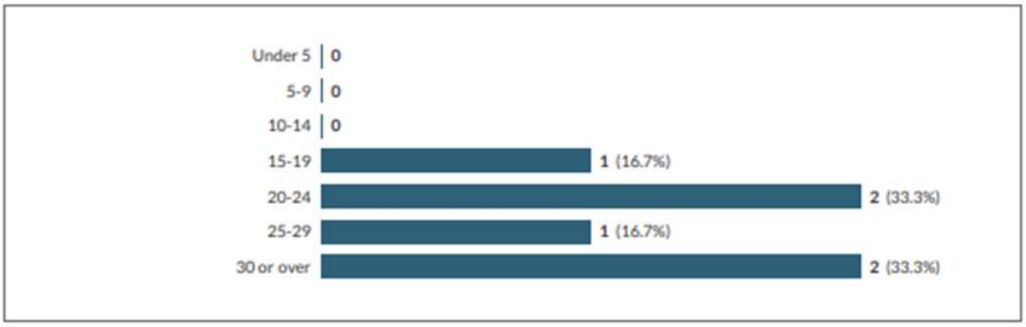
2 How old are you?



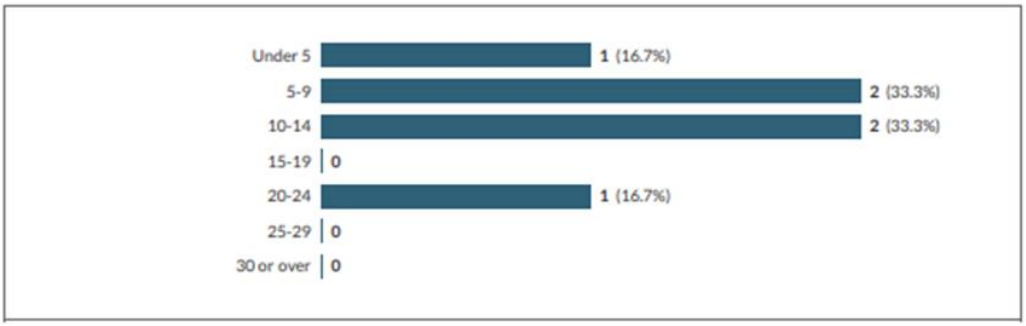
3 Where do you currently work?



4 On an average day, how many patients visit your pharmacy for minor ailments?



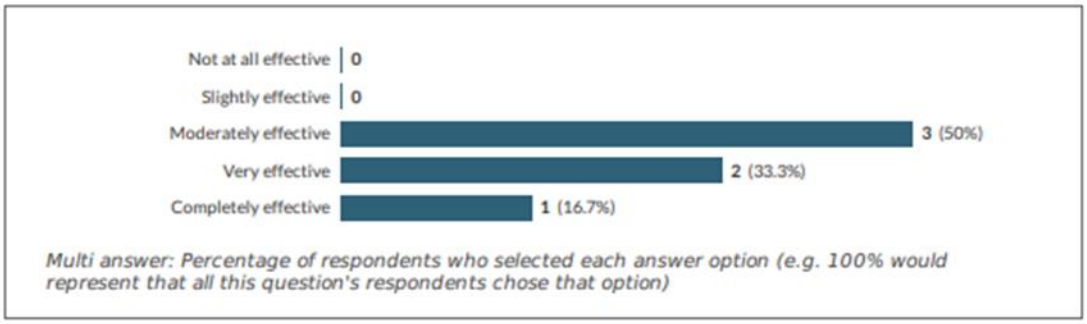
5 How many of these patients relate to sore throat?



6 On a scale of 1-5 , where 1 is not at all effective and 5 is completely effective, how effective do

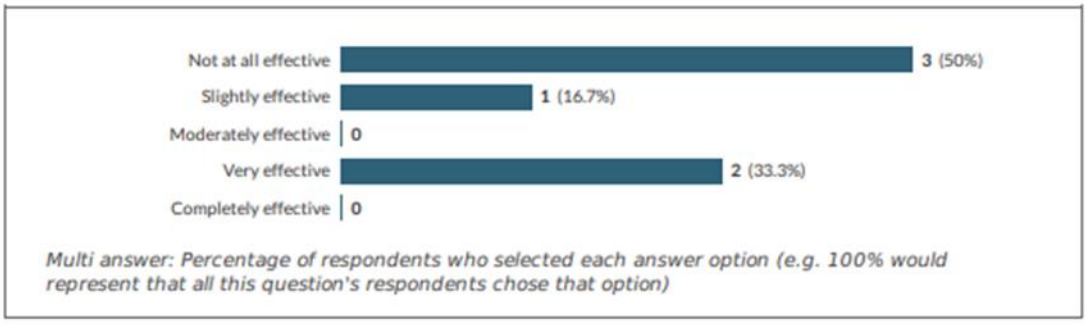
you think the following therapeutic agents and/or interventions are?

6.1 Anaesthetics



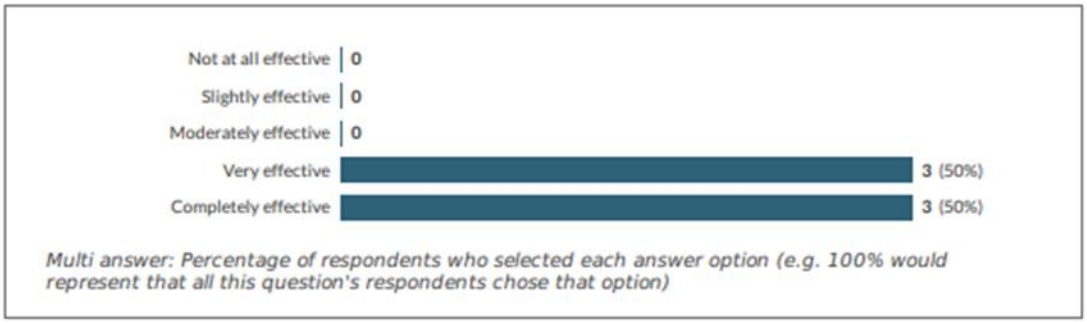
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

6.2 Antibiotics



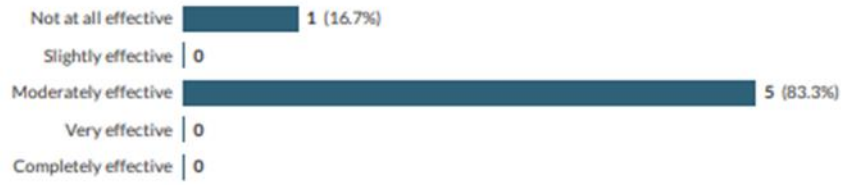
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

6.3 Anti-inflammatories/analgesics



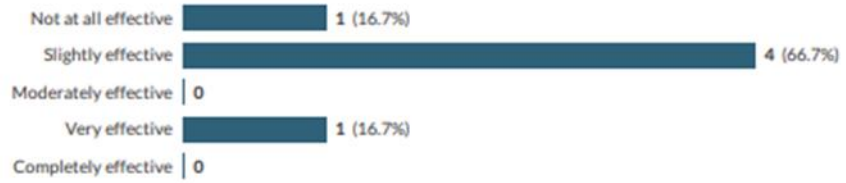
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

6.4 Antivirals



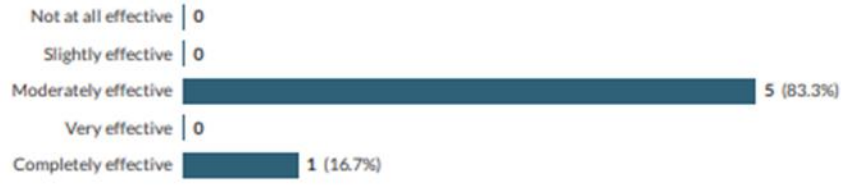
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

6.5 Home remedies (e.g. herbal tea, steam inhalation etc.)



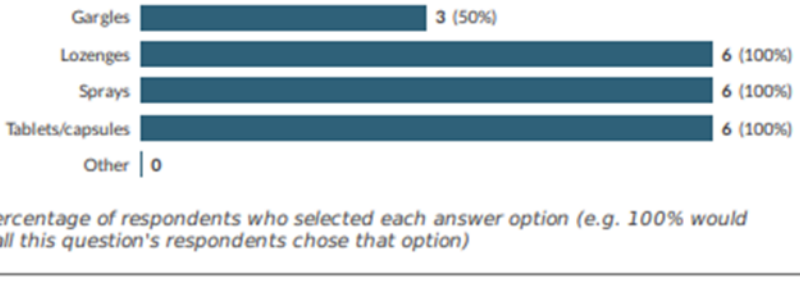
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

6.6 Topical antiseptics



Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

7 What type of products are available to buy for sore throat in the country that you are working? Please select all that apply.

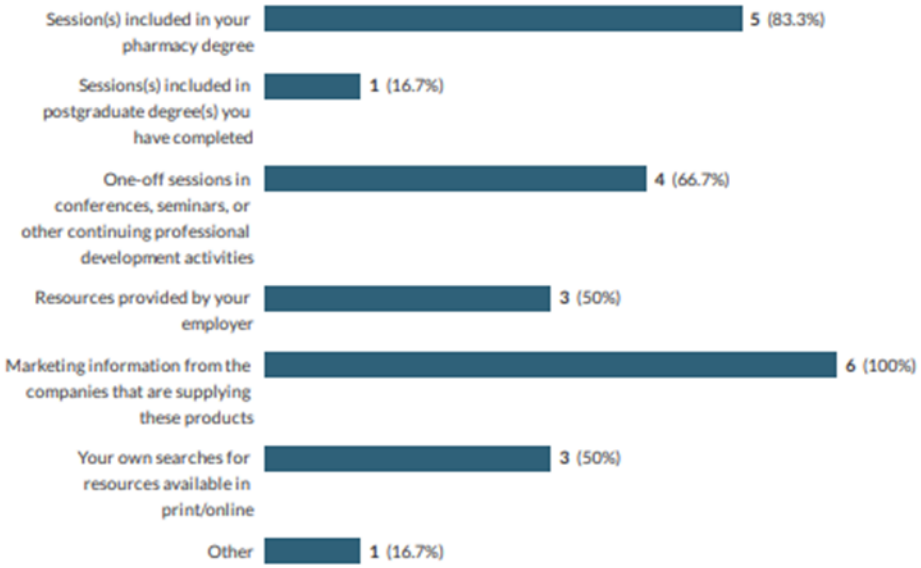


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

7.a If you selected Other, please specify:

No responses

8 How have you found out information about how effective the different therapeutic agents that are included in the different products are for managing sore throat? Please select all that apply.

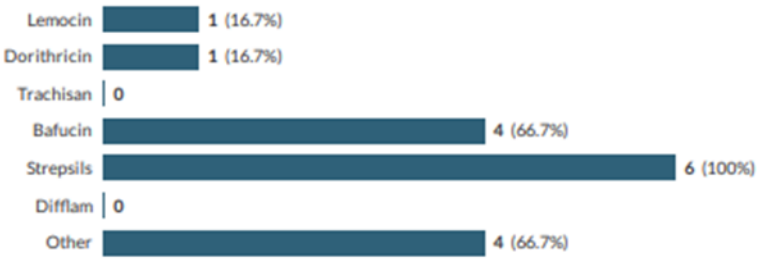


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

8.a If you selected Other, please specify:

Showing 1 response	
Marketing companies giving info lectures in the pharmacy and videos	925586-925568-98590722

9 What products come to mind when deciding how to treat a patient's sore throat in the pharmacy? Please select all that apply.

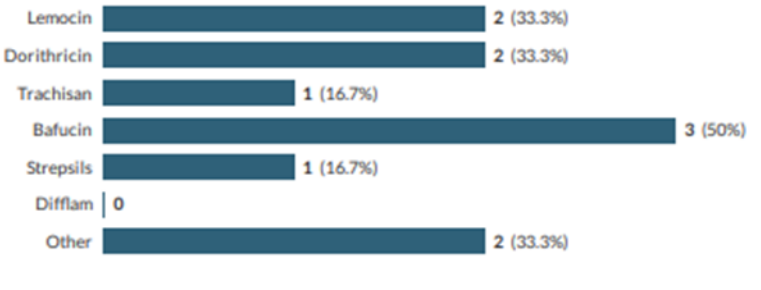


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

9.a If you selected Other, please specify:

Showing all 4 responses	
Septabene, Zyx,	925586-925568-98540158
Strefen, ZYX, Septabene and all the Ibuprofen products	925586-925568-98590722
Zyx, Septabene	925586-925568-98685227
Zyx, Strefen	925586-925568-98740351

9.b Of the products you selected above, which ones do you think contain an antibiotic? Please select all that apply.

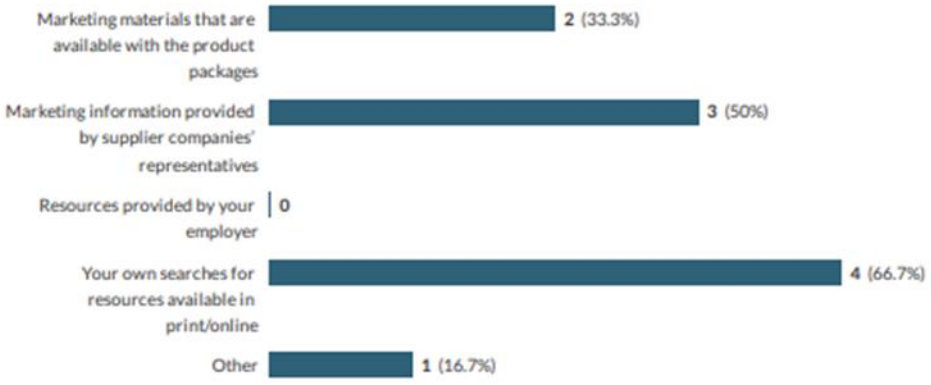


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

9.b.i If you selected Other, please specify:

Showing all 2 responses	
In Finland all other products which contains antibiotics are under prescription	925586-925568-98590722
None contain antibiotics	925586-925568-98685227

10 Please tell us how you have found out which of these products contain an antibiotic. Please select all that apply.

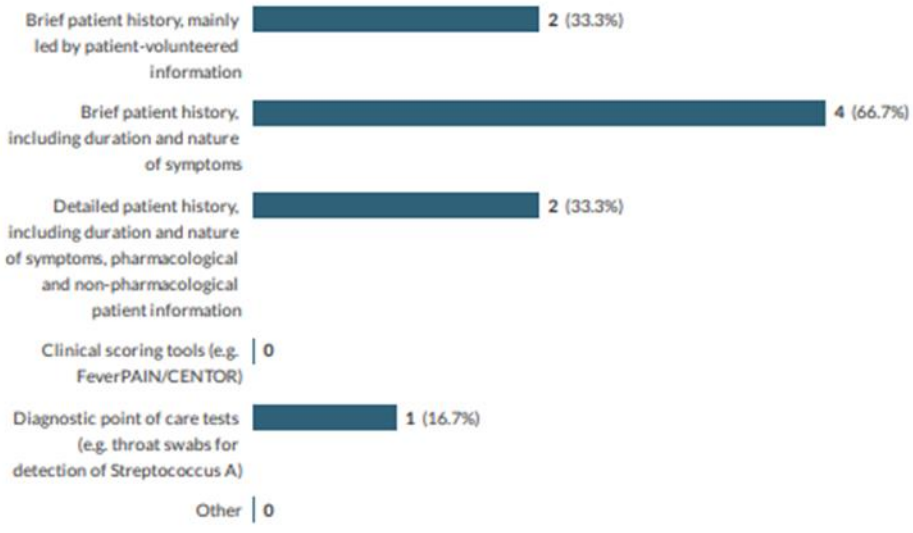


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

10.a If you selected Other, please specify:

Showing 1 response	
If I don´t know that anyone contain antibiotic, this question could be optional?	925586-925568-98685227

11 When a patient asks for advice on managing their sore throat, which of the below do you routinely complete before deciding whether to supply a product, and if so, which one? Please select all that apply.

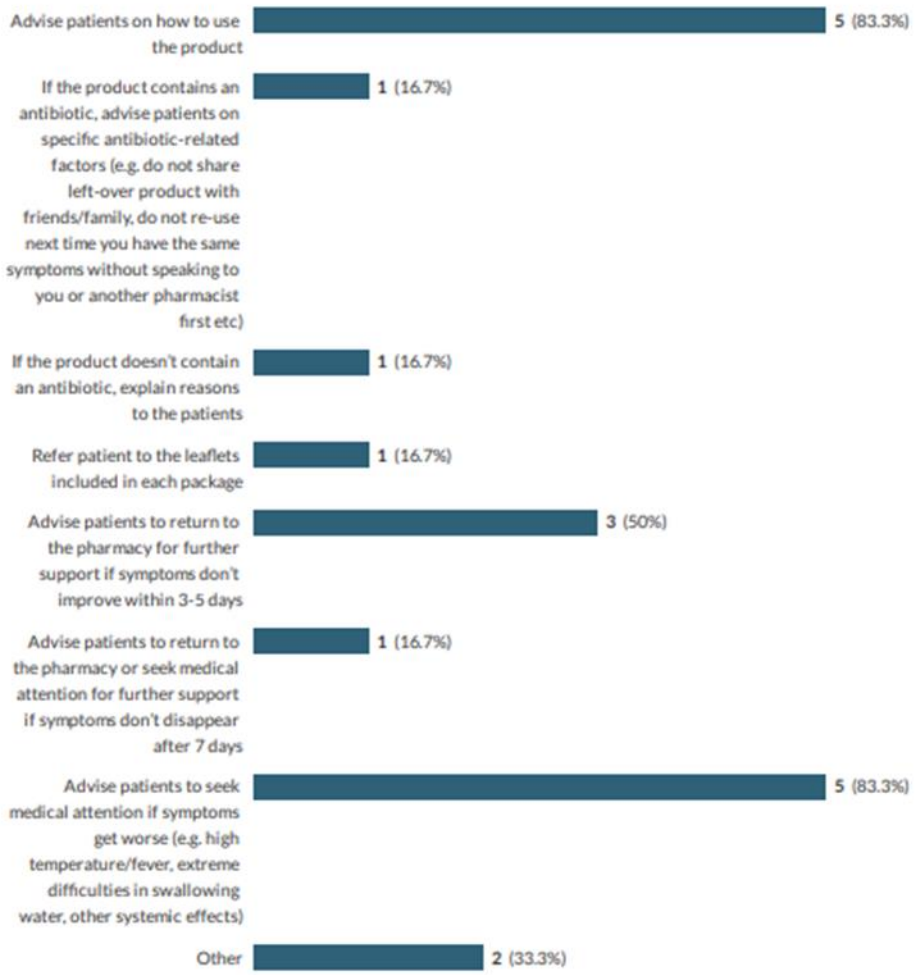


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

11.a If you selected Other, please specify:

No responses

12 Once you have decided to supply a product, which of the below do you routinely complete? Please select all that apply.

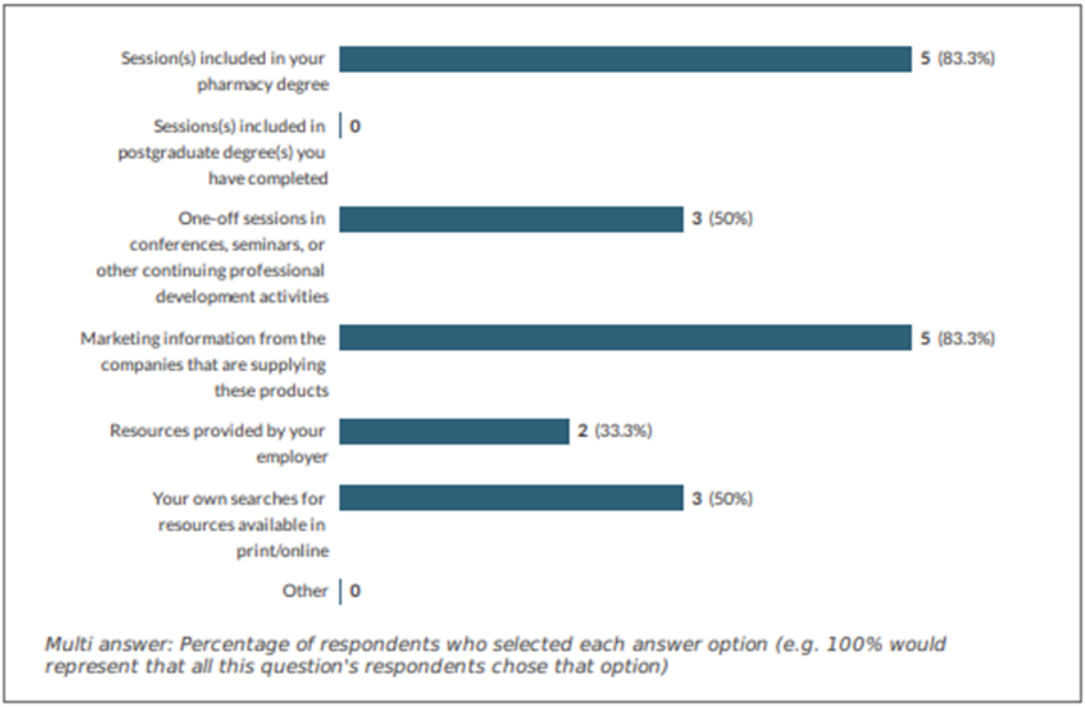


Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

12.a If you selected Other, please specify:

Showing all 2 responses	
Medical attension= go to doctor	925586-925568-98590722
AS Strefen recommend that seek doctor advice if not better after 3 day treatment that is what I inform.	925586-925568-98685227

13 How have you found what information to share with patients, depending on the product that you are supplying? Please select all that apply.



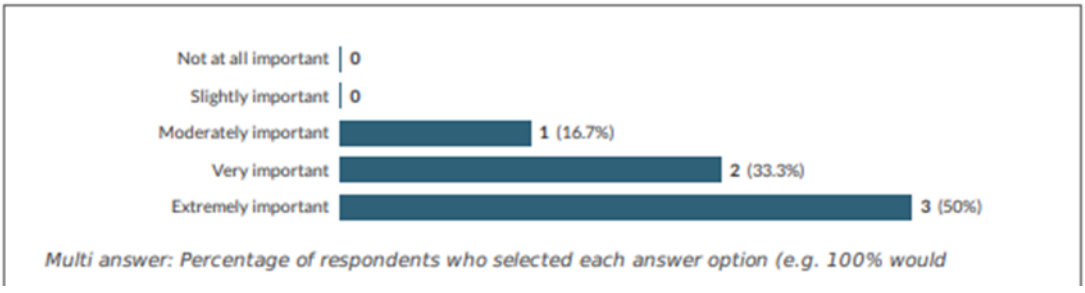
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

13.a If you selected Other, please specify:

No responses

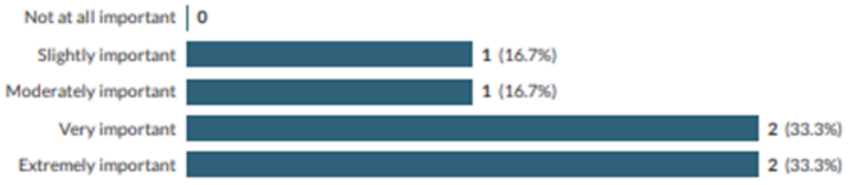
14 On a scale of 1 to 5, where 1 is not at all important and 5 is extremely important, how do you feel about the importance of the following for community pharmacists?

14.1 Additional education on products that are available for sore throats and their effectiveness



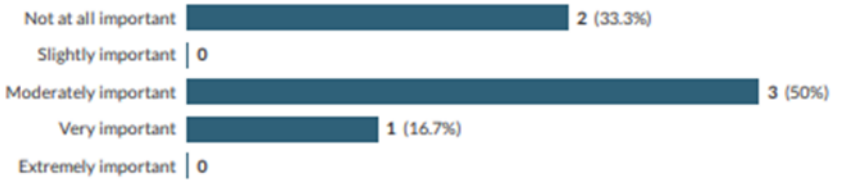
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would

14.2 Additional education on common side-effects of different products



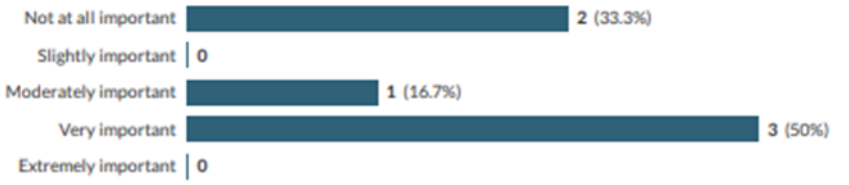
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

14.3 Diagnostic point of care tests to help decide on whether a product containing antibiotics needs to be supplied



Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

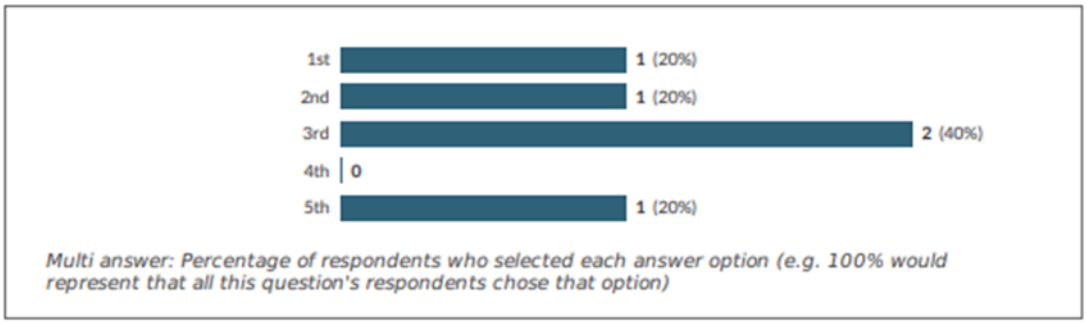
14.4 Additional education on reasons why antibiotics are not the best choice for all patients with sore throat



Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

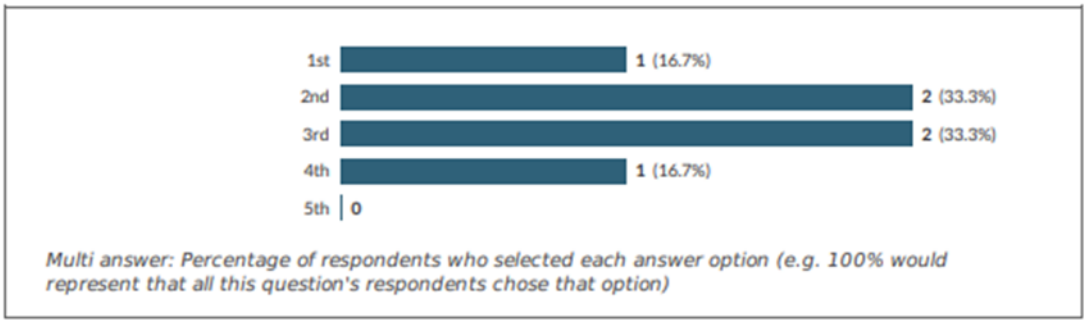
15 If you have stated that additional education on any of the areas above is important, what format would you like this to be provided as? Please rank in order of preference.

15.1 Online live webinars/sessions



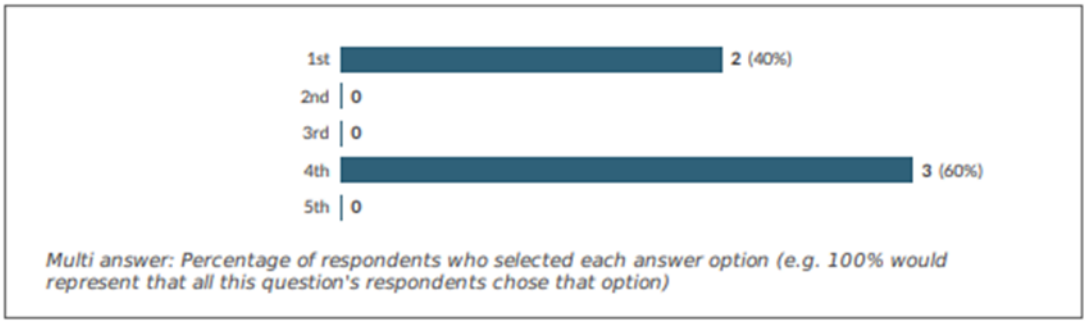
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

15.2 Face-to-face seminars/conferences



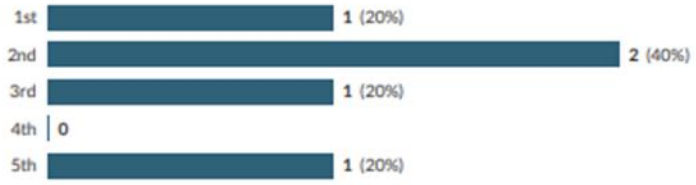
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

15.3 Online self-directed reading



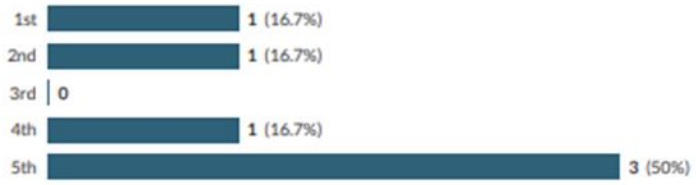
Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

15.4 Printed materials sent to your pharmacy



Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

15.5 Online resources that you can print at your convenience



Multi answer: Percentage of respondents who selected each answer option (e.g. 100% would represent that all this question's respondents chose that option)

15.a If you would like addtional information provided to you in a way not stated in the list, please let us know.

Showing all 3 responses	
N/A	925586-925568-98534887
In Finland it's always the doctors which decided the use of antibiotics. Bafucin is the only minor antibiotics in OTC	925586-925568-98590722
I also like information coming with a representative, when we are smaller groups at the pharmacy.	925586-925568-98685227

Appendix 4.1. Solvents and diluents used for MIC testing in accordance with ISO 20776-1:2020.

Antibiotic	**Solvent**	**Diluent**
Imipenem	**0,01 mol/l phosphate buffer, pH 7,2**	**0,01 mol/l phosphate buffer, pH 7,2**
Amikacin	**Water**	**Water**
Ciprofloxacin	**Water**	**Water**
Ampicillin	**0,1 mol/l phosphate buffer, pH 8,0**	**0,1 mol/l phosphate buffer, pH 6,0**
Gentamicin	**Water**	**Water**
Cefotaxime	**Water**	**Water**

Appendix 4.2. *S. aureus* passage data

Clinical Antibiotic	SXT					
Baseline	24		25		26	
P0	NZ		23		NZ	
OTC exposure	With Gramicidin			Without Gramicidin		
P1	23	22	NZ	20	19	22
P5	21	22	22	22	18	23
P10	22	21	20	22	22	19

Appendix 4.3. *A. baumannii* passage data

Clinical Antibiotic	IPM						AK					
Baseline	34		31		34		23		25		23	
P0	27		23		23		18		16		18	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin		
P1	27	24	22	27	26	30	18	18	18	16	18	17
P5	26	22	21	29	28	28	16	18	16	14	16	17
P10	30	22	23	30	27	29	17	14	16	17	15	15

Appendix 4.4. *P. aeruginosa* passage data

Clinical Antibiotic	IPM						CIP					
Baseline	23		24		23		32		30		30	
P0	18		17		21		25		25		26	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin		
P1	17	16	17	17	16	16	25	24	26	24	24	27
P5	19	17	17	17	17	17	26	24	25	26	24	27
P10	18	17	19	18	19	21	27	27	24	26	27	29

Appendix 4.5. *E. coli* passage data

Clinical Antibiotic	CIP						AMP						AMP						CN					
Baseline	34		34		35		20		20		20		20		20		20		18		18		18	
P0	24		24		29		NZ		NZ		11		NZ		NZ		NZ		15		17		NZ	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin			With Tyrothricin			Without Tyrothricin		
P1	32	27	13	26	27	25	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	15	14	16	17	16	15
P5	31	31	30	29	29	30	NZ	NZ	NZ	NZ	16	NZ	NZ	NZ	NZ	NZ	NZ	NZ	14	14	15	16	15	14
P10	29	29	27	28	25	29	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	15	17	16	15	14	17

Appendix 4.6. *K. pneumoniae* passage data

Clinical Antibiotic	IPM						CTX						CN					
Baseline	29		28		26		29		28		26		21		20		21	
P0	21		19		20		NZ		NZ		NZ		14		15		17	
OTC exposure	With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin			With Tyrothricin			Without Tyrothricin		
P1	20	19	20	21	20	22	NZ	NZ	NZ	NZ	NZ	NZ	15	NZ	17	15	14	14
P5	20	20	20	20	21	22	NZ	NZ	NZ	NZ	NZ	NZ	14	NZ	15	NZ	14	12
P10	20	21	23	22	22	23	NZ	NZ	NZ	NZ	NZ	NZ	16	NZ	15	14	17	14

Appendix 4.7. *E. cloacae* passage data

Clinical Antibiotic	CTX																	
Baseline	18		19		20		18		19		20		18		19		20	
P0	NZ		NZ		NZ		NZ		NZ		NZ		NZ		NZ		NZ	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin		
P1	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
P5	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
P10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Clinical Antibiotic	IPM																	
Baseline	23		22		20		23		22		20		23		22		20	
P0	20		20		19		20		19		20		18		19		18	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin		
P1	18	18	17	16	17	16	17	20	19	20	20	20	16	16	19	16	16	20
P5	19	19	18	21	18	19	16	16	19	17	17	17	16	17	17	14	18	19
P10	18	17	17	17	19	20	15	20	17	18	19	17	14	17	18	22	20	19

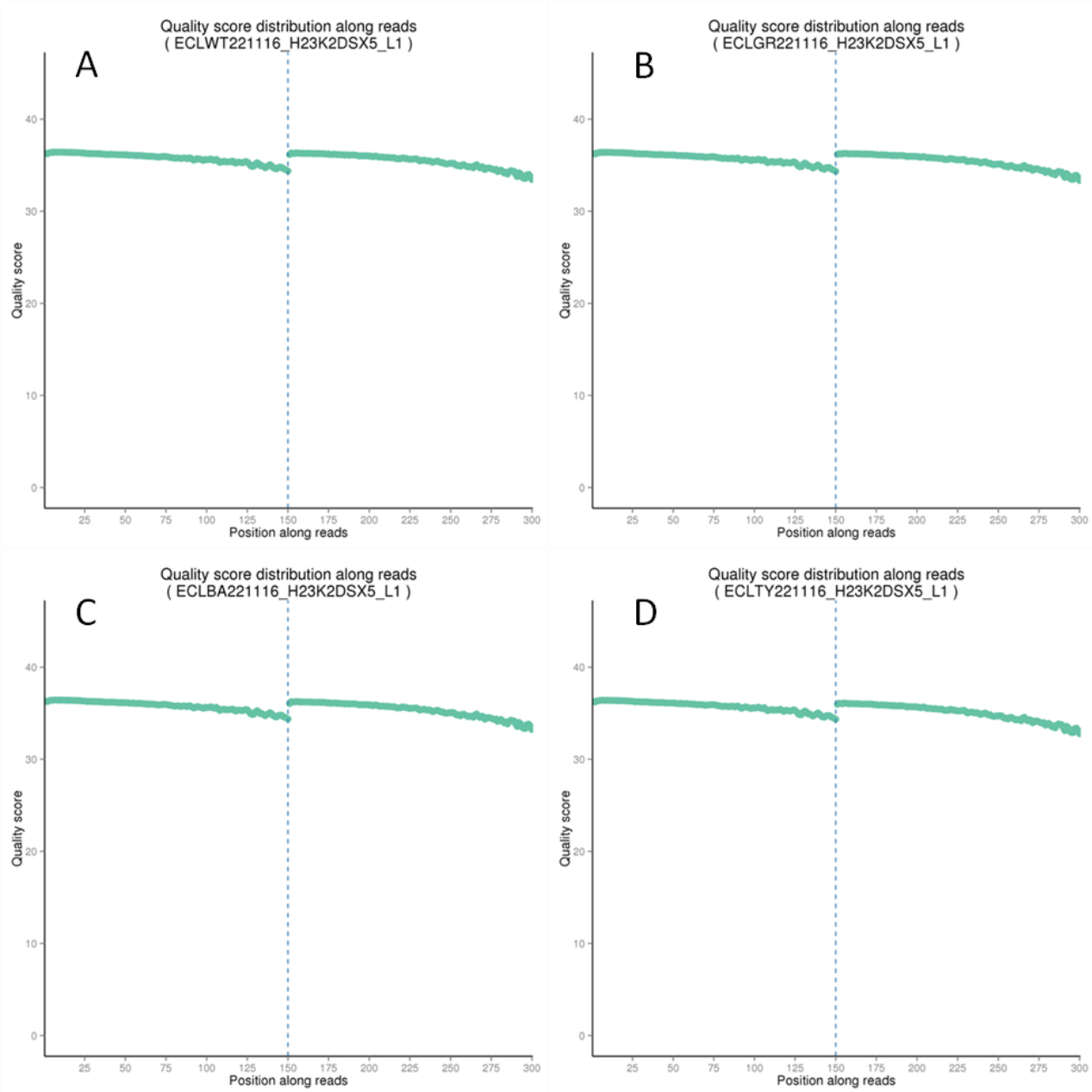
Appendix 4.7. *E. cloacae* passage data – continued

Clinical Antibiotic	CN																	
Baseline	18			18			18			18			18			18		
P0	NZ			16			15			15			15			16		
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin		
P1	15	16	16	15	15	16	15	15	15	15	14	14	14	15	16	16	15	16
P5	16	16	17	16	15	15	15	15	15	16	15	14	16	14	14	16	15	13
P10	15	15	16	15	16	14	15	15	14	15	15	14	16	15	15	13	16	16
Clinical Antibiotic	ATM																	
Baseline	24			20			25			24			20			25		
P0	16			15			13			17			15			16		
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin		
P1	15	20	15	18	15	17	18	19	14	22	15	14	18	16	16	17	17	15
P5	15	17	20	15	20	18	22	15	15	23	21	16	16	17	15	18	19	18
P10	20	16	18	21	19	17	18	15	15	22	20	21	NZ	15	14	27	23	20

Appendix 4.7. *E. cloacae* passage data – continued

Clinical Antibiotic	CIP						SXT						SXT					
Baseline	31		32		32		15		16		15		15		16		15	
P0	24		NZ		24		13		13		14		13		13		13	
OTC exposure	With Gramicidin			Without Gramicidin			With Bacitracin			Without Bacitracin			With Tyrothricin			Without Tyrothricin		
P1	23	25	26	26	22	26	12	14	14	14	14	14	13	11	12	14	14	11
P5	26	25	25	28	25	27	12	13	13	12	13	13	12	15	14	12	14	14
P10	24	24	25	25	25	26	14	13	15	13	13	13	13	16	14	12	16	14

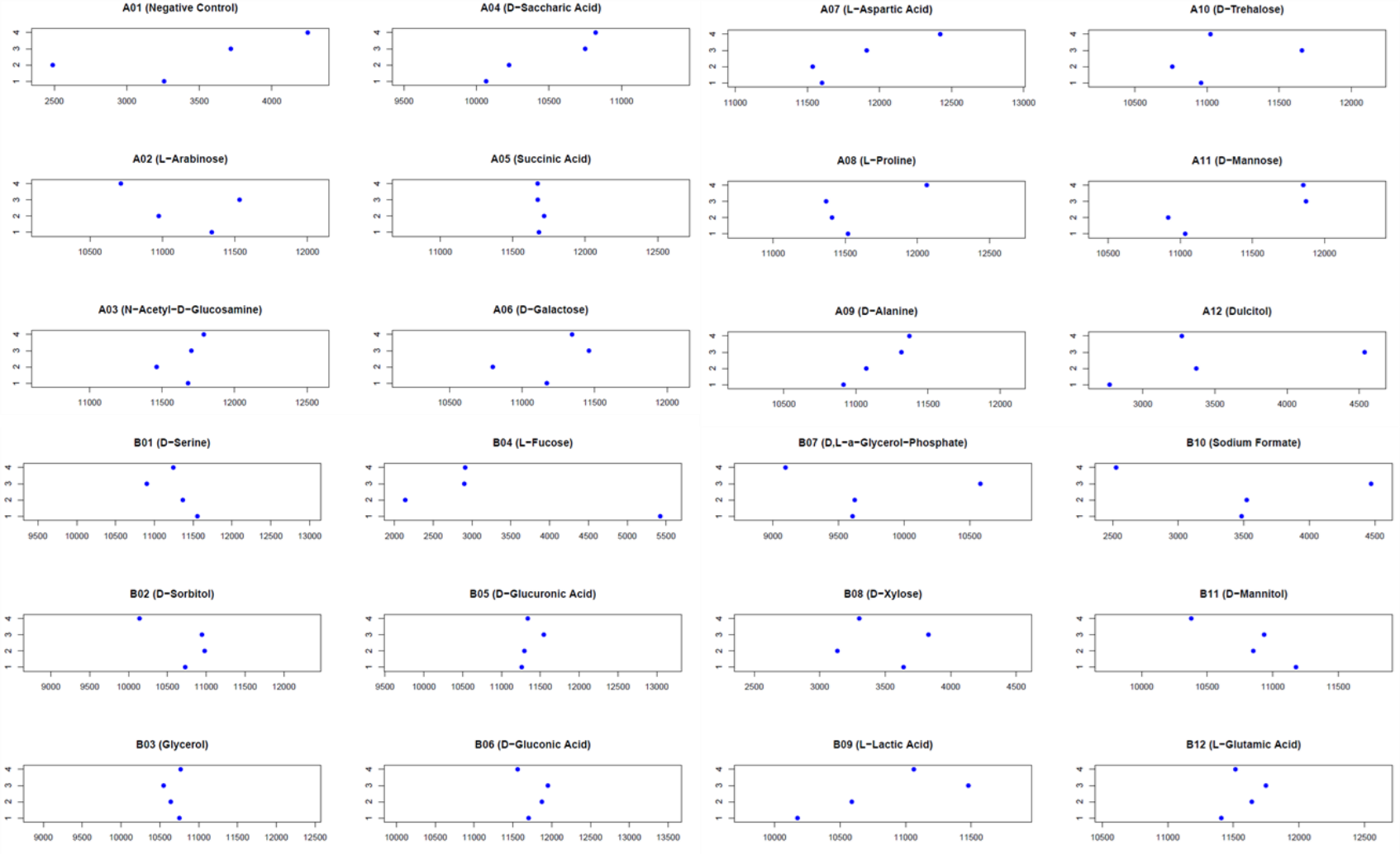
Appendix 5.1. Sequence quality data – Control (A), Gramicidin (B), Bacitracin (C) and Tyrothricin (D).



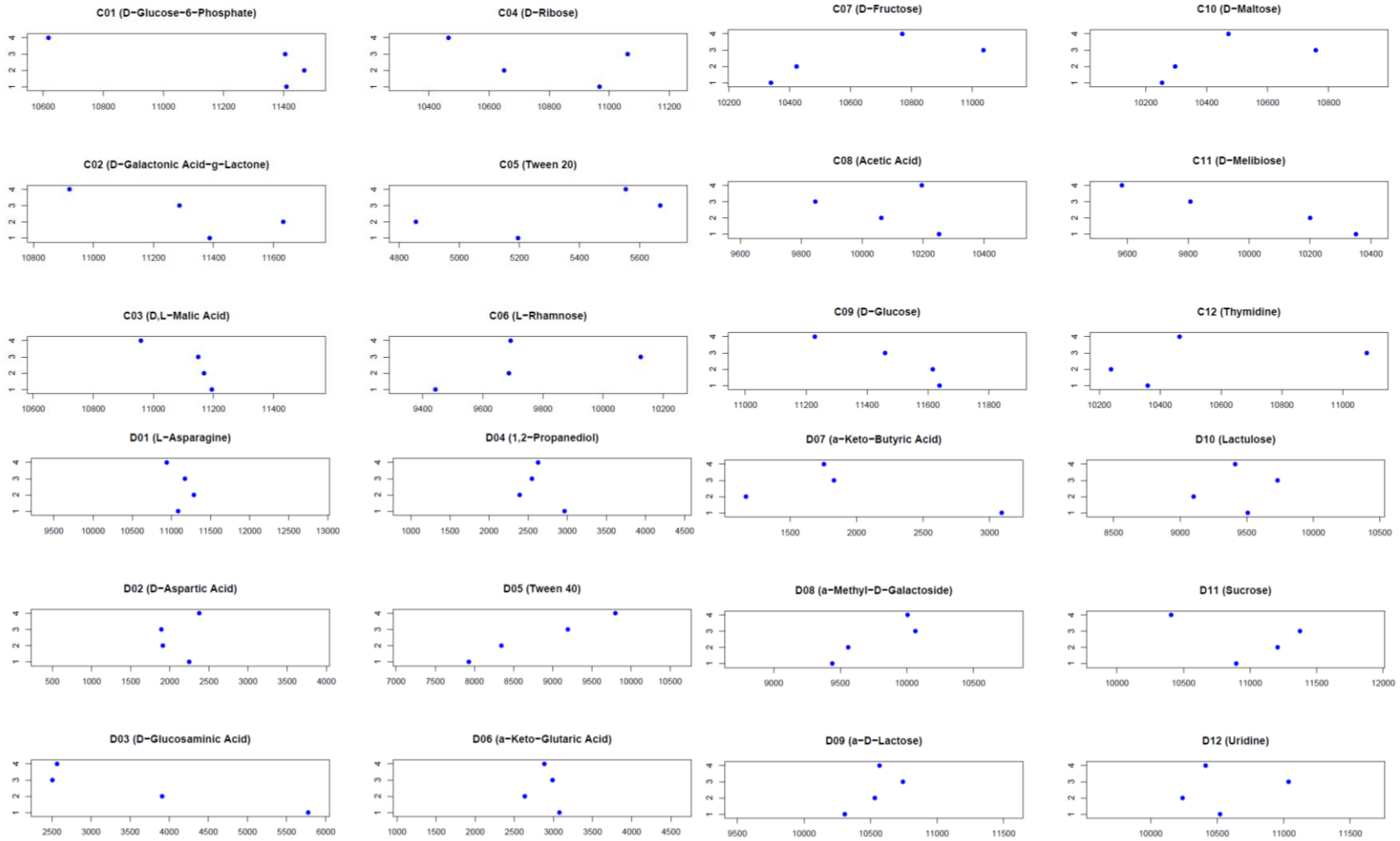
Appendix 5.2. Mapping Quality Statistics when mapped to a reference genome (CP001918)

Sample	Mapped	Total Reads	Mapping Rate	Average depth (X)	Coverage at least 1X (%)	Coverage at least 4X (%)
Control	10,721,650	11,463,321	93.53	242.1	99.99	99.98
Gramicidin	8,776,917	9,422,418	93.15	202.39	99.99	99.98
Bacitracin	9,493,214	10,181,124	93.24	218.14	99.99	99.98
Tyrothricin	8,892,394	9,572,580	92.89	207.94	99.99	99.98

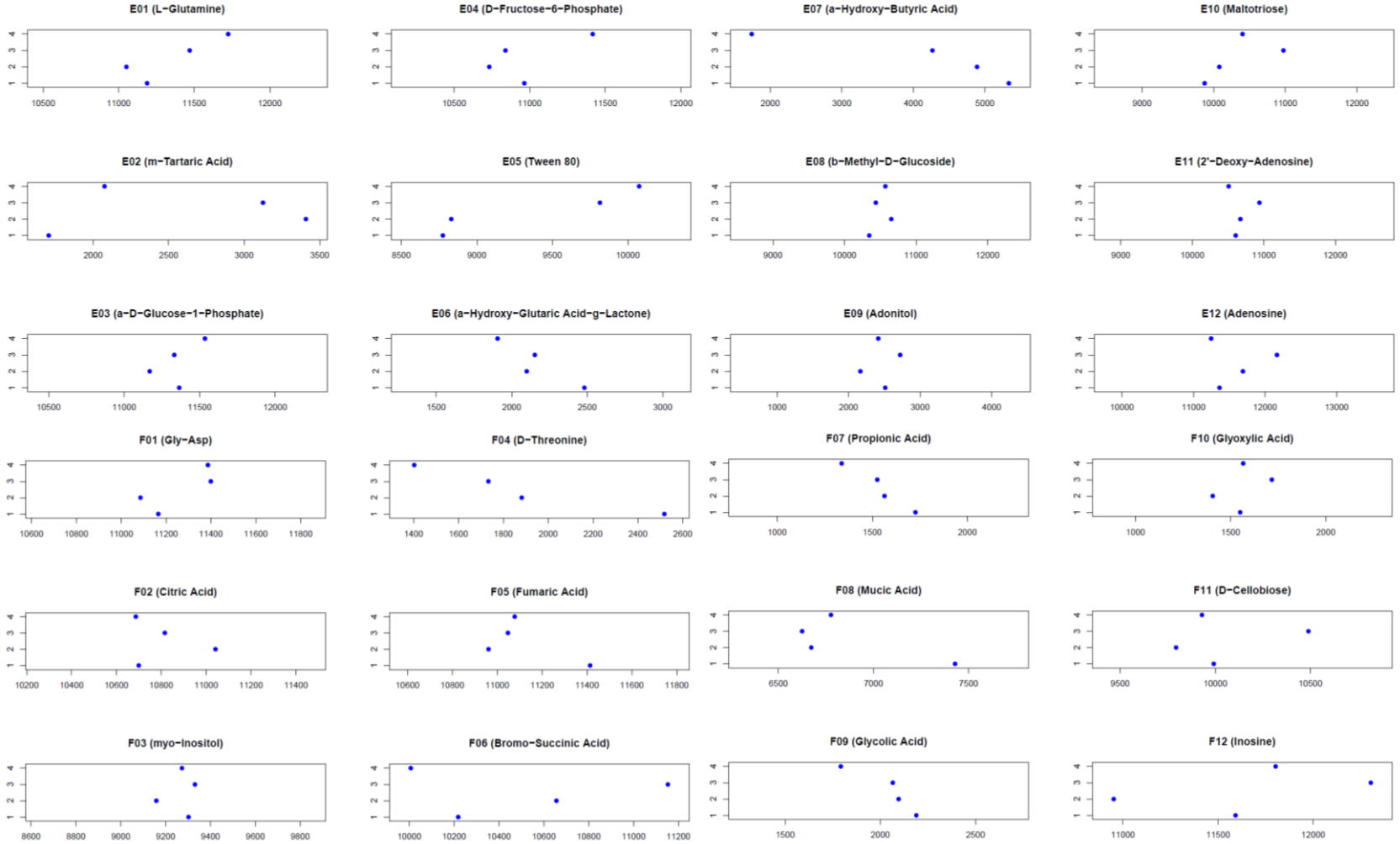
Appendix 5.3. Confidence interval plots of *E. cloacae* – Biolog PM01; A01-B12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



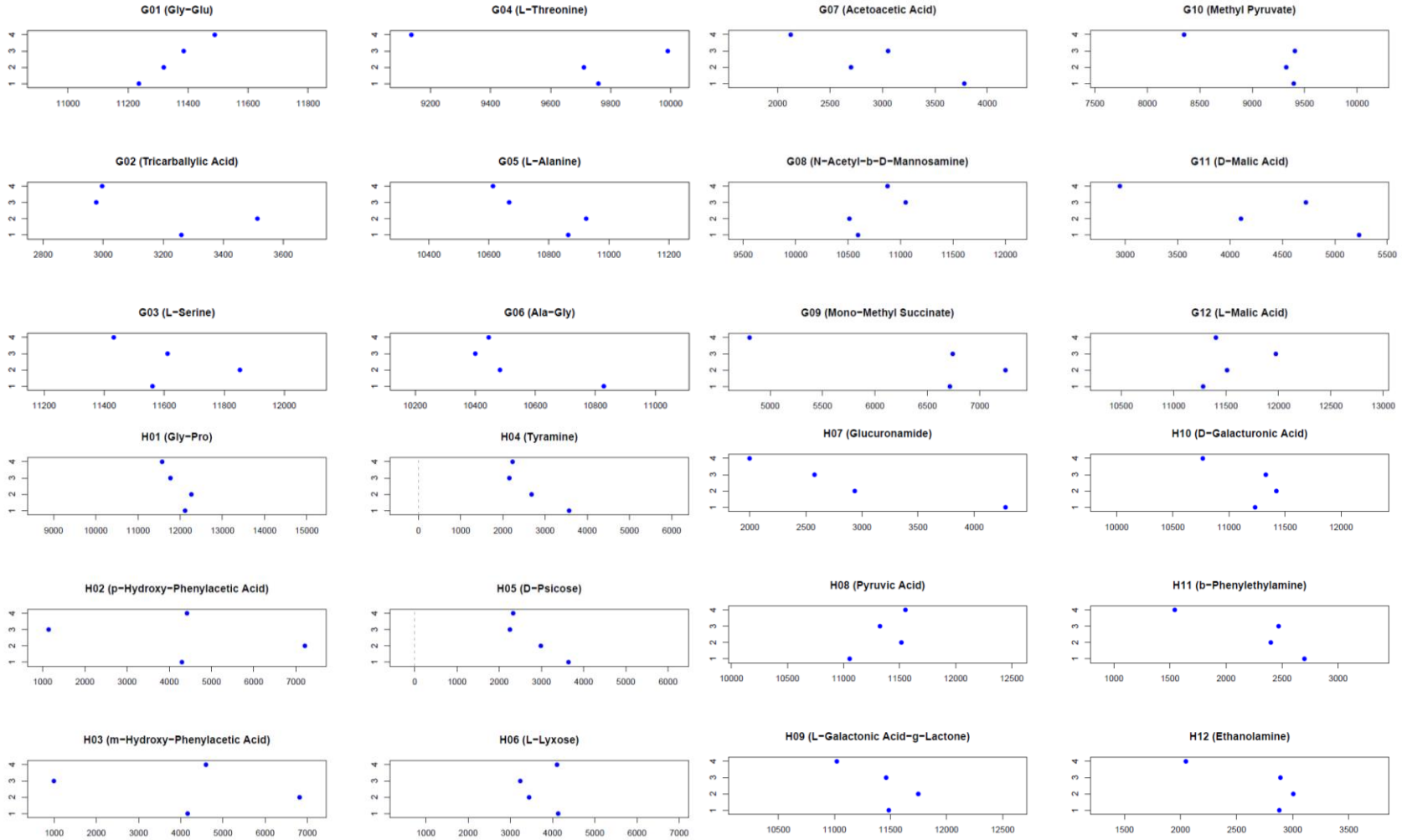
Appendix 5.4. Confidence interval plots of *E. cloacae* – Biolog PM01; C01-D12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



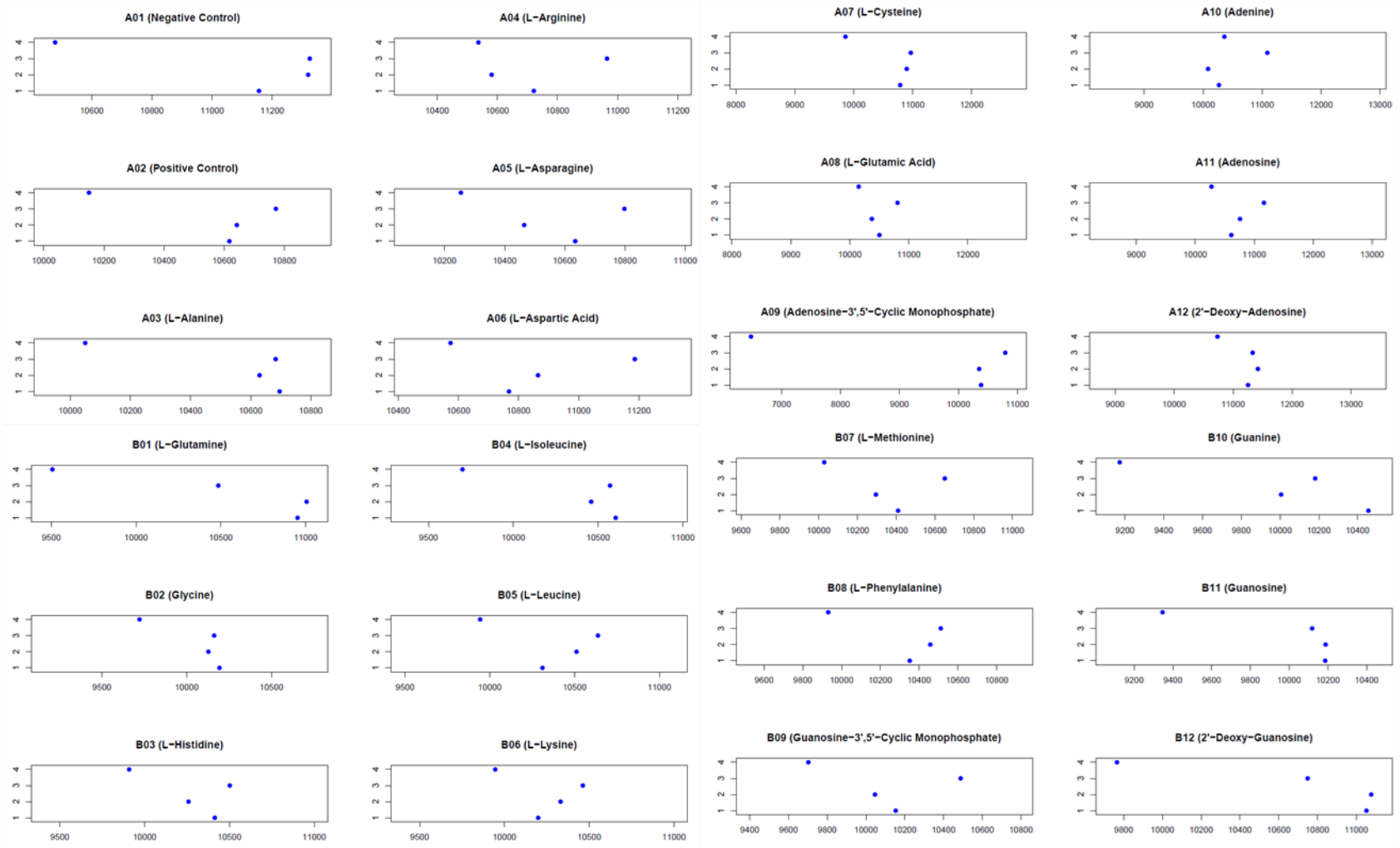
Appendix 5.5. Confidence interval plots of *E. cloacae* – Biolog PM01; E01-F12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



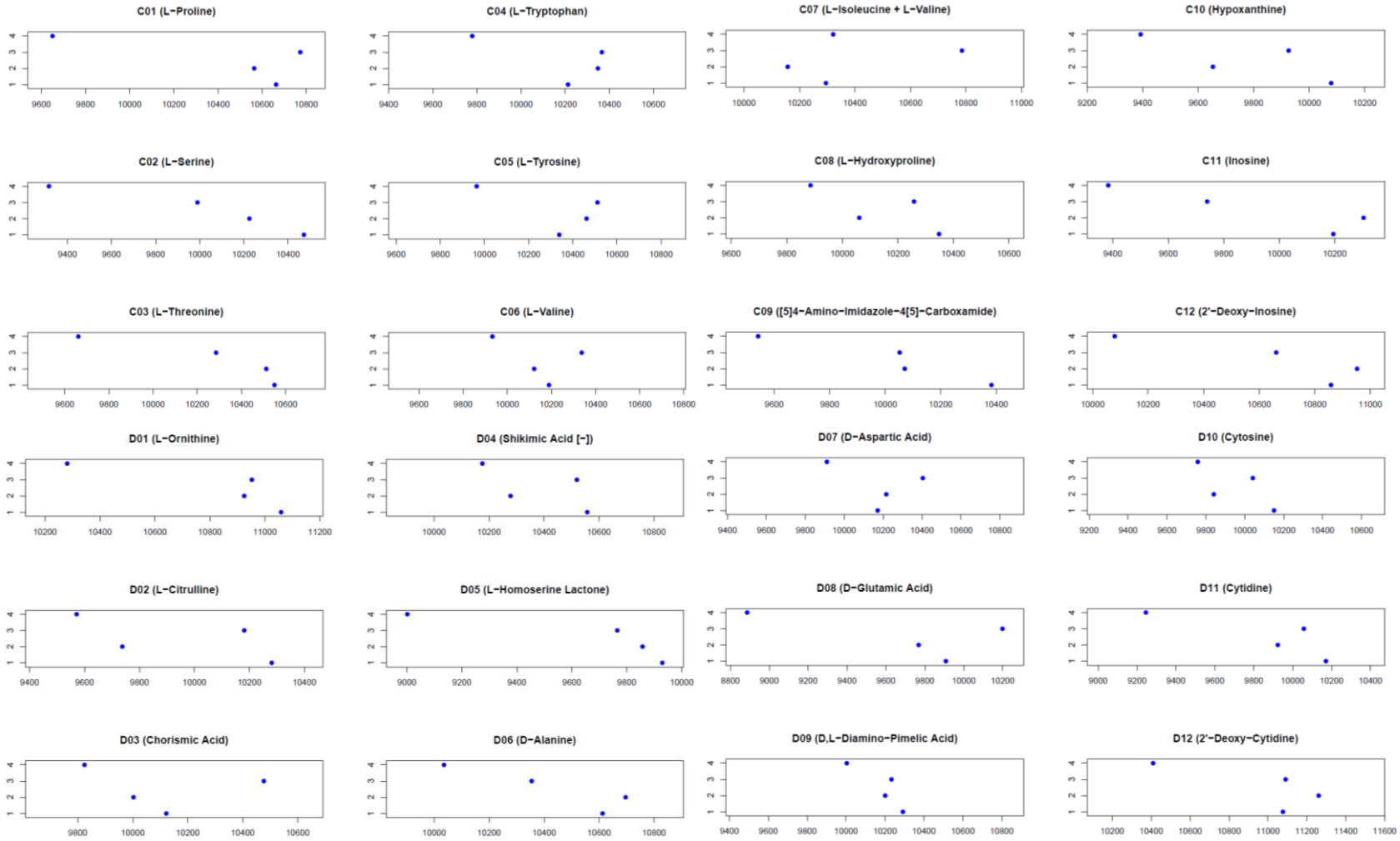
Appendix 5.6. Confidence interval plots of *E. cloacae* – Biolog PM01; G01-H12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



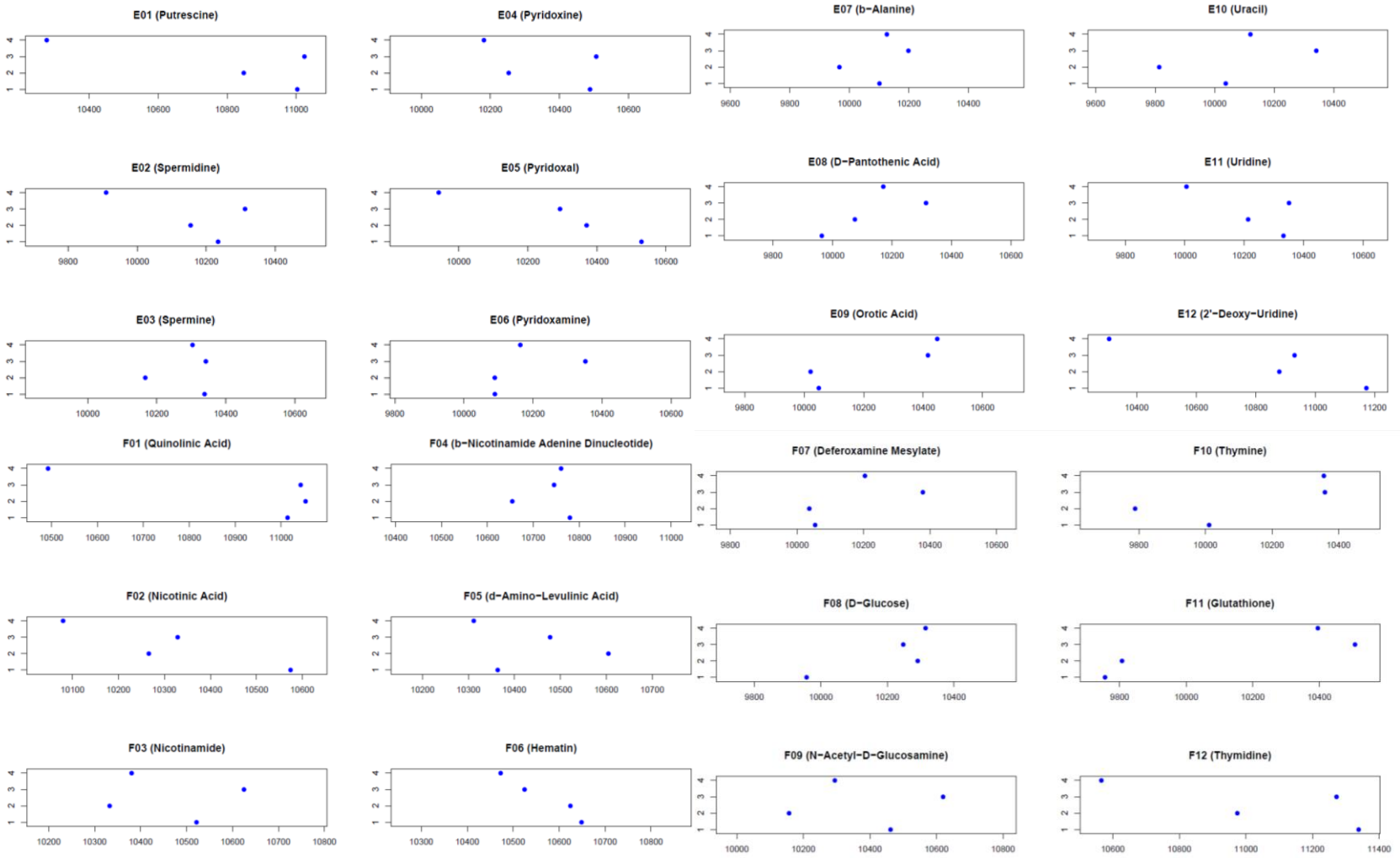
Appendix 5.7. Confidence interval plots of *E. cloacae* – Biolog PM05; A01-B12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



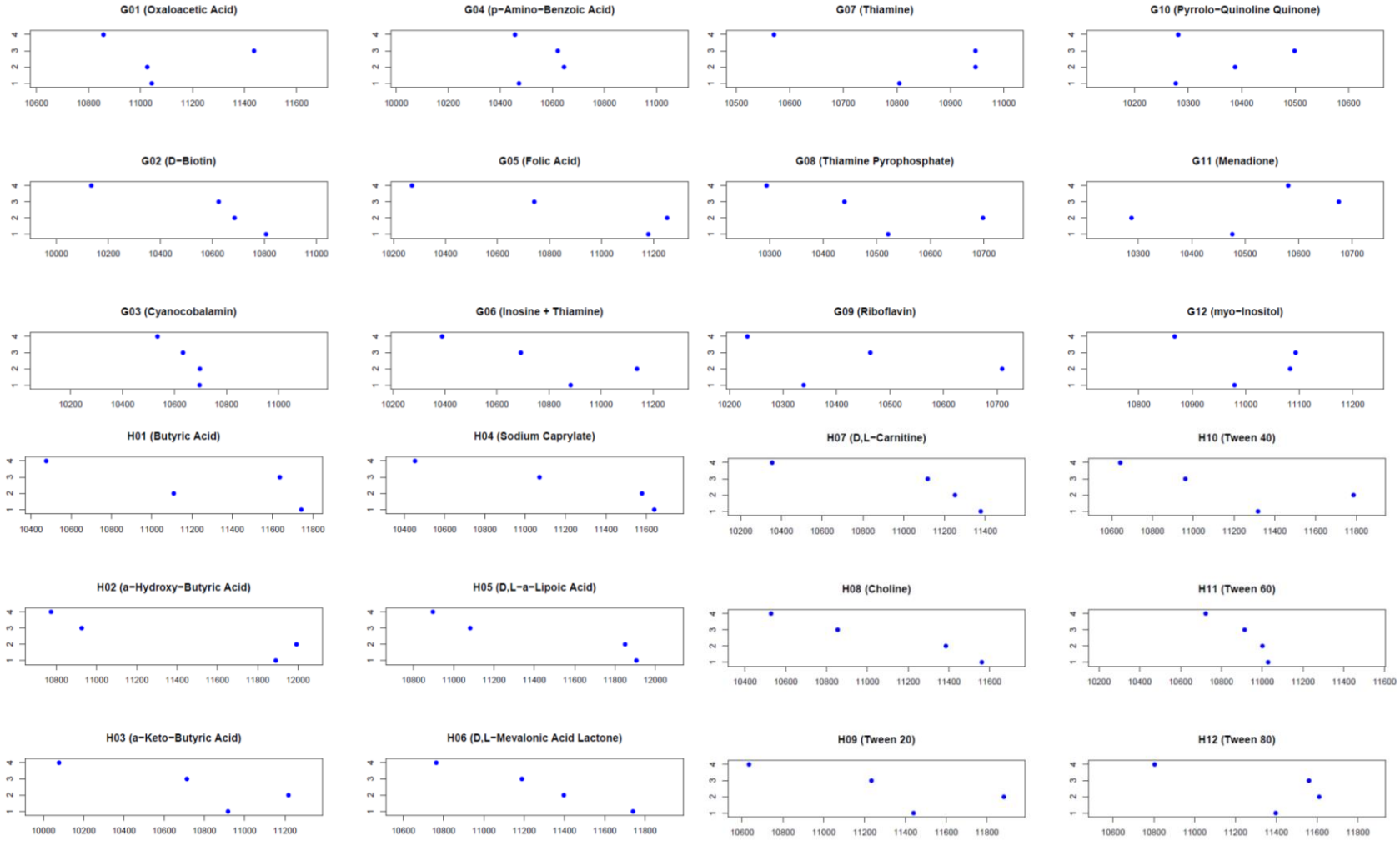
Appendix 5.8. Confidence interval plots of *E. cloacae* – Biolog PM05; C01-D12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



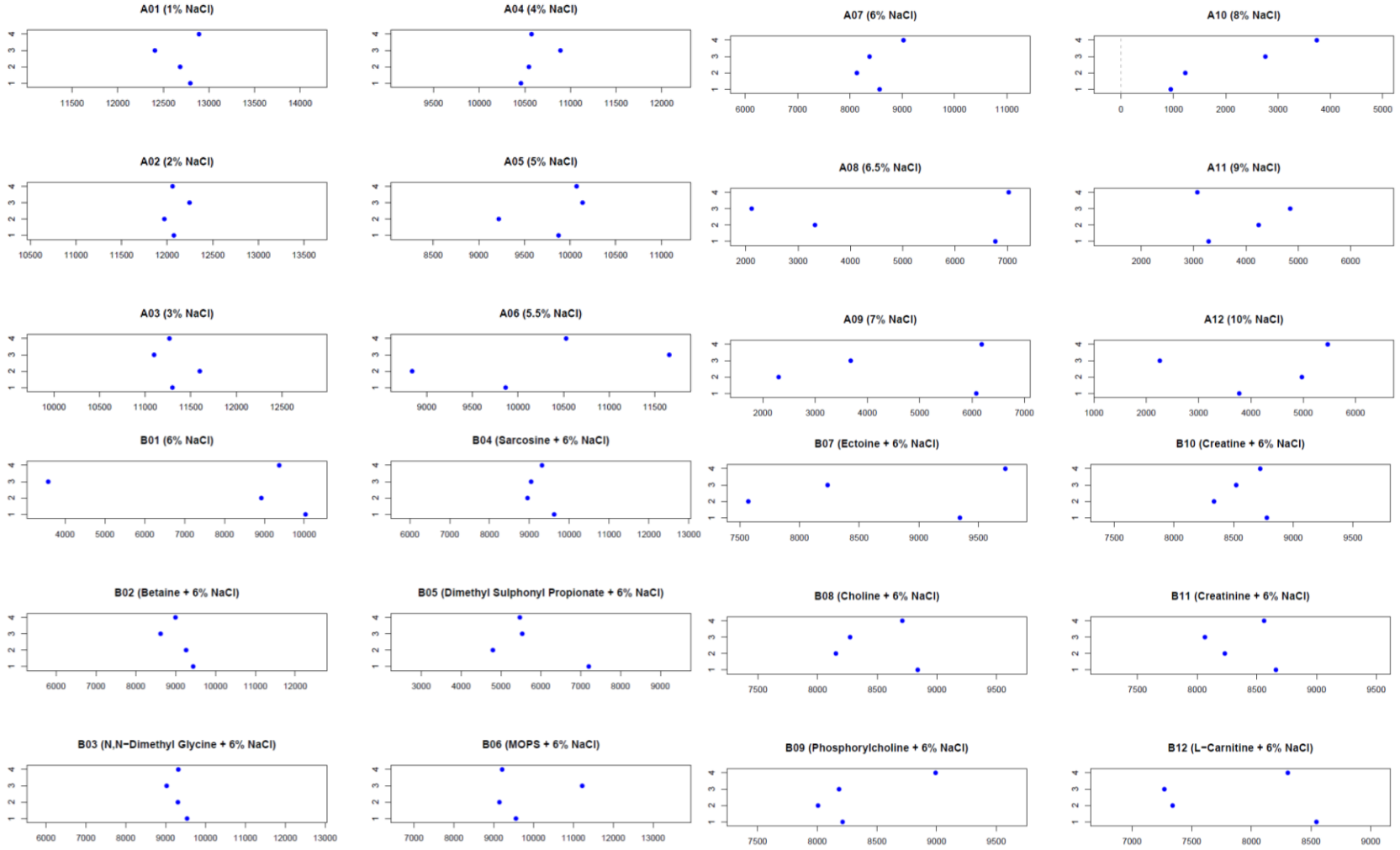
Appendix 5.9. Confidence interval plots of *E. cloacae* – Biolog PM05; E01-F12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



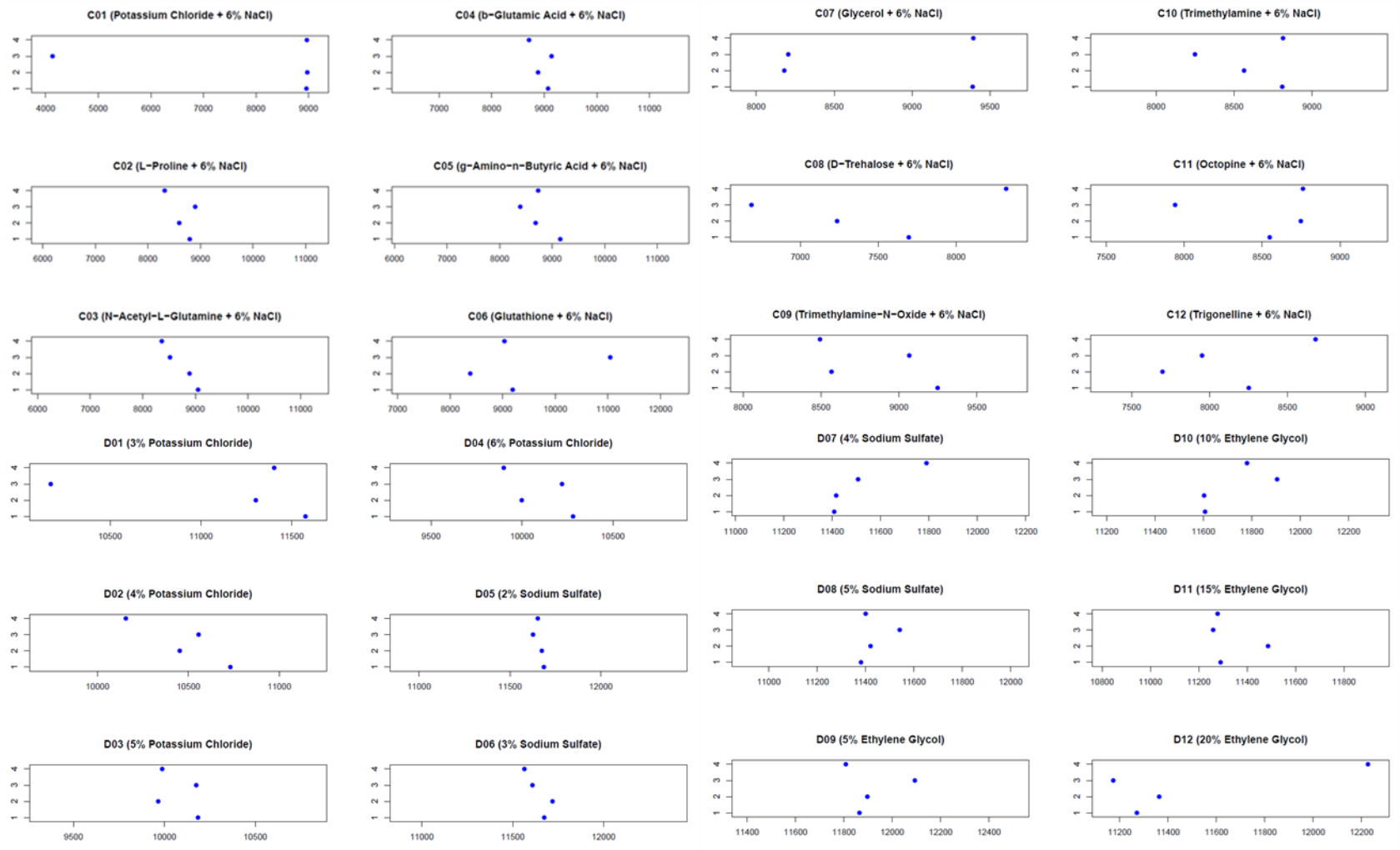
Appendix 5.10. Confidence interval plots of *E. cloacae* – Biolog PM05; G01-H12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



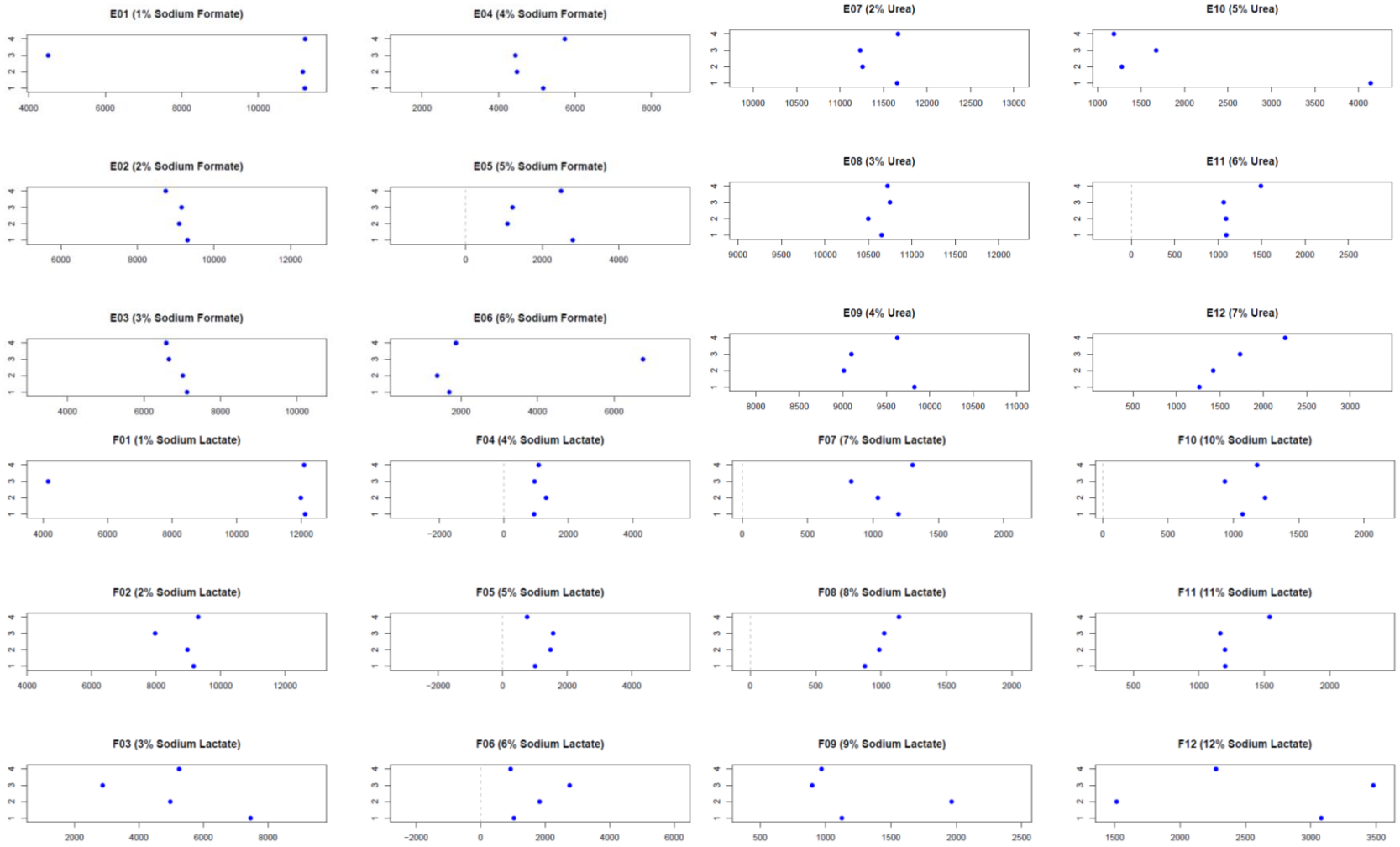
Appendix 5.11. Confidence interval plots of *E. cloacae* – Biolog PM09; A01-B12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



Appendix 5.12. Confidence interval plots of *E. cloacae* – Biolog PM09; C01-D12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



Appendix 5.13. Confidence interval plots of *E. cloacae* – Biolog PM09; E01-F12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).



Appendix 5.14. Confidence interval plots of *E. cloacae* – Biolog PM09; G01-H12. Bacitracin (5 IU/ml; 1), Untreated Control (2), Gramicidin (15 µg/ml; 3), Tyrothricin (200 µg/ml; 4).