Australian Government
Department of Health
Office of the Gene Technology Regulator

Risk Assessment and Risk Management Plan for

DIR 183

Clinical trial with genetically modified *E.coli* to reduce antibiotic resistance

Applicant: The Westmead Institute for Medical Research

July 2021

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Summary of the Risk Assessment and Risk Management Plan

for

Licence Application DIR 183

Decision

The Gene Technology Regulator (the regulator) has decided to issue a licence (DIR 183) to allow the Westmead Institute for Medical Research to conduct a clinical trial to evaluate the safety and efficacy of genetically modified (GM) *E.coli* to deliver genes that restore sensitivity to antibiotics in gut bacteria.

Clinical trials conducted in Australia must be conducted in accordance with the National Statement on Ethical Conduct in Human Research and with the Guidelines for Good Clinical Practice of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

The Regulator has prepared a Risk Assessment and Risk Management Plan (RARMP) for this application, which concludes that the proposed clinical trials pose negligible risks to human health and safety and the environment. Licence conditions have been imposed for the proposed clinical trial to manage any risk posed by the dealings.

The application

Application number	DIR 183
Applicant	The Westmead Institute for Medical Research
Project title	Clinical trial with genetically modified *E.coli* to reduce antibiotic resistance
Parent organism	*Escherichia coli* (Nissle strain) and human gut bacteria
Introduced gene and modified trait	Two antibiotic resistance plasmids (circular pieces of bacterial DNA) were modified by • Deletion of genes responsible for resistance to multiple classes of antibiotics • Deletion of genes that enable plasmids to persist in bacteria • Introduction of genes for resistance to specific antibiotics (fosfomycin or tetracycline) to enable selection for the GMO
Previous releases / approvals	First in human trial
Proposed locations	Westmead hospital
Primary purpose	The proposed trial is designed to evaluate the safety and efficacy of GM *E.coli* to deliver genes to gut bacteria that restore sensitivity to antibiotics

Risk assessment

The risk assessment concludes that risks to the health and safety of people or the environment from the proposed clinical trial are negligible. No specific risk treatment measures are required to manage these negligible risks.

The current assessment focuses on risks posed to people other than the intended treatment recipient and to the environment, including long term persistence of the GMOs, which may arise from the administration and disposal of the GMO.

The risk assessment process considers how the genetic modifications and activities conducted with the GMO might lead to harm to people or the environment. Risks are characterised in relation to both the seriousness and likelihood of harm, taking into account information in the application (including proposed controls), relevant previous approvals, current scientific/technical knowledge and advice received from a wide range of experts, agencies and authorities. Both the short and long term risks were considered.

Credible pathways to potential harm that were considered included: whether people and animals can be inadvertently exposed to the GMO, and the potential for transfer of genetic material to and from the GMO. The potential for GMO to be released into the environment and its effects was also considered.

The principal reasons for the conclusion of negligible risks are that the potential risks that could arise would be the result of genetic recombination and transmission events that are unlikely to occur given the limits and controls imposed.

Risk management plan

The risk management plan describes measures to protect the health and safety of people and to protect the environment by controlling or mitigating risk. The risk management plan is given effect through licence conditions.

As the level of risk is considered negligible, specific risk treatment is not required. However, since this is a clinical trial, the licence includes limits on the number of trial participants, location limited to hospitals and clinical trial sites, limits on the duration of the trial, as well as a range of controls to minimise the potential for the GMO to spread to non-participants. In addition, there are several general conditions relating to ongoing licence holder suitability, auditing and monitoring, and reporting requirements which include an obligation to report any unintended effects.

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Abbreviations

AICIS	Australian Industrial Chemicals Introduction Scheme
APVMA	Australian Pesticides and Veterinary Medicines Authority
AQIS	Australian Quarantine and Inspection Service
AMR	Antimicrobial resistance. AMR generally refers to resistance to antibiotics that are important for human health.
ARTG	Australian Register of Therapeutic Goods
ASTAG	Australian Strategic and Technical Advisory Group on Antimicrobial Resistance
cfu	Colony forming units
DAWE	Department of Agriculture, Water and the Environment
DIR	Dealings involving Intentional Release
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EU	European Union
FSANZ	Food Standards Australia New Zealand
FOS	Fosfomycin (an antibiotic)
g	gram
GM	Genetically modified
GM bacteria	*E.coli* or Human gut bacteria containing the GM plasmid
GMAC	Genetic Manipulation Advisory Committee
GMO	Genetically modified organism
GTTAC	Gene Technology Technical Advisory Committee
HGT	Horizontal gene transfer
HREC	Human Research Ethics Committee
IATA	International Air Transport Association
kb	Kilobase pair of DNA
LGA	Local government area
Mb	Mega base pairs
min	minute
ml	Milli litre
NSW	New South Wales
OGTR	Office of the Gene Technology Regulator
PCR	Polymerase chain reaction
QLD	Queensland
RARMP	Risk Assessment and Risk Management Plan
TET	Tetracycline (an antibiotic)
TGA	Therapeutic Goods Administration
the Act	The *Gene Technology Act 2000*
the Regulations	Gene Technology Regulations 2001

the Regulator	Gene Technology Regulator
USA	United States of America
WHO	World Health Organization

Chapter 1   Risk assessment context

Section 1   Background

1.   An application has been made under the *Gene Technology Act 2000* (the Act) for Dealings involving the Intentional Release (DIR) of genetically modified organisms (GMOs) into the Australian environment.

2.   The Act and the Gene Technology Regulations 2001 (the Regulations), together with corresponding State and Territory legislation, comprise Australia's national regulatory system for gene technology. Its objective is to protect the health and safety of people, and to protect the environment, by identifying risks posed by or as a result of gene technology, and by managing those risks through regulating certain dealings with GMOs.

3.   As the proposed trial involves the intentional introduction of a GMO into a human being, where the GMO is not a human somatic cell, the proposed dealings require a licence under Schedule 3, Part 3.1(n) of the Regulations, as amended June 2016.

4.   Section 50 of the Act requires that the Gene Technology Regulator (the Regulator) must prepare a Risk Assessment and Risk Management Plan (RARMP) in response to an application for release of GMOs into the Australian environment. Sections 50, 50A and 51 of the Act and sections 9 and 10 of the Regulations outline the matters which the Regulator must take into account and who must be consulted when preparing the RARMP.

5.   The *Risk Analysis Framework* (RAF) (OGTR, 2013b) explains the Regulator's approach to the preparation of RARMPs in accordance with the Act and the Regulations. The Regulator has also developed operational policies and guidelines that are relevant to DIR licences. These documents are available from the Office of the Gene Technology Regulator (OGTR) website.

6.   Figure 1 shows the information that is considered, within the regulatory framework above, in establishing the risk assessment context. This information is specific for each application. Potential risks to the health and safety of people or the environment posed by the proposed release are assessed within this context. Chapter 1 provides the specific information for establishing the risk assessment context for this application.

RISK ASSESSMENT CONTEXT

The GMO
Modified genes
Novel traits

Parent organism (comparator)
Origin and taxonomy
Cultivation and use
Biology

Receiving environment
Environmental conditions: abiotic and biotic factors
Production practices
Related organisms
Similar genes and proteins

Proposed GMO dealings
Activities
Limits
Controls

Previous releases
Australian approvals
International approvals

Figure 1. Summary of parameters used to establish the risk assessment context, within the legislative requirements, operational policies and guidelines of the OGTR and the RAF.

7.   In accordance with Section 50A of the Act, this application is considered to be a limited and controlled release application, as the Regulator was satisfied that it meets the criteria prescribed by

the Act. Therefore, the Regulator was not required to consult with prescribed experts, agencies and authorities before preparation of the RARMP.

8.   Section 52 of the Act requires the Regulator to seek comment on the consultation RARMP from agencies - the Gene Technology Technical Advisory Committee (GTTAC), State and Territory Governments, Australian Government authorities or agencies prescribed in the Regulations, Australian local councils and the Minister for the Environment and from the public. The advice from the prescribed experts, agencies and authorities, and how it was taken into account, is summarised in Appendix A. Two public submissions were received and their consideration is summarised in Appendix B.

1.1   Interface with other regulatory schemes

9.   Gene technology legislation operates in conjunction with other regulatory schemes in Australia. The GMOs and any proposed dealings conducted under a licence issued by the Regulator may also be subject to regulation by other Australian government agencies that regulate GMOs or GM products, including Food Standards Australia New Zealand (FSANZ), the Australian Pesticides and Veterinary Medicines Authority (APVMA), the Therapeutic Goods Administration (TGA), the Australian Industrial Chemical Introduction Scheme (AICIS) and the Department of Agriculture, Water and the Environment (DAWE).

10.   Medicines and other therapeutic goods for use in Australia are required to be assessed for quality, safety and efficacy under the *Therapeutic Goods Act 1989* and must be included in the Australian Register of Therapeutic Goods. The TGA is responsible for administering the provisions of this legislation. Clinical trials of therapeutic products that are experimental and under development, prior to a full evaluation and assessment, are also regulated by the TGA through the Clinical Trial Approval (CTA) scheme or the Clinical Trial Notification (CTN) scheme.

11.   For clinical trials, the TGA has regulatory responsibility for the supply of unapproved therapeutic products. In terms of risk to individuals participating in a clinical trial, the TGA (as the primary regulatory agency), the trial sponsor, the investigators and the Human Research Ethics Committee (HREC) at each trial site all have roles in ensuring participant's safety under the *Therapeutic Goods Act 1989*. However, where the trial involves a GMO, authorisation is also required under gene technology legislation. To avoid duplication of regulatory oversight, and as risks to trial participants are addressed through the above mechanisms, the Regulator's focus is on assessing risks posed to people other than those participating in the clinical trial, and to the environment. This includes risks to people preparing and administering the GMO, and risks associated with import, transport and disposal of the GMO.

12.   The International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use – Guidelines for Good Clinical Practice (ICH-GCP) is an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects (ICH, 2016). The guideline was developed with consideration of the current good clinical practices of the European Union, Japan, and the United States of America (USA), as well as those of Australia, Canada, the Nordic countries and the World Health Organization (WHO). The TGA has adopted the Integrated addendum to ICH E6(R1): Guideline for good clinical practice E6(R2) (Therapeutic Goods Administration), which provides overarching guidance for conducting clinical trials in Australia which fall under TGA regulation.

13.   The National Health and Medical Research Council (NHMRC) has issued the *National Statement on Ethical Conduct in Human Research* (National Health and Medical Research Council et al., 2018). This document sets the Australian standard against which all research involving humans is reviewed. The Therapeutic Goods Act 1989 requires that the use of a therapeutic good in a clinical trial must be in accordance with the ethical standards set out in this document.

14.   Approval by a Human Research Ethics Committee (HREC) is also a fundamental requirement of a clinical trial. HRECs conduct both ethical and scientific assessment of the proposal and in addition often consider issues of research governance. Other elements of governance of clinical trials that are

considered by HRECs include appropriate informed consent, specific inclusion and exclusion criteria, data monitoring, accounting and reconciliation of investigational product.

15.   The DAWE regulates products imported into Australia to protect Australia from biosecurity risks. Under the *Biosecurity Act 2015*, the importation of biological material such as live GM vaccines and treatments requires a permit from DAWE.

16.   All clinical trial sites would be located at medical facilities including out-patient settings, hospitals and associated pharmacies. Analysis of biological samples collected from trial participants administered with the GMO would occur at clinical trial sites, pathology laboratories or certified facilities.

17.   The state and territory governments regulate hospitals and other medical facilities in Australia. All public and private hospitals and day procedure services need to be accredited to the National Safety and Quality Health Service (NSQHS) Standards developed by the Australian Commission on Safety and Quality in Healthcare (the Commission) and endorsed by the state and territory Health Ministers. The Commission coordinates accreditation processes via the Australian Health Service Safety and Quality Accreditation (AHSSQA) scheme. The NSQHS Standards provide a quality assurance mechanism that tests whether relevant systems are in place to ensure that the minimum standards of safety and quality are met. The safety aspects addressed by the NSQHS Standards include the safe use of sharps, disinfection, sterilisation and appropriate handling of potentially infectious substances. Additionally, the Commission has developed the National Model Clinical Guidance Framework, which is based on, and builds on NSQHS Standards to ensure that clinical governance systems are implemented effectively and to support better care for patients and consumers.

18.   The National Pathology Accreditation Advisory Council (NPAAC) advises Commonwealth, State and Territory health ministers on matters relating to the accreditation of pathology laboratories. NPAAC plays a key role in ensuring the quality of Australian pathology services and is responsible for the development and maintenance of standards and guidelines for pathology practices. The standards include safety precautions to protect the safety of workers from exposure to infectious microorganisms in pathology laboratories. While compliance with NPAAC standards and guidelines is not mandatory, there is a strong motivation for pathology services to comply, as Medicare benefits are only payable for pathology services if conducted in an appropriate Accredited Pathology Laboratory (APL) category, by an Approved Pathology Practitioner (APP) employed by an Approved Pathology Authority (APA). Accreditation of pathology services is overseen by Services Australia (formerly Department of Human Services), and currently, the only endorsed assessing body for pathology accreditation is the National Association of Testing Authorities (NATA).

19.   Hospitals and pathology laboratories, including their workers, managers and executives, all have a role in making the workplace safe and managing the risks associated with handling potentially infectious substances including the proposed GMO. There are minimum infection prevention practices that apply to all health care in any setting where health care is provided. These prevention practices were initially developed by the Centers for Disease Control and Prevention (CDC), and are known as the standard precautions for working with potentially infectious material. The standard precautions are described in the Australian Guidelines for the Prevention and Control of Infection in Healthcare (2019).

20.   To avoid duplication of regulatory oversight, risks that have been considered by other regulatory agencies would not be re-assessed by the Regulator. The Regulator will assess risks to people as a consequence of conducting these activities and risks from persistence of the GMOs in the environment.

1.2   Summary of the proposed dealings

21.   The Westmead Institute for Medical Research have applied to conduct a clinical trial to evaluate the safety and efficacy of GM *E.coli* to deliver genes to gut bacteria that restore sensitivity to antibiotics.

22.   The dealings assessed by the Regulator are:

      a. Conduct experiments with the GMO

          i. administer of the GMO to clinical trial participants

          ii. collect and analyse the GMO

      b. transport from the clinical trial site to the site of analysis

      c. dispose of the GMO

and the possession (including storage), supply or use of the GMO for the purposes of, or in the course of, any of the above.

23.   The figure below gives an overview of the proposed clinical trial and subsequent sections provide more detail.

1. Some bacteria have become resistant to the antimicrobials commonly used to treat them. This resistance (AMR) is often carried on bacterial plasmids.

5. Inside participant's gut GM plasmid spreads to many gut bacteria through conjugation.
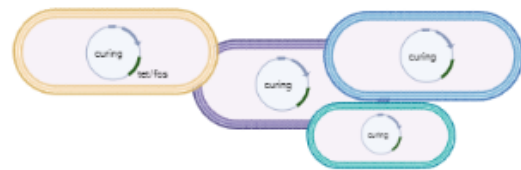
GMO 2. Human gut bacteria that contain the GM plasmid

2. Genetic modification was used to create "curing plasmids".

GMO 1. modified E.coli

6. AMR plasmid is swapped for curing plasmid because bacteria can't hold two overly similar plasmids and there is now a selective advantage for Tet resistance

Once AMR plasmid is removed gut bacteria are sensitive to normal antibiotics

3. Participant with target AMR plasmid is identified
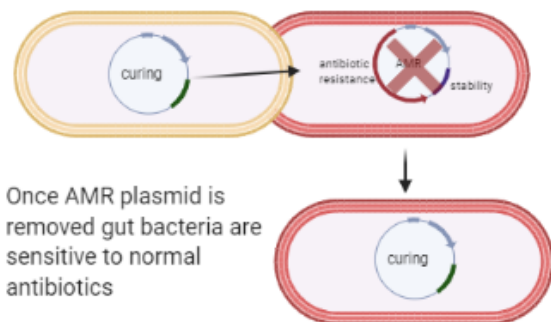
7. Participant can now be treated with a common antibiotic

Eg: β-lactam Gentamicin ect.

4. GMO *E. coli* carrying curing plasmid and selective antibiotic is administered to participant

+
Tetracycline or Fosfomycin

8. Faecal/rectal samples to monitor GMO 1, GMO2 and AMR

Created in BioRender.com bio

Figure 2. Simplified overview of the clinical trial.

1.3 Details of the proposed dealings

24.   To participate in this trial, participants would need to be identified as carrying one of the two targeted antimicrobial resistance plasmids (pJIBE401 or pJIE512b-like) by pathology services connected to Westmead Hospital in Sydney, NSW. For example, participants may be drawn from one of the following three groups

25.   Patients who have the target AMR plasmids and a mild-moderate active infection with *enterobacteriaceae*. This would include patients who suffer therapeutic failure of an antibiotic and are then screened and discovered to have one of the target AMR plasmids. These infections are most likely to be located in the gut, perineal skin, urinary tract or in wounds.

      i.   Patients who are discovered to have the AMR target plasmids as part of pre-surgical or pre-treatment screening conducted at Westmead. Such as, but not limited to, patients scheduled for solid organ (e.g. kidney, pancreas) or bone marrow transplantation or treatment for leukaemia or lymphoma.

      ii.   Patients with life threating infections caused by *enterobacteriaceae* who have been admitted to the Westmead intensive care unit or a screened critical care unit such as haematology or transplant unit, and who have the targeted AMR.

26.   Patients with abnormal renal function, neuromuscular disease or other neuromuscular junction blocking agents, inflammatory bowel disease, aminoglycoside allergy or who are, or might be, pregnant would be excluded.

27.   The default method of administration would be ingestion of a single daily dose of 10^8 cfu *E.coli* Nissle strain (containing a GM plasmid) in normal saline for three consecutive days. This will be administered in a disposable cup by trained medical and nursing staff wearing appropriate PPE (disposable gloves, gowns, and eye protection). The dose size, frequency or duration may change depending on HREC approval of the trial. Participants who are identified as having the target AMR plasmid pJIBE401 will be administered the curing plasmid pJIMK46 in *E.coli* Nissle strain. Participants who are identified as having target AMR plasmids like pJIE512b will be administered the curing plasmid pJIMK56 in *E.coli* Nissle strain. If the patient is already receiving other treatments via nasogastric or nasoduodenal tube, the GMO will be administered via the tube.

28.   The antibiotic tetracycline or fosfomycin will be co-administered with the GMO. Tetracycline would be administered for 3 days and the dosage would be the standard dose used for the specific formulation (tetracycline or doxycycline). Fosfomycin would be given as a single dose of 3 grams. Tetracycline would be preferentially used unless there is known or suspected tetracycline resistance and/or a clinical indication that already exists for use of fosfomycin.

29.   Most participants would be subsequently treated with a second antibiotic after administration of the final dose of the GMO. The choice, dosage and duration of the second antibiotic administered to the participant will depend on their clinical presentation. For patients in intensive care unit, with life-threating infections, the treating physician may decide that waiting 72 hours for the course of tetracycline/fosfomycin is ill-advised and may administer the second powerful antibiotic earlier. For this reason, patients with life threating conditions are disfavoured participants. In cases where the participant does not have an active infection caused by *enterobacteriaceae,* a second antibiotic may not be administered.

30.   The participants would stay at the clinical trial site for 4 days after the last administration of the GMO or earlier if two consecutive faecal samples are shown to be free of GMOs. Faecal and/or rectal swabs would be taken at regular intervals and analysed to monitor the success of the treatment.

31.   Samples that are likely to contain the GMO would be transported from the clinical site to the site of analysis in line with the Regulator's *Guidelines for the Transport, Storage and Disposal of GMOs*.

32.   The Australian/New Zealand Standard *AS/NZ 2243.3:2010 Safety in laboratories Part 3: Microbiological safety and containment* (2010) classifies some of the bacterial genera expected to be present in faecal/rectal samples (such as *Klebsiella* spp.) as Risk Group 2 and specifies that any dealing involving these bacteria be conducted in a PC2 facility, using PC2 work practices.

33.   Analysis of the samples would be conducted in PC2 certified facilities. Analysis could include culturing human gut bacteria on media containing various antibiotics such as fosfomycin, tetracycline and/or β-lactam antibiotics. PCR based analysis may also be used for the detection, quantification and characterisation of bacteria and target and GM curing plasmids. If the GM plasmid is detected in fosfomycin or tetracycline-resistant colonies either by subculture or by direct PCR, sampling from particpants will be repeated at two week intervals until two consecutive samples are negative.

34.   For the first 4 days after the last administration of the GMO participant samples will be collected at the clinical trial site. When discharged from the clinical trial site, participants would be provided with clinical containers, safety protocols and instructions for sample collections. If any GM plasmid is detected in fosfomycin or tetracycline-resistant colonies either by subculture or by direct PCR, sampling will be repeated at two week intervals until two consecutive samples are negative.

35.   Disposal of single-use items (such as cups, spoons, tubing) as well as unused/expired GMO preparation at the medical facilities would be discarded into clinical waste. This waste would be then decontaminated by a method approved by the Environmental Protection Agency or Health Department of NSW.

36.   The parent organism of the GMO in this licence application is *E.coli* and therefore other dealings connected to this clinical trial such as production of GM *E.coli* and transport from the site of production to the site of administration, are permitted under an NLRD issued by the relevant IBC under schedule 3, Part 2, 2.1(d) of the *Gene Technology Regulations 2001*.

37.   Dealings associated with a clinical trial may be classified as a DIR or a DNIR depending on whether or not GMOs are expected to be released into the environment, which is generally related to the potential for shedding of GMOs by trial participants. This application is being assessed as a DIR because participants will be shedding GM bacteria in their faeces for a short time after treatment.

Section 2   Parent organism

38.   The parent organism is the bacterium *E.coli* Nissle strain. The bacteria belongs to the family *enterobacteriaceae* in the order enterobacteriales. As per the Australian and New Zealand Standards 2243:3:2010, this bacteria can be defined as a microorganism that is unlikely to cause human or animal disease and can therefore be classified as a Risk Group 1 organism (Standards Australia/New Zealand, 2010). The characteristics of the parent organism provide a baseline for comparing the potential for harm from dealings with GMOs. As such, the relevant biological properties of *E.coli* Nissle strain will be discussed here.

39.   The classification of enterobacteriales was changed in 2016 (Adeolu et al., 2016). The family *enterobacteriaceae* now includes 21 different genera. This includes the potential pathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and multiple species of *Salmonella* and *Shigella*. *E.coli, K. pneumoniae and E. cloacae* can be the cause of many different infections including urinary tract infections, post-surgical infections and sometimes results in life-threating septicaemia. Antibiotics are used to treat these infections but increasingly *enterobacteriaceae*, including *E.coli*, are showing resistance to antibiotics.

2.1   Basic biology of *E.coli*

40.   *Escherichia coli (E.coli)* was first described by Theodor Escherich in 1885 (Lim et al., 2010). *E.coli* are facultative anaerobic, gram negative, non-sporulating rod shaped bacteria. Facultative anaerobes can survive both in aerobic as well as in anaerobic conditions. *E.coli* can be either non-motile or

motile, with a flagellum, and grow best at 37°C. *E.coli* can either live inside a host or in the environment. Inside a host *E.coli* can either be commensal or pathogenic.

2.1.1   Commensal E.coli

41.   When *E.coli* lives inside a host, their relationship is usually commensal. In commensal relationships, one of the two organisms benefits from the interaction between them whereas the other is neither harmed nor helped. *E.coli* strains derive a steady supply of nutrients as well as protection and dissemination from the host. This interaction, however, provides some benefits for the host as *E.coli* microbiota prevents colonizing and growth of pathogens by producing bacteriocins and other mechanisms (Rastegarlari et al., 1990; Vollaard and Clasener, 1994; Hudault et al., 2001; Schamberger et al., 2004). *E.coli* have a wide host range colonising mammals, birds, reptiles and amphibians (Berg et al., 1983).

42.   Commensal *E.coli* strains that reside in the human digestive tract are located in the large intestine, especially in the caecum and the colon. The mucus layer covering the epithelial cells throughout the tract provides the main habitat for *E.coli*. They are shed into the intestinal lumen with degraded mucus components and are excreted in the faeces. It is estimated that there are 10^7-10^9 *E.coli* in each gram of human faeces (Tenaillon et al., 2010).

43.   *E.coli* has adapted to its ecological niche and competes with other bacteria for nutrients (Poulsen et al., 1994; Licht et al., 1999; Rang et al., 1999). The effect of this competition is demonstrated by a generation time of 30 minutes *in vitro* (no competition), 40 – 80 min in the intestines of streptomycin-treated mice (minimal competition) and 120 minutes after mice are 'conventionalized' by removing streptomycin and feeding of mouse caecal content (maximum competition)(Rang et al., 1999).

2.1.2   Pathogenic E.coli

44.   Most strains of *E.coli* are non-pathogenic and are commensal residents of the human gut (Gordon and Cowling, 2003). However, some *E.coli* can cause diseases. *E.coli* is estimated to cause hundreds of thousands of deaths a year (Russo and Johnson, 2003). Pathogenic *E.coli* have virulence factors that are not present in commensal *E.coli*, such as toxins, adhesins, protective coats and invasins.

45.   Certain types of pathogenic *E.coli* can cause infection within the gut and can be classified based on the symptoms they cause such as enteropathogenic *E.coli* (EPEC), enterohemorrhagic *E.coli* (EHEC), enterotoxigenic *E.coli* (ETEC) and and enteroaggregative *E.coli* (EAEC) (Vila et al., 2016). Most commonly, infections with these *E.coli* cause diarrhoea or gastroenteritis and are often acquired though eating contaminated food. Some EHEC have a virulence factor that leads to the production of a toxin called shiga, so they are also called shiga toxin-producing *E.coli* (STEC). STEC infections can cause bloody diarrhoea, abdominal cramps, vomiting and sometimes a serious condition called haemolytic uraemic syndrome which can be fatal (Lim et al., 2010). Large outbreaks of STEC sometimes occur in developed countries, but are relatively uncommon in Australia. 822 STEC infections were notified in Australia between 2000 and 2010 along with 169 cases of haemolytic uraemic syndrome (Vally et al., 2012). Antibiotics are not recommended for STEC infections and may be harmful (2018).

46.   Pathogenic *E.coli* can sometimes cause disease outside of the gut and are therefore called extraintestinal pathogenic *E.coli* (ExPEC). These *E.coli* have often previously colonised the human gut without causing issues, but become a problem when they are able to spread to other body sites. Some of the same virulence factors (such as P fimbriae and specific capsules) that make these *E.coli* damaging when they are outside of the gut, help them to successfully colonise the human gut (Vila et al., 2016).

47.   ExPEC are the most common cause of urinary tract infections, and are sometimes called urinary pathogenic *E.coli* (UPEC). Urinary tract infections (UTI) include infection of the bladder, urethra, ureters and kidneys. They are normally treated with antibiotics, but if left untreated (or if the

antibiotics used are ineffective) can lead to serious complications (healthdirect, 2020). *E.coli* that cause UTIs have multiple virulence factors; adhesins that help them stick to cells, toxins that help them spread into tissues and evade the immune system, the ability to form biofilms, and iron acquisition mechanisms that help them get nutrients (Vila et al., 2016).

48.   A large study into skin and soft tissue infections found *E.coli* was the third most common cause of infection (*Staphylococcus aureus* was the most common cause)(Moet et al., 2007). When these infections are not self-limiting, they are treated with antibiotics to ensure the infection does not spread or enter the bloodstream.

49.   Under certain conditions including after surgical operations or immunosuppression, previously commensal *E.coli* can act pathogenically. Bloodstream infections are the most common and life-threating complication after solid organ transplants, and about 37% of these are caused by *E.coli (AAP, 2018)*.

2.1.3   *Free-living E.coli*

50.   It is estimated that half of the *E.coli* population resides in water and sediments (Savageau, 1983). The oral – faecal route is the main mode of transmission and distribution of *E.coli* and its presence in water is often used as an indicator of faecal pollution (Savageau, 1983; Russell and Jarvis, 2001). However, more recent reports show that some *E.coli* are naturalised to soil, sand and sediments (Jang et al., 2017).

2.1.4   *E.coli Nissle strain*

51.   The parent organism *E.coli* Nissle strain was first isolated by Alfred Nissle in 1915 (despite being called *E.coli* Nissle 1917), and is the most frequently used probiotic *E.coli* strain (Wassenaar, 2016). This strain of *E.coli* has 5324 genes, is motile with flagella, It has 3 different types of fimbriae that help it to attach to surfaces and multiple iron incorporation systems. It is commercially available as a probiotic in capsules, and is mostly used to treat inflammatory bowel disease. The maximum daily dose is 10^{11} cfu, and treatment is usually well tolerated and does not cause significant changes to stool in healthy people, but can reduce constipation. Some ingested bacteria just pass through the gut whereas bacteria that live in the gut for a significant amount of time are considered to have colonised the gut. A systematic review of multiple studies using the *E.coli* Nissle strain suggests that it is not very efficient at colonising the adult human gut long term (Wassenaar, 2016), but this is debated with others claiming it is a good coloniser (Lodinová-Zádniková and Sonnenborn, 1997; Lasaro et al., 2009). The Nissle strain does not have any native conjugative plasmids or genes that carry resistance to antibiotics.

E.coli are bacteria from the enterobacteriaceae family that can be a normal part of the human digestive system, pathogenic or free living in the environment.

2.2   Genetics of *E.coli*

52.   The genome size varies widely across *E.coli* with the average genome containing around 5000 genes. Only 1700 genes are conserved among all strains (these are commonly referred to as 'strict core') and 3000 genes are conserved in at least 95% of the strains (commonly referred to as 'soft core') (Kaas et al., 2012). Hence each strain contains genes from the core genome and genes from an extended pool of genes of approximately 8000 genes. This provides a high level of plasticity in the genome and also reflects the adaptive nature of the organism (Tenaillon et al., 2010).

2.2.1   *Horizontal gene transfer*

53.   In addition to a large gene pool, *E.coli* is capable of exchanging genetic elements with other compatible bacteria present in the surrounding environment. Genetic elements are thought to move horizontally (to compatible bacteria) and vertically (to offspring) as they can help bacteria adapt to changing environments (Kaper et al., 1995) and contributes to the development of novel strains and pathotypes.

54.   *E.coli* carry genetic material in two different forms; chromosomes and plasmids. Chromosomes contain the essential genetic material of *E.coli* and are generally inherited vertically from the parent to offspring. Plasmids are usually smaller packets of DNA that exist separately from the bacterial chromosomes and can replicate independently of chromosomes. Enterobacterales, which include *E.coli*, often carry multiple plasmids simultaneously (Garcia et al., 2007; Dionisio et al., 2019).

55.   There are four main genetic mechanisms that enable the horizontal transfer of genetic elements in *E.coli*: conjugation, transformation, transposition and transduction.
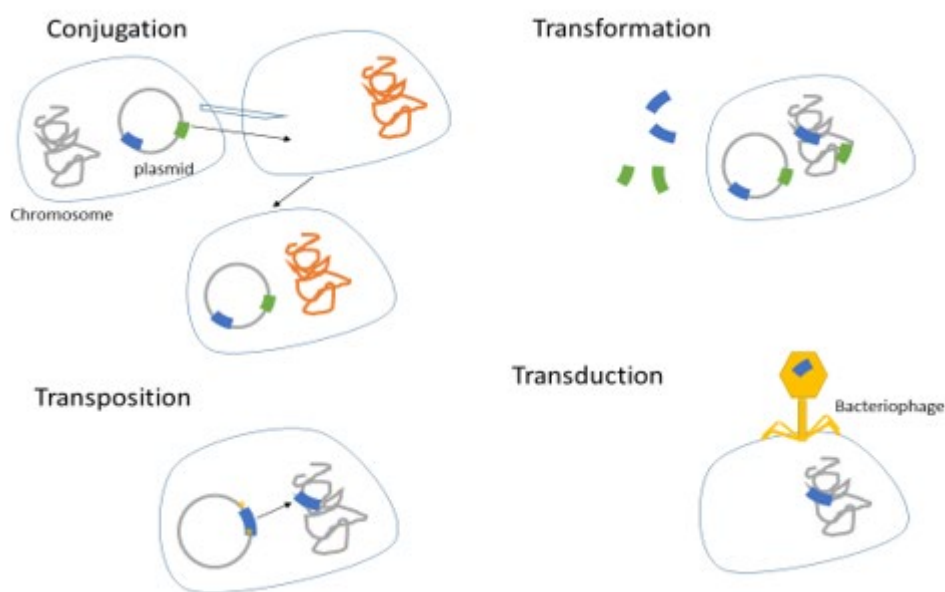
Figure 3. Common mechanisms of horizontal gene transfer in bacteria

56.   **Conjugation** describes the direct transfer of DNA from one bacteria to another and is arguably the most important mechanism of horizontal gene transfer in bacteria (Sørensen et al., 2005; Norman et al., 2009). Conjugation involves two bacteria coming into physical contact and forming a mating pair. The donor bacteria produces a filamentous pilus that allows a copy of the plasmid to travel across to into the recipient bacteria. Both the donor and acceptor now have a copy of the plasmid.

57.   **Transformation** in *E.coli* involves the induction of competence, DNA binding followed by fragmentation of the DNA, uptake and stable maintenance of the DNA by either integration in the genome (recombination) or recircularization of plasmid DNA (Sørensen et al., 2005; Harrison and Brockhurst, 2012).

58.   **Transduction** is the movement of genetic material with the help of bacteriophages. Erroneously packed host DNA can be transferred to another bacteria upon its infection with the phage. In theory, any region of the bacterial genome can be transferred in that way, including plasmids, but the DNA will not be retained by the host unless the phage integrates into the bacterial genome (prophage). The regions co-integrated with prophage DNA are commonly the flanking regions of the prophage insert site (Berg et al., 1983).

59.   **Transposition** describes the translocation of a discrete segment of DNA (the transposable element or transposon) from a donor site to non-homologous target sites. Transposable elements encode the machinery required to execute such rearrangements in addition to other determinants such as antibiotic resistance genes and genes for virulence factors. In general, transposition is an infrequent event probably because of its capacity for deleterious effects in the host. Usually, a transposon is translocated onto a plasmid upon conjugation. This may be followed by the integration of the transposon into the chromosome. For many transposons, however, plasmids rather than the bacterial chromosome appear to be the preferred target (Craig, 2014).

2.2.2   The genetics of bacterial plasmids

60.   Plasmids are packets of DNA that exist separately from the bacterial chromosomes and can replicate independently of chromosomes. Plasmids cannot replicate outside of a bacterial host but can be transferred between different species of host, and therefore are somewhat separate from their initial host organism. Unlike viruses, plasmids generally do not harm their host and seek to stay associated with a bacteria throughout its life. Most plasmids replicate only the number of times needed to ensure that daughter cells of their host will continue to carry the plasmid into subsequent generations in the same number as carried by the parent. Bacterial plasmids are a diverse group and it is beyond the scope to discuss all aspects; this section focuses only on the genetic features of plasmids most relevant to this application. The following paragraphs briefly explain (i) factors that make bacteria maintain plasmids; (ii) the frequency of carrying multiple plasmids; (iii) plasmid incompatibility groups; and (iv) features that enable a plasmid to spread to new bacterial populations.

2.2.2.1 Plasmid persistence

61.   It is metabolically expensive for bacterial populations to maintain plasmids because when plasmids replicate they use the host's resources to do so. Large plasmids are more expensive to maintain than small plasmids. However, plasmids that provide a selective advantage to the host bacteria, such as the resistance to an antibiotic, are maintained despite the fitness cost. Plasmids may also be maintained because losing them is harmful to the host, even in the absence of selection. This is sometimes called "plasmid addiction" because the bacteria can't survive without the plasmid.

62.   The most common systems involve a stable toxin and an unstable antitoxin. If a daughter cell doesn't inherit the plasmid encoding the antitoxin, it is killed by the leftover toxin from the parent cell.

63.   If there is no reason to maintain a given plasmid and it is expensive to do so, then that plasmid doesn't persist in the bacteria population. The plasmid is lost because it is not passed to daughter cells when the original bacteria divides. Given the speed of cell division in bacteria this process can occur quickly.

2.2.2.2 Plasmid incompatibility groups

64.   Carrying multiple plasmids at the same time is common. In a study of over 200 Australian isolates, 63% of *enterobacteriaceae* were found to contain one or more plasmids. On average *E.coli* carried 2.5 different plasmids per cell (Sherley et al., 2003). However, some combinations of plasmids are incompatible and cannot stably be inherited in the same bacterial population. This happens when the two plasmids use replication and/or segregation strategies that are too similar (Novick, 1987). In simple terms, to remain present in a bacterial population, a plasmid needs to replicate itself at least twice for every cell cycle of the host bacterium, and distributes one copy of the plasmid on either side of the bacterial cell division boundary. In *enterobacteriaceae,* the main plasmid incompatibility groups are called IncF, IncA/C, IncL/M, IncI, IncH12/s and IncN (Shintani et al., 2015). Two plasmids that

belong to the same Inc group are incompatible with each other and cannot both persist in the same bacterium.

2.2.2.3 Host range for conjugative plasmids

65.   Conjugative plasmids are self-transmitting because they encode everything they need to be transferred horizontally between bacteria by conjugation. Another type of plasmid, mobilizable plasmids, can only be transferred by conjugation with help. The presence of a conjugative plasmid can help transfer a mobilizable plasmid. Some plasmids have a relatively narrow host range and can only inhabit specific families of bacteria, while others have the ability to spread across broad taxonomic groups. The host range is often linked to the Inc group.

Plasmids contain extra bacterial genes. Conjugative plasmids can spread to, and establish in, any nearby bacteria that are suitable hosts (such as other enterobacteriaceae). Bacteria can often carry multiple plasmids, but some plasmids are incompatible with each other. In these cases only one of the two plasmids will be maintained in subsequent generations.

2.3   Human gut bacteria (the second parental organism)

66.   The human gut naturally contains a wide range of bacteria as well as archaea, viruses, phages, yeast and fungi. The human colon has been estimated to contain about 1.5 kg of bacteria (Sender et al., 2016). The composition of the human gut bacteria varies between individuals and is affected by diet, lifestyle, medical conditions and treatments, as well as geographical location. In health, the gut microbiota clearly plays roles in training the immune system, protecting against colonisation by pathogens, biosynthesizing vitamins, energy generation, endocrine function and metabolising drugs and bile salts (Lynch and Pedersen, 2016). There are many other proposed interactions between the microbiome and the host.

67.   In healthy adults, 90% of the gut bacteria are Firmicutes and Bacteroidetes. There are smaller amounts of Actinobacteria, Proteobacteria (including *E.coli*), and Verrucomicrobia (Rinninella et al., 2019).

68.   Microbiome diversity generally increases with age. The infant gut microbiome is affected by the way they are delivered, antibiotic use and feeding patterns. Babies born vaginally have a gut microbiome similar to that around their mother's birth canal while those delivered by c-section carry bacteria similar to their mother's skin, but these differences reduce over time (Yang et al., 2016) . Additionally there are commensal bacteria in healthy human breast milk that are passed from mother to child to help the infant build a healthy microbiome (Murphy et al., 2017) . The gut microbiomes of infants may be more easily persistently colonised than adult microbiomes. A study that supplemented breast-fed infants with *B.infantis* EV001 for 28 days found that this bacterium was still the dominant species 60 days later (Frese et al., 2017). Studies of probiotics in adults tend not to show such a dramatic and persistent effect (Zmora et al., 2018). Children are thought to develop a microbiome more similar to adults by around age three (Yang et al., 2016).

69.   *Enterobacteriaceae* in the human gut include several species of *Citrobacter*, *E.coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter gergoviae*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Morganelle morganii*, *Pantoea agglomerans*, *Proteus mirabilis*, *Serratia marcescens*, *Serratia plymuthica*. Just like *E.coli*, many of these *enterobacteriaceae* are usually commensal but can be pathogenic under certain circumstances. Among the gut bacterial species listed above the most prominent potential human pathogens are, *E.coli* strains (other than the Nissle strain), *Klebsiella sp.* and *Enterobacter sp.* All three of these bacteria are common sources of hospital acquired infections

and can sometimes cause life threatening sepsis. They can also cause other diseases such as food poisoning, wound infections, urinary tract infections, and pneumonia. *K. pneumonia* is one of the most common causes of hospital acquired pneumonia, and is often resistant to treatment with antibiotics (Chung, 2016).

The human gut naturally contains a diverse and dynamic population of bacteria. Plasmids can be spread between bacterial populations present in the human gut and sometimes to other body sites.

Section 3   Antibiotic resistance

70.   Antibiotics are medications that kill or slow down the growth of bacteria. Antibiotic resistance occurs when some bacteria are able to survive treatment by medications that are used to eradicate them. Over time an antibiotic tends to become less effective at treating a particular type of infection because resistance becomes more common. The main drivers of antibiotic resistance are the use of antibiotics and the horizontal gene transfer abilities of bacteria. Genes that mediate antibiotic resistance are often found on plasmids allowing them to spread rapidly through bacterial populations. The growing levels of antibiotic resistance are a serious concern for human and animal health. Infection with antibiotic resistant bacteria can lead to longer stays in hospital and a higher mortality rate. Globally AMR is estimated to account for 700,000 deaths a year.

71.   To address the growing threat of antibiotic resistance, the Australian Government has released *Australia's National Antimicrobial Resistance Strategy - 2020 and Beyond*, which was endorsed by the Council of Australian Governments (2020).

3.1.1 Types of antibiotics used in Australia

72.   Antibiotics are grouped into families based on their chemical structure and function. It is beyond the scope of this document to describe all of the classes of antibiotics, only those most commonly used or, of particular relevance, are mentioned. Figure 4 shows the 10 most commonly prescribed antibiotics in Australia.
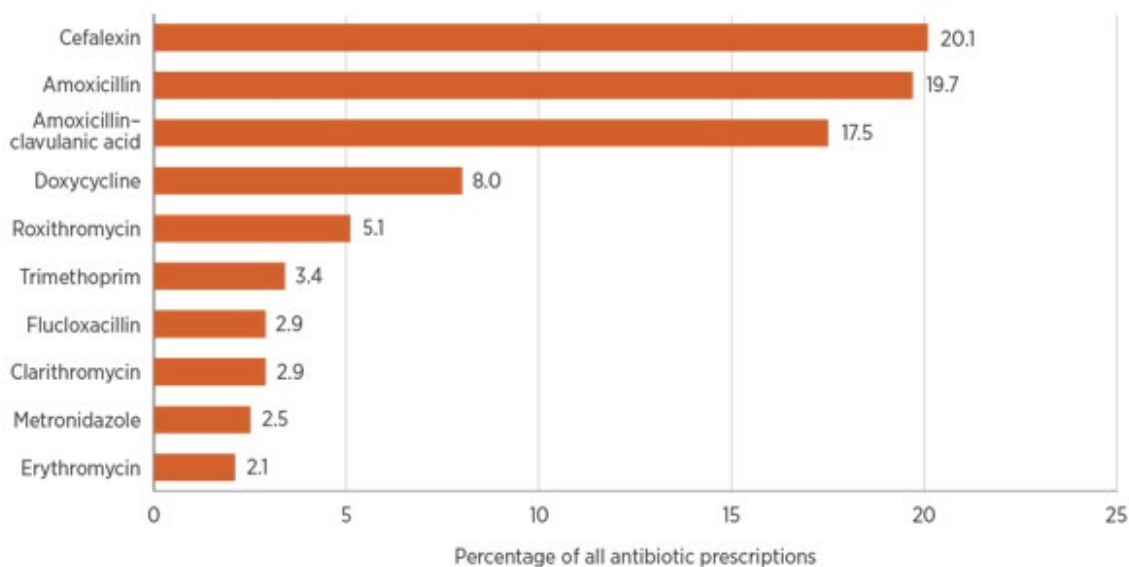

Figure 4. The 10 most commonly dispensed antibiotics under the PBS/RPBS, by percentage of all antibiotic prescriptions, 2017. Image from AURA 2019 (Australian Commission on Safety and Quality in Health Care (ACSQHC), 2019).

73. Bacteria can be divided into two large groups called Gram-positive and Gram-negative based on their cell wall. Gram positive bacteria have a thick cell wall of peptidoglycan. Gram negative bacteria have a thinner layer of peptidoglycan surrounded by a lipopolysaccharide membrane. Many antibiotic classes work either only on Gram-positive or only on Gram-negative bacteria. *Enterobacteriaceae,* including *E.coli,* are Gram-negative bacteria so this discussion focuses primarily on antibiotics used to treat them.

3.1.1.1 β-lactam antibiotics

74. β-lactam antibiotics include penicillins, cephalosporins, monobactams, and carbapenems (Pandey and Cascella, 2020). This class of antibiotics actively kill bacteria and so are considered bactericidal. Most work on a broad range of Gram-negative bacteria by stopping the synthesis of the cell wall. These are the most widely used antibiotics for human health. Of all the antibiotics prescribed in Australia in 2017, 20% were for cephalexin (a cephalosporin) and another 20% were for amoxicillin and 3% for flucloxacillin (both penicillins)(Australian Commission on Safety and Quality in Health Care (ACSQHC), 2019).

3.1.1.2 Tetracyclines

75. Tetracyclines were discovered in the late 1940s and have been used extensively against both Gram-positive and Gram-negative bacteria, prophylactically against protozoa, and in some countries as a growth promoter in animal feed (Chopra and Roberts, 2001). This class includes tetracycline, doxycycline, minocycline, and two newer drugs tigecycline and ervacycline (Shutter and Akhondi, 2020). Tetracyclines work by stopping the bacterial cell's ability to make new proteins, by binding to ribosomes. They are bacteriostatic because they stop the growth of bacteria instead of killing them outright. In 2017, 8% of the antibiotic prescriptions in Australia were for doxycycline. Doxycycline is usually used to treat pneumonia, bronchitis, prostatitis or chlamydia (healthdirect, 2021). It can also be used to control acne or as an anti-malarial for travellers.

3.1.1.3 Aminoglycoside antibiotics

76. The aminoglycoside antibiotics include gentamicin, tobramycin, amikacin, neomycin, plazomicin, paromomycin, and streptomycin. Aminoglycosides are bactericidal against a broad range of aerobic bacteria by causing incorrect protein synthesis. They tend to have poor oral absorption compared to other antibiotics but still have many uses (Reyhanoglu and Reddivari, 2020). Serious infections by *E.coli*, *K. pneumoniae* and *E. cloacae* are often empirically treated with intra venous aminoglycosides, especially gentamicin (Australian Commission on Safety and Quality in Health Care (ACSQHC), 2019). Amikacin is used to treat drug resistant tuberculosis while neomycin is used for skin infections.

3.1.1.4 Amphenicol antibiotics

77. Of the amphenicol antibiotics, chloramphenicol is the only one used in humans in Australia. Generally it is either used in a topical form for eye infections or in cases of life treating bacterial infections (such as meningitis) when other options are not available (ASTAG, 2018). Systemic administration of chloramphenicol can have fatal side effects and is therefore rarely used (Oong and Tadi, 2020).

3.1.1.5 Fosfomycin

78. Fosfomycin was discovered in 1969 and works by inhibiting an enzyme bacteria need to make cell walls. It is the only antibiotic in its class. Fosfomycin wasn't used widely in the USA or Europe until an oral formulation was developed in 1995 for use on antibiotic-resistant urinary tract infections (Gardiner et al., 2019). In Australia, the antibiotic used for UTIs resistant to trimethoprim is usually nitrofurantoin, but fosfomycin can also be used (ASTAG, 2018). Oral fosfomycin was registered in 2017

for use in acute uncomplicated lower urinary tract infections in females over 12 years old (ARTG). Fosfomycin in other forms may be available through the Special Access Scheme[1].

3.1.1.6 Other commonly used antibiotics

79.   Other commonly used classes of antibiotics are macrolides, dihydrofolate reductase inhibitors and nitroimidazoles. Macrolides work by inhibiting bacterial protein synthesis, and include roxithromyin, clarithromycin and erythromycin. They are usually used for infections with Gram-positive bacteria, and collectively accounted for about 10% of all antibiotic prescribing in 2017. Trimethoprim is part of a class called dihydrofolate reductase inhibitors, and is often the drug of choice to treat urinary tract infections. It works by inhibiting the synthesis of folic acid, which bacteria need to grow. Metronidazole is part of a class called a nitroimidazoles, which infuse into bacteria and damages bacterial DNA. It can be used on anaerobic bacteria but is also used for infections by protozoa (Weir and Le, 2020).

3.1.2 Prevalence and mechanisms of antibiotic resistance

80.   Resistance to a given antibiotic tends to be detected within 5-20 years of it becoming widely used. Some gene products provide resistance to one antibiotic while others mediate resistance to multiple different antibiotics within a class, and sometimes across classes. Generally there are multiple genes that can encode resistance to a given antibiotic. These antibiotic resistance genes can be on the bacterial chromosomes or on mobile elements such as plasmids or transposons. Mobile versions of an antibiotic resistance gene are more concerning because they have the ability to spread rapidly.

81.   For the purpose of this document AMR is generally referring to resistance to the antibiotics that doctors would normally use to treat a specific infection. Many factors go into treatment decisions, but frequently for infections with *enterobacteriaceae,* the chosen antibiotics are members of the β-lactam or aminoglycoside class of antibiotics.

3.1.2.1 Resistance to β-lactam antibiotics

82.   One of the most important events of antibiotic resistance is the emergence of resistance to the β-lactam antibiotics. β-lactamases are enzymes that counteract β-lactam antibiotics by cleaving the β-lactam ring rendering the drug ineffective. There are three severities of β-lactamase, "original" β-lactamases (BL), extended-spectrum β-lactamases (ESBL), and metallo- β-lactamases (MBL). The original β-lactamases provide resistance to penicillins and became wide-spread by the late 1970s (Bush, 2018). To solve this issue in the 1980s, pharmaceutical companies developed β-lactamase inhibitors such as clavulanic acid, and new drugs called 1st-generation cephalosporins and carbapenems. But then the next level of β-lactamases, called extended spectrum (because they can also inactivate cephalosporins and sometimes overcome the inhibitors), began to appear more frequently (see figure 5 for the frequency of ESBLs in Australia). As resistance to cephalosporins became more common, carbapenems started to be used more frequently. The most dangerous β-lactamases are the MBLs that provide resistance to penicillins, β-lactamase inhibitors, cephalosporins and carbapenems (Bush, 2018). MBLs were first detected in the early 1990s (Iovleva and Doi, 2017). Serious infections with bacteria carrying MBLs are very hard to treat and can be fatal.

83.   For example, Ampicillin is a penicillin that has been widely used in Australia, but in 2013 more than 50% of *E.coli* were resistant to it. More than 20% of *E.coli* infections are now also resistant to the combination of amoxicillin–clavulanic acid (a β-lactamase inhibitor). Amoxicillin–clavulanic acid is often still prescribed in cases where the infection is unlikely to become life threating and account for more than 17% of all antibiotics prescribed in Australia (see figure 4). The level of resistance to

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[1] The Special Access Scheme is regulated by the TGA and allows certain health practitioners to access therapeutic good that are not in the ARTG for a single patient.

cephalosporins in *E.coli* depends on the specific drug within this group, and the specific geographical location, but are on average somewhere around 5-10%.
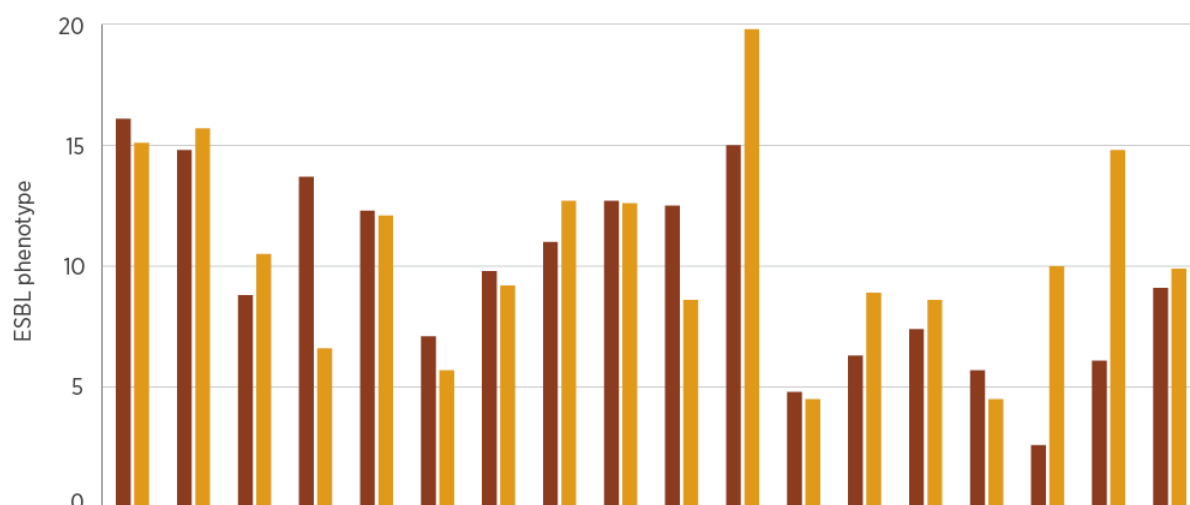
Year	NSW	Vic	Qld	SA	WA	Tas	NT	ACT	Aus	NSW	Vic	Qld	SA	WA	Tas	NT	ACT	Aus
					E.coli									*K. pneumoniae*				
2016, %	16.1	14.8	8.8	13.7	12.3	7.1	9.8	11.0	12.7	12.5	15.0	4.8	6.3	7.4	5.7	2.6	6.1	9.1
2017, %	15.1	15.7	10.5	6.6	12.1	5.7	9.2	12.7	12.6	8.6	19.8	4.5	8.9	8.6	4.5	10.0	14.8	9.9

Figure 5. Percentage of Escherichia coli and Klebsiella pneumoniae with extended-spectrum β-lactamase (ESBL) phenotype, by state and territory and nationally, 2016–17 .Note: ESBL phenotype refers to strains that are resistant to ceftriaxone and/or ceftazidime (MIC > 1 mg/L). Image modified from (Australian Commission on Safety and Quality in Health Care (ACSQHC), 2019)

84.   Carbapenem-resistant *enterobacteriaceae* (CRE) are a cause of global concern. The level of resistance in Australia is currently low, about 0.3% in 2019 (Coombs G et al., 2019), but it is of critical importance that it does not increase. Enzymes that can hydrolyse carbapenem antibiotics are sometimes called carbapenemases, this includes but is not limited to the MBLs discussed earlier. Some studies have found the in-hospital mortality of CRE infections to be over 40% (Patel et al., 2008; Wang et al., 2016). A study of a local outbreak that occurred in a Melbourne ICU from January to July 2004 followed 16 patients with hospital acquired infections with MBL (specifically bla$_{IMP-4}$) bacteria, of which 6 died during the study (Peleg et al., 2005). In Australia, bloodstream infection with Carbapenem-resistant *enterobacteriaceae* (CRE) is estimated to cost hospitals an additional $5.8 million a year (Wozniak et al., 2019). *The Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia* rate carbapenems as being of high importance for the mitigation of antibacterial resistance (ASTAG, 2018).

3.1.2.2 Resistance to aminoglycoside antibiotics

85.   As mentioned above, serious infections by *enterobacteriaceae* are often initially treated with intravenous aminoglycosides (Australian Commission on Safety and Quality in Health Care (ACSQHC), 2019). Resistance to aminoglycoside antibiotics is lower than many other classes of antibiotics. Resistance is usually mediated by enzymes that modify the drug to neutralise it. The three main classes of these enzymes are aminoglycoside acetyltransferases, aminoglycoside phosphotransferases, and aminoglycoside nucleotidyltransferases. *The Importance Ratings and Summary of Antibacterial*

Uses in Human and Animal Health in Australia rate neomycin as low, gentamicin and tobramycin as medium and amikacin as high importance for the mitigation of antibacterial resistance.

3.1.2.3 Resistance to tetracyclines

86.   Tetracycline resistance started to be abundant by the mid-1950s and now limits the use of this class of antibiotics (Chopra and Roberts, 2001). There are at least 36 different genes that can give resistance to tetracycline, the two most common are tetA and tetB (Grossman, 2016). Tetracycline is not routinely used to treat *enterobacteriaceae* in Australia, so the proportion that are resistant to tetracyclines is not measured in large scale surveillance efforts. International data shows that a substantial proportion of *enterobacteriaceae* are resistant to tetracyclines. In a European surveillance program across 21 countries, 43.5% of *E.coli* and 27.3% of *Klebsiella spp.* and 12.3% of *Enterobacter spp*. were resistant to tetracycline (Jones et al., 2014). Tetracycline resistance is frequently found in combination with extended-spectrum β-lactamases. The same study found that, 66.9% of ESBL isolates were also tetracycline resistant (Jones et al., 2014). Another large study of *E.coli* infections found that in North America 31.3% were resistant to tetracycline, while in Latin America it is 54.4%. *The Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia* rate most tetracyclines (including doxycycline) as being of low importance for the mitigation of antibacterial resistance. However, tigecycline is rated as high importance (ASTAG, 2018).

3.1.2.4 Resistance to fosfomycin

87.   In Australia, UTIs account for around 2.5 million GP visits and 75,000 hospital stay a year (Outbreak Project, 2020). Trimethoprim is the first choice antibiotic for UTIs but resistance to it is now about 30% (Bell, 2019), so alternatives such as fosfomycin and nitrofurantoin are sometimes used. There appear to be no large scale systematic studies of fosfomycin resistance (Gardiner et al., 2019). However, one Australian study in 2019, found only 2 out of 1033 *E.coli* urinary tract infections tested were resistant to fosfomycin (less than 0.2%) (Mowlaboccus et al., 2020). Both of the two isolates were also resistant to some β-lactam antibiotics and one was also resistant to aminoglycosides. Resistance to fosfomycin was carried on a plasmid and encoded by the gene *fosA4* and associated with IS26 transposon elements. International data suggests that generally less than 2% of *E.coli* are resistant to fosfomycin, even among AMR bacteria (Cattoir and Guerin, 2018). Resistance to fosfomycin occurs through three different mechanisms; mutations that reduce drug uptake, production of enzymes that inactivate the drug, and mutation that change the drugs target. Fosfomycin resistance is most frequently caused by mutations that impair drug uptake which are encoded on the chromosomes (Cattoir and Guerin, 2018), but some fosfomycin resistance genes are part of transposons or plasmids including *fosA3* and *fosA4*. *The Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia* rate fosfomycin as an antibiotic of high importance for the mitigation of antibacterial resistance due to it's potential to be used in cases where few options remain (ASTAG, 2018).

3.1.2.5 Polymyxins

88.   Polymyxins, including Polymyxin B and Polymyxin E (also called colistin) are reserve antibiotics for serious Gram-negative infections that are resistant to other antibiotics. They work by damaging the bacterial lipopolysaccharide membrane colistin was discovered in 1949 but its use was stopped in the 1970s due to renal and neural side-effects. However, colistin is now sometimes used, often in combination with other antibiotics, to treat life-threatening infections with Carbapenem-resistant *enterobacteriaceae* (CRE) (Stefaniuk and Tyski, 2019). As colistin use increases resistance to it becomes more common. Colistin resistance is mediated by several different genes, some of which (mcr-1 to mcr-9) can be found on plasmids. A study based at Westmead hospital found that among *enterobacteriaceae* that were resistant to 4th generation cephalosporins, 2.1% were also resistant to colistin (Ellem et al., 2017).

3.1.3   Antimicrobial resistance in the human gut

89.   Most people have probably carried various AMR plasmids in their gut bacteria without suffering any ill effects. However when a serious infection develops, AMR plasmids seem to be able to spread rapidly from the commensal gut bacteria to the pathogenic bacteria and lead to therapeutic failure (de Smet et al., 2009; van Hal et al., 2009). In a case study of a patient admitted to a Sydney ICU, his *E.coli* blood infection went from being sensitive to β-lactams and gentamycin to only 20 hours later being resistant to both, presumably though acquisition of an AMR plasmid that was native to the *Serratia spp.* in his gut (van Hal et al., 2009). Removing a plasmid from a bacterial population is described as plasmid curing (Buckner et al., 2018). Therefore one approach to reducing serious AMR infections might be to cure the gut of AMR plasmids thus removing a potential reservoir of antibiotic resistant genes.

3.1.4   Antibiotics and antibiotic resistance in animals

90.   Antibiotics are also used in animals. Some antibiotic are used in both humans and animals (including doxycycline), while some are reserved for human use (such as carbapenems), and others are only used in animals. Additionally, there are specific rules about which antibiotics can be used in food animals compared to companion animals. Animals can harbour antibiotic resistant bacteria. Limited studies have detected AMR *E.coli* in Australian pigs, poultry, cattle, cats and dogs (APVMA, 2017). A study of companion animals across three European countries found that *E.coli* were resistant to ampicillin and tetracycline in around 20% and 15% of samples respectively (Joosten et al., 2020).

Some bacteria have gained genes that allow them to resist treatment with antibiotics (AMR). It's harder to treat infections caused by these bacteria. When these genes are found on conjugative AMR plasmids, they can easily be spread through bacterial populations.

Section 4   Nature and effect of the genetic modification

4.1   The genetic modification

91.   Genetic modifications were made to conjugative plasmids which are designed to self-transmit from the initial host *E.coli* (GMO1) to other gut bacteria (GMO2). No genetic modifications were made to the *E.coli* chromosomal genome.

92.   Antimicrobial resistance (AMR) is often encoded on a stable self-transmitting plasmid that is passed between related bacterial species through a process called conjugation. In this clinical trial, the objective is to replace resident AMR plasmids ("target plasmid") with genetically engineered "curing plasmids" for the purpose of reducing antibiotic resistance. The curing plasmids were modified from naturally occurring AMR plasmids by (i) deletion of genes responsible for the resistance to multiple classes of antibiotics (ii) deletion of genes that enable plasmids to persist in bacteria (iii) introduction of genes for resistance to specific antibiotics (fosfomycin or tetracycline), which will be used to select for the GMO. The approach used in this proposed clinical trial relies on plasmid incompatibility. An individual participant, carrying the target AMR plasmid in their gut, would be given the "curing" plasmid of the same incompatibility group (Inc). The following paragraphs describe the modifications in more detail.
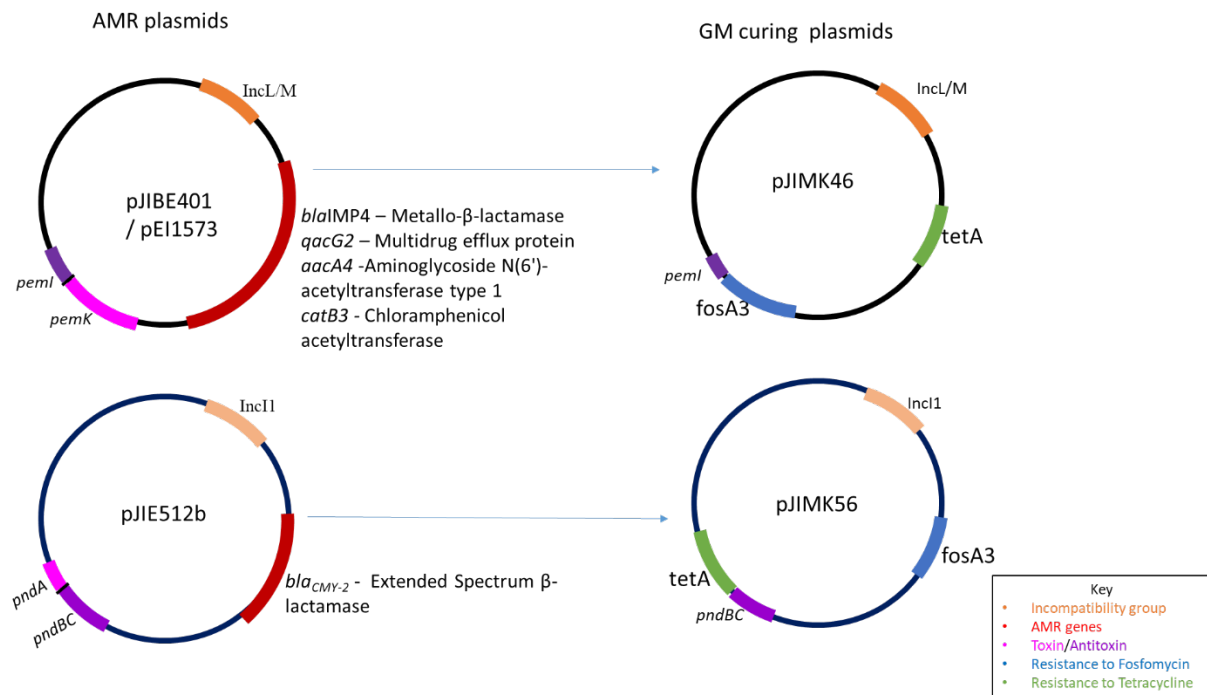
Figure 6. Construction of the GM plasmids. Orange denotes the incompatibility group of the plasmid, Red denotes genes that mediate antibiotic resistance to antibiotics in common clinical usage, and purple/pink denotes systems for plasmid persistence. Locations around the circle show approximately which regions were replaced. Full details of plasmid construction are described in (Kamruzzaman et al., 2017)

4.1.1   Target AMR plasmids

93.   To be eligible for this clinical trial participants have to be carrying the plasmid pJIBE401 or pJIE512b, both of these are AMR plasmids often detected in clinical settings in Sydney. If the participant has pJIBE401, they would be treated with pJIMK46. If the participant has pJIE512b, they would be treated with pJIMK56. For ease of understanding, pJIBE401 and pJIE512b are collectively referred to as the target AMR plasmids, while pJIMK46 and pJIMK56 are the curing plasmids.

94.   pJIBE401 was identified in Sydney from clinical isolate K. pneumoniae Kp1239, but is essentially identical to pEI1573 isolated from *E.coli* (Espedido et al., 2005; Kamruzzaman et al., 2017). pJIBE401 is a large IncL/M conjugative plasmid. It contains a *blaIMP-4-qacG2-aacA4-catB3* cassette.

   i.   *bla*$_{IMP4}$ encodes a metallo-β-lactamase (MBL) which provides resistance to penicillins, cephalosporins and carbapenems.

   ii.   The *qacG2* gene encodes a multidrug efflux protein. Efflux proteins pump drugs out of the bacterial cell. This may make it harder for antibiotics to reach an effective concentration within the bacteria The pump encoded by *qacG2*

   iii.   The *aacA4* (Aminoglycoside N(6')-acetyltransferase type 1) gene gives resistance to genetamicin and tobramycin

   iv.   The *catB3 (*Chloramphenicol acetyltransferase) gene gives resistance to chloramphenicol. pJIBE401 is also resistant to sulfa drugs and quinolone antibiotics.

95.   The presence of pJIBE401 plasmid in a bacterial infection makes most of the antibiotics likely to be prescribed to patients with serious Gram-negative infections less effective. Resistance to carbapanems is of particular concern because ASTAG describes these as reserve agents for serious infections.

96.   pJIBE401 also carries the *pemIK* type-I toxin-antitoxin system. The toxin is *pemK* (for killer) and the antitoxin is *pemI* (for inhibitor). See section 2.2.2.1 for a general explanation of toxin-antitoxin systems. The *pemIK* system ensures pJIBE401 persists long after any of the antibiotic treatment it

confers resistance to have been stopped, i.e. it persists in the absence of selection. In mice, pJIBE401 persists for at least 3 weeks without selection (Kamruzzaman et al., 2017).   IncL/M plasmids are considered to have a broad host range. pJIBE401 has been identified in the following species by a Sydney based laboratory: *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *E.coli*, *Serratia marcescens*, *Enterobacter gergoviae*, *Morganelle morganii*, *Pantoea agglomerans*, *Proteus mirabilis*, *Serratia plymuthica* and several species of *Citrobacter* (data supplied by applicant).

97.   pJIE512b, the second targeted plasmid, is a conjugative IncI1 plasmid from clinical isolate *E.coli* JIE512b (HG970648) first sequenced at Westmead Hospital (Tagg et al., 2014). It contains bla_{CMY-2}, an extended spectrum β-lactamase (ESBL) which provides resistance to penicillins and cephalosporins. Extended spectrum β-lactamases do not provide as broad a resistance as metallo-β-lactamases because they are often still sensitive to carbapenems. pJIE512b carries the *pndABC* type-II toxin-antitoxin system which ensures its persistence. IncI plasmids are considered to have a narrow host range, meaning there are fewer bacterial species it can inhabit.

4.1.2   Details of the construction of the GM curing plasmids

98.   The target AMR plasmid pJIBE401 was modified to produce the curing plasmid pJIMK46. Genes responsible for the resistance to multiple classes of antibiotics (the *blaIMP-4-qacG2-aacA4-catB3* cassette) were replaced with *tetA* which mediates resistance to the tetracycline family of antibiotics. Secondly, *pemK* which encodes the toxin half of a type-II toxin-antitoxin was replaced with *fosA3* which mediates resistance to the antibiotic fosfomycin (Kamruzzaman et al., 2017). It is anticipated that pJIMK46 would have the same host range as pJIBE401.

99.   The target AMR plasmid pJIE512b was modified to give the curing plasmid pJIMK56. The gene responsible for resistance to extended spectrum β-lactam antibiotics (bla_{CMY-2}) was replaced by *fosA3* which mediates resistance to the antibiotic fosfomycin. Subsequently, *tetA* that mediates resistance to the tetracycline family of antibiotics was used to disrupt *pndA* the toxin component of a type-I toxin-antitoxin system (Kamruzzaman et al., 2017). pJIMK56 should have the same host range as pJIE512b.

4.1.3   Reducing the ability of the GM plasmids to persist in bacterial populations

100.   In both curing plasmids, the toxic component of their toxin-antitoxin system has been removed. This eliminates the negative selection mechanism that maintains the plasmids in bacterial populations, allowing the emergence of plasmid free bacteria. The antitoxin component is kept because it is thought to promote the loss of the target plasmid (Hale et al., 2010; Kamruzzaman et al., 2017). As a result, the curing plasmid is unstable and does not persist in the absence of antibiotic selection in the daughter cells. The target AMR plasmids can persist in bacterial populations for over 100 days in the absence of antibiotic selection. In mice experiments, the GM curing plasmids were undetectable 10 days after the removal of antibiotic selection (Kamruzzaman et al., 2017). Bacteria were still present but they no longer contained the GM curing plasmid.

4.1.4   Specifics of the resistance to tetracycline and fosfomycin added to the curing plasmids.

101.   The gene providing resistance to tetracycline in the GM curing plasmids is *tetA,* which encodes an efflux pump that pumps tetracycline out of bacterial cells stopping the drug from reaching therapeutic concentrations. *TetA* will provide resistance to tetracycline, doxycycline and minocycline, and may somewhat reduce sensitivity to the newer family member's eravacycline and tigecycline (Grossman, 2016).

102.   The fosfomycin resistance gene incorporated into the GM plasmids in this application (*fosA3*) encodes an enzyme that inactivates FOS and is carried on a plasmid. The gene *fosA3* is often found in a composite IS26-type transposon on a conjugative plasmid that also carries bla_{CTX-M} genes (Yang et al., 2019). The version of *fosA3* inserted in the GM plasmids does not include the flanking regions that allow for transposition and therefore is less genetically mobile than naturally occurring *fosA3*. Fosfomycin is the only antibiotic in its class so *fosA3* doesn't convey resistance to any other drugs.

4.2   Effect of the genetic modification

103.   The addition of the GM plasmid to the *E.coli* Nissle strain (GMO1) gives this bacteria the ability to conjugate and provides transient resistance to TET and FOS.

104.   When the GM plasmid is passed by conjugation to a gut bacteria that carries the target AMR plasmid, this would result in a cell with two incompatible plasmids. When this cells divides only one daughter will carry the target AMR plasmid while the other daughter will have the GM curing plasmid. Selection with TET will kill the daughter cell that got the AMR plasmid. As long as TET is present, the daughter cells would inherit the curing plasmid. Once tetracycline selection is removed, the GM curing plasmid would not confer a competitive advantage for the bacterial cell but constitute a metabolic cost for the bacteria. The advantage of this technology is that the microbiome diversity should be maintained (figure 7). Bacteria that carried the target AMR plasmid would be cured of this plasmid and therefore regain sensitivity to multiple antibiotics.
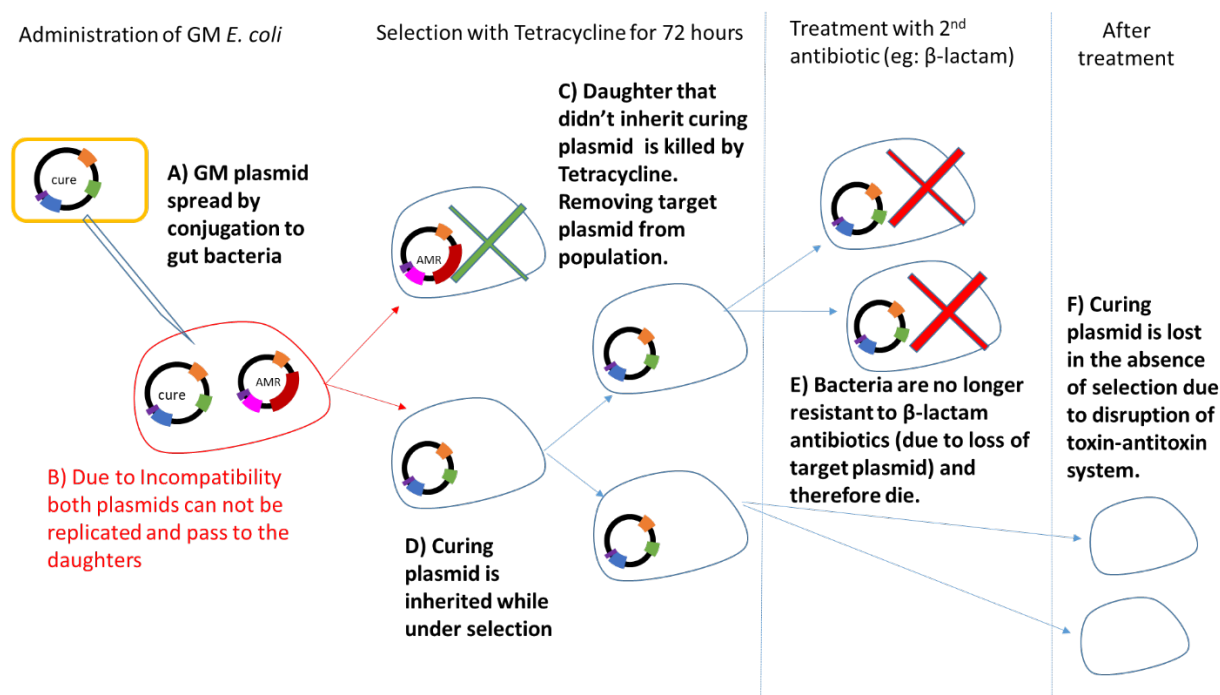

Figure 7. Intended consequences on bacteria carrying the target plasmid. In some cases FOS may be used instead of TET. Most participants will receive treatment with a second antibiotic but some may not in which case the plasmid would be lost over time after the removal of the selective antibiotic.

105.   Where clinically recommended by treating physicians, a 2nd round of antibiotic treatment (with an antibiotic other than TET or FOS) will accelerate the clearance process by killing all bacteria carrying the GM curing plasmid.

106.   The effects of the addition of the GM plasmid to human gut bacteria that did not have the target AMR plasmid (GMO2) depends on the genetics of the receiving bacteria. Some human gut bacteria will not receive the GM plasmid. Others may gain transient resistance to TET and FOS and the ability to conjugate.

4.2.1   Pre-clinical studies with the GMOs

107.   This application is for the first use of these GMOs in humans. As discussed in section 2.1.4, the *E.coli* Nissle strain is a safe and widely used probiotic, which has often been used in higher concentrations (up to 10^{11} cfu than those considered here (10^8 cfu). In this application a GM plasmid would be added to the *E.coli* before administration.

108.   Pre-clinical experiments were conducted with the GMOs in mice. Initially, adult mice were fed *E.coli* carrying one of the target AMR plasmids along with a β-lactam antibiotic (cefotaxime) so that bacteria bearing the target plasmid were established in the mice's gut. Mice could consume

approximately 2x 10^6 cfu *E.coli* containing the target plasmid over the three days. Faecal samples taken from the mice now contained large amounts of *E.coli* that were resistant to a β-lactam antibiotic (see figure 8). Then these mice were "cured" of the target plasmid by feeding them *E.coli* containing the GM curing plasmid along with the selective antibiotic tetracycline for three days. Mice could consume approximately 6x 10^7 cfu *E.coli* containing the curing plasmid over the three days. Over the three days of the treatment, the faecal samples contained increasing numbers of bacteria resistant to tetracycline and decreasing numbers of bacteria resistant to the β-lactam antibiotic. The number of bacteria resistant to the β-lactam antibiotic decreased over 1,000 fold by day 3 and were undetectable by day 5. On day 4, administration of *E.coli* and tetracycline stopped, this removes selection for the GMO. Between days 4 and 10 the number of bacteria resistant to tetracycline decreased. By day 7 the amount of tetracycline resistant bacteria had reduced over 100 fold and by day 10 they were undetectable. As discussed in section 4.1.3, the GM plasmids are designed to have low persistence. Between days 10 and 18 samples were taken but no bacteria resistant to either the β-lactam or tetracycline were detected. To check for complete eradication of the AMR target, the mice were given a β-lactam antibiotic on day 16 and no AMR plasmid returned. Molecular tags were put on the two different *E.coli* so that the author could verify that conjugation had occurred and that the curing plasmid had moved into bacterial populations that previously contained the target plasmid. Three mice were colonised with pEI1573 and then cured with pJIMK46, another three mice were colonised with pJIE512b and cured with pJIMK56. Full details of this experiment, including the control groups, are published (Kamruzzaman et al., 2017).
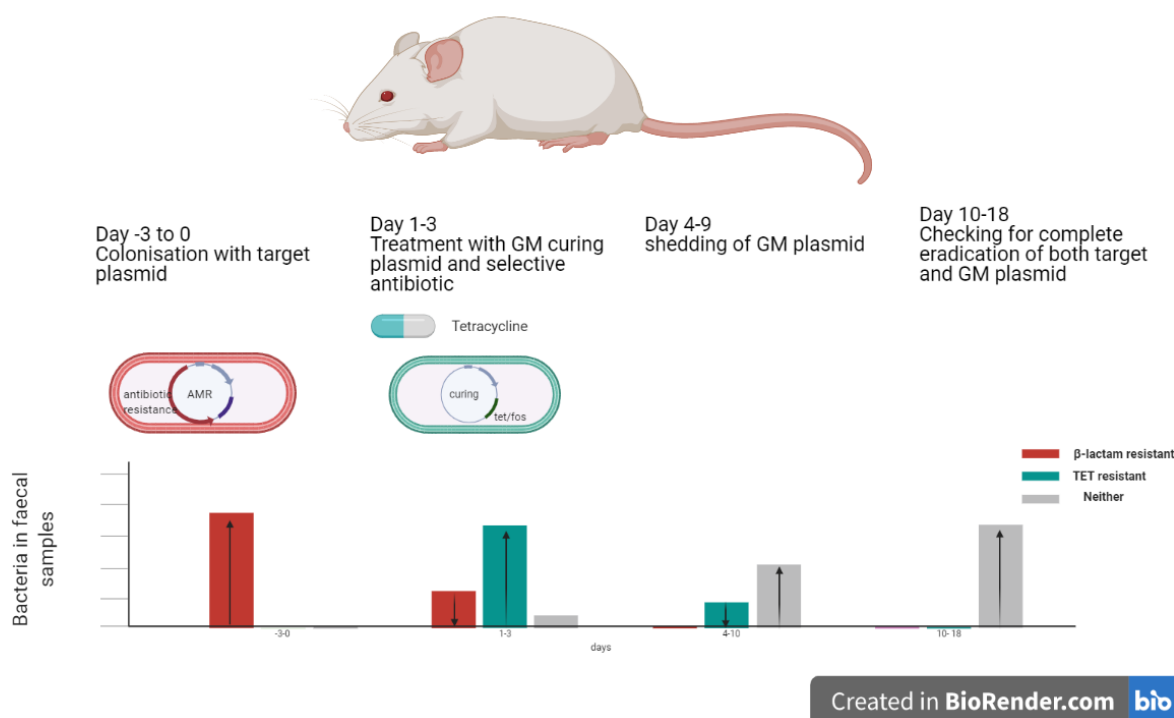

Figure 8. Simplified diagram of preclinical experiments in mice. Adult mice were first fed the AMR plasmid (in E.coli) then they were fed the curing plasmid (in E.coli). The GM curing plasmid was able to displace the target plasmid. The GM curing plasmid was shed for a short time after treatment. Full details are published (Kamruzzaman et al., 2017). Image created using BioRender.

109.   In the mouse experiments, no bacteria were detected that were simultaneously resistant to both the β-lactam antibiotic and tetracycline. This suggests that horizontal gene transfer events that could result in both the AMR and *tetA* genes being brought together did not occur. Section 5.5 and figure 9 discuss various ways in which hybrid plasmids or the transfer of resistances to the genome could occur.

110.   It is uncertain how well these mice results will transfer to clinical trial in humans. The mice were bred in controlled laboratory conditions and were selectively colonised with *E.coli* containing the AMR

plasmid with the help of a β-lactam antibiotic. The human participants are likely have a more varied microbiome based on their age, diet, medical history and other factors. It is also uncertain how well the shedding results obtained in mice can be applied to human participants. The whole gut transit time for mice is estimated to be around 6 hours (Padmanabhan et al., 2013). Unlike the mice, where clinically indicated, human participants may receive a second dose of antibiotics on the equivalent of day 4. This would be likely to result in faster clearance of the GMO.

E.coli will be used to deliver GM plasmids to a participant's guts. The GM plasmids have been designed to replace plasmids that carry resistance to multiple antibiotics (AMR) by using plasmid incompatibility. This should allow antibiotic resistant bacteria to regain sensitivity to antibiotic treatment. The GM plasmids would not persist in trial participant once the therapy is completed.

Section 5   The receiving environment

111.   The receiving environment forms part of the context for assessing risks associated with dealings with GMOs (OGTR, 2013b). It informs the consideration of potential exposure pathways, including the likelihood of the GMOs spreading or persisting outside the site of release.

5.1   Site of release – the human gut

112.   The primary environment receiving the GM *E.coli* would be the gastrointestinal (GI) tract of the trial participant. See section 2.3 for a discussion of bacteria in the human gut.

113.   Whole gut transit time has been estimated to be between 10-73 hours, consisting of 2-5 hours for gastric emptying, 2-6 hours to transit the small bowel and 10-59 hours for the colon (Lee et al., 2014). A study using a motility capsule technology found that the intestinal transit time of ICU patients was fairly similar to that of healthy volunteers but the rate of gastric emptying was slower in ICU patients (Rauch et al., 2009). A meta-analysis of the effects of probiotics on intestinal transit time found they were moderately efficacious in reducing intestinal transit time, but *E.coli* based probiotics were not included (Miller et al., 2016).

114.   Antibiotic use impacts the gut microbiome. The effect depends on the class, dosage and duration of the antibiotic treatment as well factors to do with the individual patient. As well as decreasing the amount of bacteria in the gut, broad spectrum antibiotics can change the balance between bacterial species (Rinninella et al., 2019).

5.2   Shedding and transmission

115.   The principal route by which the GM bacteria may enter the wider environment following inoculation is via shedding. Further, GM bacteria could also enter the environment via accidental spilling of unused GMO preparation.

116.   Human faeces is estimated to contain about 10^{12} bacteria per gram (Sender et al., 2016) and healthy adults produce in the order of 100 g of faeces a day in western countries (Cummings et al., 1992). So approximately 10^{14} bacteria per person a day may enter sewage. Around 90% of these bacteria will be Firmicutes and Bacteroidetes (Rinninella et al., 2019), it has been estimated that there are about 10^8 cfu of *E.coli* per gram of faeces (Zuo et al., 2011) or 10^7-10^9 *E.coli* (Tenaillon et al., 2010).

117.   Human gut microbiota is excreted into sewage and wastewater, where it is removed through standard waste treatment processes, prior to the water being released back into the environment. The sewage treatment is also likely to be effective at removing the GM bacteria from sewage. However, due to variable levels of sewage treatment in the wastewater plants (Toze et al., 2012), this could result in varying amount of bacteria in the sewage and could hypothetically result in disposal of some GM bacteria directly into rivers or marine environment.

5.3   Secondary site of release, sewage

118.   Bacterial populations in raw sewage include human faecal bacteria, bacteria resident in the sewer system infrastructure, and environmental bacteria originating from grey water and surface runoff (Shanks et al., 2013). In untreated sewage samples collected from 13 wastewater treatment plants in the United States, the most abundant bacterial phyla were Proteobacteria, which includes *E.coli* (average 62%), Firmicutes (average 21%) and Bacteroidetes (average 13%) (Shanks et al., 2013). Similarly, in activated sludge samples collected from 14 wastewater treatment plants in east Asia and North America, the most abundant phyla were Proteobacteria (35-65%), Firmicutes (averaging 8%), Bacteroidetes (averaging 7%) and Actinobacteria (averaging 7%) (Zhang et al., 2012)

119.   In urban areas most waste water is processed at centralised wastewater treatment plants (WWTPs). WWTP vary but generally the waste water undergoes a primary treatment process involving sedimentation followed by a secondary treatment where aeration is used to allow bacteria to digest organic matter. Some, but not all, WWTPs use tertiary treatment to disinfect the water further via chlorination, ozonation, UV treatment or other methods. After treatment most waste water is returned to the ocean, a lake, or a river. A large UK study of 162 WWTP found that primary treatment did not reduce the concentration of faecal indicator bacteria much, but secondary treatment reduced faecal indicator bacteria by 95-99%, and tertiary treatments reduce this even further by another 93-97% (Kay et al., 2008). Overall this is a reduction in bacteria of up to 3000-fold.

120.   An analysis of four wastewater treatments plants across Australia found an average of 126 different genera of bacteria were present (Ahmed et al., 2017). The 10 most abundant genera were; *Pseudomonas, Arcobacter, Bacteroides, Paludibacterium, Conchiformibius, Flavobacterium, Polynucleobacter, Acinetobacter, Parabacteroides,* and *Cloacibacterium.* A study into 4 WWTPs in Queensland found sometimes human pathogenic *E.coli* could survive tertiary treatment and reach the environment (Anastasi et al., 2010). Determining the number of *E.coli* in the environment that came from waste water is complicated by birds and other animals carrying similar *E.coli* to humans (Anastasi et al., 2012).

121.   Some human waste does not enter commercial waste water treatment but is instead subject to various types of on-site-treatment. These include septic systems, aerated wastewater treatment system and dry composting toilets. Generally these treatments are less effective at killing bacteria compared to waste water treatment plants.

5.4   Presence of similar genes in the environment

122.   All of the genes in the GM plasmids can be found on naturally occurring bacterial plasmids. As discussed in section 4, antibiotic resistance genes are already present on conjugative bacterial plasmids in the environment. However the exact order and combination of genes on the GM plasmids could be novel.

123.   There appears to be no available data on how frequently a combination of TET and FOS resistance occur in Australian bacterial isolates. Tetracycline resistance is frequently found in combination with extended-spectrum β-lactamases (ESBL). In a European surveillance program 66.9% of ESBL isolates were also tetracycline resistant (Jones et al., 2014). Conjugative plasmids encoding combinations of fosfomycin resistance and ESBL genes have been identified previously. The gene *fosA3* is often found in a composite IS26-type transposon on a conjugative plasmid that also carries *bla*$_{CTX-M}$ genes (Yang et al., 2019). However, it is unknown how frequently this combination occurs in Australia.

5.5   Possible interactions with genes in the environment

As discussed previously there is a large diversity of bacterial species and plasmids present in the two receiving environments. The genetic mechanisms of particular importance to the application are summarised below and in figure 9.

1. The ability of the curing plasmids to become co-resident in bacteria with a compatible plasmid. The compatible plasmids could include plasmids encoding for antibiotic resistance (other than the target plasmid) or carrying virulence genes.

2. Homologous recombination between the GM plasmids and native plasmids in the receiving environment

3. The formation of a cointegrant, a structure where two plasmids combine into one very large plasmid

4. Transformation of bacteria with fragments of DNA originating from the GM plasmid.
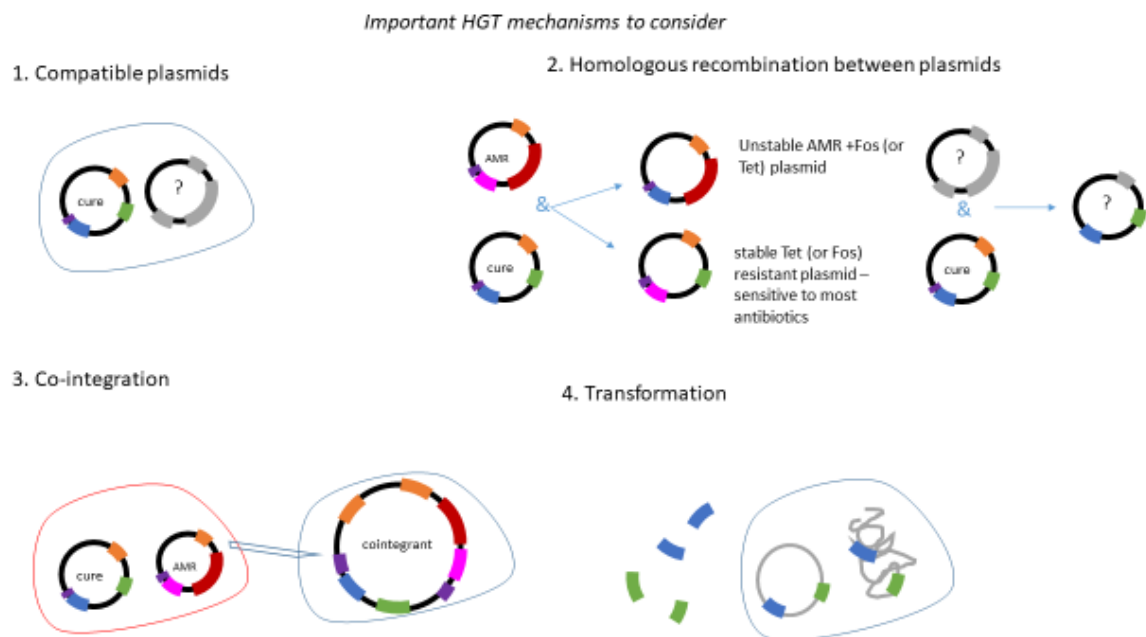

Figure 9. important horizontal gene transfer mechanisms to this application. plasmid with grey genes represents all the unknown plasmids that are present in the participant's gut bacteria. Blue represents fosA3, green represents TetA and red represents the AMR cassette.

Addition of the GM plasmid might also displace native plasmids (other than the target plasmid) that have the same Inc group.

5.6   Stability in the environment and decontamination

124.   The GM plasmids have been altered to reduce their ability to persist in bacteria. In mice experiments, the GM curing plasmids were undetectable 10 days after the removal of antibiotic selection (Kamruzzaman et al., 2017).

125.   Methods of decontamination effective against the parent organism, *E.coli* Nissle strain, are expected to be equally effective against the GMO. Methods of decontamination against human gut bacteria should work equally well on those with or without the GM plasmid. In the absence of antibiotic selection, the GM imposes a fitness cost on the bacterial host making them unable to outcompete matched wild-type bacteria.

126.   All bacteria can be killed by autoclaving or high-temperature incineration (Rutala et al., 2008). Ethanol (60-80%), formaldehyde (4%) and Virkon (1%) are effective disinfectants for vegetative bacteria but lack sporicidal action or require long contact time (2 – 20 hours for tested species) to kill bacterial spores. Hypochlorite (0.5%) kills both vegetative bacteria and spores within 10 minutes contact time but is less effective in the presence of organic matter (Russell, 1990; Rutala et al., 2008).

Bacteria containing the GM plasmid will be shed into the waste water though the faeces of participants for a short time after treatment.

Chapter 2   Risk assessment

Section 1   Introduction

127.   The risk assessment identifies and characterises risks to the health and safety of people or to the environment from dealings with GMOs, posed by or as the result of gene technology (Figure 5). Risks are identified within the established risk assessment context (Chapter 1), taking into account current scientific and technical knowledge. A consideration of uncertainty, in particular knowledge gaps, occurs throughout the risk assessment process.
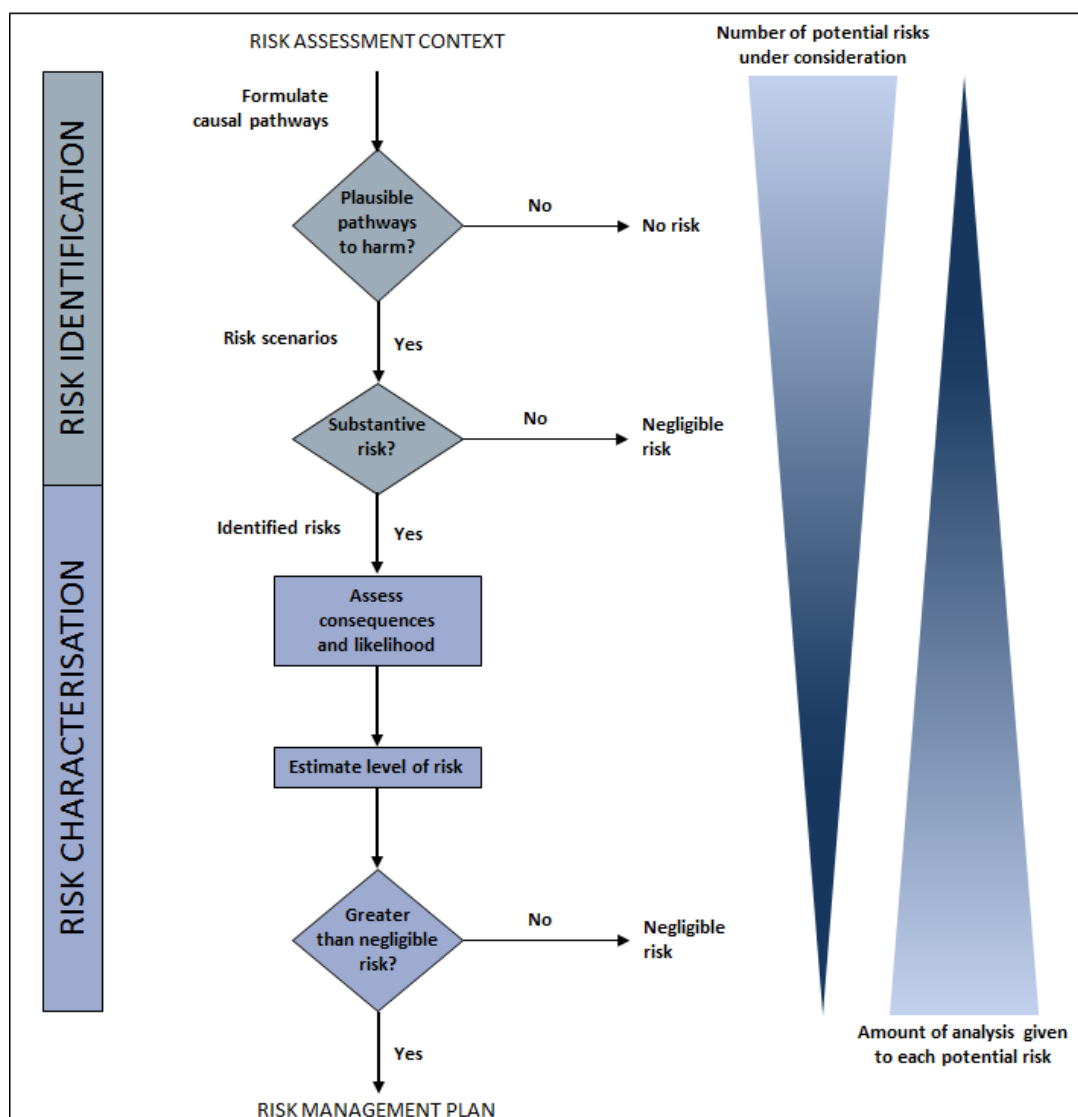

Figure 10. The risk assessment process

128.   The Regulator uses a number of techniques to identify risks, including checklists, brainstorming, previous agency experience, reported international experience and consultation (OGTR, 2013b).

129.   Risk identification first considers a wide range of circumstances in which the GMO, or the introduced genetic material, could come into contact with people or the environment. This leads to postulating causal pathways that may give rise to harm for people or the environment from dealings with a GMO. These are called risk scenarios.

130.   Risk scenarios are screened to identify substantive risks, which are risk scenarios that are considered to have some reasonable chance of causing harm. Risk scenarios that could not plausibly

occur, or do not lead to harm in the short and long term, do not advance in the risk assessment process (Figure 5), i.e. the risk is considered no greater than negligible.

131.   Risk scenarios identified as substantive risks are further characterised in terms of the potential seriousness of harm (Consequence assessment) and the likelihood of harm (Likelihood assessment). The consequence and likelihood assessments are combined to estimate the level of risk and determine whether risk treatment measures are required. The potential for interactions between risks is also considered.

Section 2   Risk identification

132.   Postulated risk scenarios are comprised of three components (Figure 6):

  i.   The source of potential harm (risk source)

  ii.   A plausible causal linkage to potential harm (causal pathway), and

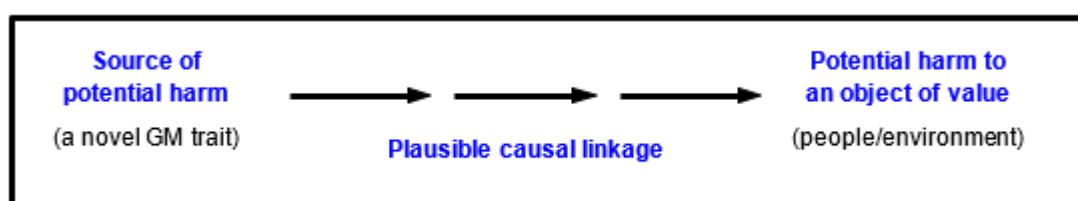 iii.   Potential harm to people or the environment.

Figure 11. Components of a risk scenario

133.   When postulating relevant risk scenarios, the risk context is taken into account, including the following factors detailed in Chapter 1:

- the proposed dealings
- the proposed limits including the extent and scale of the proposed dealings
- the proposed controls to limit the spread and persistence of the GMO and
- the characteristics of the parent organism(s).

2.1   Risk source

134.   The parent organism of GMO1 is the commensal *E.coli* Nissle strain, while the parental organism for GMO2 is the participant's gut bacteria. Details of the properties of these GMOs can be found in chapter 1 section 4. The GMO includes genes conferring resistance to the antibiotics tetracycline and fosfomycin which are the main risk source.

135.   Potential sources of harm can be intended novel GM traits associated with one or more of the introduced genetic elements or unintended effects/traits arising from the use of gene technology. Unintended effects can arise through horizontal gene transfer (HGT), the stable transfer of genetic material from one organism to another without reproduction. All genes within an organism, including those introduced by gene technology, can be transferred to another organism by HGT. A gene transferred through HGT could confer a novel trait to the recipient organism. The novel trait may result in negative, neutral or positive effects on the fitness of the recipient organism.

2.2   Causal pathway

136.   The following factors are taken into account when postulating plausible causal pathways to potential harm:

- the proposed dealings,
- characteristics of the parent organism,
- routes of exposure to the GMOs, the introduced gene(s) and gene product(s),

- potential effects of the introduced gene(s) and gene product(s) on the properties of the organism,

- potential exposure of other organisms to the introduced gene(s) and gene product(s) from other sources in the environment,

- potential exposure of other organisms to the GMOs in the environment,

- the release environment,

- spread and persistence of the GMOs (e.g. dispersal pathways and establishment potential),

- environmental stability of the organism (tolerance to temperature, UV irradiation and humidity),

- gene transfer by horizontal gene transfer,

- unauthorised activities, and

- practices before and after administration

137.   Although all of these factors are taken into account, some are not included in the risk scenarios below as they may have been considered in previous RARMPs and a plausible pathway to harm could not be identified.

138.   As discussed in Chapter 1, the TGA, the trial sponsor, the Investigators and HREC all have roles in ensuring the safety of trial participants under the *Therapeutic Goods Act 1989*, and human clinical trials must be conducted in accordance with the *National Statement on Ethical Conduct in Human Research (National Health and Medical Research Council et al., 2018)*. Therefore, risk scenarios in the current assessment focus primarily on risks posed to people, other than those participants in the trial, and to the environment. It does not access the likelihood of the experimental treatment benefiting participants.

139.   The GMOs and samples containing the GMO are proposed to be transported and stored in line with the Regulator's *Guidelines for the Transport, Storage and Disposal of GMOs*. These are standard protocols for the handling of GMOs to minimise exposure to the GMOs, so risks associated with such transport will not be further assessed.

140.   The Act provides for substantial penalties for unauthorised dealings with GMOs or non-compliance with licence conditions, and also requires the Regulator to have regard to the suitability of an applicant to hold a licence prior to the issuing of the licence. These legislative provisions are considered sufficient to minimise risks from unauthorised activities. Therefore, unauthorised activities will not be considered further.

2.3 Potential harm

141.   In addition, the following factors are taken into account when postulating relevant risk scenarios for this licence application:

- harm to the health of people including disease in humans

- the potential for establishment of the GM *E.coli* or GM gut bacteria in the environment

- harm to animals other than humans

2.4 Postulated risk scenarios

142.   Four risk scenarios were postulated and screened to identify substantive risk. These scenarios are summarised in Table 2 and discussed in depth in this Section.

143.   In the context of the activities proposed by the applicant and considering both the short and long term, none of the four risk scenarios gave rise to any substantive risks that could be greater than negligible.

144.   Scenario 1 and 2 discuss potential risks from unaltered forms of GMO1 and GMO2, while scenario 3 and 4 discuss the possible risks in cases where recombination has occurred. Scenario 2 and

3 discuss the risks to close contacts of the participant. Scenario 4 discusses risks associated with dispersal in the wider environment through the waste water system.

Table 1   Summary of risk scenarios from dealings with GM plasmids

Risk scenario	Risk source	Causal pathway	Potential harm	Substantive risk	Reason
1	GM *E.coli* Nissle strain (GMO1)	Exposure of clinical trial staff to GMO1 via contact, inhalation/ ingestion during (a) Preparation and administration of the GMO (b) Analysis of the GMO (c) Disposal of the GMO ⬇ local infection with GM bacteria in permissive environment ⬇ illness caused by *E.coli* resistant to the antibiotics tetracycline and fosfomycin	*Illness caused by E.coli resistant to the antibiotics tetracycline and fosfomycin*	No	• Only trained personnel wearing PPE would prepare, administer and analyse the GMO • The dose of GMO received through accidental exposure during administration would be low • *E.coli* Nissle strain lacks pathogenic genes • Acquired resistance to TET and FOS would be transient • TET and FOS are not routinely used for potential infections
2	GM human gut bacteria (GMO2)	Administration of GMO to participant ⬇ Transit through participant's gut ⬇ Within the participant's gut the GM plasmid spreads by conjugation to resident bacteria with the potential to be pathogenic ⬇ These bacteria are shed by the participant ⬇ Exposure of medical staff, carers or pets to bacteria shed by the participant ⬇ Infection with bacteria with introduced resistance to TET and FOS ⬇ Illness caused by gut bacteria resistant to the antibiotics tetracycline and fosfomycin	*Illness caused by gut bacteria resistant to the antibiotics tetracycline and fosfomycin*	No	• Participant will stay in the hospital for 4 days after the last dose of GMO, while shedding is highest. During this time only trained and experienced hospital personal wearing PPE will help with personal care. • Carers and participants will be advised to follow good hygiene practices, including covering any compromised skin, in the weeks following treatment. • The dose received through accidental exposure to shedding would be far smaller than that administered • Acquisition of the GM plasmid does not enhance the ability of gut bacteria to cause infections. • As described in scenario 1

Risk scenario	Risk source	Causal pathway	Potential harm	Substantive risk	Reason
					o Acquired antibiotic resistance will be short lived
					o TET and FOS not routinely used for these infections
3	GM *E.coli* Nissle strain (GMO1) and human gut bacteria containing the GM plasmid (GMO 2)	Administration of GMO to participant ⬇ Transit through participant's gut ⬇ Within the participant's gut the GM plasmid spreads by conjugation to resident bacteria with the potential to be pathogenic or which are carrying non-targeted AMR plasmids ⬇ Within these bacteria a recombination event occurs resulting in a new hybrid plasmid that carries resistance to TET and FOS in addition to its pre-existing characteristics ⬇ These bacteria are shed by the participant ⬇ Exposure of medical staff, carers or pets to bacteria shed by the participant ⬇ Infection with bacteria with introduced resistance to TET and FOS ⬇ Illness caused by gut bacteria additionally resistant to the antibiotics tetracycline and fosfomycin	*Illness caused by bacteria resistant to the antibiotics tetracycline and fosfomycin in addition to pre-existing antibiotic resistances*	No	• The worst case scenario is the addition of stable TET and FOS resistance to gut bacteria that already carry multiple other resistances or pathogenic characteristics • Hybridisation can only occur during the transit of the bacteria through the participant's gut, and only in the bacteria that take up the plasmid. So there is limited opportunity for it to occur. • The trial involves a low number of participants which reduces the opportunity for a potentially harmful hybrid plasmid to form. • No hybrid plasmids were detected during the pre-clinical experiments • Some features that promote horizontal gene transfer have been removed from the GM plasmids • In the absence of antibiotic selection, wild-type strains are likely to outcompete the GM bacteria • Bacteria resistant to TET and/or FOS as well as β-lactamases already exist in the environment • Pets are often treated with Doxycycline but are unlikely to become infected if they are healthy. • As described in scenario 2

Risk scenario	Risk source	Causal pathway	Potential harm	Substantive risk	Reason
					o 4 days in hospital after the last administration of the GM bacteria, trained staff and PPE
					o Lower amount shed
					o Hygiene practices
					o No enhanced ability to cause infection
					o TET and FOS not routinely used of these infections.
4	GM bacteria	Administration of GMO to participant ⬇ Transit through participant's gut ⬇ Within the participant's gut the GM plasmid spreads by conjugation to resident bacteria with the potential to be pathogenic or which are carrying non-targeted AMR plasmids ⬇ Within these bacteria a recombination event occurs resulting in a new hybrid plasmid that carries resistance to TET and FOS in addition to its pre-existing characteristics ⬇ These bacteria are shed by the participant ⬇ Bacteria enter waste water system ⬇ Bacteria survive treatment at WWTP ⬇ A vunerable person or animal comes into contact with the bacteria ⬇ Illness caused by gut bacteria additionally resistant to the antibiotics	*Illness caused by bacteria resistant to the antibiotics tetracycline and fosfomycin in addition to pre-existing antibiotic resistances*	No	• As described in scenario 3 o Lower amount shed o No enhanced ability to cause infection, healthy people unlikely to become infected. o TET and FOS not routinely used of these infections. o Potentially harmful hybridisation is disfavoured o No hybrids were detected in pre-clinical experiments o Similar genes are already present in the environment o In the absence of selection hybrids will be outcompeted • Bacteria are substantially diluted upon entry to waste water and 95-99% are likely to be killed by secondary waste water treatment • This dilution makes it even more unlikely any exposed person or animal would develop an infection.

Risk scenario	Risk source	Causal pathway	Potential harm	Substantive risk	Reason
		tetracycline and fosfomycin			

Risk scenario 1

Risk source	GM bacteria
Causal pathway	Exposure of clinical trial staff to GMO1 via contact or ingestion during (d) Preparation and administration of the GMO (e) Analysis of the GMO (f) Disposal of the GMO ⬇ local infection with GM bacteria in permissive environment ⬇ illness caused by *E.coli* resistant to the antibiotics tetracycline and fosfomycin
Potential harm	Illness caused by *E.coli* resistant to the antibiotics tetracycline and fosfomycin

Risk source

145.   The source of potential harm for this postulated risk scenario is GMO1, the *E.coli* Nissle strain containing the GM curing plasmid.

Causal Pathway

146.   During administration nurses/doctors/ scientists could potentially be exposed to GMO1 in an number of ways

   i.   by ingesting the *E.coli* residues from items used to prepare the treatment or by direct contact with contaminated surfaces. These could also occur during disposal of GMO1.

   ii.   by touching face or eyes during administration

   iii.   through a spill of GM *E.coli*

   iv.   through contact with spit/vomit immediately after administration.

147.   Only trained and experienced personnel would prepare, administer and analyse the GMO. Use of PPE (e.g. gown, gloves, mask and eye protection) minimises the potential for exposure to staff handling the GMO. Staff are trained in procedures designed to prevent the spread of infection within hospitals. Patients with active AMR infections are treated with special care by hospitals in order to avoid spreading the infection, so the GMO will be administered carefully. These work practices would reduce the likelihood of exposure to cuts or mucous membranes.

148.   Any exposure via these pathways would only involve low levels of the GMO which is therefore unlikely to result in any negative effects or ill-health.

149.   If GMO1 did come in contact with one of these body surfaces, an immune reaction could occur which is likely to clear the infection. *E.coli* Nissle strain lacks virulence genes such as those that allow pathogenic strains of *E.coli* to evade or counteract the immune system. *E.coli* are highly abundant and most humans are exposed to them at birth and therefore will have prior immunity.

150.   In the event of ingestion of a small amount of GMO1, a large proportion of the bacteria would be killed by stomach acid. Even repeated high dose *E.coli* probiotics often fail to achieve bacterial colonisation of the gut of healthy adult individuals (Wassenaar, 2016), so it is very unlikely that a single accidental exposure to a low dose would result in colonisation of the GMO. Without colonisation, any

E.coli ingested would soon be excreted. Deliberate consumption of 10^{11} cfu of *E.coli* Nissle strain has been shown to be safe (Wassenaar, 2016), so there is no risk to ingesting small amounts.

151.   In the unlikely event that accidentally consumed GMO1 colonised the gut and was able to pass the GM plasmid to other bacteria, any acquired antibiotic resistance would be transient due to the low persistence of the plasmid.

152.   Most people harbour *E.coli* with resistance to particular antibiotics as part of their microbiome with no ill effects. Antibiotic resistance is only relevant when an infection develops. The GM plasmid has been modified to prevent persistence in the absence of antibiotic selection with TET or FOS and therefore will be lost quickly. In preclinical experiments, the resistance to TET and FOS was lost after ten days. Unless an infection develops shortly after exposure and antibiotic treatment is needed, it is unlikely that TET and FOS resistance would be acquired through an exposure to GMO1.

Potential harm

153.   If clinical trial staff were exposed to GMO1 this could result in illness caused by *E.coli* which would be resistant to treatment with TET or FOS. TET and FOS are not routinely used for *E.coli* infections that could arise as a result of accidental exposure during administration. Therefore, transient resistance to these antibiotics is likely to have no impact.

154.   In the unlikely event of an illness developing as result of accidental exposure to GMO1, this infection could be transiently resistant to treatment with TET or FOS, but would be sensitive to other antibiotics. While a member of the tetracycline class of antibiotics called doxycycline is commonly prescribed (see section 3.1.1.2), it is not routinely used to treat human *E.coli* infections. FOS is generally only used to treat uncomplicated UTIs with *E.coli*, but other antibiotics (such as trimethoprim or nitrofurantoin) could be used instead. A UTI is unlikely to develop in an individual accidently exposed to the GMO as it would involve a succession of unlikely steps including the successful colonisation of a small amount GMO1 (discussed above), the proliferation of GMO1 in the gut and the migration of this non-pathogenic bacteria to the urinary tract to produce an infection.

Conclusion

155.   Scenario 1 is not identified as a risk that could be greater than negligible. Therefore, it does not warrant further detailed assessment.

Risk scenario 2

Risk source	GM bacteria
Causal pathway	Administration of GMO to participant ⬇ Transit through or colonisation of the participant's gut ⬇ Within the participant's gut, the GM plasmid spreads by conjugation to resident pathogenic bacteria ⬇ These bacteria are shed by the participant in faeces ⬇ Exposure of medical staff, carers, household members or pets to bacteria shed by the participant ⬇ contact with abraded skin or mucous membranes, or ingestion ⬇ Infection with bacteria with introduced resistance to TET and FOS ⬇ Illness caused by enterobacteriaceae resistant to the antibiotics tetracycline and fosfomycin
Potential harm	*Illness caused by gut bacteria resistant to the antibiotics tetracycline and fosfomycin*

Risk source

156.   The source of potential harm for this postulated risk scenario is GMO2, human gut bacteria that have received the GM plasmid by conjugation. Transfer of the GM curing plasmid from GMO1 to other gut bacteria is an intentional part of the clinical trial.

157.   The range of the curing plasmid should be the same as that of the corresponding target AMR plasmid. The plasmid pJIBE401 has been identified in the following species by a Sydney based laboratory *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *E.coli (strains other than the Nissle strain)*, *Serratia marcescens*, *Enterobacter gergoviae*, *Morganelle morganii*, *Pantoea agglomerans*, *Proteus mirabilis*, *Serratia plymuthica* and several species of *Citrobacter*.

158.   Among the gut bacterial species listed above the most prominent potential human pathogens are, *E.coli* strains (other than the Nissle strain), *Klebsiella sp.* and *Enterobacter sp.* These *enterobacteriaceae* are consistently being excreted from every human being, but only lead to disease in particular contexts.

Causal Pathway

GM plasmid passes from GMO1 to participant's gut bacteria forming GMO2

159.   The participant would ingest 10^8 cfu of GMO1 on three sequential days. A significant amount of the *E.coli* will be killed by the participant's stomach acid but some will reach the participant's gut. GMO1 will be diluted at least thousands-fold by the other gut bacteria in the intestines. However, GMO1 may be able to colonise the participant's gut and multiply within it. GMO1 is more likely to be able to colonise the gut of participants who have compromised microbiomes. Selection with the antibiotic TET or FOS will promote colonisation with GMO1.

160.   During transit though, or colonisation of, the participant's gut, the GM plasmid is passed to other *enterobacteriaceae* such as those listed above. In order for the GM plasmid to be passed by conjugation, GMO1 needs to come into close physical contact with a gut bacteria that is a suitable

host for the plasmid. A mating pair needs to form with a pilus forming a bridge between the two bacteria and then a copy of the GM plasmid passes from the donor to the recipient. The proportion of gut bacteria that will receive the plasmid is not known but is likely to be higher in cases where colonisation of GMO1 has occurred.

161.   The effect of gaining the GM plasmid on the recipient gut bacteria depend on the pre-existing properties of that bacteria.

i.   Some of the gut bacteria that will gain the GM plasmid will have the target AMR. In these cases the curing plasmid is anticipated to replace the target plasmid. This results in gut bacteria that are now resistant to fewer antibiotics because although they gain transient resistance to TET and FOS, they have lost resistance to β-lactam antibiotics (and the others included in the AMR cassette).

ii.   Some of the gut bacteria may have a compatible conjugative plasmid to the GM plasmid and therefore may carry both plasmids. These bacteria would gain transient resistance to TET and FOS.

iii.   Some of the gut bacteria may have had mobilisable plasmids. If this bacteria gain the GM plasmid in addition to transient resistance to TET and FOS, they also become conjugative (see section 2.2.2). The addition of any conjugative plasmid would have this effect and many resident bacteria already have conjugative plasmids.

iv.   Some of the gut bacteria may have already been resistant to TET and or/FOS in which case the situation has not changed. There is no selective advantage for these bacteria to receive the GM plasmid so they are less likely to do so.

v.   Some of the gut bacteria might carry incompatible plasmids with functions unrelated to AMR. These bacteria might trade their native plasmids for the GM curing plasmid and gain transient resistance to TET and FOS. This trade could be a net-positive or a net-negative.

vi.   Some of the gut bacteria will not receive the GM plasmid and their properties will be unchanged.

162.   The outcome with the greatest potential for harm is a potentially pathogenic gut bacteria transiently acquiring resistance to TET and FOS, so this is considered further.

Bacteria shed from the participant infects a close contact

163.   GMO1 and GMO2 will be shed by participants for a short time after treatment. Human gut bacteria are shed in faeces. The shedding of GMO1 and GMO2 will be most intense during the first few days after treatment and will then reduce over subsequent days. In preclinical experiments, detailed in section 4.2.1, neither GMO was detectable in faecal samples ten days after treatment began. As discussed in chapter 1, section 2.3, *enterobacteriaceae* are only a small percentage of the human gut bacteria that is excreted. If clinical trial staff or a close contact of the participant was exposed to the GMO that had been shed, the amount would be small and is therefore unlikely to cause infection in a healthy individual.

164.   Analysis of clinical trial samples likely to contain GMOs will be conducted in a PC2 facility, by individuals who are trained in PC2 work practices, including the appropriate use of PPE.

165.   During the 4 days after the last administration of the GMO, the participant will stay at the clinical site and any necessary personal care will be provided by trained medical staff. Trained medical staff are familiar with procedures to prevent the spread of infection when interacting with patients, and more specifically procedure to adopt to prevent exposure to human faecal bacteria. Patients with active AMR infections are treated with special care by hospitals in order to avoid spreading the infection. These measures generally include (i) isolation in individual rooms with private bathroom facilities and have impermeable surfaces that are bleach decontaminated (ii) all staff and visitors are required to wear impermeable gloves and gowns that are disposed of after each interaction. While these measure are in place to protect staff and family members from the AMR target, they would also

further reduce the likelihood of the GMO being spread. Depending on their health status, some participants may stay in hospital for longer than 4 days after the last administration of the GMO. Some participants will also be treated with a second round of antibiotics, to treat the infection affecting them prior to the clinical trial (using an antibiotic other than TET or FOS), which would be likely to speed up elimination of the GMOs.

166.   While most of the shedding is likely to occur during the first week of treatment, some low level of shedding may continue for more than 4 days after the last administration of the GMO. In order to reduce the chance of the shed GMO being transmitted to household members (including pets) after being discharged from the clinical trial site, participants will be instructed to follow good hygiene practices. Good hygiene is essential to avoid faecal-oral transmission and the contamination of wounds. If shed GMO2 is ingested, it is unlikely to achieve colonisation of the gut of a household contact of the participant due to the small amount present in contaminated surfaces or skin.

167.   Close contacts of patients could potentially develop a UTI shortly after treatment. For this to occur, GMO2 would have to be consumed and then effectively colonise the gut of a non-participant, the GM bacteria would then have to migrate to the urinary tract and cause infection. Alternatively GMO2 could be transferred through sexual intercourse into the urinary tract and then cause a UTI. Acquisition of the GM plasmid would not increase the ability of bacteria to adhere to or invade the urinary tract.   If an infection did occur, and the bacteria were already resistant to trimethoprim, other antibiotics such as nitrofurantoin could be used instead. All of this would need to happen before the transient resistance to FOS was lost.

168.   The GM plasmid should have no effect on the types of bacteria that are routinely treated with doxycycline in Australia. Doxycycline is mostly used against Gram-positive bacteria which are very different from Gram-negative bacteria. Conjugation in Gram-positive bacteria has a different mechanism, so the GM plasmid should not be passed to Gram-positive bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA), would not obtain the GM plasmid because they are Gram-positive bacteria. Additionally, Gram-positive and Gram-negative bacteria tend to occupy different niches in the gut.

169.   GMO2 has no enhanced ability to cause infection relative to the parental bacteria. Acquiring the GM plasmid will not increase the virulence of gut bacteria. It will only provide resistance to the antibiotics TET and FOS. While some of the potential host *enterobacteriaceae* can cause disease, the GM plasmid doesn't change the likelihood of an infection developing.

170.   Most people harbour *enterobacteriaceae* with resistance to particular antibiotics as part of their microbiome with no ill effects. Antibiotic resistance is only relevant when an infection develops. The GM plasmid has been modified to prevent persistence in the absence of antibiotic selection with TET or FOS and therefore will be lost quickly in the absence of selection. TET and FOS are not commonly used to treat *E.coli* infection as mentioned in Risk Scenario 1. In preclinical experiments the resistance to TET and FOS was lost after ten days. Unless an infection develops shortly after exposure, it is unlikely to have TET and FOS resistance caused by GMO2.

171.   Pets could come into contact with low amounts of GMO2 shed once the participant returns home. Trial participants are instructed to practice good hygiene which would minimise the risk of exposure. As stated above, in the event a pet is exposed to GMO2, the GM plasmid do not enhance the potential for the parental organism to cause an infection but would only confer a transient resistance to TET and FOS. This acquired resistance is only relevant when an infection develops and antibiotic treatment is prescribed including TET and FOS. In other circumstances, there is likely to be no effect. *E.coli* infections in animals are sometimes called Colibacillosis and are most serious in new born animals. In a study of 3 European countries, 5% of the antibiotics prescribed for cats and dog were doxycycline. The *E.coli* isolated from the cats and dogs were resistant to tetracycline in about 15% the samples collected (Joosten et al., 2020). Tetracycline resistance gene in domestic animal is therefore already present. Fosfomycin is not used in animals.

Potential harm

172.   In the unlikely event of an illness developing as result of accidental exposure to GMO1 or GMO2, this infection could be transiently resistant to treatment with TET or FOS, but would be sensitive to other antibiotics.

173.   An infection being resistant to tetracycline only causes harm when tetracycline would have been used to treat that infection. While a member of the tetracycline class of antibiotics called doxycycline is commonly prescribed (see section 3.1.1.2), it is not routinely used to treat human *enterobacteriaceae* infections. As discussed in section 3.1.2.3 *enterobacteriaceae* are often already resistant to tetracycline. Other antibiotics would be available to treat a potential infection.

174.   Fosfomycin is generally only used to treat uncomplicated UTIs with *E.coli* resistant to trimethoprim. However, if a UTI could be treated with other antibiotics such as trimethoprim, or if the bacteria were already resistant to trimethoprim, other antibiotics such as nitrofurantoin could be used instead.

Conclusion

175.   Scenario 2 is not identified as a risk that could be greater than negligible. Therefore, it does not warrant further detailed assessment.

Risk scenario 3

Risk source	GM bacteria
Causal pathway	Administration of GMO to participant ⬇ Transit through or colonisation of the participant's gut ⬇ Within the participant's gut the GM plasmid spreads by conjugation to resident bacteria with the potential to be pathogenic or which are carrying non-targeted AMR plasmids ⬇ Within the participant's gut a HGT event occurs resulting in a new GM bacteria that carries resistance to TET and FOS in addition to its pre-existing characteristics ⬇ These bacteria are shed by the participant ⬇ Exposure of medical staff, carers, household members or pets to bacteria shed by the participant ⬇ contact with abraded skin or mucous membranes, or ingestion ⬇ Infection with bacteria with introduced resistance to TET and FOS ⬇ Illness caused by gut bacteria additionally resistant to the antibiotics tetracycline and fosfomycin
Potential harm	*Illness caused by gut bacteria resistant to the antibiotics tetracycline and fosfomycin*

Risk source

176.   The source of potential harm for this postulated risk scenario is GMO2, human gut bacteria that have received the GM plasmid by conjugation. Transfer of the GM curing plasmid from GMO1 to other gut bacteria is an intentional part of the clinical trial. This postulated risk scenario considers a hypothetical *enterobacteriaceae* bacteria that has acquired stable expression of tetracycline and/or fosfomycin in addition to the antibiotic resistances it carried previously. This scenario discusses the ways that this unintended event could theoretically occur and the potential consequences.

Causal Pathway

GM plasmid passes from GMO1 to participant's gut bacteria forming GMO2

177.   The initial pathway for risk scenario 3 are the same as scenario 2, described above.

Within the participant's gut, a HGT event occurs resulting in a new GM bacteria that carries resistance to TET and FOS in addition to its pre-existing characteristics

178.   A bacteria could obtain the combination of resistance to TET and FOS in addition to the AMR genes it carried previously by either

i.   Addition of *fosA3* and/or *tetA* to the bacterial genome

ii.   The creation of a novel stable hybrid plasmid that now carries *fosA3* and/or *tetA* in addition to the previous AMR genes.

179.   As discussed in section 2.2.1, the main mechanisms of horizontal gene transfer in bacteria are transformation, transduction, transposition and conjugation. The first three of these can result in a

gene from a foreign source being transferred to the bacterial genome or on to a plasmid. Transposition is unlikely because the GM plasmid lacks the repeat elements that promote transposition.

180.   Transduction would require a bacteriophage to infect a bacteria carrying the GM plasmid and then randomly carry *tetA* or *fosA3* (instead of any of the other genes from the lysed bacteria) to a second bacteria. The phage would need to integrate into the second bacteria's genome as a prophage. Transduction is not high frequency and bacteria often have defence mechanisms against phages because integration into the chromosome has the potential to kill the bacteria if it occurs in the wrong location.

181.   Transformation involves the uptake of released DNA fragments from the environment by bacteria that are competent. In order to be competent bacteria have to have specific genes to allow them to take in DNA and receive the correct environmental signals (Blokesch, 2016). In this case, DNA fragments containing *tetA* or *fosA3* could come from dead GMO1 or GMO2. Free DNA is unlikely to survive for long in the participant's gut due to the presence of deoxyribonucleases and ribonucleases in the small intestine. These enzymes are present to digest the nucleic acids consumed in food. The short time during which the GM plasmid is being selected for (3 days) and the low number of participants (only 100) makes the fairly rare and random events needed for transduction or transformation possible, but unlikely, to occur.

182.   Conjugation is a more deliberate mechanism of horizontal gene transfer involving bacterial plasmids instead of the genome. The mechanism of conjugation is discussed in section 2.2.1 and the explanation of compatible vs incompatible plasmids can be found in section 2.2.2.2. Because bacteria can carry multiple plasmids at once, there is the potential for homologous recombination to occur between plasmids within the same cell.

183.   When a bacterial cell is carrying the target AMR plasmid and the GM curing plasmid, the most likely recombinations are (i) an unstable AMR plasmid that carries *tetA* or *fosA3* (ii) a stable plasmid that has replaced the AMR with either *tetA* or *fosA3*. Figure 12 shows which pairings of plasmids could produce which recombinants. The target AMR plasmid and the GM curing plasmid are incompatible plasmids so they will only co-exist in the same bacterial cell for about one cell cycle. This significantly reduces the opportunity for homologous recombination to occur between these plasmids. All of these potential combinations are less dangerous to the participant than continuing to carry the original stable target AMR plasmid. While TET and FOS are not routinely used to treat infections by *enterobacteriaceae,* the antibiotics that the AMR provides resistance to are important treatment for these infections, especially in the case of life-threatening infections. Participants carrying pJIBE401 have, or at risk of developing, infections that are resistant to the main β-lactams (including carbapenems) and aminoglycosides. Section 3.1.2.1 discusses the danger of carbapenem resistant *enterobacteriaceae.*

1. Homologous recombination between Target AMR and GM curing plasmids (Incompatible plasmids)
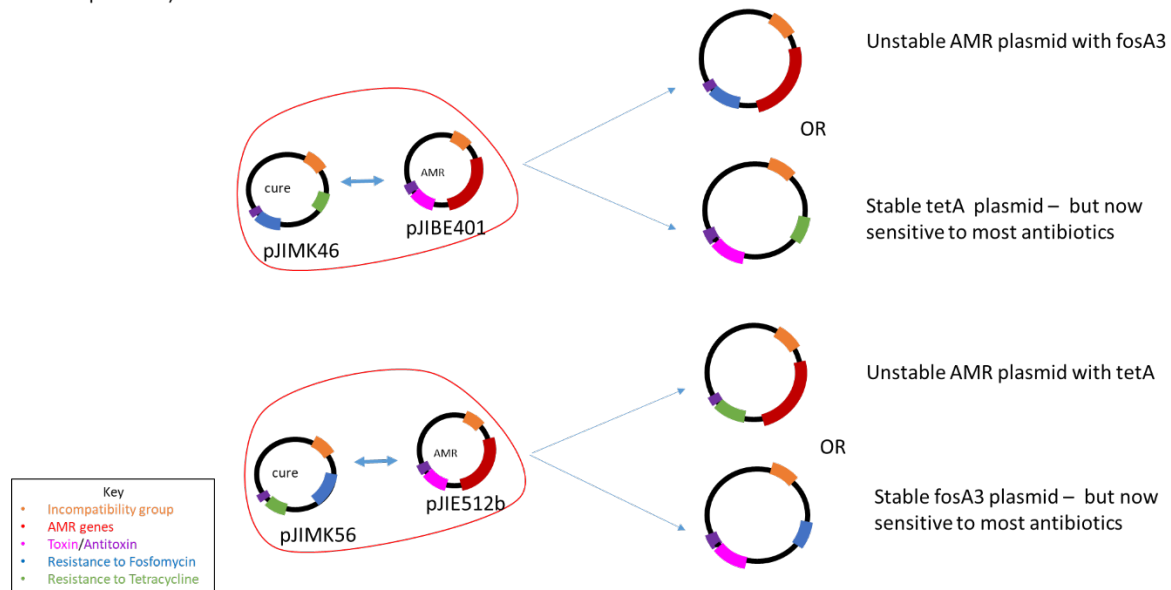

Figure 12. The most likely hybrid plasmids.

184. Occasionally during conjugation, a pair of plasmids are passed together as a large hybrid plasmid called a cointegrant. Figure 13 includes an illustration of a cointegrant. If this were to happen between the target AMR plasmid and the GM curing plasmid, it could result in a plasmid carrying the original AMR and *tetA* and *fosA3* resistance genes. Cointegrants are quite disfavoured because they are metabolically expensive to a cell due to their large size. They tend to be resolved back into two individual plasmids though a process called multimer resolution after cell division. The presence of two copies of the antitoxin gene may further destabilise the cointegrant (Kamruzzaman et al., 2017). In the preclinical experiments described in section 4.2.1**,** no plasmids of this kind were identified. In the case of participants given first tetracycline and then a β-lactam there is an argument that the cointegrant is being selected for but it is unlikely to persist once selection is removed.
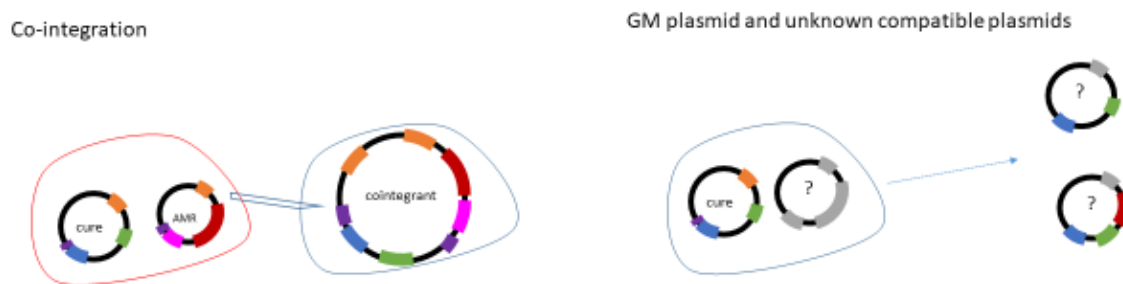

Figure 13. Other hypothetically possible hybrid plasmids. A co-integrant could occur between the target and GM curing plasmid. Alternatively the GM plasmid could combine with an unknown plasmid. This unknown plasmid could carry an AMR that is either the same or different from the target plasmid, or it could carry a pathogenic gene.

185. In addition to the target AMR plasmid, the participant's gut will contain a large number of uncharacterised plasmids with unknown homology relative to the GM plasmid. Each participant in the study will have a somewhat different set of plasmids within their gut *enterobacteriaceae.* Some of these plasmid are likely to be compatible with the GM curing plasmids (section 2.2.2.2 discuses plasmid incompatibility groups). For example, the most abundant group in bacteria are IncF plasmids which would be compatible with either of the GM curing plasmids which are IncL/M and IncI. Some AMR plasmids are carried by IncF plasmids. In figure 13, the genes on these plasmids are represented in grey to illustrate their unknown content. The homology between the unknown plasmids and the GM curing plasmids would clearly be lower than that between the GM and target plasmid, but beyond

this, the regions of homology are impossible to quantify or characterised for these unknown plasmids. The lower the homology the less likely homologous recombination is to occur.

186.   In summary, there are several molecular pathways through which a bacteria could obtain the combination of resistance to TET and FOS in addition to AMR genes or pathogenic genes it carried previously. Although, theoretically possible, no data are currently available demonstrating these recombination events are likely. However, the low number of participants in this trial and the limited opportunity for recombination to occur within the trial participant reduce the likelihood of these recombination occurring.

187.   For context, it is important to know that bacteria which are resistant to TET or FOS in combination with the AMR genes that provide the broadest antibiotic resistance (MBLs) already exist in Australia. As discussed in section 3.1.2.3, it is not uncommon for bacteria resistant to β-lactams to also be resistant to tetracycline. A study into UTIs found that all of those that were resistant to fosfomycin were also resistant to other antibiotics and had ESBL genes (Mowlaboccus et al., 2020). One of the isolates had resistance to TET and FOS and some β-lactams. Genes that provide resistance to FOS are sometimes found as part of a transposable element called IS26, as was the case in this study. fosA3 (or fosA4) that is part of IS26, is more mobile and therefore more likely to recombine than the fosA3 from the GM plasmid in this application. Bacteria that have ESBL genes and fosA3 in have been found in multiple contexts, including pet and food animals, in Japan, China, Taiwan, Korea and Hong Kong (summarised in (Yang et al., 2019)).

Bacteria shed from the participant infects a close contact

188.   The main difference in this step between scenario 2 and scenario 3 is that the resistance to TET and FOS in scenario 3 is more persistent due to genetic recombination. The same measures to reduce the risk of spreading the GMO to contacts described in scenario 2 apply to scenario 3.

Potential harm

189.   In the unlikely event of an illness developing as result of accidental exposure to a bacteria where stable TET or FOS resistance had been acquired though recombination, the infection would be resistant to TET and/or FOS in addition to previously carried resistances.

190.   The addition of TET and FOS resistance is not likely to affect treatment because as is described for scenario 2, these antibiotics have only limited use against infections with *enterobacteriaceae.* The treatment used in these cases would be determined by the previously carried AMR genes.

Conclusion

191.   Scenario 3 is not identified as a risk that could be greater than negligible. Therefore, it does not warrant further detailed assessment.

Risk scenario 4

Risk source	GM bacteria
Causal pathway	Administration of GMO to participant ⬇ Transit through participant's gut ⬇ Within the participant's gut the GM plasmid spreads by conjugation to resident bacteria with the potential to be pathogenic or which are carrying non-targeted AMR plasmids ⬇ Within these bacteria a HGT event occurs resulting in a new hybrid plasmid that carries resistance to TET and FOS in addition to its pre-existing characteristics ⬇ These bacteria are shed by the participant ⬇ Bacteria enter waste water system ⬇ Bacteria survive waste water treatment and enter the environment ⬇ A vulnerable person or animal comes into contact with the bacteria ⬇ Illness caused by gut bacteria additionally resistant to the antibiotics tetracycline and fosfomycin
Potential harm	Illness caused by gut bacteria resistant to the antibiotics tetracycline and fosfomycin

Risk source

192.   The source of potential harm for this postulated risk scenario is GMO2, human gut bacteria that have received the GM plasmid by conjugation. Transfer of the GM curing plasmid from GMO1 to other gut bacteria is an intentional part of the clinical trial. This postulated risk scenario considers a hypothetical *enterobacteriaceae* bacteria that has acquired stable expression of tetracycline and/or fosfomycin in addition to the antibiotic resistances it carried previously. Scenario 3 discusses this bacteria being passed to close contacts of the participant while Scenario 4 discusses potential dispersal in the wider environment.

Causal Pathway

193.   The steps up until the shedding of bacteria into the waste water system are as described in scenario 3. The introduced resistance to TET and FOS in GMO2 will be transient due to the low persistence of the GM plasmid. Only in the case of the unlikely recombination described in scenario 3 would a GM bacteria capable of persisting for the medium term be created.

194.   During the hospital stay (at least four days after the last administration of the GMO) and once the participant returns home, most of the gut bacteria they excrete will enter the local human waste treatment systems.

195.   Initially, the GMOs can potentially outcompete wild-type bacteria and proliferate due to high levels of tetracycline or fosfomycin in untreated hospital waste that could maintain a selective pressure for these bacteria containing the plasmids. This could potentially result in a longer persistence of the GMO in the environment. However, the selective pressure would only be applied transiently and would disappear once the waste proceeds further into the sewage system.

196.   Subsequently, the bacteria would be highly diluted by the water used in the general sewage system. Sydney Water states that waste water is comprised of 99% water, so the bacterial content that is shed from the participant will be diluted about 100-fold. Waste water treatment plants are designed to remove faecal bacteria. As discussed in Chapter 1, section 5.3, wastewater treatment plants should remove at least 95% of the bacteria. Based on location of the clinical trial site, waste water from Westmead hospital is likely to be treated in a WWTP that performs tertiary treatment (see Sydney Water interactive maps webpage), which would reduce the bacterial content even further. Westmead hospital treats approximately 100,000 patients a year. A maximum of 100 participants will be treated with the GMO over the course of 5 years. The nearby WWTPs treat hundreds of millions of litres of water each year. There are some studies that suggest that the initial stages of hospital sewage may be a site of AMR proliferation due to the mix of bacteria and the presence of excreted antibiotics (Cahill et al., 2019). AMR does not enhance bacteria's ability to survive disinfection protocols and the WWTPs around Westmead hospital have tertiary treatment.

197.   Studies reviewed by Nappier, et al. generally agree that the treatment of waste water can reduce the concentration of bacteria but does not considerably reduce the proportion of resistant bacteria (Nappier et al., 2020). Hence, despite wastewater treatment, occasionally some bacteria do make it into the final effluent that enters a river, lake or the sea and could potentially be a source of ARBs and ARGs in the environment (Fouz et al., 2020; Nappier et al., 2020). However, as discussed in Chapter 1, Section 3.1.2.4 and Section 3.1.2.4, tetracycline and fosfomycin resistant genes are already present in the environment.

198.   The ideal temperature for most *enterobacteriaceae* is 37°C so they are not very well adapted to cold temperatures, and do not proliferate well in waterways or the ocean (Bogosian et al., 1998). UV irradiation from the sun also kills *enterobacteriaceae.* Therefore, the amount of GM *enterobacteriaceae* in these environments would remain low.

199.   As mentioned previously, the presence of antibiotics such as tetracycline and fosfomycin can act as a selective pressure in aquatic environments, resulting in the persistence of the GM bacteria. The use of antibiotics and antimicrobials in animal husbandry and aquaculture could potentially contribute to the presence of high levels of tetracycline in the environment. In Australia, the use of antibiotics for veterinary and aquaculture is regulated by the APVMA. Currently, tetracycline is only registered for use in aquarium fish and ornamental birds; and fosfomycin is not registered for veterinary use or aquaculture (APVMA, 2021b). A similar class of antibiotics, oxytetracycline is registered for the use in cattle, pigs and sheep. The APVMA can issue permits on request to allow limited short term use of antibiotics such as tetracycline and fosfomycin. No permits for the use of tetracycline and fosfomycin are currently valid (APVMA, 2021a) and the last permit for the use of oxytetracycline issued for aquaculture expired in 2018 (APVMA, 2021a). In addition, FSANZ sets maximal residual limits on the presence of antibiotics in food. The tight regulation of the use of antibiotics such as tetracycline in agriculture, veterinary and aquaculture further reduces the presence of tetracycline and fosfomycin in the environment and therefore the selective pressure and risk of persistence of the GMOs in the environment.

200.   If a person or animal were to accidently ingest water from these environments, it is highly unlikely the GM bacteria would be at a sufficient concentration to cause illness. The chance of illness caused by shed GM bacteria in waterways is even further reduced by their being only 100 participants in the study. Fish and other aquatic animals generally cannot be colonised by human gut bacteria due to their lower body temperatures (Del Rio-Rodriguez et al., 1997).

201.   The GM plasmid, or any potential derivative, would not increase the ability of bacteria to survive the waste water treatment process or its ability to survive in the environment. Bacteria carrying the GM plasmid are likely to be outcompeted by wild-type bacteria that are not paying the metabolic cost to maintain a plasmid that does not confer any advantage in the aquatic environment. Conjugation requires physical contact between bacteria so dilution drastically reduces the chance of the GM plasmid being passed to new bacteria in the environment.

202.   Wild birds and other animals might come into contact with waste water during the sewage treatment process. However because these animals are generally not treated with antibiotics, the presence of the GM plasmid, or any derivatives, conferring resistance to TET and FOS is unlikely to be of direct practical consequence. Wild animals including sea birds have previously been shown to carry the AMR plasmids. A study of silver gulls in Wollongong were found to carry bla_{IMP4}-$qacG$-$aac4$-$catB3$, which is the AMR cassette found in the target plasmid JIBE401 (Dolejska et al., 2016). It is thought that these gulls obtained AMR plasmids through interaction with sewage treatment.

203.   While resistance to some antibiotics is detected in stock animals and poultry, this is more likely to come from the use of antibiotics in these animals than exposure to human gut bacteria through waste water. This idea is supported by resistance to antibiotics which are used exclusively in humans, such as colistin, not being detected in Australian food animals. Resistance to tetracycline, which can be used in animals, is high among faecal isolates from Australian food animals. More than 50% of isolates from pigs showed tetracycline resistance (Australian Pork Limited, 2017). FOS is not registered for use in animals. Adequate food preparation should kill bacteria before human consumption.

204.   Some human waste does not enter commercial waste water treatment but is instead subject to various types of on-site-treatment. These include septic systems, aerated wastewater treatment system and dry composting toilets. Generally these treatments are less effective at killing bacteria compared to waste water treatment plants. Competition and dilution still occur in these system but may be to a lower extent. Individuals may also use non-standard toilets during activities such as camping. While wild animals could come into direct contact with shed GMO they are unlikely to develop an infection and even more unlikely to be treated with relevant antibiotics. The applicant has proposed that the participants stay at a clinical site for four days after the last administration when shedding is most intense, so these systems would only be applicable after this time.

205.   The reduction and dilution that would occur in the wastewater treatment process make it very unlikely that someone swimming or accidentally consuming water at the site where the effluent is released would become infected or have their gut colonised by GM bacteria.

Potential harm

206.   In the unlikely event of an illness developing in people or animals as result of accidental exposure to a bacteria that has gained stable resistance to TET and FOS through recombination in the environment, the infection could be resistant TET and/or FOS in addition to previously carried resistances or toxin.

207.   The addition of TET and FOS resistance is not likely to affect treatment because as is described for scenario 2, these antibiotics have only limited use against infections with *enterobacteriaceae.* The treatment used in these cases would be determined by the previously carried AMR genes.

Conclusion

208.   Scenario 4 requires all the unlikely events in scenario 3 to occur followed by further unlikely events. Therefore it is not identified as a risk that could be greater than negligible and as such it does not warrant further detailed assessment.

Section 3   Uncertainty

209.   Uncertainty is an intrinsic part of risk analysis[2]. There can be uncertainty in identifying the risk source, the causal linkage to harm, the type and degree of harm, the likelihood of harm or the level of

[2] A more detailed discussion is contained in the Regulator's *Risk Analysis Framework* available from the OGTR website or via Free call 1800 181 030.

risk. In relation to risk management, there can be uncertainty about the effectiveness, efficiency and practicality of controls.

210.   Uncertainty is addressed by approaches such as balance of evidence, conservative assumptions, and there are several types of uncertainty in risk analysis (Clark and Brinkley, 2001; Hayes, 2004; Bammer and Smithson, 2008). These include:

- uncertainty about facts:
  o   knowledge – data gaps, errors, small sample size, use of surrogate data
  o   variability – inherent fluctuations or differences over time, space or group, associated with diversity and heterogeneity
- uncertainty about ideas:
  o   description – expression of ideas with symbols, language or models can be subject to vagueness, ambiguity, context dependence, indeterminacy or under-specificity
  o   perception – processing and interpreting risk is shaped by our mental processes and social/cultural circumstances, which vary between individuals and over time.

211.   Uncertainty is addressed by approaches such as balance of evidence, conservative assumptions, and applying risk management measures that reduce the potential for risk scenarios involving uncertainty to lead to harm. If there is residual uncertainty that is important to estimating the level of risk, the Regulator will take this uncertainty into account in making decisions.

212.   As clinical trials are designed to gather data, there are generally data gaps when assessing the risks of a clinical trial application involving GMOs. However, clinical trial applications are required to be limited and controlled. Even if there is uncertainty about the characteristics of a GMO, limits and controls restrict exposure to the GMO, and thus decrease the likelihood of harm.

213.   For DIR-183, uncertainty is noted in relation to the extent and duration of GMO shedding from human participants. As this is a first in human trial, the applicant has conducted non-clinical studies relating to these subject matters in a model species as described in section 4.2.1. The transferability of results from mice to humans is always somewhat uncertain. To account for this risk scenarios 2, 3 and 4 consider shedding beyond the length of time observed in the pre-clinical studies. Due to the underlying uncertainty about how frequently recombination occurs, risk scenario 3 and risk scenario 4 take into account theoretically possible unfavourable events that were not observed in the pre-clinical studies.

214.   Additional data, including information to address these uncertainties, may be required to assess possible future applications with reduced limits and controls, such as a larger scale trial or the commercial release of these GMOs. Section 4 discusses information that may be required for future release.

Section 4   Risk evaluation

215.   Risk is evaluated against the objective of protecting the health and safety of people and the environment to determine the level of concern and, subsequently, the need for controls to mitigate or reduce risk. Risk evaluation may also aid consideration of whether the proposed dealings should be authorised, need further assessment, or require collection of additional information.

216.   Factors used to determine which risks need treatment may include:

- risk criteria
- level of risk
- uncertainty associated with risk characterisation
- interactions between substantive risks.

217.   Four risk scenarios were postulated whereby the proposed dealings might give rise to harm to people or the environment. In the context of the limits and controls proposed by the applicant, and considering both the short and long term, none of these scenarios were identified as substantive risks. The principal reasons for these conclusions are:

- Suitability of limits and controls proposed by the applicant.

- The GMO is designed to be self-limiting and persist for only a short period of time. However consideration was given to events that may result in the GM becoming stable.

- Limited ability and opportunity for the GM to be transferred by horizontal gene transfer mechanisms

- The GM would not be a novel addition to the receiving environment

218.   Therefore, risks to the health and safety of people, or the environment, from the proposed release of the GMO into the environment are considered to be negligible. The *Risk Analysis Framework* (OGTR, 2013a), which guides the risk assessment and risk management process, defines negligible risks as risks of no discernible concern with no present need to invoke actions for mitigation. Therefore, no additional controls are required to treat these negligible risks. Hence, the Regulator considers that the dealings involved in this proposed release do not pose a significant risk to either people or the environment.

Chapter 3   Risk management plan

Section 1   Background

219.   Risk management is used to protect the health and safety of people and to protect the environment by controlling or mitigating risk. The risk management plan addresses risks evaluated as requiring treatment and considers limits and controls proposed by the applicant, as well as general risk management measures. The risk management plan informs the Regulator's decision-making process and is given effect through licence conditions.

220.   Under Section 56 of the Act, the Regulator must not issue a licence unless satisfied that any risks posed by the dealings proposed to be authorised by the licence can be managed in a way that protects the health and safety of people and the environment.

221.   All licences are subject to three conditions prescribed in the Act. Section 63 of the Act requires that each licence holder inform relevant people of their obligations under the licence. The other statutory conditions allow the Regulator to maintain oversight of licensed dealings: Section 64 requires the licence holder to provide access to premises to OGTR inspectors and Section 65 requires the licence holder to report any information about risks or unintended effects of the dealing to the Regulator on becoming aware of them. Matters related to the ongoing suitability of the licence holder must also be reported to the Regulator.

222.   The licence is also subject to any conditions imposed by the Regulator. Examples of the matters to which conditions may relate are listed in Section 62 of the Act. Licence conditions can be imposed to limit and control the scope of the dealings and to manage risk to people or the environment. In addition, the Regulator has extensive powers to monitor compliance with licence conditions under Section 152 of the Act.

Section 2   Risk treatment measures for substantive risks

223.   The risk assessment of risk scenarios listed in Chapter 2 concluded that there are negligible risks to people and the environment from the proposed clinical trial of GMO. These risk scenarios were considered in the context of the scale of the proposed clinical trial, the proposed controls, and the receiving environments, and considering both the short and the long term. The risk evaluation concluded that no specific risk treatment measures are required to treat these negligible risks. Limits and controls proposed by the applicant and other general risk management measures are discussed below.

Section 3   General risk management

224.   The limits and controls proposed in the application were important in establishing the context for the risk assessment and in reaching the conclusion that the risks posed to people and the environment are negligible. Therefore, to maintain the risk context, licence conditions have been imposed to limit the number of trial participants, location limited to hospitals and clinical trial sites, limits on the duration of the trial, as well as a range of controls to restrict the spread and unlikely persistence of the GMOs and their genetic material in the environment. The conditions are discussed and summarised in this Chapter and listed in detail in the licence.

3.1   Limits and controls on the clinical trial

225.   Many of the proposed controls are discussed in the four risk scenarios considered in chapter 2. The appropriateness of the limits and controls is considered further in the following sections.

3.1.1   *Consideration of limits and controls proposed by the applicant*

226.   The proposed clinical trial would involve a maximum of 100 participants at Westmead hospital facilities, and most dealings with the GMOs would take place in medical facilities such as clinical trial units, hospitals and analytical laboratory facilities. Activities that would occur outside of medical facilities include preparation, transport, storage and analysis of the GMOs. Preparation and transport to the clinical trial site is permitted under an NLRD. Administration of the GMO must be conducted within the clinical trial site and a condition is included in the licence to reflect this. Transport and disposal of trial participant samples likely to contain GMOs, will be conducted according to the OGTR guidelines for *Transport, Storage and Disposal* which are included in the licence. Analysis of trial samples likely to contain the GMOs will be conducted in PC2 certified facilities. The applicant has proposed to complete the study within 5 years of commencement. Conditions maintaining the risk context and proposed limits of the trial such as the maximum number of trial participants and duration of the study and have been included in the licence.

227.   There are proposed inclusion and exclusion criteria for both trial participants and staff as listed in section 1.3. The inclusion and exclusion criteria for trial participants would be subject to approval by a HREC, who would consider the safety of the individuals involved in the trial. The licence requires that trial participants who are pregnant or breastfeeding are excluded. As discussed in section 2.3 infants often acquire part of their microbiome from their mothers. Additionally, the guts of infants are often more easily colonised than adults and antibiotic use is higher in young children (Yang et al., 2016). This has been included in the licence.

228.   Once at the clinical trial site, access to the GMO would be restricted to appropriately trained personnel. The applicant advised that the GMO would be administered to trial participants on three consecutive days via either ingestion or a pre-existing nasogastric or nasoduodenal tube. The applicant has also proposed that clinical staff would wear PPE including gown, gloves, mask and eye protection. These practices would minimise exposure of people handling and administering GMO1 (Risk scenario 1) and have been included in the licence conditions.

229.   Conditions are included in the licence requiring the licence holder to ensure that all GMOs, including material or waste that has been in contact with the GMO, within the clinical trial site, are decontaminated by autoclaving, chemical treatment or by high-temperature incineration. Licence conditions require that the licence holder must ensure that the GMO, or material or waste that has been in contact with the GMO, that is to be destroyed by external service providers, is through a clinical waste stream. This is considered satisfactory, provided that the licence holder is only permitted to engage persons who can adhere to appropriate standards to conduct the dealings, as described in Paragraph 233.

230.   The Industry Code of Practice for the Management of Clinical and Related Wastes details requirements for clinical waste including waste segregation, packaging, labelling, storage, transport and accountability (Biohazard Waste Industry, 2010). The clinical waste stream typically involves destruction of infectious waste by incineration or autoclaving, which are considered appropriate for disposal of the GMO. Given that *E.coli* can persist in the environment disposal measures such as burial or maceration would not ensure containment. Therefore, the licence also requires waste disposal by external service providers to be by autoclaving or high-temperature incineration. These measures would limit the exposure of people or other animals to the GMOs.

231.   The applicant has proposed to provide participants with instructions on good hand hygiene practices because these will limit the potential for shed GMO to be passed to close contacts. Where relevant these instructions will be given to the participant's carer. This has been included in the licence.

232.   The applicant has proposed to give participants instructions for taking samples at home where applicable. The applicant will provide sufficient containers and sealable plastic bags to ensure

transport between the participant's home and the site of analysis meet the Regulator's *Guidelines for the Transport, Storage and Disposal of GMOs*. A condition to ensure the licence holder obtain written agreement from the trial participant to follow instruction provided following administration has been included in the licence as well as a condition requiring the licence holder to provide the applicant with appropriate containers for the collection of samples.

233.   A standard condition is included in the licence requiring the licence holder to ensure that dealings are conducted so as to ensure containment of the GMO, not compromise the health and safety of people and minimise unintentional exposure to the GMO. A note written under the condition explains that compliance may be achieved by only engaging persons who are required to adhere to appropriate standards to conduct the dealings.

234.   Other conditions included in the licence are standard conditions that state that only people authorised by the licence holder are covered by the licence, and that the licence holder must inform all people dealing with the GMOs, other than external service providers, of applicable licence conditions.

3.1.2   *Summary of licence conditions to be implemented to limit and control the clinical trial*

235.   A number of licence conditions have been imposed to limit and control the proposed clinical trial, based on the above considerations. These include requirements to:

- limit the trial to 100 trial participants, which are to be conducted at clinical trial sites (Westmead Hospital)
- restrict access to the GMO
- ensure personnel involved in the trial are appropriately trained and follow appropriate behavioural requirements
- ensure appropriate PPE is used
- restrict personnel permitted to administer the GMO
- requiring decontamination of the GMO and materials and equipment that have been in contact with the GMO at clinical trial sites using effective disinfectants or disposal using a certified waste contractor in accordance with standard clinical waste disposal practices, as required by the relevant Australian and state legislation
- clinical waste stream to be used by external service providers to destroy untreated GMO and GMO-related waste

3.2   Other risk management considerations

236.   All DIR licences issued by the Regulator contain a number of conditions that relate to general risk management. These include conditions relating to:

- applicant suitability
- contingency plans
- identification of the persons or classes of persons covered by the licence
- reporting requirements
- access for the purpose of monitoring for compliance.

3.2.1   *Applicant suitability*

237.   In making a decision whether or not to issue a licence, the Regulator must have regard to the suitability of the applicant to hold a licence. Under Section 58 of the Act, matters that the Regulator must take into account include:

- any relevant convictions of the applicant
- any revocation or suspension of a relevant licence or permit held by the applicant under a law of the Commonwealth, a State or a foreign country
- the capacity of the applicant to meet the conditions of the licence.

238.   If a licence were issued, the conditions would include a requirement for the licence holder to inform the Regulator of any information that would affect their suitability.

239.   In addition, the applicant organisation must have access to an IBC and be an accredited organisation under the Act.

3.2.2   Contingency plans

240.   Should a licence be issued, The Westmead Institute of Medical Research is required to submit a contingency plan to the Regulator before commencing dealings with the GMOs. This plan will detail measures to be undertaken in the event of:

- the unintended release of the GMOs, including spills
- exposure of, or transmission to persons other than trial participants
- a person exposed to the GMOs developing a serious adverse response.

3.2.3   Identification of the persons or classes of persons covered by the licence

241.   If issued, the persons covered by the licence would be the licence holder and employees, agents or contractors of the licence holder and other persons who are, or have been, engaged or otherwise authorised by the licence holder to undertake any activity in connection with the dealings authorised by the licence. Prior to dealings with the GMOs, The Westmead Institute of Medical Research is required to provide a list of people and organisations that are covered by the licence, or the function or position where names are not known at the time.

3.2.4   Reporting requirements

242.   If issued, the licence would require the licence holder to immediately report any of the following to the Regulator:

- any additional information regarding risks to the health and safety of people or the environment associated with the dealings
- any contraventions of the licence by persons covered by the licence
- any unintended effects of the clinical trial.

243.   A number of written notices are also required under the licence regarding dealings with the GMO, to assist the Regulator in designing and implementing a monitoring program for all licensed dealings. The notices include:

- identification of the clinical trial sites where administration of the GMO to trial participants would take place
- expected date of administration with the GMOs for each clinical trial site
- cease of administration with the GMOs for each clinical trial site

3.2.5   Monitoring for compliance

244.   The Act stipulates, as a condition of every licence, that a person who is authorised by the licence to deal with a GMO, and who is required to comply with a condition of the licence, must allow inspectors and other persons authorised by the Regulator to enter premises where a dealing is being undertaken for the purpose of monitoring or auditing the dealing.

245.   If monitoring activities identify changes in the risks associated with the authorised dealings, the Regulator may also vary licence conditions, or if necessary, suspend or cancel the licence.

246.   In cases of non-compliance with licence conditions, the Regulator may instigate an investigation to determine the nature and extent of non-compliance. The Act provides for criminal sanctions of large fines and/or imprisonment for failing to abide by the legislation, conditions of the licence or directions from the Regulator, especially where significant damage to the health and safety of people or the environment could result.

Section 4   Issues to be addressed for future releases

247.   Additional information has been identified that may be required to assess an application for a commercial release of the GMO, or to justify a reduction in limits and controls. This includes:

- Quantity and duration of shedding of GMO bacteria in participants faeces
- Characterisation of bacteria where recombination resulting in stable expression of *tetA* or *fosA3* has occurred.

Section 5   Conclusions of the consultation RARMP

248.   The risk assessment concludes that the proposed clinical trial of the GMOs poses negligible risks to the health and safety of people or the environment as a result of gene technology. The general increase of bacteria resistant to antibiotics poses a risk to health and the environment, but the gene technology used in this application does not appreciably increase this risk. These negligible risks do not require specific risk treatment measures.

249.   If a licence is issued, conditions are imposed to limit the trial to the proposed scale, location and duration, and to restrict the spread and persistence of the GMOs and its genetic material in the environment, as these were important considerations in establishing the context for assessing the risks.

References

AAP (2018). Report warns superbug E.coli driving Australia sepsis rates. (HealthTimes: https://healthtimes.com.au/hub/infection-control/33/news/aap/report-warns-superbug-ecoli-driving-australia-sepsis-rates/3202/).

Adeolu, M., Alnajar, S., Naushad, S., and S. Gupta, R. (2016). Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. Int J Syst Evol Microbiol *66*, 5575-5599.

Ahmed, W., Staley, C., Sidhu, J., Sadowsky, M., and Toze, S. (2017). Amplicon-based profiling of bacteria in raw and secondary treated wastewater from treatment plants across Australia. Applied microbiology and biotechnology *101*, 1253-1266.

Anastasi, E.M., Matthews, B., Gundogdu, A., Vollmerhausen, T.L., Ramos, N.L., Stratton, H., Ahmed, W.*, et al.* (2010). Prevalence and persistence of Escherichia coli strains with uropathogenic virulence characteristics in sewage treatment plants. Appl Environ Microbiol *76*, 5882-5886.

Anastasi, E.M., Matthews, B., Stratton, H.M., and Katouli, M. (2012). Pathogenic Escherichia coli found in sewage treatment plants and environmental waters. Appl Environ Microbiol *78*, 5536-5541.

APVMA (2017). Antibiotic resistance in animals (The Australian Pesticides and Veterinary Medicines Authority).

APVMA (2021a). Agricultural and Veterinary Permits Search. Accessed: 22 June 2021.

APVMA (2021b). Public Chemical Registration Information System (PubCRIS) Search. Accessed: 22 June 2021.

ASTAG (2018). Importance Ratings and Summary of Antibacterial Uses in Human and Animal Health in Australia. (The Commonwealth of Australia).

Australian Commission on Safety and Quality in Health Care (ACSQHC) (2019). AURA 2019: third Australian report on antimicrobial use and resistance in human health. In ACSQHC, D.o. Health, ed. (Sydney).

Australian Pork Limited (2017). Surveillance for antimicrobial resistance in enteric

commensals and pathogens in Australian pigs (http://australianpork.com.au/wp-content/uploads/2019/02/Summary-AMR-surveillance-study.pdf).

Bammer, G., and Smithson, M. (2008). Uncertainty and risk: Multidisciplinary perspectives (London: Earthscan).

Bell, J., M. Fajardo Lubian, Alicia. Partridge, Sally. Gottlieb,Thomas. Iredell, Jonathan. Daley, Deneise, A. Coombs, Geoffrey, W. (2019). Australian Group on Antimicrobial Resistance (AGAR) Australian Gram-negative Sepsis Outcome Programme (GNSOP) Annual Report 2019.

Berg, C.M., Grullon, C.A., Wang, A., Whalen, W.A., and Berg, D.E. (1983). Transductional instability of Tn5-induced mutations: Generalized and specialized transduction of Tn5 by bacteriophage P1. Genetics *105*, 259-263.

Biohazard Waste Industry (2010). Industry Code of Practice for the Management of Clinical and Related Wastes, 6 edn.

Blokesch, M. (2016). Natural competence for transformation. Current Biology *26*, R1126-R1130.

Bogosian, G., Morris, P.J.L., and O'Neil, J.P. (1998). A Mixed Culture Recovery Method Indicates that Enteric Bacteria Do Not Enter the Viable but Nonculturable State. Appl Environ Microbiol *64*, 1736-1742.

Buckner, M.M.C., Ciusa, M.L., and Piddock, L.J.V. (2018). Strategies to combat antimicrobial resistance: anti-plasmid and plasmid curing. FEMS Microbiol Rev *42*, 781-804.

Bush, K. (2018). Past and Present Perspectives on β-Lactamases. Antimicrobial Agents and Chemotherapy *62*, e01076-01018.

Cahill, N., O'Connor, L., Mahon, B., Varley, Á., McGrath, E., Ryan, P., Cormican, M.*, et al.* (2019). Hospital effluent: A reservoir for carbapenemase-producing Enterobacterales? Science of The Total Environment *672*, 618-624.

Cattoir, V., and Guerin, F. (2018). How is fosfomycin resistance developed in Escherichia coli? Future Microbiol *13*, 1693-1696.

Chopra, I., and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev *65*, 232-260 ; second page, table of contents.

Chung, P.Y. (2016). The emerging problems of Klebsiella pneumoniae infections: carbapenem resistance and biofilm formation. FEMS Microbiology Letters *363*.

Clark, A.J., and Brinkley, T. (2001). Risk management: for climate, agriculture and policy. (Canberra: Commonwealth of Australia).

Coombs G, B.J., Daley D, Collignon P, Cooley L, Gottlieb T, Iredell J, Warner M, Nimmo, and, G.a.R.J.o.b.o.t.A.G.o.A.R., and Care", A.C.o.S.a.Q.i.H. (2019). Australian Group on Antimicrobial Resistance Sepsis Outcomes Programs: 2019 Report. (Sydney).

Craig, N. (2014). Molecular Biology - Principles of Genome Function, 2nd Edition edn.

Cummings, J.H., Bingham, S.A., Heaton, K.W., and Eastwood, M.A. (1992). Fecal weight, colon cancer risk, and dietary intake of nonstarch polysaccharides (dietary fiber). Gastroenterology *103*, 1783-1789.

de Smet, A.M.G.A., Kluytmans, J.A.J.W., Cooper, B.S., Mascini, E.M., Benus, R.F.J., van der Werf, T.S., van der Hoeven, J.G.*, et al.* (2009). Decontamination of the Digestive Tract and Oropharynx in ICU Patients. N Engl J Med *360*, 20-31.

Del Rio-Rodriguez, R.E., Inglis, V., and Millar, S.D. (1997). Survival of Escherichia coli in the intestine of fish. *28*, 257-264.

Dionisio, F., Zilhão, R., and Gama, J.A. (2019). Interactions between plasmids and other mobile genetic elements affect their transmission and persistence. Plasmid *102*, 29-36.

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., Carlile, N.*, et al.* (2016). High prevalence of Salmonella and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. J Antimicrob Chemother *71*, 63-70.

Ellem, J.A., Ginn, A.N., Chen, S.C., Ferguson, J., Partridge, S.R., and Iredell, J.R. (2017). Locally Acquired mcr-1 in Escherichia coli, Australia, 2011 and 2013. Emerg Infect Dis *23*, 1160-1163.

Espedido, B., Iredell, J., Thomas, L., and Zelynski, A. (2005). Wide dissemination of a carbapenemase plasmid among gram-negative bacteria: implications of the variable phenotype. Journal of clinical microbiology *43*, 4918-4919.

Fouz, N., Pangesti, K.N.A., Yasir, M., Al-Malki, A.L., Azhar, E.I., Hill-Cawthorne, G.A., and Abd El Ghany, M. (2020). The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. Trop Med Infect Dis *5*.

Frese, S.A., Hutton, A.A., Contreras, L.N., Shaw, C.A., Palumbo, M.C., Casaburi, G., Xu, G.*, et al.* (2017). Persistence of Supplemented Bifidobacterium longum subsp. infantis EVC001 in Breastfed Infants. mSphere *2*.

Garcia, A., Navarro, F., Miro, E., Villa, L., Mirelis, B., Coll, P., and Carattoli, A. (2007). Acquisition and diffusion of bla CTX-M-9 gene by R478-IncHI2 derivative plasmids. FEMS Microbiol Lett *271*, 71-77.

Gardiner, B.J., Stewardson, A.J., Abbott, I.J., and Peleg, A.Y. (2019). Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. Aust Prescr *42*, 14-19.

Gordon, D.M., and Cowling, A. (2003). The distribution and genetic structure of Escherichia coli in Australian vertebrates: Host and geographic effects. Microbiology (Reading) *149*, 3575-3586.

Grossman, T.H. (2016). Tetracycline Antibiotics and Resistance. Cold Spring Harb Perspect Med *6*, a025387.

Hale, L., Lazos, O., Haines, A., and Thomas, C. (2010). An efficient stress-free strategy to displace stable bacterial plasmids. Biotechniques *48*, 223-228.

Harrison, E., and Brockhurst, M.A. (2012). Plasmid-mediated horizontal gene transfer is a coevolutionary process. Trends in Microbiology *20*, 262-267.

Hayes, K.R. (2004). Ecological implications of GMOs: robust methodologies for ecological risk assessment. Best practice and current practice in ecological risk assessment for genetically modified organisms. (Tasmania: CSIRO Division of Marine Research).

healthdirect (2020). Urinary tract infection (UTI). (https://www.healthdirect.gov.au/urinary-tract-infection-uti).

healthdirect (2021). Doxycycline. (https://www.healthdirect.gov.au/doxycycline).

Hudault, S., Guignot, J., and Servin, A. (2001). Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. Gut *49*, 47-55.

Iovleva, A., and Doi, Y. (2017). Carbapenem-Resistant Enterobacteriaceae. Clinics in laboratory medicine *37*, 303-315.

Jang, J., Hur, H.G., Sadowsky, M.J., Byappanahalli, M.N., Yan, T., and Ishii, S. (2017). Environmental Escherichia coli: ecology and public health implications-a review. J Appl Microbiol *123*, 570-581.

Jones, R.N., Flonta, M., Gurler, N., Cepparulo, M., Mendes, R.E., and Castanheira, M. (2014). Resistance surveillance program report for selected European nations (2011). Diagn Microbiol Infect Dis *78*, 429-436.

Joosten, P., Ceccarelli, D., Odent, E., Sarrazin, S., Graveland, H., Van Gompel, L., Battisti, A.*, et al.* (2020). Antimicrobial Usage and Resistance in Companion Animals: A Cross-Sectional Study in Three European Countries. Antibiotics (Basel) *9*.

Kaas, R.S., Friis, C., Ussery, D.W., and Aarestrup, F.M. (2012). Estimating variation within the genes and inferring the phylogeny of 186 sequenced diverse Escherichia coli genomes. BMC Genomics *13*, 577-Article.

Kamruzzaman, M., Shoma, S., Thomas, C.M., Partridge, S.R., and Iredell, J.R. (2017). Plasmid interference for curing antibiotic resistance plasmids in vivo. PLOS ONE *12*, e0172913.

Kaper, J.B., Morris, J.G., Jr., and Levine, M.M. (1995). Cholera. Clinical microbiology reviews *8*, 48-86.

Kay, D., Crowther, J., Stapleton, C.M., Wyer, M.D., Fewtrell, L., Edwards, A., Francis, C.A.*, et al.* (2008). Faecal indicator organism concentrations in sewage and treated effluents. Water Res *42*, 442-454.

Lasaro, M.A., Salinger, N., Zhang, J., Wang, Y., Zhong, Z., Goulian, M., and Zhu, J. (2009). F1C Fimbriae Play an Important Role in Biofilm Formation and Intestinal Colonization by the Escherichia coli Commensal Strain Nissle 1917. APPLIED AND ENVIRONMENTAL MICROBIOLOGY *75*, 246-251.

Lee, Y.Y., Erdogan, A., and Rao, S.S.C. (2014). How to assess regional and whole gut transit time with wireless motility capsule. Journal of neurogastroenterology and motility *20*, 265-270.

Licht, T.R., Tolker-Nielsen, T., Holmstrom, K., Krogfelt, K.A., and Molin, S. (1999). Inhibition of Escherichia coli precursor-16S rRNA processing by mouse intestinal contents. Environmental Microbiology *1*, 23-32.

Lim, J.Y., Yoon, J., and Hovde, C.J. (2010). A brief overview of Escherichia coli O157:H7 and its plasmid O157. Journal of microbiology and biotechnology *20*, 5-14.

Lodinová-Zádniková, R., and Sonnenborn, U. (1997). Effect of preventive administration of a nonpathogenic Escherichia coli strain on the colonization of the intestine with microbial pathogens in newborn infants. Biol Neonate *71*, 224-232.

Lynch, S.V., and Pedersen, O. (2016). The Human Intestinal Microbiome in Health and Disease. N Engl J Med *375*, 2369-2379.

Miller, L.E., Zimmermann, A.K., and Ouwehand, A.C. (2016). Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit. World J Gastroenterol *22*, 5122-5131.

Moet, G.J., Jones, R.N., Biedenbach, D.J., Stilwell, M.G., and Fritsche, T.R. (2007). Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998-2004). Diagn Microbiol Infect Dis *57*, 7-13.

Mowlaboccus, S., Daley, D., Pang, S., Gottlieb, T., Merlino, J., Nimmo, G.R., George, N.*, et al.* (2020). Identification and characterisation of fosfomycin resistance in Escherichia coli urinary tract infection isolates from Australia. International Journal of Antimicrobial Agents *56*, 106121.

Murphy, K., Curley, D., O'Callaghan, T.F., O'Shea, C.A., Dempsey, E.M., O'Toole, P.W., Ross, R.P.*, et al.* (2017). The Composition of Human Milk and Infant Faecal Microbiota Over the First Three Months of Life: A Pilot Study. Sci Rep *7*, 40597.

Nappier, S.P., Liguori, K., Ichida, A.M., Stewart, J.R., and Jones, K.R. (2020). Antibiotic Resistance in Recreational Waters: State of the Science. Int J Environ Res Public Health *17*.

Norman, A., Hansen, L.H., and Sorensen, S.J. (2009). Conjugative plasmids: vessels of the communal gene pool. Philos Trans R Soc Lond B Biol Sci *364*, 2275-2289.

Novick, R.P. (1987). Plasmid incompatibility. Microbiol Rev *51*, 381-395.

NSW Goverment (2018). Shiga toxigenic Escherichia coli (STEC) Infection and Haemolytic Uraemic Syndrome (HUS).

OGTR (2013a). Risk Analysis Framework 2013, 4th edn (Canberra: Office of the Gene Technology Regulator, available online: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/raffinal5-toc).

OGTR (2013b). Risk Analysis Framework 2013. (Office of the Gene Technology Regulator) Accessed: July 2020.

Oong, G.C., and Tadi, P. (2020). Chloramphenicol. In StatPearls (Treasure Island (FL)).

Outbreak Project (2020). New research reveals Australia's multi-billion dollar superbug crisis. (https://outbreakproject.com.au/2020/11/17/australias-multi-billion-dollar-superbug-crisis/).

Padmanabhan, P., Grosse, J., Asad, A.B.M.A., Radda, G.K., and Golay, X. (2013). Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT. EJNMMI research *3*, 60-60.

Pandey, N., and Cascella, M. (2020). Beta Lactam Antibiotics. In StatPearls (Treasure Island (FL)).

Patel, G., Huprikar, S., Factor, S.H., Jenkins, S.G., and Calfee, D.P. (2008). Outcomes of Carbapenem-Resistant Klebsiella pneumoniae Infection and the Impact of Antimicrobial and Adjunctive Therapies. Infection Control & Hospital Epidemiology *29*, 1099-1106.

Peleg, A.Y., Franklin, C., Bell, J.M., and Spelman, D.W. (2005). Dissemination of the Metallo-β-Lactamase Gene blaIMP-4 among Gram-Negative Pathogens in a Clinical Setting in Australia. Clinical Infectious Diseases *41*, 1549-1556.

Poulsen, L.K., Lan, F., Kristensen, C.S., Hobolth, P., Molin, S., and Krogfelt, K.A. (1994). Spatial Distribution of Escherichia coli in the Mouse Large Intestine Inferred from rRNA In Situ Hybridization. Infection and Immunity *62*, 5191-5194.

Rang, C.U., Licht, T.R., Midtvedt, T., Conway, P.L., Chao, L., Krogfelt, K.A., Cohen, P.S.*, et al.* (1999). Estimation of growth rates of Escherichia coli BJ4 in streptomycin-treated and previously germfree mice by in situ rRNA hybridization. Clinical and Diagnostic Laboratory Immunology *6*, 434-436.

Rastegarlari, A., Gold, F., Borderon, J.C., Laugier, J., and Lafont, J. (1990). Implantation and In-Vivo Antagonistic Effects of Antibiotic-Susceptible Escherichia-Coli Strains Administered to Premature Newborns. Biology of the Neonate *58*, 73-78.

Rauch, S., Krueger, K., Turan, A., Roewer, N., and Sessler, D.I. (2009). Determining small intestinal transit time and pathomorphology in critically ill patients using video capsule technology. Intensive Care Med *35*, 1054-1059.

Reyhanoglu, G., and Reddivari, A.K.R. (2020). Tobramycin. In StatPearls (Treasure Island (FL): StatPearls, LLC).

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.A.D., Gasbarrini, A., and Mele, M.C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms *7*, 14.

Russell, A.D. (1990). Bacterial spores and chemical sporicidal agents. Clin Microbiol Rev *3*, 99-119.

Russell, J.B., and Jarvis, G.N. (2001). Practical mechanisms for interrupting the oral-fecal lifecycle of Escherichia coli. Journal of Molecular Microbiology and Biotechnology *3*, 265-272.

Russo, T.A., and Johnson, J.R. (2003). Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. Microbes and Infection *5*, 449-456.

Rutala, W.A., Weber, D.J., and Healthcare Infection Control Practices Advisory Committee (2008). Guideline for Disinfection and Sterilization in Healthcare Facilities. (United States: Centres for Disease Control and Prevention).

Savageau, M.A. (1983). Escherichia-Coli Habitats Cell Types and Molecular Mechanisms of Gene Control. American Naturalist *122*, 732-744.

Schamberger, G.P., Phillips, R.L., Jacobs, J.L., and Diez-Gonzalez, F. (2004). Reduction of Escherichia coli O157:H7 populations in cattle by addition of colicin E7-producing E. coli to feed. Applied and Environmental Microbiology *70*, 6053-6060.

Sender, R., Fuchs, S., and Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol *14*, e1002533.

Shanks, O.C., Newton, R.J., Kelty, C.A., Huse, S.M., Sogin, M.L., and McLellan, S.L. (2013). Comparison of the microbial community structures of untreated wastewaters from different geographic locales. Appl Environ Microbiol *79*, 2906-2913.

Sherley, M., Gordon, D.M., and Collignon, P.J. (2003). Species differences in plasmid carriage in the Enterobacteriaceae. Plasmid *49*, 79-85.

Shintani, M., Sanchez, Z.K., and Kimbara, K. (2015). Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front Microbiol *6*, 242.

Shutter, M.C., and Akhondi, H. (2020). Tetracycline. In StatPearls (Treasure Island (FL)).

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., and Wuertz, S. (2005). Studying plasmid horizontal transfer in situ: a critical review. Nature Reviews Microbiology *3*, 700-710.

Standards Australia/New Zealand (2010). Safety in laboratories - Microbiological safety and containment AS/NZS 2243.3:2010.

Stefaniuk, E.M., and Tyski, S. (2019). Colistin Resistance in Enterobacterales Strains - A Current View. Polish journal of microbiology *68*, 417-427.

Tagg, K.A., Iredell, J.R., and Partridge, S.R. (2014). Complete Sequencing of IncI1 Sequence Type 2 Plasmid pJIE512b Indicates Mobilization of bla-CMY-2 from an IncA/C Plasmid. Antimicrobial Agents and Chemotherapy *58*, 4949-4952.

Tenaillon, O., Skurnik, D., Picard, B., and Denamur, E. (2010). The population genetics of commensal Escherichia coli. Nature Reviews Microbiology *8*, 207-217.

The Department of Health (2020). The Australian Government has released Australia's National Antimicrobial Resistance Strategy - 2020 and Beyond Health, ed. (The Commonwealth of Australia).

Toze, S., Hodgers, L., Matthews, B., Stratton, H., Ahmed, W., Collins, S., Schoeder, S.*, et al.* (2012). Presence and removal of enteric microorganisms in South East Queensland wastewater treatment plants. . (Urban Water Security Research Alliance; 2012. http://hdl.handle.net/102.100.100/100932?index=1).

Vally, H., Hall, G., Dyda, A., Raupach, J., Knope, K., Combs, B., and Desmarchelier, P. (2012). Epidemiology of Shiga toxin producing Escherichia coli in Australia, 2000-2010. BMC Public Health *12*, 63.

van Hal, S.J., Wiklendt, A., Espedido, B., Ginn, A., and Iredell, J.R. (2009). Immediate appearance of plasmid-mediated resistance to multiple antibiotics upon antibiotic selection: an argument for systematic resistance epidemiology. J Clin Microbiol *47*, 2325-2327.

Vila, J., Sáez-López, E., Johnson, J.R., Römling, U., Dobrindt, U., Cantón, R., Giske, C.G.*, et al.* (2016). Escherichia coli: an old friend with new tidings. FEMS Microbiol Rev *40*, 437-463.

Vollaard, E., and Clasener, H. (1994). Mini-review: Colonization resistance. Antimicrobial Agents and Chemotherapy *38*, 409-414.

Wang, Q., Zhang, Y., Yao, X., Xian, H., Liu, Y., Li, H., Chen, H.*, et al.* (2016). Risk factors and clinical outcomes for carbapenem-resistant Enterobacteriaceae nosocomial infections. Eur J Clin Microbiol Infect Dis *35*, 1679-1689.

Wassenaar, T.M. (2016). Insights from 100 Years of Research with Probiotic E. Coli. European journal of microbiology & immunology *6*, 147-161.

Weir, C.B., and Le, J.K. (2020). Metronidazole. In StatPearls (Treasure Island (FL)).

Wozniak, T.M., Bailey, E.J., and Graves, N. (2019). Health and economic burden of antimicrobial-resistant infections in Australian hospitals: a population-based model. Infect Control Hosp Epidemiol *40*, 320-327.

Yang, I., Corwin, E.J., Brennan, P.A., Jordan, S., Murphy, J.R., and Dunlop, A. (2016). The Infant Microbiome: Implications for Infant Health and Neurocognitive Development. Nursing research *65*, 76-88.

Yang, T.-Y., Lu, P.-L., and Tseng, S.-P. (2019). Update on fosfomycin-modified genes in Enterobacteriaceae. Journal of Microbiology, Immunology and Infection *52*, 9-21.

Zhang, T., Shao, M.F., and Ye, L. (2012). 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J *6*, 1137-1147.

Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiardes, S., Kotler, E.*, et al.* (2018). Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. Cell *174*, 1388-1405.e1321.

Zuo, H.-J., Xie, Z.-M., Zhang, W.-W., Li, Y.-R., Wang, W., Ding, X.-B., and Pei, X.-F. (2011). Gut bacteria alteration in obese people and its relationship with gene polymorphism. World journal of gastroenterology *17*, 1076-1081.

Appendix A: Summary of submissions from prescribed experts, agencies and authorities on the consultation RARMP

The Regulator received a number of submissions from prescribed experts, agencies and authorities on the consultation RARMP. All issues raised in submissions that related to risks to the health and safety of people and the environment were considered in the context of the currently available scientific evidence and were used in finalising the RARMP that formed the basis of the Regulator's decision to issue the licence. Advice received is summarised below.

Submission	Summary of issues raised	Comment
1	Agrees: • all plausible risk scenarios have been identified; • the proposed limits and controls are appropriate; • with the overall conclusions of the RARMP. The committee did not identify additional information to be considered.	Submission has been noted.

2	Agrees that it is unlikely bacteria will be present in high enough amounts to cause infection or direct harm to organisms in the environment due to the limited number of trial participants and potentially reduction of GMOs shed by trial participants in waste.
	
	Suggested including more information to address the uncertainties regarding:
	
	• Recombination, HGT and shedding duration in humans.
	
	    ○ *Risk scenario 3 should provide data to support the conclusion that HGT is highly unlikely or may 'never occur' in the human gut, or clearly identify uncertainty,, particularly around the applicability of mice data to humans*
	
	    ○ *Recommends the applicant samples for recombinants and HGT in participants as well as presence of GMOs.*
	
	• Entry and persistence in the environment due to selective pressure and potential risk of exposure.
	
	    ○ *The RARMP should further discuss the likelihood that*
		Submission has been noted.
		
		• Uncertainties in relation to HGT in risk scenarios 2, 3 and 4 have been highlighted in Chapter 2, Section 3. This uncertainty has also been mentioned in risk scenario 3.
		
		• The RARMP has indicated that additional information listed below are required for a potential commercial release of the GMO, or to justify a reduction in limits and controls:
		
		    ○ Quantity and duration of shedding of GMO bacteria in participant faeces; and
		
		    ○ Characterisation of GM bacteria resulting from this therapy.
		
		• Additional information has been included to risk scenario 4 to clarify the:
		
		    ○ entry and persistence of ARBs in the environment;

Submission	Summary of issues raised	Comment

	GMOs will persist due to the presence of antibiotic selective pressure and their potential ability to outcompete wild type strains in the environment.
	    ○ *Clarify and address the risk of tetracycline use in agriculture and aquaculture potentially resulting in the selection and persistence of the GM bacteria in the environment.*
	• Survival and persistence of GMOs in waste after treatment
	    ○ *Risk scenario 4 should include recent data and further discuss the potential for ARBs and ARG plasmids to survive waste treatments and enter the environment and persist.*
	    ○ *Waste treatments may significantly reduce the concentration of ARBs but not the relative abundance or proportion of ARBs in treated waste.*
	    ○ *Waste treatments do not reduce ARGs or ARG plasmids.*
		○ persistence of ARGs or ARG plasmids; and
		
		the regulation of antibiotic use by the APVMA and FSANZ.

3	Based on the information provided they do not have any concerns about the work.	Submission has been noted.

Appendix B: Summary of submissions from the public on the consultation RARMP

The Regulator received 2 submissions from the public on the consultation RARMP. The issue raised in the submission is summarised in the table below. All issues that related to risks to the health and safety of people and the environment were considered in the context of currently available scientific evidence in finalising the RARMP that formed the basis of the Regulator's decision to issue the licence.

Submission	Summary of issues raised	Comment
1	Submitter stated, "Playing God!"	Submission has been noted.