Insects as models to study the impact of antibiotics and microbiota therapies on the human gut microbiome: reducing the use of animals in research

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Abstract

The human microbiome is rapidly becoming recognised as a central player in human health, with both a metabolic and immunological function. The makeup of the gut microbiome, especially in infants, can be a major factor predisposing individuals to diseases such as IBD, diabetes, and necrotising enterocolitis. Recent improvements in sequencing technology and computational analysis of sequencing data have allowed scientists to see in detail how changes in the microbiome accompany different lifestyles and health conditions, but the causality and mechanisms of these associations are still not fully understood. There is also a need to develop and test potential therapeutics targeting the microbiome.

Mice are the most used *in vivo* model for the microbiome but pose issues in terms of cost, time and ethics. This project's aim has been to investigate the potential of using the Greater Wax Moth, *Galleria mellonella,* as an alternative model for the human infant gut microbiome. Galleria has been gaining popularity as a model host due to its ease of experimentation, simpler regulatory framework and fast life cycle.

In this thesis I have shown that, using antibiotics, *Galleria* can be cleared of its native gut bacteria. Larvae, both those that have been antibiotic-treated and those that have not, can then be colonised with a range of foreign bacteria. I have shown this is possible through both feeding and injection of bacterial culture, and from faecal slurry through feeding. Larvae colonised with labelled *Enterococcus mundtii* pass those bacteria onto the next generation.
This thesis also describes the native *Galleria* microbiome of both lab-reared and wild larvae, which is informs the use of *Galleria* as a model. Through this investigation, we discovered a novel species of *Enterococcus* and carried out a characterisation of this species.

These results will help guide any development of *Galleria* as a model host for human commensal bacteria.

Table of Contents

List of figures

List of tables

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Chapter 1

1 Introduction

The study of human health and biology requires the use of surrogate systems to carry out experiments that would be either impractical or unethical to carry out on humans. There are a wide range of model organisms that are used for this purpose, depending on the nature of the research to be done. One such organism that has been gaining popularity is *Galleria mellonella.* The aim of this project has been to determine how suitable *Galleria* is as a model for the human infant gut microbiome.

1.1 Galleria mellonella

1.1.1 *Galleria* ecology

The greater wax moth, *Galleria mellonella* (**Figure 1.1**)*,* is a member of the order Lepidoptera and is most commonly known as a pest of beehives (Kwadha et al. 2017). Wax moths generally do not lead to the collapse of healthy colonies but can cause significant damage to weak colonies ('Wax moth: a beekeeping pest' 2022) and are thus the cause of significant economic loss (Kwadha et al. 2017).

The life cycle of *Galleria* includes egg, larva, pupa and adult (**Figure 1.2**). Eggs are laid into crevices by adult females in clusters of 50-150. Depending on temperature these take 3-30 days to hatch into small larva 1-3 mm in length. The larval stage lasts 28 days to 6 months depending on diet (Jorjão et al. 2018) and temperature. By pupation the larvae reach a length of 12-20mm. Pupae take 1-9 weeks to emerge as adults. Shortly after emerging, adult moths mate and females begin to lay eggs. Females live for around 12 days after emerging; males live to about 21 days (Kwadha et al. 2017).

Galleria can grow for several generations on artificial food (Ignasiak and Maxwell 2018), are easily inoculated with bacteria, and can grow at 37°C, crucial to their use as a model host for human commensal bacteria (Champion, Titball, and Bates 2018). *Galleria* larvae are also capable of digesting polyethylene (Bombelli, Howe, and Bertocchini 2017) and have attracted much interest as a result.

Figure 1.1: A healthy, final instar, Galleria mellonella larva

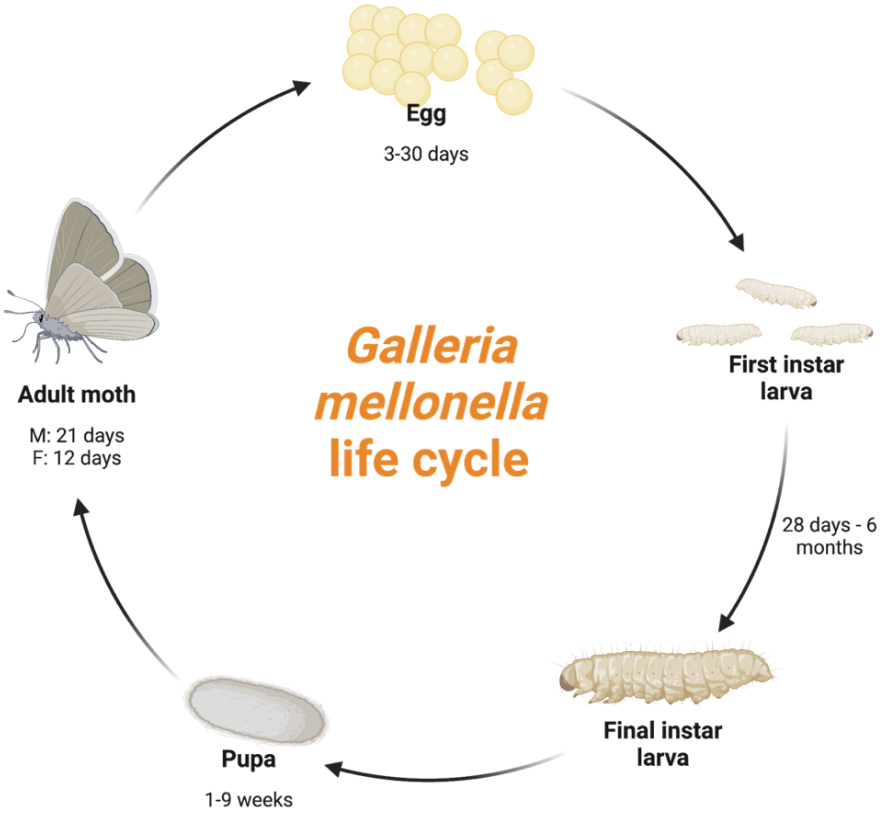


Figure 1.2 The Galleria mellonella life cycle: *length of stages taken from Kwadha et al. 2017 (Kwadha et al. 2017). Created with Biorender.*

1.1.2 Use of *Galleria* as a model

Since the turn of the millennium, *Galleria* (**Figure 1.3**) has been gaining popularity as a model host to study pathogenesis (Tsai, Loh, and Proft 2016; Fallon, Kelly, and Kavanagh 2012; Kling 2020). Its advantages include a short life cycle, easy rearing in the lab, few ethical issues, an absence of the regulations that restrict mammalian studies, and low cost. Previously, most studies have used larvae supplied from pet food shops, which may have been grown with antibiotics and hormones, but recently there have been attempts to standardise *Galleria* as a model (Jorjão et al. 2018; Ellis, Graham, and Mortensen 2013), including the establishment of Biosystems Technology, a company selling standardised larvae (TruLarv™), grown without hormones or antibiotics (Champion, Titball, and Bates 2018).

Figure 1.3: Galleria on artificial food*. Left: two Galleria mellonella larva feeding on artificial food. Right: Adult male and female Galleria moths*

Galleria has continued to gain momentum as a model in recent years: a recent Pathogens and Disease thematic issue focused on the use of *Galleria* (Junqueira, Mylonakis, and Borghi 2021). It included several reviews and some recent papers focussing on standardising *Galleria* as a model. A review from Pereira et al. (Pereira et al. 2020) reports on the growing popularity of *Galleria* and offered some useful suggestions for increasing reproducibility. Recommended factors to consider included: larval diet; any preincubation of larvae at low temperatures or without food, which compromises the immune response; inoculum preparation and delivery; definition of larval death; and number of larvae used. It recommends using groups of 10 larvae for each treatment, with three experimental and three biological replicates.

Lange et al. demonstrated use of *Galleria* to study gut commensals and pathogens by force-feeding bacterial suspensions of *Bacteroides vulgatus* and *Escherichia coli* (Lange, Schäfer, and Frick 2019). Larvae were not treated with antibiotics beforehand. The load of introduced bacteria decreased until no bacteria were seen after 24 hr. Russo and MacDonald (2020) found that the *Galleria* model

cannot differentiate between hypervirulent and classical *Klebsiella pneumoniae* while a murine model can, although the larvae used were not research grade but bought from a bait shop, which may have limited the power of the study (Russo and MacDonald 2020). Vilela et al. found that injecting probiotic *Lactobacillus acidophilus* into *Candida* infected larvae increased the survival of the larvae (Vilela et al. 2015).

Piatek et al. published a useful review of *Galleria* as a model for studying *in vivo* activity of antimicrobials (Piatek, Sheehan, and Kavanagh 2020). The review discusses the use of oral gavage in comparison to injection into the hemacoel, noting that it may be better for the study of enteropathogens and commensals, and that the survival of *Galleria* larvae following gavage of a toxin or pathogen is higher than survival following injection of the same toxin or pathogen. All reviews stress that *Galleria* cannot completely replace mammalian models and are most useful for rapid and high-throughput testing, or pre-screening prior to the use of mammalian models.

A review from Dinh et al., 'Microbiology's next top model: Galleria in the molecular age' (Dinh et al. 2021), discusses the potential future for *Galleria* as a model. This includes the use of multi-omics methods to elucidate host-pathogen interactions, and the use of genetic and molecular techniques to functionally characterise genes using *Galleria,* such as mutant screens. The review also briefly discusses the use of *Galleria* to elucidate host-microbiome interactions, including generating 'germ free' larvae using antibiotics or surface-sterilisation of eggs. Recent uses of *Galleria* to test safety and efficacy of probiotics and prebiotics are also reviewed.

The relationship *Galleria* has with the commensal enterococci of its gut may make it a useful model to study *Enterococcus* commensal bacteria from humans. *Galleria* is a well-established *Enterococcus* infection model and is used to assess virulence (Hanin et al. 2010), characterise virulence factors (Lebreton et al. 2009; Gaspar et al. 2009) and test potential treatments (Chibebe Junior et al. 2013). I have yet to see use of *Galleria* to study human commensal *Enterococcus* species outside of merely differentiating them from pathogenic species.

1.1.3 Galleria biology

Insects, including *Galleria*, lack an adaptive immune response but their innate immune response is very similar to that of mammals. It consists of cellular and humoral immunity. Cellular immunity is mediated by haemocytes, of which there are 8 types: prohaemocytes, plasmatocytes, granular cells, coagulocytes, spherulocytes and oenocytoids. Plasmatocytes and granular cells carry out phagocytosis, encapsulation and nodule formation. Phagocytosis by plasmatocytes is similar to

neutrophils in mammals and generally ends by killing with active ROS. Humoral immunity again is similar to mammals and involves opsonins, antimicrobial peptides and the phenoloxidase pathway, which results in the melanisation of the cortex of infected larvae (Tsai, Loh, and Proft 2016).

The structure of the lepidopteran gut consists of foregut, midgut and hindgut. In the foregut food is mixed with salivary fluids, in the midgut it is digested and absorbed, and in the hindgut, water is reabsorbed. The foregut and hindgut are ectodermal in origin and secrete cuticle continuous with that covering the outside of the body. This is called the intima (Chapman 1998). The midgut is lined with the peritrophic membrane, a structure secreted by midgut epithelial cells composed of chitin and glycoproteins. It is semipermeable and serves as a barrier between gut contents and epithelial cells. The pore size of the peritrophic membrane in most lepidopterans is 21-29 nm, and therefore would not permit the transmission of bacteria (Lehane 1997). The epithelial cells lining the midgut include columnar, goblet and stem cells (Lehane 1996). Lepidopteran goblet cells are similar in appearance to mammalian goblet cells but very little is known about their function (Wu et al. 2016). Oxygenation levels in the insect gut vary depending on size and species (Engel and Moran 2013) but the guts of small caterpillars are generally anoxic (Johnson and V. Barbehenn 2000). The lepidopteran midgut is known to be highly alkaline thanks to the active transport of ions across the epithelium (Dow 1992). It is not known where the gut bacteria in *Galleria* are most abundant and which niches they may inhabit in the gut. Any of these differences in gut architecture and conditions could impact the survival of the human commensal bacteria in the *Galleria* gut.

1.1.4 *Galleria* microbiome

Many insects are obligate symbionts and cannot live without the bacteria that they host. These are often endosymbionts such as Wolbachia or Spiroplasma (Engel and Moran 2013). There are also many insects that have very sparse microbiomes that do not play any role in metabolism or development.

There is huge variety in the nature of the relationship that insects have with their microbiomes. The microbiome of many insects, unlike that of humans, does not always play a role in metabolism or development and in most insects is quite sparse (Engel and Moran 2013). However, many insects are obligate symbionts and cannot survive without the bacteria they host. These are often endosymbionts and live in specialised cells or organs within the insect (Takeshita and Kikuchi 2017). Many insects have symbiotic relationships with ectosymbiotic gut bacteria: the termite microbiome contributes to lignocellulose digestion and nitrogen metabolism (Warnecke et al. 2007); in honeybees, the gut microbiome protects against parasites and assists in pollen degradation (Koch and Schmid-Hempel

2011; Engel, Martinson, and Moran 2012); in the locust *Schistocerca gregaria*, the gut commensal *Pantoea agglomerans* produces components of the aggregation pheromone (Engel and Moran 2013; Dillon, Vennard, and Charnley 2002).

It has been theorised that lepidopteran caterpillars lack a resident gut microbiome, on the basis that disrupting the microbiome using antibiotic-treated feed has no impact on survival and development (Phalnikar, Kunte, and Agashe 2019) and that, in wild leaf-feeding caterpillars, the bacteria found in the guts are more associated with the bacterial found on the leaves the caterpillars eat than the species of the caterpillar (Hammer et al. 2017).

The bacterial population in the *Galleria* gut is low in both diversity and abundance, although I'm not convinced *Galleria* lacks a gut microbiome entirely. Most studies report the gut microbiome to primarily be composed of *Enterococcus* (Allonsius et al. 2019; Johnston and Rolff 2015; Ignasiak and Maxwell 2018), (with some exceptions (Cassone et al. 2020)). *Lepidoptera* are known to have a long relationship with enterococci. 145 million years ago there was a lateral gene transfer of a gene from an *Enterococcus* species to a common ancestor of many *Lepidoptera,* including *Galleria* (Wheeler, Redding, and Werren 2013).

The species seen most commonly in *Galleria* is *Enterococcus mundtii* (Johnston and Rolff 2015). In the related species *Spodoptera littoralis, E. mundtii* produces a bacteriocin that suppresses colonisation of the gut by competitor bacteria (Shao et al. 2017). This also appears to be true in *Galleria* (Jarosz 1979), where *E. mundtii* has been shown to work cooperatively with host lysozyme to prevent colonisation of the gut by pathogens during metamorphosis (Johnston and Rolff 2015). The female passes *E. mundtii* to the next generation by spreading bacteria over the surface of the egg (Bucher 1963). Other than this, little else is known about the role the microbiome plays in *Galleria* biology, and no studies have been carried out on wild larvae.

Allonsius et al. studied the bacteria present in the fat body, haemolymph, faeces and skin of bait shop and research-grade *Galleria* larvae and found that *Enterococcus* dominated in all cases, but that the diversity was lower for research-grade larvae (Allonsius et al. 2019). They used 16S rRNA amplicon sequencing to investigate the composition of the *Galleria* microbiome and found that the microbiome is dominated by *Enterococcus,* that bait grade larvae have a lower abundance and greater diversity of bacteria than research grade larvae, and that the research-grade larvae they studied were dominated by a single *Enterococcus* taxon at all body sites.

Lou et al. investigated the impact of the gut microbiome on digestion of polyethylene and polystyrene and the impact of diet on the gut microbiome. They found that the *Galleria* gut microbiome was mainly dominated by *Enterococcus,* with an increase in *Okibacterium* and *Anoxybacillus* abundance in the guts of larvae fed bran and an increase in *Anoxybacillus* and *Geobacillus* abundance in the guts of larvae fed beeswax(Lou et al. 2020).

Mazumdar et al. elucidated the adaption of common *Galleria* commensal *E. mundtii* to the *Spodoptera littoralis* gut and found that *E. mundtii* genes involved in the response to alkaline stress are differentially regulated during gut passage, which helps to deal with the highly alkaline parts of Lepidopteran gut (Mazumdar et al. 2020). They also found that pathways for biofilm production, two component signalling systems, oxidative stress responses and quorum sensing were upregulated.

1.2 The human gut microbiome

Commensal bacteria can be found in a wide range of sites on the body but are by far the most abundant and diverse in the gut. The human gut plays host to trillions of bacteria, with which we have a well-established evolutionary relationship (Ley et al. 2008). The human gut microbiome plays a role in our metabolism, immune system, and endocrinology. The make-up of the microbiome varies massively between individuals and depends on many factors, including geography, diet, antibiotic treatment, health, and genetics (Rooks and Garrett 2016).

As in many other mammals, a major effect of the gut microbiome in humans is to expand the range of foods that we can digest and receive significant nutritional value from. Many foods that would otherwise be too toxic or nutrient-poor to digest with the human repertoire of digestion enzymes can be easily digested by the gut bacteria. For example, plant fibre is mostly digested by the gut microbiome, which then provides the human host with energy through the release of metabolites. The gut microbiome changes in response to diet, which poses a difficulty if an individual consumes too much or too little food. Both malnutrition and obesity decrease the diversity of the gut microbiome and can lead to changes in the gut that reinforce the effects of poor diet (Voreades, Kozil, and Weir 2014).

The gut microbiome also plays a role in priming and maintaining the immune system. Short chain fatty acids, for example acetate, proponate and butyrate, produced from anaerobic fermentation suppress inflammation and promote immune tolerance (Trompette et al. 2014; Voltolini et al. 2012; Vinolo et al. 2011). They act as inhibitors of histone deacetylases (Chang et al. 2014; Smith et al. 2013; Furusawa

et al. 2013) and ligands for G protein-coupled receptors (Maslowski et al. 2009; Singh et al. 2010; Singh et al. 2014). They also improve intestinal epithelial cell barrier function, which prevents leakage of bacteria and toxins from the gut lumen into the circulation (Gaudier et al. 2004; Fukuda et al. 2011; Wrzosek et al. 2013; Willemsen et al. 2003). This is one of several ways the gut microbiome can influence the immune system. Through immune responses, the immune system also influences the microbiota, resulting in reciprocal cross-talk (Rooks and Garrett 2016).

An imbalance in the relative abundance of bacteria in the gut of sick animals when compared to healthy animals is referred to as dysbiosis, which can result from illness, diet or antibiotics, among other causes. It is associated with obesity and some autoimmune diseases and is thought to contribute to many health conditions (Riva et al. 2017; Frank et al. 2011; Maier et al. 2018).

There have also been calls for caution and scepticism, to ensure that any attempt to causally link the microbiome with disease has a solid scientific basis ('Hype or hope?' 2019). Of particular relevance is a systematic review (Walter et al. 2020) revealing the limitations of human-microbiota associated (HMA) rodents in establishing causal relationships between the microbiome and disease, which found that "95% of published studies (36/38) on HMA rodents reported a transfer of pathological phenotypes to recipient animals, and many extrapolated the findings to make causal inferences to human diseases." The authors argued that this proportion of positive results is "implausible and likely stems from a combination of insufficient rigor in experimental designs, inappropriate statistical analyses, and bias."

1.3 The infant gut microbiome

The fetal gut is generally agreed to be sterile (Perez-Munoz et al. 2017), and if there is any bacterial presence in the fetal gut, it is very limited (Rackaityte et al. 2020). The gut microbiome is seeded by the mother during birth, transferring vaginal and faecal bacteria to the infant gut. The first colonisers are facultative anaerobes that consume oxygen and produce metabolites that change the gut conditions to allow colonisation by strict anaerobes (Voreades, Kozil, and Weir 2014). These early anaerobic colonisers are almost entirely comprised of members of the genus *Bifidobacterium* (and some *Bacteriodes*) in healthy infant guts (Stewart et al. 2018). The growth of *Bifidobacterium* species is promoted by the presence of prebiotics in the form of complex human milk oligosaccharides (HMOs) (Rodriguez et al. 2015). HMOs cannot be digested by human metabolism and are instead mainly broken down by bifidobacteria (Thomson, Medina, and Garrido 2018). The resulting degradation products then promote the growth of other beneficial bacteria, ensuring the development of a healthy

microbiome as it diversifies (Lawson et al. 2020). Breastfeeding also introduces beneficial bacteria to the infant gut through the milk and areolar microbiomes (Pannaraj et al. 2017). Following this initial development phase, the gut microbiome diversifies, with an increase in the abundance and diversity of members of the Firmicutes phylum, such as *Enterococcus* and *Streptococcus.* By three years of age, the microbiome has generally stabilised (Stewart et al. 2018).

Preterm babies are more likely to be born by C-section, undergo medical interventions, have less contact with the mother, be given antibiotics and spend time in a hospital environment. Early-life antibiotic treatment and hospitalization of preterm infants has a long-lasting impact on the composition of the gut microbiome, including an increased presence of antibiotic-resistance genes and carriage of multi-drug resistant *Enterobacteriaceae* (Gasparrini et al. 2019). This disruption to the 'normal' assembly of the infant gut microbiome leads to an increased risk of necrotising enterocolitis, a severe inflammatory disease of the bowel affecting 7% of low-birth-weight preterm babies (Rodriguez et al. 2015).

Understanding of the role the gut microbiome plays in health has led to the development of therapies that target the microbiome. These fall into either the category of probiotic, which were defined by the World Health Organisation in 2001 as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Schlundt 2001) or prebiotic, which was defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' (Gibson et al. 2017). When a prebiotic and a probiotic are administered simultaneously, to improve delivery and survival in the gut, this is referred to as a 'synbiotic' (Greenhalgh et al. 2019). A postbiotic, by the ISAPP definition, is 'a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host' (Salminen et al.). There is limited understanding of how efficacious many of these treatments are in promoting a healthy gut microbiome and of any potential negative effects they might have. Similarly, more understanding is required of the effect antibiotics have on the microbiome.

1.4 Techniques used to study microbiomes

A large part of the reason that the microbiome field has expanded so much in recent decades is due to the advent of next-generation sequencing. Many of the bacteria in the human microbiome are difficult to culture and are therefore not detectable by culture-dependent methods, and ecosystems like the human microbiome contain far too wide a diversity of bacteria to characterise the entire community through culture-dependent methods alone (Hugenholtz and Tyson 2008). Metagenomics

refers to the study of microbial ecosystems through the sequencing of, ideally, all the genomes in a sample (Quince et al. 2017). The contents of these genomes can then be analysed, for example to characterise their functional capacity. Metagenomic sequencing is generally shotgun sequencing, but in recent years long-read sequencing methods like Nanopore have improved enough in accuracy and cost to be a desirable alternative (Latorre-Pérez et al. 2020).

16S rRNA amplicon sequencing, in contrast, only involves the sequencing of (regions of) the 16S rRNA gene (Weisburg et al. 1991). 16S amplicon sequencing is cheaper than metagenomic sequencing, but cannot be used to carry out functional analyses, and is more limited in the resolution of species that can be identified (Jovel et al. 2016). It almost exclusively targets bacteria, so cannot be used to study eukaryotic microbes, but is therefore useful to study samples with large amounts of contaminating eukaryotic cells (Jousselin et al. 2016).

Sequencing can characterise the contents of the microbiome but it cannot necessarily provide information on the health effects of varying microbiome profiles. For this, model organisms are needed. A germ-free animal – generally a mouse – will be colonised with human gut bacteria through the feeding of faecal slurry (Gootenberg and Turnbaugh 2011; Hugenholtz and de Vos 2018). The humanised microbiomes of these animals can then be used to study, for example, how the gut microbiome shifts in response to diet or treatment (Hildebrandt et al. 2009; Morgan et al. 2014), or the effect of gut microbiomes of differing compositions on the health of the host animal (Turnbaugh et al. 2008). However, the use of animal models comes with obvious ethical concerns and should be avoided where possible, with an effort made to develop more ethical alternatives. In this thesis I investigate the potential of *Galleria* as one such alternative.

Fluorescence labelling is a technique by which bacteria are transformed with a plasmid containing a fluorescence gene. The fluorescent protein will then fluoresce under light of the correct wavelength. This technique has been used for pathogen research in *Galleria,* allowing the progression of infection to be followed non-invasively (Ramarao, Nielsen-Leroux, and Lereclus 2012).

1.5 Previous work in the Maxwell Lab

A previous student of the Maxwell lab, Katarzyna Ignasiak, investigated antibiotic resistance in the guts of insects. She published a paper in 2017 titled “Antibiotic-resistant bacteria in the guts of insects feeding on plants: prospects for discovering plant-derived antibiotics” (Ignasiak and Maxwell 2017a), which studied the gut bacteria of insects that exclusively fed on a specific plant. If the gut bacteria of

these insects were unusually resistant to antibiotics, it could indicate that that plant produces an antibacterial compound and prompt further investigation, making it easier to find new antibiotics. The study also intended to establish how common antibiotic resistance is in the guts of insects. The insects studied were giant lime stick insect feeding on eucalyptus, diamondback moth feeding on Chinese cabbage, cinnabar moth feeding on ragwort, rosemary beetle feeding on lavender, death's-head hawkmoth feeding on potato leaves and beet armyworm feeding on Madagascar periwinkle.

She discovered that while some bacteria did show antibiotic resistance, many were no more resistant than the relevant type strain. This could be partially explained by some type strains being clinical isolates, but others were not. She also had difficulty in isolating and identifying the active components that had antibacterial activity. In the case of Madagascar periwinkle, she managed to identify vindoline, a precursor to the chemotherapy drug vinblastine, as having antibiotic activity. Another issue she discovered is that a comparison of sequencing and metagenomic analysis with culture-dependent methods showed that <5% of bacterial species could be identified by culture-dependent methods.

Ignasiak published another paper in 2017 titled '*Galleria mellonella* (greater wax moth) larvae as a model for antibiotic susceptibility testing and acute toxicity trials' (Ignasiak and Maxwell 2017b). She used *Galleria* larvae to test the efficacy and toxicity of various antibiotics according to OECD guidelines and found that there was a good correlation between the results in the larvae and the results in mice and rats.

The third paper Ignasiak published, in 2018, was titled "Oxytetracycline reduces the diversity of tetracyline-resistance genes in the *Galleria mellonella* gut microbiome." (Ignasiak and Maxwell 2018), *Galleria mellonella* larvae were fed oxytetracycline and then their guts dissected and investigated for the presence of antibiotic resistance genes. She discovered that in the absence of antibiotics the insect gut microbiome can maintain a diverse pool of tetracycline resistance genes which are then selected for when exposed to oxytetracycline. This reduces the diversity of resistance genes in the gut. Overall, diversity of operational taxonomic units (OTUs) increases because colonisation resistance by the native microbiome is disrupted.

Over the course of this experiment Ignasiak showed that *G. mellonella* larvae can be raised on artificial food, including antibiotics, for over 5 generations and that the microbiome can be sampled. She found that the gut microbiome is dominated by *E. mundtii* with a few other *Enterococcus* strains present,

consistent with other findings (Johnston and Rolff 2015). This laid the groundwork for my project by establishing *G. mellonella* in the Maxwell lab and showing that it is possible to rear the larvae and analyse their gut microbiome.

1.6 Project aims

This project is funded by NC3Rs: the National Centre for the Replacement, Refinement and Reduction of Animals in Research. This project's aim is partial replacement, in that *Galleria* may be able to be used for some experimental purposes that would previously have used mice. Using the previous experience at the John Innes Centre and in the Maxwell lab of rearing *Galleria,* and infant faecal samples provided by the Hall lab, I aimed to investigate if and how *Galleria* could be used as a model for the human infant gut microbiome. Our hypothesis was that the native *Galleria* microbiota could be cleared and replaced with human infant gut bacteria using faecal slurry, and that these 'humanised' larvae could then be used for experimentation.

A previous research assistant in the Maxwell lab, Marjorie Labédan, did work proving the potential of this model including experiments clearing the native microbiome with antibiotics, establishing individual strains of human gut bacteria in the *Galleria* gut and determining the stability of the modified microbiome over generations. However, many of these experiments were only partially completed and required repeating in a more rigorous and comprehensive manner.

The objective of my project was to develop *Galleria mellonella* as a model for the infant gut microbiome. This includes establishing protocols for generating sterile larvae, for colonising larvae with human commensals, and for characterising the composition of the larval gut microbiome. I have assessed the extent to which *Galleria* is suitable for this work and identified problems that would have to be solved to use *Galleria* for certain experiments.

First I had to establish a *Galleria* colony in the Maxwell lab. While *Galleria* is starting to see widespread use as a model, many papers report having used *Galleria* either bought as animal feed or from sources like TruLarv™ (Champion, Titball, and Bates 2018) and used immediately. I have used my own colony to ensure consistency in genetics and rearing and tested different diets and containment to optimise growth and survival (Chapter 3).

I have tested protocols for clearing the native microbiome of the *Galleria* larvae through treatment with antibiotics. I then tested several protocols for colonising *Galleria* with commensal bacteria, be it

through injection of culture (Chapters 3 and 6) or feeding of faecal slurry (Chapter 4). In Chapter 6 I have extended this to using fluorescently labelled bacteria that I have then tracked to the next generation. I have also investigated the native microbiome of the *Galleria* larvae to help inform its use as a model both for this and other studies (Chapter 5). This has also been a useful means by which to test protocols for the characterisation of the *Galleria* microbiome.

I have also used *Galleria* for its typical use, testing the toxicity and efficacy of novel compounds (Chapter 7) from the Maxwell lab and collaborators.

Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial strains and growth conditions

The bacterial strains used in this project are listed in ***Table 2.4***. Strains received from culture collections were resuspended according to instructions and prepared as glycerol stocks.

Bacterial cells were incubated at 37°C with agitation (when using liquid medium) or without agitation (when using medium supplemented with agar). Aerobic cultures were cultivated on Brain-Heart Infusion (BHI) media (Merck). Faecal slurry and the guts of faecal-slurry-fed larvae were additionally plated on MacConkey agar (Sigma). Anaerobic cultures were cultivated on Reinforced Clostridial Agar (RCA) (Sigma) and De Man, Rogosa and Sharpe (MRS) media (Sigma) supplemented with 0.5 g/L cysteine (Sigma). To distinguish enterococci from other genera, cells were grown on Rapid Enteroccocus Chromoselect Agar (Sigma).

2.1.2 Larvae

Galleria mellonella larvae were obtained from a colony grown from larvae originally sourced from Livefood UK Ltd and maintained at the John Innes Centre Entomology Facility (Norwich, UK). Where specified, *Galleria* larvae (TruLarv™) purchased from BioSystems Technology were also used.

2.1.3 Diet and rearing

Galleria larvae and eggs were provided by the JIC insectary and used to set up a colony within the lab. Larvae were kept either in clear plastic boxes (Watkins and Doncaster), 90 mm Petri dishes, 140 mm Petri dishes or Pint-sized Insect Pots (BugDorm). They were fed one of two diets (**Table 2.1**). The dry contents were mixed and autoclaved.

Diet A	*Diet B*
20 g brown sugar (Sainsbury's dark soft brown sugar)	40 g honey (clear honey, Sainsbury's)
40 mL glycerol (Sigma)	40 g glycerol (Sigma)
20 g milk powder (Dried Skimmed Milk Powder, Marvel)	30 g yeast extract (Merck)
20 g wholemeal flour (Strong Stoneground 100% Wholemeal Flour, Sainsbury's)	20 g soy flour (Neal's Yard Wholefoods Natural Wheatgerm)
10 g yeast extract (Merck)	20 g powdered milk (Dried Skimmed Milk Powder, Marvel)
10 g wheat germ (Neal's Yard Wholefoods Natural Wheatgerm)	
40 g bran (Neal's Yard Wholefoods Natural Wheat Bran)	

Table 2.1: Ingredients in the Galleria diet

2.1.4 Primers

Primers were designed using Geneious and ordered from Sigma. Sequences are listed in **Table 2.2**.

Primer	*Sequence*
8F	AGAGTTTGATCATGGCTCAG
1492R	TACGGTTACCTTGTTACGACT
pMV158F	GCTCTCCCTTATGCGACTCC
pMV158R	ACGACCTTCTGCACGTTCAT

Table 2.2: Primers

2.1.5 Antibiotics

Antibiotics were stored as recommended by manufacturers.

Antibiotic	*Solvent*	*Stock concentration (mg/mL)*	*Manufacturer*
Oxytetracycline	Ethanol	15	Sigma
Spectinomycin	H_2O	50	Sigma
Streptomycin	H_2O	15	Sigma
Tetracycline	Ethanol	15	Sigma
Vancomycin	H_2O	100	Sigma

Table 2.3: Antibiotic stock solutions

2.1.6 Faecal slurry samples and stock

Faecal slurry samples were provided by Lindsay Hall from the BAMBI study, Fecal collection from NNUH and Rosie Hospital was approved by the Faculty of Medical and Health Sciences Ethics Committee at the University of East Anglia (UEA), and followed protocols laid out by the UEA

Biorepository (License no: 11208). Samples were taken from infants of between 2 and 3 months of age. A faecal slurry stock was made using 30 unique 1 g samples, and a matching 150 mg aliquot was taken for each of these samples. Under sterile, anaerobic conditions the thirty 1 g samples were combined into a single 50 mL Falcon tube, to which 20 mL of sterile PBS (Formedium) was added. The slurry was then vortexed until homogenous. 1 mL aliquots were taken, flash-frozen in liquid nitrogen, and stored in cryo-vials at -80°C.

Species	*Source*	*Provided by*	*Strain identifier*	*Plasmid*	*Resistance*	*Reference*
Pseudomonas fluorescens		Alba Pocheco-Moreno (JIC)	SBW25	None		Pacheco-Moreno, A., et al. (2021). "Pan-genome analysis identifies intersecting roles for Pseudomonas specialized metabolites in potato pathogen inhibition." eLife 10: e71900.
Pseudomonas protegens		Alba Pocheco-Moreno (JIC)	PS682	None		(Pacheco-Moreno et al. 2021)
Enterococcus faecalis	Blood	Dzung Diep (NMBU)	SL04 (MMH594)	pSL101P$_{16S}$	Spectinomycin (150 mg/L)	La Rosa SL, Diep DB, Nes IF, Brede DA. Construction and application of a luxABCDE reporter system for real-time monitoring of *Enterococcus faecalis* gene expression and growth. Appl Environ Microbiol. 2012;78(19):7003-7011. doi:10.1128/AEM.02018-12
Enterococcus faecalis	Faeces	Lindsay Hall (QIB)	P46G 41	None		
Bifidobacterium infantis	Labinic	Lindsay Hall (QIB)		None		
Escherichia coli	Faeces	Lindsay Hall (QIB)		None		
Staphylococcus capitis	Faeces	Lindsay Hall (QIB)		None		
Lactobacillus acidophilis	Labinic	Lindsay Hall (QIB)		None		
Escherichia coli	E. coli Genetic Stock Centre	Maxwell lab (JIC)	MG1655	None		
Enterococcus faecalis	Urine	NCIMB	13280	None		
Enterococcus faecalis		NCIMB	8260	None		
Serratia marcescens	Pond water	NCIMB	9155	None		
Bacillus cereus		NCIMB	8012	None		
Bacillus thuringiensis	*Ephestia kuhniella*	NCIMB	9134	None		
Enterococcus mundtii	Soil	NCIMB	13132	None		
Enterococcus mundtii	Soil	NCIMB		pMV158$_{mCherry}$	Tetracycline (15 g/L)	

Staphylococcus aureus		NCIMB	8625	None		
Ralstonia picketii	Tracheotomy patient	NCIMB	13142	None		
Staphylococcus aureus		Stephane Mesnage (University of Sheffield)		$pMV158_{GFP}$	Tetracycline (15 g/L)	
Staphylococcus aureus		Stephane Mesnage (University of Sheffield)		$pMV158_{mCherry}$	Tetracycline (15 g/L)	
Staphylococcus aureus		Stephane Mesnage (University of Sheffield)		pAT18	Erythromycin	
Enterococcus casseliflavus	*Galleria mellonella*	This project	GAL2	None	Vancomycin (4 g/L)	
Enterococcus casseliflavus	*Galleria mellonella*	This project	GAL3	None	Vancomycin (4 g/L)	
Enterococcus casseliflavus	*Galleria mellonella*	This project	GAL5	None	Vancomycin (4 g/L)	
Enterococcus casseliflavus	*Galleria mellonella*	This project	GAL6	None	Vancomycin (4 g/L)	
Enterococcus innesii	*Galleria mellonella*	This project	GAL7	None	Vancomycin (4 g/L)	Gooch, H. C. C., et al. (2021). "*Enterococcus innesii* sp. nov., isolated from the wax moth Galleria mellonella." International Journal of Systematic and Evolutionary Microbiology 71(12): 005168.
Enterococcus casseliflavus	*Galleria mellonella*	This project	GAL8	None	Vancomycin (4 g/L)	
Enterococcus innesii	*Galleria mellonella*	This project	GAL9	None	Vancomycin (4 g/L)	(Gooch et al. 2021)
Enterococcus innesii	*Galleria mellonella*	This project	GAL10	None	Vancomycin (4 g/L)	(Gooch et al. 2021)
Enterococcus casseliflavus	*Galleria mellonella*	This project	TL1	None	Vancomycin (4 g/L)	
Enterococcus innesii	*Galleria mellonella*	This project	TL2	None	Vancomycin (4 g/L)	(Gooch et al. 2021)
Enterococcus casseliflavus	*Galleria mellonella*	This project	TL3	None	Vancomycin (4 g/L)	
Enterococcus casseliflavus	*Galleria mellonella*	This project	TL4	None	Vancomycin (4 g/L)	

Table 2.4: Strains used

2.2 Methods

2.2.1 Egg handling

Eggs were removed from where they were laid around the lid of the box using a razor. Eggs were placed onto food in plastic boxes (Watkins and Doncaster) and incubated at 30°C. Once hatched, larvae were reared at either 30°C or 37°C.

After pupation, pupae were removed from boxes containing food and placed into new boxes, allowed to emerge as adults and lay eggs. No food was required for adults, as they do not eat (Ellis, Graham, and Mortensen 2013).

2.2.2 Dissection

Larvae were transferred to an empty sterile Petri dish for 2 hours of starvation before dissection to enrich the gut contents in bacteria by excreting some of the gut contents. Dissection was carried out under sterile conditions in a biological safety cabinet, using a sterile Petri dish as a dissection surface (**Figure 2.1**). Forceps and blades were sterilised by dipping in bleach followed by sterile water then 70% ethanol. Larvae were transferred individually to small centrifuge tubes and killed by flash-freezing in liquid nitrogen. Immediately after removal from liquid nitrogen, when the larva was still frozen, the head was cut off and a cut was made down the ventral side. The gut contents were then be removed with forceps and placed in a sterile 2 mL tube. Three guts were placed in each tube.

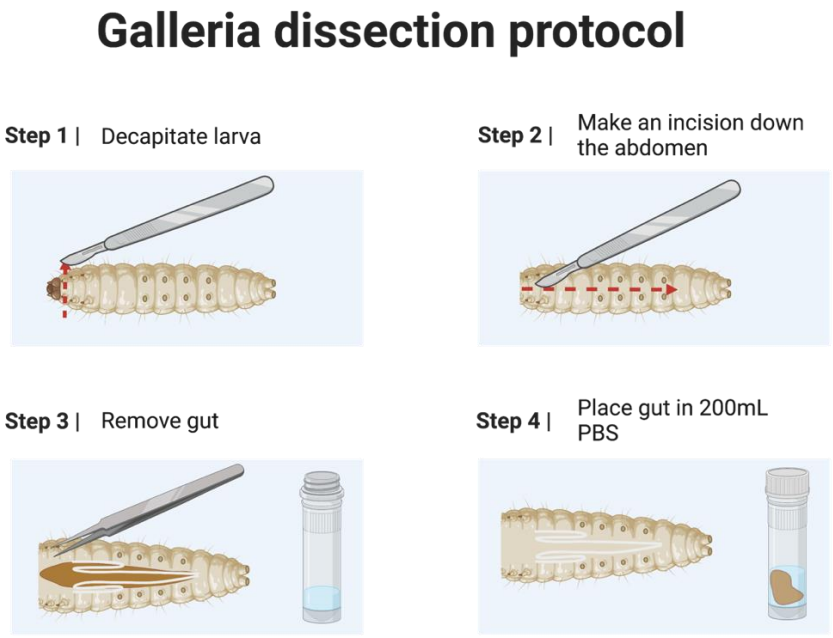


Figure 2.1: Galleria dissection protocol. *Larvae should be flash-frozen in liquid nitrogen to start and dissection should be carried out under sterile conditions.. Created with Biorender.*

2.2.3 Homogenisation

Both gut and whole larval samples were homogenised using an MP Biomedicals FastPrep-24™ homogeniser with 2 mL Lysing Matrix D tubes, for 40 seconds on speed 6.0.

2.2.4 Egg hatching

To reduce variation in the age of eggs, the first set of eggs laid were removed from the box containing adults. Two days later all eggs were again removed and placed into six pre-weighed sterile universal tubes. 0.1 g of eggs was placed in each tube. Each tube was then placed in a different environment: 4°C fridge, 6-8°C cold room, 18°C incubator, one left on the bench and one in a 30°C incubator. Each day, unhatched eggs were removed from the vial, larvae were counted and cleared from the vial, and the eggs were returned, until no more larvae were hatching.

2.2.5 Life cycle

Eggs were collected as above. 50 mg of eggs were collected and placed on Diet A and incubated at 30°C for 30 days before being weighed and counted.

2.2.6 Egg bleaching

Eggs were collected from the rim of the boxes (Watkins and Doncaster) in strips of 10-30 mm up to 100 mg. Using forceps, the eggs were either dipped in 2.5% sodium hypochlorite and then in sterile Milli-Q water or just dipped in the water. The eggs were placed in 20 mL Universal vials and incubated at 30°C. Each day, unhatched eggs were removed from the vial, larvae were counted and cleared from the vial, and the eggs were returned, until no more larvae were hatching.

2.2.7 Antibiotic treatment

To clear the guts of the native microbiota, larvae were fed on food containing 15 mg streptomycin and 15 mg oxytetracycline per 100 g of food for 0, 1, 5 or 10 days. Larvae were then dissected, guts removed, homogenised and plated. 16S amplification through PCR was carried out on gut homogenate and the products run on 1% agarose gel stained with ethidium bromide to see if bacteria were present in the gut.

All future antibiotic treatment of larvae consisted of 10 days treatment with 15 mg streptomycin and 15 mg oxytetracycline per 100 g of food

2.2.8 DNA purification from gut samples

DNA from gut contents was purified with the FastDNA® SPIN Kit for Soil according to instructions but with two extra homogenisation cycles followed by an extended 15-minute centrifugation step.

2.2.9 PCR

Qiagen Taq Polymerase was used for all PCR. PCR was carried out in 25 μL final volume containing 2.5 μL Qiagen PCR 10x buffer, 1 μL $MgCl_2$, 0.5 μL dNTP (10 mM), 0.75 μL of each primer (10 μg/mL), 0.125 μL Taq (5 U/μL), 2 μL DNA and 17.5 μL Milli-Q H_2O. Initial denaturation was carried out for 10 minutes at 95°C, followed by 35 cycles of denaturation (95°C for 60 seconds), annealing (58°C for 60 seconds), and extension (72°C for 90 seconds). The final extension was carried out at 72°C for 10 minutes.

2.2.10 Gel electrophoresis

Samples were loaded in 1% (w/v) agarose gels made in TAE buffer (40 mM Tris-Base, 20 mM Acetic Acid, 1 mM Disodium EDTA) and run for ~45 minutes at 120 V. The gel was stained in a 1 μg/mL ethidium bromide bath for 10 minutes and visualised using a Syngene G:BOX Gel Doc system.

2.2.11 16S rRNA amplicon sequencing of isolates

To identify isolates, colonies were picked and added to a PCR. Following the PCR, DNA was purified using a QIAquick PCR Purification Kit, sequenced using EuroFins Mix2Seq, and species were identified using BLAST.

2.2.12 16S rRNA amplicon sequencing of guts

Library preparation for 16S rRNA amplicon sequencing of whole *Galleria* guts was carried out by David Baker at QIB according to Illumina protocols (Illumina 2013). V3-V4 amplicon sequencing was carried out by Novogene using the Novoseq 6000 PE150 platform. Taxa were assigned using Centrifuge (v0.15).

2.2.13 Shotgun sequencing

Three larvae from the insectary, three commercial research grade larvae (TruLarv™), four larvae from one wild colony in Norfolk (Peter Sutherland) and two from another (Tom Johnson) were dissected and their guts removed. Three guts were pooled per sample. DNA was purified from the guts using the MP Biomedicals™ FastDNA™ SPIN Kit for Soil. DNA concentration was measured using

QuBit, library preparation was carried out by David Baker, and samples were sent to Novogene for Illumina paired-end shotgun sequencing.

2.2.14 Metagenomic analysis

Metagenomic analysis was carried out by Rebecca Ansorge at QIB. Sequences were assessed for quality using fastQC (v0.11.9) and multiQC (v1.9). Taxonomic labels were assigned to reads using Kraken (v2.1.1) and visualised using Krona (v2.7.1). Genomes were also assigned to host or symbiont using the whole genome sequence of *Galleria* and the isolates from the *Galleria* gut that I had previously sequenced.

2.2.15 Isolation of *E. innesii* and initial short-read sequencing

Larvae were flash frozen in liquid nitrogen; their whole guts were dissected out under sterile conditions and three guts were pooled into each single sample. Each sample was then homogenised in 200 μL of PBS (Formedium), diluted 1/100 in PBS and then 50 μL was spread on BHI agar plates and incubated at 37°C for 48 hours. Individual colonies were selected and grown up in 20 mL BHI media for 48 hours. Ten isolates were taken from larvae from the John Innes Centre colony and four were isolated from TruLarv™ larvae. The culture was centrifuged at 6,000 x g for 10 minutes and DNA purified using the MP Biomedicals™ FastDNA™ SPIN Kit according to manufacturer's instructions.

Sequencing was performed by the Wellcome Sanger Institute using Illumina HiSeq. Assembly was performed by Raymond Kiu at QIB using SPAdes (v3.14.1) (Bankevich et al. 2012). Phylogenetics and species assignment were carried out using the Type Strain Genome Server (Meier-Kolthoff and Göker 2019).

2.2.16 Purification of high-MW DNA for Nanopore sequencing

DNA for long-read sequencing was purified using the MP Biomedicals™ FastDNA™ SPIN Kit for Soil using a modified protocol, described below.

200 μL of culture was added to 2 mL Lysing Matrix E tubes. 980 μL of Sodium Phosphate Buffer and 120 μL of MT Buffer was added to each sample. Samples were mixed by inverting the tubes a few times, then stirred at 4000rpm at 50˚C for 10 minutes. Samples were centrifuged at 10,000 x g for 15 minutes to pellet debris. The supernatant was added to 500 μL of Protein Precipitation Solution in fresh catch tubes. Samples were mixed by inverting tubes 10 times. Samples were centrifuged at

10,000 x g for 10 minutes to pellet the precipitate. The supernatant was added to 5 mL Eppendorf tubes containing 2 mL Binding Matrix and inverted for 2 minutes by hand. The tubes were left to sit for 3 minutes at room temperature to allow settling of silica matrix. 1 ml of the supernatant from each 5 mL Eppendorf was discarded, being careful to avoid the settled Binding Matrix. Binding Matrix was resuspended in the remaining supernatant and 750 µL of the suspension was added to spin filter tubes. Samples were centrifuged at 10,000 x g for 4 minutes then the catch tubes were emptied. 750 µL of the remaining suspension was resuspended again in the 5 mL Eppendorfs and added to the spin filter tubes. The samples were centrifuged at 10,000 x g for 4 minutes and the catch tubes were again emptied. 500 µL of prepared SEWS-M was added to the spin filter tubes, and the pellet was gently resuspended. Samples were centrifuged at 10,000 x g for 10 minutes, and catch tubes again emptied. All samples were centrifuged a second time without any addition of liquid at 10,000 x g for 10 minutes to get rid of residual wash solution. The catch tubes were discarded and replaced with new clean labelled catch tubes. Samples were incubated for 15 minutes at 37˚C. 65 µL of DNase/Pyrogen-Free Water (DES) was added to the samples then they were incubated at room temperature for 5 minutes. Finally, the samples were centrifuged at 10,000 x g for 4 minutes to bring eluted DNA into the catch tubes.

DNA concentration was measured using Qubit and DNA size was measured using Tapestation.

2.2.17 Nanopore sequencing & genome assembly

The genomes of *E. innesii* GAL7^{T}, *E. innesii* GAL9, *E. innesii* GAL10 and *E. innesii* TL2 were sequenced using the Nanopore MinION sequencing platform.

The sequencing library was prepared via a modified Illumina Nextera Flex low input tagmentation approach using symmetrical 24 base barcoded primers (Baker et al. 2021). Libraries were pooled and stringently size selected on a sageELF 0.75 % cassette and fractions from 4 kb and above were pooled and put into a standard Nanopore Ligation reaction using the SQK-LSK109 kit and protocol and loaded onto a MinION following the recommended loading guidelines and run for 48 h. Basecalling was performed using Guppy version 3.6.0 (Oxford Nanopore Technologies) in high accuracy mode (model dna_r9.4.1_450bps_hac). Subsequently, high-quality pure culture genomes (genome size range: 3.6–3.8 Mb) were assembled via Unicycler version 0.4.9 (Wick et al. 2017) and further polished using Racon version 1.3.1 in the Unicycler pipeline, with a range of 13–18 in contigs and G+C content of ~42 mol%. Genomes were further annotated using Prokka version 1.13.

2.2.18 Genomic characterisation of *E. innesii*

The 16S rRNA sequences of 61 validated *Enterococcus* species (60 were *Enterococcus* type strains) were obtained from the web server of List of Prokaryotic names with Standing in Nomenclature (LPSN; May 2021) (Parte 2014; Parte et al. 2020). Using *in silico* approaches, near-full-length 16S rRNA sequences (~1.5 kb) of *E. innesii* were extracted via bactspeciesID version 1.2 (Kiu 2020a), aligned with 16S rRNA sequences of other 61 public genomes using MUSCLE version 3.8.31 (Edgar 2004), and a 16S rRNA-based maximum-likelihood phylogenetic tree was reconstructed via IQ-TREE version 2.0.5 with the GTR model at 1000 bootstrap replications while visualized with iTOL version 6 (Letunic and Bork 2019; Minh et al. 2020). Digital DNA–DNA hybridization (dDDH) was carried out via the Type Strain Genome Server (TYGS) and average nucleotide identity (ANI) analysis was carried out via fastANI v1.3.

Ten closest-related *Enterococcus* strains (vs *E. innesii*) were identified by TYGS. Antibiotic resistance genes were screened, using the *resfinder* database, for the four novel *E. innesii* strains (Bortolaia et al. 2020) and these 10 related strains. The pangenome of these 14 strains were investigated using Roary version 3.12.0 (Page et al. 2015) at BLASTp threshold at 70% identity for inference of core genes. Next, a core-gene alignment was generated and further supported by single nucleotide polymorphism (SNP) analysis carried out using snp-dists version 0.7.0.

2.2.19 AntiSMASH & BAGEL

Identification of biosynthetic gene clusters was carried out using antiSMASH (v5.2.0), and identification of genes involved in bacteriocin synthesis and secretion was carried out using BAGEL4 (v1.1). The identified bacteriocin sequence was used to search PFAM for matches.

2.2.20 Comparison of efficiency of bacterial extraction

Bacteria were extracted from three different sample types using three different homogenisation methods. The sample types were whole larvae, flash frozen in liquid nitrogen; dissected guts, flash frozen; and dissected guts, thawed. The homogenisation methods were bead beating using Lysing Matrix D tubes and a Fastprep-24™, for 40 seconds on speed 6.0; grinding using a Pellet Pestle™; and grinding using a ceramic pestle and mortar. The beads and pellet pestle were sterilised by autoclaving. The ceramic pestle and mortar were sterilised by washing with 70% ethanol. Following homogenisation, the samples were serially diluted and spread on BHI agar plates and incubated for 16-20 hours at 37°C before colonies were counted.

2.2.21 Motility tests

Motility tests were carried out on *E. innesii* GAL7^T using motility test medium (Merck). Media were prepared according to manufacturer's instructions in 20 mL Universal vials. *Enterococcus innesii* (GAL7), *Enterococcus casseliflavus* (GAL2), *Pseudomonas fluorescens* (SBW25), *Escherichia coli* (MG1655) and *Enterococcus faecalis* (NCIMB 13280) were cultured overnight. Sterile loops were used to stab the culture into the media. Vials were incubated for 48 h at 37°C before photographing.

2.2.22 MIC assays

The susceptibility of *E. innesii* GAL7^T to antibiotic vancomycin was evaluated using MIC assays on BHI agar plates (carried out in three biological replicates) as described previously (Andrews 2001) . 4 μL of 1/10 serial dilutions of an OD_{600} = 1 culture were spotted on BHI agar Petri dishes supplemented with 2-fold dilutions of an antibiotic. Plates were incubated 20-22 h at 37°C. The MIC value was defined as the lowest antibiotic concentration in which there was no visible growth in the OD_{600} = 0.01 spot (~10,000 cells).

2.2.23 Biochemical characterisation

Biochemical characterisation of *E. innesii* was carried out by the Identification Service, Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Biochemical characteristics were determined using API 50CHE strips for carbohydrate utilization profiles, after incubation for up to 48 h at 37 °C. Further phenotypic features were determined using the API rapidID32 STREP system on single strain *E. innesii* GAL7^T. Pyruvate utilization was tested by culturing with sodium pyruvate as sole carbon source in mineral salt medium for 6 days at 37 °C. Aesculin hydrolysis was tested by culturing in Bacto-Peptone media with 1 g/L aesculin.

Cellular fatty acids were analysed after conversion into fatty acid methyl esters (FAMEs) using a modified protocol by Miller (Miller 1982). Mixtures of the FAMEs were then separated by gas chromatography and detected by a flame ionization detector using the Sherlock Microbial Identification System (MIDI) based on TSBA6 database.

2.2.24 Growth inhibition assays

Enterococcus mundtii (NCIMB 13132), *Bacillus thuringiensis* (NCIMB 9134), *Bacillus cereus* (NCIMB 8012), *Serratia marcescens* (NCIMB 9155), *Enterococcus faecalis* (NCIMB 8260) and *Staphylococcus aureus* (NCIMB 8625) were obtained from NCIMB. *Pseudomonas fluorescens* (SBW25) and

Pseudomonas protegens (PS682) were obtained from Alba Pacheco-Moreno at JIC. *Enterococcus innesii* (GAL7) and *Enterococcus casseliflavus* (GAL2) were isolated from *Galleria*. *Escherichia coli* (MG1655) was taken from lab stocks.

Cultures of test and indicator strains were grown up in 10 mL BHI overnight at 37°C. Assays were carried out on BHI agar plates.

Test strains were streaked across the plate in two parallel streaks with a sterile toothpick, then incubated at 30°C for 24 hours. Indicator strains were then streaked perpendicular to the test strains and again incubated for 24 hours at 30°C.

A separate assay was carried out in which, test strains were streaked, left to dry, and then indicator strains cross-streaked immediately after. Plates were incubated for 48 hours at 30°C.

For the radial streak assays, 20 μL of test strain was dropped in the middle of the plate, left to dry, then incubated at 30°C for 48 hours. Indicator strains were then streaked radially inwards towards the centre with a sterile toothpick, without contacting the centre spot. Plates were incubated for 24 hours at 30°C.

2.2.25 Colonising guts with bacterial cultures

Staphylococcus capitis, *Escherichia coli*, *Bifidobacterium longum* subsp. *infantis* and *Lactobacillus acidophilus* were provided by the Hall lab at QIB. *S. capitis* and *E. coli* were cultured in LB broth in a shaking incubator at 37°C. *B. infantis* and *L. acidophilus* were cultured in MRS broth supplemented with 0.5% cysteine in a static incubator at 37°C. Either 1 mL, 2 mL or 5 mL of culture was added to approx. 25 g of food and placed in a 90 mm Petri dish.

Larvae that had been treated with antibiotics were placed on this food and allowed to feed for a further 5 days. They were then dissected, and their guts homogenised and plated.

2.2.26 Initial faecal slurry colonisation attempt

Two 1 g samples were separately diluted with 1 mL PBS (Formedium) and mixed until homogenous. The slurry was mixed with 20 g of food each. This food-slurry mix was placed in Petri dishes. 20 g of food mixed with 2 mL PBS was also placed in a Petri dish. Some of this food-slurry mix was placed in a separate Petri dish and incubated at 37°C. At 0, 1 and 24 h, ~200 μg of food was sampled, diluted

with an equal volume of PBS, vortexed for 10 s and briefly centrifuged. The supernatant was diluted 10^{-3} and 50 μL was plated on BHI, MacConkey and RCA agar.

Ten antibiotic-treated larvae for each dish (30 total), were fed sterile food without antibiotics for 2 days were placed on the faecal slurry food/control food and kept in an incubator at 37°C. After 2 and 4 days three larvae from each group were dissected, the guts homogenised and then plated. Plates were incubated aerobically and anaerobically at 37°C for 24 h. Four individual colonies from each plate were picked and streaked, then colonies from these plates were used for 16S colony PCR.

2.2.27 Gut colonisation with faecal slurry stock

A 1 g aliquot of faecal slurry was mixed with 10 g of food in a Petri dish. 1 mL of PBS was mixed with 10 g of food in a Petri dish. 12 larvae previously treated with antibiotics were placed in each dish and left in a 37°C incubator for 8 days. Their guts were then dissected, homogenised, and plated on BHI agar.

2.2.28 Colonisation using faecal slurry

1 g of faecal slurry was mixed into 10 g of sterile food in each of 3 Petri dishes. 12 larvae were placed on the food then incubated at 37°C. On day 0, guts were dissected from 9 antibiotic-treated larvae, followed by 9 larvae each from the control and faecal slurry groups on days 1, 2, 4 and 8. Guts were pooled 3 per sample and homogenised using glass beads in an Omni Bead Ruptor on Speed 4 for 2x 60 seconds with a 30 second dwell time. Samples were diluted 1000x in PBS, spread on BHI plates and incubated for 24 hours at 37°C.

2.2.29 Oral colonisation using *Enterococcus*

Cultures of *E. faecalis* (P46G 41)*, E. casseliflavus* (GAL2) and *E. innesii* (GAL7) were grown overnight in 10 mL BHI broth at 37°C. Culture was diluted to OD_{600} = 0.5 (approx. 10^8 cfu/mL *Enterococcus)* and then 1 mL of culture was mixed into 10 g of sterile food in a Petri dish. 10-12 larvae were placed on the food then incubated at 37°C. On day 0, guts were dissected from 9 antibiotic-treated larvae, followed by 9 larvae each from the control and faecal slurry groups on days 1, 2, 4 and 8. Guts were pooled three per sample and homogenised using glass beads in an Omni Bead Ruptor on Speed 4 for 2x 60 seconds with a 30 second dwell time. Samples were diluted 1000x in PBS, spread on BHI plates and incubated for 24 hours at 37°C.

2.2.30 Through injection

Cultures were grown up overnight in 10 mL BHI broth. 1 mL of overnight culture was spun in a microcentrifuge at 13,000 rpm for 10 minutes then resuspended in 100 μL PBS. Cultures were diluted to OD_{600} = 0.5 (approx. 10^8 cfu/mL *Enterococcus)* and then 10 μL was injected into antibiotic-treated larvae.

Mortality was assessed using a health index from Champion, Titball and Bates (2018). Larvae were homogenised using glass beads in an Omni Bead Ruptor on Speed 4 for 2x 60 seconds with a 30 second dwell time. This homogenate was diluted by 10^3 in PBS and spread on BHI agar then incubated at 37°C for 48 hours.

Category	*Description*	*Score*
Activity	No movement	0
	Minimal movement on stimulation	1
	Move when stimulated	2
	Move without stimulation	3
Cocoon formation	No cocoon	0
	Partial cocoon	0.5
	Full cocoon	1
Melanisation	Black larvae	0
	Black spots on brown larvae	1
	≥3 spots on beige larvae	2
	<3 spots on beige larvae	3
	No melanisation	4
Survival	Dead	0
	Alive	2

Table 2.5: The Galleria mellonella health index scoring system *(Champion, Titball, and Bates 2018)*

2.2.31 Plasmid extraction and quantification of DNA

Enterococcus faecalis pMV158-mCherry was provided by Stephane Mesnage of the University of Sheffield. The strain was streaked on BHI agar plates and incubated overnight at 37°C. A single colony was used to inoculate 10 mL of BHI broth. The culture was centrifuged for 15 minutes at 6000

rpm at 4°C. To extract the plasmid, a QIAprep Spin Miniprep Kit (QIAGEN) was used with 5 μg mutanolysin added for the initial lysis step.

2.2.32 Generation of electrocompetent *Enterococcus mundtii* cells

Three buffers were prepared from autoclave-sterilised materials. Medium A (2.5 mL 20% glycine, 5 mL 1 M sucrose, 42.5 mL BHI); Medium B (5 mL 2x BHI, 5 mL 1 M sucrose); washing buffer (10 mL 1 M sucrose, 10 mL 20% glycerol). Washing buffer was stored and used at 4°C.

Enterococcus mundtii (NCIMB 13132) was taken from a glycerol stock and incubated in 3 mL BHI at 37°C overnight. 30 μL of the overnight culture was added to 25 mL medium A and again incubated at 37°C overnight.

The culture was centrifuged at 4500 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 25 mL of pre-warmed medium A before a 1 hour incubation at 37°C. The culture was again centrifuged at 4500 rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in 10 mL of washing buffer. This 10 mL was divided into six 2 mL microcentrifuge tubes. These were centrifuged at 12000 rpm for 1 minute and the supernatant was discarded. The cells were resuspended in 2 mL washing buffer, centrifuged again at 12000 for 1 minute, and the supernatant discarded. The pellets were resuspended in 200 μL of washing buffer each and all six tubes recombined in a single tube. This was then separated into 50 μL aliquots that were flash-frozen in liquid nitrogen and stored at -80°C until use.

2.2.33 Transformation using pMV158-mcherry

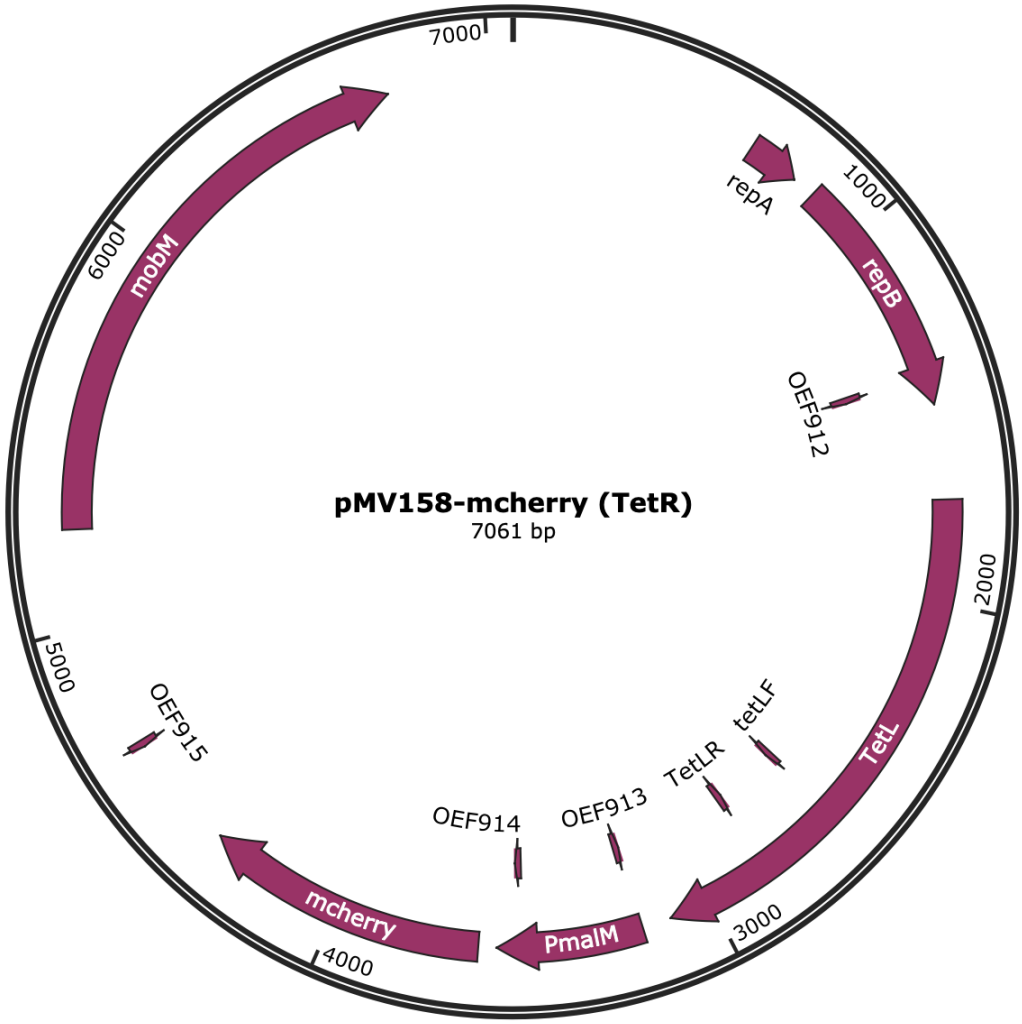


Figure 2.2: pMV158-mcherry plasmid map *(SnapGene). Showing the fluorescence gene mCherry and the tetracyline resistance gene TetL*

50 μL of electrocompetent *E. mundtii* cells were defrosted. 3 μL of pMV158 mCherry (100 ng/μL) (**Figure 2.2**) was added to the 50 μL cells. The mixture was moved to a cuvette (2 mm gap) and kept on ice for 20 minutes. Electroporation was carried out at 2.5 kV. The cells were resuspended in the cuvette in 1.8 mL medium B at room temperature, moved to a new 2 mL microcentrifuge tube, and incubated at 37°C for 2 hours. The culture was centrifuged at 12,000 rpm for 1 minute, and all but 100 μL of supernatant removed. The pellet was resuspended. 20 μL was spread on a BHI agar plate with 15 μg/mL tetracycline and incubated at 37°C for 2 days. Colonies were picked and cultured in broth with 15 μg/mL tetracycline. Transformation was verified using PCR.

2.2.34 Loss of the pMV158 plasmid

Ten (10) μL of pMV158 *E. mundtii* culture was added to 10 mL of non-selective BHI broth and cultured for 8 hours at 37°C before serial dilution and plating on BHI agar plates with and without 100 μg/mL tetracycline.

2.2.35 Injection of labelled *E. mundtii*

Antibiotic-treated and untreated larvae were injected with 10 μL of labelled bacteria at OD_{600} = 0.5 and incubated at 37°C for 2 and 4 days. Larvae were homogenised and the homogenate serially diluted and plated on BHI agar plates.

2.2.36 Passage of labelled *E. mundtii* over generations

Antibiotic-treated and untreated larvae were injected with 10 μL of labelled bacteria at OD_{600} = 0.5 and incubated at 37°C for 2 and 4 days. Larvae were allowed to pupate and lay eggs, eggs were hatched and larvae sampled again after 4 weeks incubation at 30°C. Larvae were homogenised and the homogenate serially diluted and plated on BHI agar plates.

2.2.37 Horizontal transfer of the pMV158 plasmid from *E. mundtii* to *E. innesii in vitro*

E. innesii and pMV158-labelled *E. mundtii* were cultured to OD_{600} = 0.5. 0.5 μL of each were added to 5 mL of BHI broth and cultured at 37°C for 8 hours. The co-culture was serially diluted and plated on BHI agar plates containing 15 μg/mL tetracycline, 4 μg/mL vancomycin, both tetracycline and vancomycin, and no antibiotic.

Initial toxicity assaysFive pale, fast moving larvae (200 – 300 mg each) were selected for each compound for each step of the assay. All of the larvae were weighed and the average weight was used to calculate dosage. Test
compounds were suspended in 10% DMSO with PBS buffer and diluted if necessary. Compounds were injected through the last left proleg (Hamilton syringe 705 SN SYR, 22s gauge, bevel point) while held over a p1000 tip. The syringe was rinsed with sterile water between injections and 70% ethanol and sterile water between compounds. Larvae were incubated at 30°C in the dark and mortality was recorded after 48 hours.

The toxicity testing procedure was taken from Ignasiak, K. and A. Maxwell (2017):
“A flowchart, adapted from the OECD guidelines for acute toxicity (OECD 2002), was used to select the toxic dose of test compounds. The acute toxicity testing was started by injecting five larvae with

the initial dose of a compound (5 mg/kg body weight). Larval mortality was recorded daily. If three or more larvae died, the compound was assigned the highest toxicity class (GHS 1). If three or more larvae survived for 5 days, the toxicity testing was continued by re-testing the initial dose (5 mg/kg body weight) on a new cohort of larvae. If three or more larvae of the second cohort survived, a higher dose (25 mg/kg body weight) was tested in five fresh larvae. The experiment was continued until a toxic dose was established. If a compound was not toxic at the highest dose tested (2000 mg/kg body weight), the compound was classified as non-toxic. The obtained toxic dose was compared to a dose reported in Material Safety Data Sheet (MSDS) for the compound. Where possible the reported value used for the comparison was from mouse or rat via an intraperitoneal injection, when such data was absent the data from oral toxicity tests in a mammalian system was used. Each step in the procedure included three control groups: untreated control, traumatized control (cuticle was pierced with a needle) and buffer-injected control."

2.2.38 Extract toxicity assay

Plant extract was supplied by Martin Goldberg (BCU). Larvae were injected with 10 μL of extract at varying concentrations of extract and DMSO and mortality was scored each day for 5 days.

2.2.39 *Staphylococcus aureus* infectivity assay

Staphylococcus aureus (NCIMB 8625) was streaked from a glycerol stock onto a BHI agar (Sigma) plate and cultured overnight. A single colony was taken and used to inoculate 5 mL of BHI broth (Sigma) until OD_{600} = 0.5. 1 mL of culture was centrifuged at 13,200 rpm for 1 minute. The supernatant was resuspended in 100 μL of PBS. This was then diluted 1/10 and 1/100. 20 μL of each was serially diluted, plated on BHI agar (Sigma) plates and incubated overnight at 37°C. Colonies were counted the following day.

For each dilution, five larvae of mass 180-220 mg were selected. 10 μL of cells was injected into each larva. Larvae were incubated for 5 days at 37°C and mortality was scored.

2.2.40 Microbial efficacy assay

For each assay, 40 larvae of mass 180-220 mg were selected. 1.6 mg plant extract was supplied by Martin Goldberg (BCU) and suspended in 400 μL DMSO.

Staphylococcus aureus (NCIMB 8625) was cultured overnight at 37°C in BHI broth. 20 μL of culture was used to inoculate 5 mL of BHI broth, which was incubated at 37°C until OD_{600} = 0.5. The plant extract was diluted 1/10 in PBS to a final concentration of 0.4 mg/mL.

Fifteen (15) μL of culture or 15 μL of broth was injected into each larva. Larvae were incubated at 37°C for 2 hours. 7 μL of extract (0.4 mg/mL) or 7 μL of 10% DMSO PBS was injected into each larva. Larvae were incubated at 37°C for 5 days. Each day mortality and health index (bottom) (Champion, Titball, and Bates 2018) were scored.

2.2.41 Uppsala compounds toxicity assays

Compounds (IDs: 1488, 1529, 1532, 2086) were supplied from Uppsala University, suspended in DMSO, and diluted 1/10 in PBS. 5 healthy larvae of mass 180-200 mg were selected per per compound tested. 5 larvae were injected with each compound in 10 μL at 10x the MIC (w/w in larvae), and 5 additional larvae were injected with 10% DMSO PBS. Mortality was scored after 5 days.

Chapter 3

3 The Care and Keeping of Waxmoths

3.1 Introduction

At the start of the project, there was no current colony of *Galleria* being maintained in the lab, so the first task was to establish the basics of how the colony would be maintained and experiments would be carried out. Eggs were supplied by the Insectary and taken to the lab, where they were reared in in clear plastic lunchboxes, in incubators at 30°C and 37°C. Next it was necessary to develop methods to clear *Galleria* larvae of their own gut microbiome and replace it with bacteria from humans. Ideally, larvae would be colonised with bacteria over a period of days without causing the larvae harm. Both feeding and injection were used as methods to achieve this.

Some of this preliminary work had already been carried out by Marjorie Labédan, who worked in the Maxwell lab for 6 months before I arrived. She had investigated how to clear the *Galleria* larvae of their own microbiome and how to colonise them with faecal slurry. However, her work was very preliminary, so it was deemed necessary to repeat it.

3.2 Results

3.2.1 A *G. mellonella* artificial diet

The first task I had was to determine which diet would be most appropriate for my project. This has been previously tested by Jorjão et al. (Jorjão et al. 2018). Diets 1 to 3 as tested in Jorjão et al. are shown in **Table 3.1**. Diet A is based on the diet that the Insectary at JIC had been using, which they had found to be effective. This diet is similar to Diet 1 from Jorjão et al. I decided to compare Diet A to Diet 3 from Jorjão et al. I did not test Diet 2 as it contains dried (living) yeast, which I thought might be an obstacle to my microbiology work, despite the fact it would be autoclaved. I made some modifications to the insectary diet and Diet 3. I chose not to use beeswax as I didn't know what effect it might have on the human gut commensals I was trying to introduce to the larvae and I wanted to keep the diet as similar to a human's as possible.

I reared larvae from eggs to pupae on each diet to compare how practical they were for my project. The two diets I tested, A and B, are shown in Table 3.2 below.

Diet 1	Diet 2	Diet 3
20 g of brown sugar	300 g oat flakes	250 g of corn meal
80 g of glycerol	300 g of whole wheat flour	150 g of yeast extract
400 g of powder milk	60 g dried yeast	100 g of soy flour
120 g yeast extract	120 mL of glycerol	100 g of powder milk
200 g of whole wheat flour	120 mL of honey	200 g of honey
200g wheat bran	Beeswax blocks	200 g of glycerol
200 g of wheat germ		Beeswax blocks
Beeswax blocks		

Table 3.1: Diets tested by Jorjão et al. *(Jorjão et al. 2018)*

Diet A	Diet B
20 g brown sugar	40 g honey
10 mL water	40 g glycerol
40 mL glycerol	30 g yeast extract
20 g powdered milk	20 g soy flour
20 g wholemeal flour	20 g powdered milk
10 g yeast extract	
10 g wheat germ	
40 g bran	

Table 3.2: Composition of diets tested *(Diet B here approximately corresponds to Diet 3 in Jorjão et al. (Jorjão et al. 2018)*

I made up the two diets as described, put 10 g of each into Petri dishes and then placed unhatched eggs on each. I observed that Diet A had a far lower mortality rate than Diet B, which may be due to nutrients or to Diet B being denser and stickier, therefore harder for first instar larvae to move through. I also found in future experiments that Diet A it is still not sticky after the addition of liquid, which is useful given the number of experiments that require feeding *Galleria* by mixing liquid with the food. Diet B, in contrast, becomes so sticky after the addition of even 0.5 mL of liquid to 10 g that even large larvae are prone to drowning in it. Therefore I decided that Diet B was not suitable for my project and decided to use Diet A from this point forward.

3.2.2 Antibiotic treatment to clear the gut bacteria from *G. mellonella* larvae

Before attempting to replace the gut microbiome of *Galleria* I had to first clear the native microbiome. Marjorie Labédan had previously attempted this and found that after 5 days of treatment with 15 mg/100 g of each of oxytetracycline and streptomycin, no more colonies grew when she plated the homogenised guts and she stopped seeing bands after 16S rRNA PCR amplification and electrophoresis. I wanted to confirm this result for my own experiments. The antibiotics oxytetracycline and streptomycin were chosen because using two broad-spectrum antibiotics maximises the likelihood that the bacteria in the larvae will be susceptible.

I fed larvae 15 mg oxytetracycline and streptomycin per 100 g of food for 0, 1, 5 and 10 days, using 10 larvae for each timepoint. The guts were dissected out, homogenised, and the DNA purified. Prior to DNA purification I also plated the homogenate at 1/100 dilution on BHI plates.

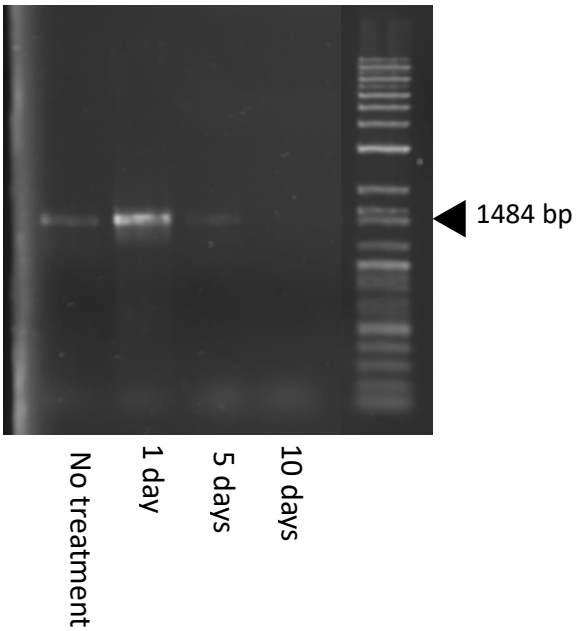


***Figure 3.1: 16S PCR of purified DNA from gut extracts following antibiotic treatment**. Larvae were fed 15 mg of each of streptomycin and oxytetracycline for 0, 1, 5 or 10 days before being killed by flash freezing. Guts were dissected out and homogenised using bead-bashing. DNA was purified and 16S PCR carried out. The PCR products are shown here by 1% agarose gel electrophoresis.*

No colonies grew on the plates from the 5-day or 10-day treated gut samples. **Figure 3.1** shows PCR products of 16S rRNA amplification of the purified DNA. A band can be seen for the 1-day and 5-day samples but not from the 10-day, showing that 10 days is probably how long it takes to clear the guts. This is contrary to Marjorie's findings, that it only took 5 days, but is only a small discrepancy that could be the result of different growth conditions or PCR protocols. Many of Marjorie's later results also suggest that the gut had not in fact been fully cleared, as many of the isolates she

identified after faecal slurry feeding were more typical of the *Galleria* microbiome than that of a human infant.

3.2.3 Life cycle

I sought to investigate whether the conditions I was using were producing larvae with masses and life spans similar to the known means (Jorjão et al. 2018; Kwadha et al. 2017) for lab-reared *Galleria* larvae. I recorded the time taken for the eggs to hatch by collecting the eggs on the day they were laid and incubating them at 30°C in a Universal vial, checking them daily until they hatched. Eggs started hatching 5 days after being laid and the final eggs hatched 10 days after being laid.

I collected another set of eggs and incubated 50 mg of them on Diet A, as described above, at 30°C in a lunchbox and recorded the time between them hatching and pupating.

After 30 days I weighed and counted the larvae. There were 60 larvae and their average mass was 235±26 mg. I checked how many larvae had pupated each day. On day 36 44/60 of the larvae had pupated, giving a median larval phase length of 36 days. Of the remaining larvae, 6 were healthy: pale and fast-moving. The remaining 10 larvae were smaller and brown from melanisation. The average mass of the healthy larvae was 292 mg, within normal bounds for final-stage *Galleria* larvae (Jorjão et al. 2018).

3.2.4 Egg treatment and storage

Sterilising eggs is a common technique used to rear germ-free insects but there is currently no published method for doing so with *Galleria*. It is known (Bucher 1963) that bacteria are passed from adult moths to offspring by being spread over the surface of the egg, so I tried to sterilise the eggs by bleaching them. I dipped 100 mg of eggs in 2.5% bleach, then in double-distilled water, then I placed the eggs in Universal vials and incubated them at 30°C. Each day I counted the number of hatched larvae before then removing the eggs, clearing the vial of larvae, and returning the eggs to the vial.

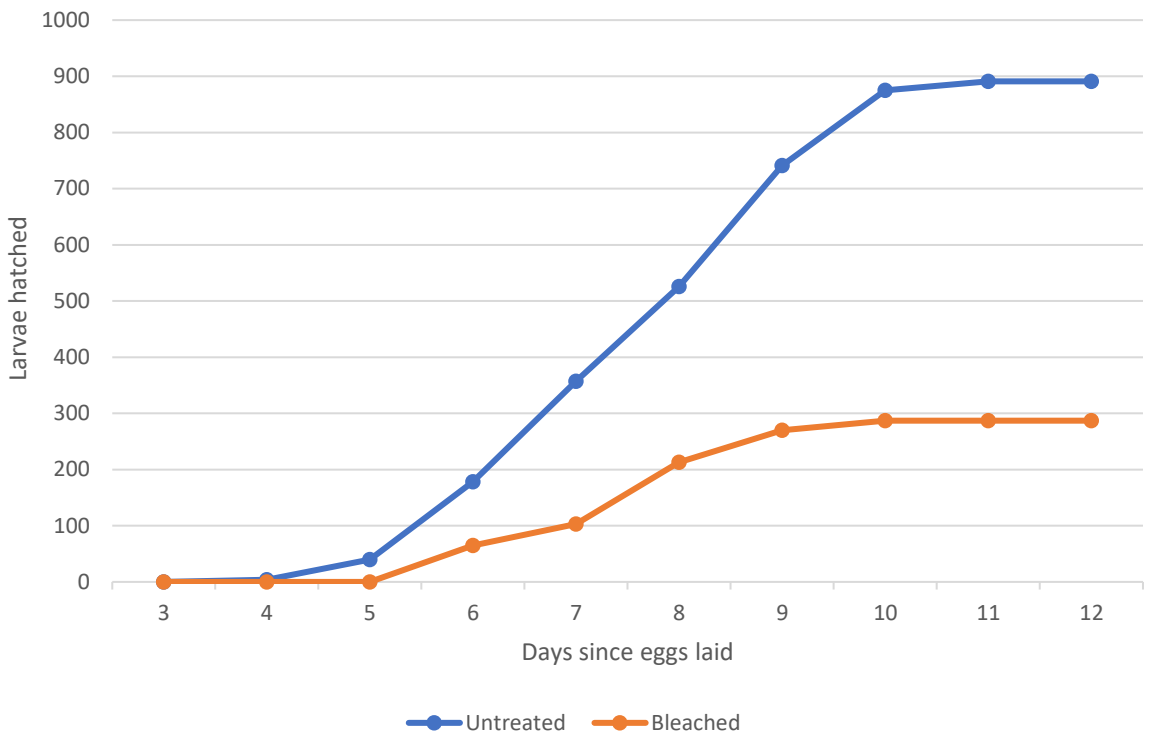


Figure 3.2: The effect of bleaching on Galleria egg hatch rates. *0.1 g of eggs were either left untreated (blue) or rinsed in 2.5% bleach (orange), before being placed in a vial and incubated at 30 ℃. The number of hatched larvae was counted every day. One replicate*

As seen in **Figure 3.2**, eggs rinsed with bleach have a hatch rate ⅓ that of untreated eggs at 30°C. However, I found issues after hatching as I didn't have any way of containing the larvae that was both secure enough and sterile enough: small larvae will crawl out of Petri dishes and get stuck to the tape, but the Watkins and Doncaster boxes I had been using could not be sufficiently sterilised to keep larvae free of bacteria, so they succumbed to infection before they could grow large enough to be collected and homogenised. As a result I did not continue using this method.

3.2.5 Comparison of efficiency of bacterial extraction

I carried out an experiment to test if the method by which I was extracting bacteria from the larvae was optimal. I tried several methods from the literature and from what was available to me: bead beating using glass beads and a Fastprep-24™, a Pellet Pestle™, and a ceramic pestle and mortar. The beads and pellet pestle were sterilised by autoclaving, whereas the ceramic pestle and mortar could only be sterilised by washing with ethanol. I used these methods on whole larvae flash frozen in liquid nitrogen, frozen larval guts and larval guts that had not been re-frozen following dissection. I carried out each method on 5 larvae each. I then serially diluted the homogenates and plated them on BHI agar plates.

The quantities of bacteria isolated can be seen in **Figure 3.3**. Keeping the gut material frozen and bead beating led to the highest number of bacteria extracted from the gut. This is the method I had been using up to this point, so I continued to use this method. It is unsurprising that the whole larva yielded more bacteria than just the gut, given that whole larvae are larger, and can be prepared with minimal time at room temperature. During the dissection of the gut, the gut is exposed to the open air at room temperature, during which time many bacteria may die.

In contrast to the other two methods, the quantities of bacteria resulting from homogenisation using the ceramic pestle and mortar did not change meaningfully depending on the substrate. This may reflect the difficulty in sterilising the ceramic pestle and mortar, as I only cleaned it using ethanol, which cannot effectively remove all the bacteria. Thus, there will be a high degree of contamination which results in the appearance of more bacteria having been extracted, even when the substrate itself does not have many, i.e. the thawed gut samples.

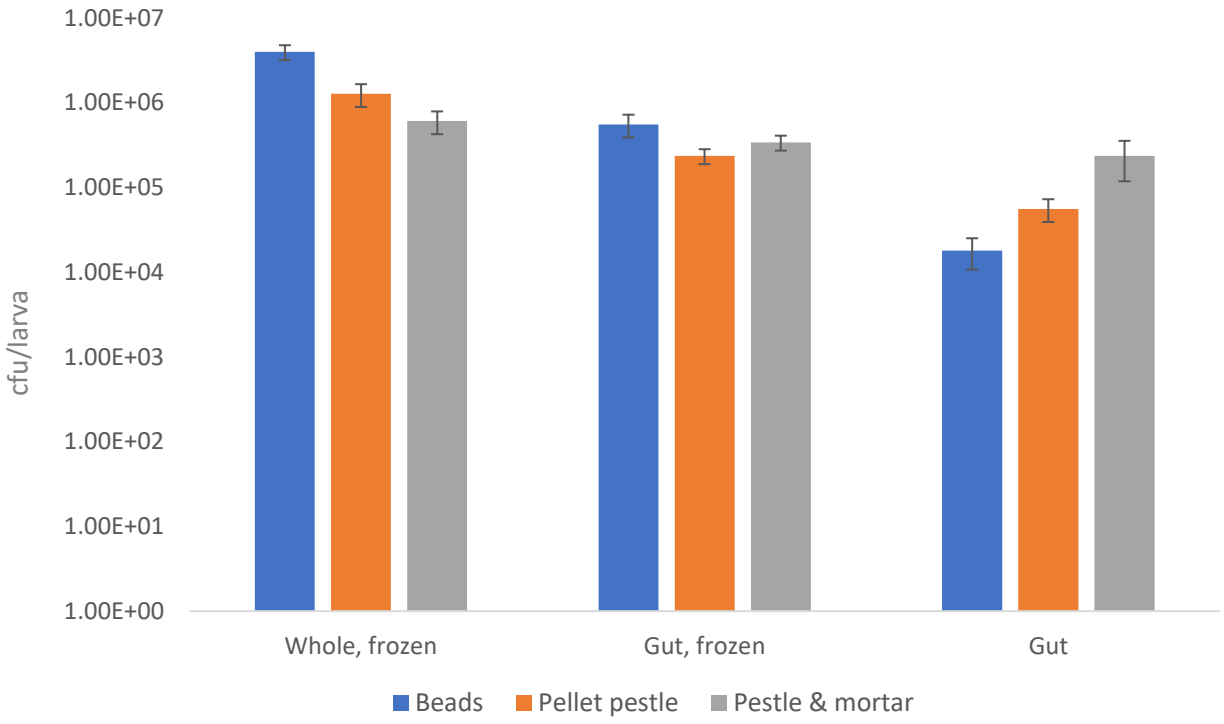


Figure 3.3: Comparison of different protocols for the isolation of bacteria from larvae. *Three different substrates: whole larvae flash frozen in liquid nitrogen; larval guts homogenised while frozen; and larval guts homogenised while thawed, were subject to three different homogenisation protocols: bead-bashing; grinding using a Pellet Pestle™; and grinding using a ceramic mortar and pestle. Homogenates were serially diluted and plated on BHI agar plates. Plates were incubated at 37℃ for 24 hours and colonies counted*

3.2.6 Introducing bacteria to recently hatched larvae

I attempted to colonise newly hatched larvae by putting 100 mg of eggs on food mixed with faecal slurry directly before they hatched. As I found in the egg-bleaching experiment (**Figure 3.2**), 100 mg

of eggs is approximately 1000 eggs. After 1 week of incubation at 37°C only 3 live larvae could be seen in one of the dishes and none in the other. I suspect the rest died on the food, perhaps due to the food being too wet, or because of the higher temperature. I think that recently hatched larvae may be too fragile to attempt to colonise them.

3.2.7 Colonising *G. mellonella* larvae with pure bacteria

I decided that initially it might be best to try to colonise the *Galleria* gut with individual strains of bacteria from culture by mixing the bacterial culture into the food. I tried this by mixing different quantities (1 mL, 2 mL, 5 mL) of mid-log-phase culture into 25 g of food. I then left 10 treated larvae on the food and incubated them at 37°C for 5 days. After 5 days I dissected out the guts, homogenised them, diluted 1/1000, and plated them on BHI plates for aerobic incubation and RCA plates for anaerobic. I also purified the DNA from the samples and carried out 16S rRNA PCR. I did this with *E. coli, S. hominis, B. infantis* and *L. acidophilus* isolates I had been given from the Hall lab, all of which were isolates from either the infant gut or from infant probiotics.

I did this several times and never got any colonies or any amplification from the PCR. This is probably because I did not leave sufficient time between when I took them from the antibiotic food and when I put them on the bacterial food. This will have meant that the larvae would still have high concentrations of antibiotic in their gut, which I suspect would have prevented any colonisation of the gut by bacteria. I did not test how long the bacteria survived on the food for, but I imagine that by the time the antibiotic concentration in the gut had reduced enough to allow colonisation, not enough bacteria would have remained alive on the food to colonise the gut upon ingestion.

3.2.8 Oral colonisation with Enterococci

Later, I decided to try the oral colonisation route again, this time giving the larvae a 2-day break to ensure that they no longer contained inhibitory levels of antibiotic, and using enterococci, the genus dominant in the native *Galleria* microbiome. I used *E. innesii*, isolated from my larvae, *E. mundtii,* a known *Galleria* commensal (Johnston and Rolff 2015), and an *E. faecalis* isolate from the infant gut.

I took larvae that I had treated with antibiotics and gave them a 2-day break. I then mixed 1 mL of bacterial culture at OD_{600}=0.5 (approx. $1x10^8$ cfu) in 10 g of food. I fed that to 10 larvae per group for 2 days while incubating at 37°C before dissecting out their guts, homogenising them, serially diluting them and plating them on BHI plates. As shown in **Figure 3.4**, I found that for all 3 species, the gut could be colonised with around 10^5 cfu per larva, with no observable harm done to the larvae.

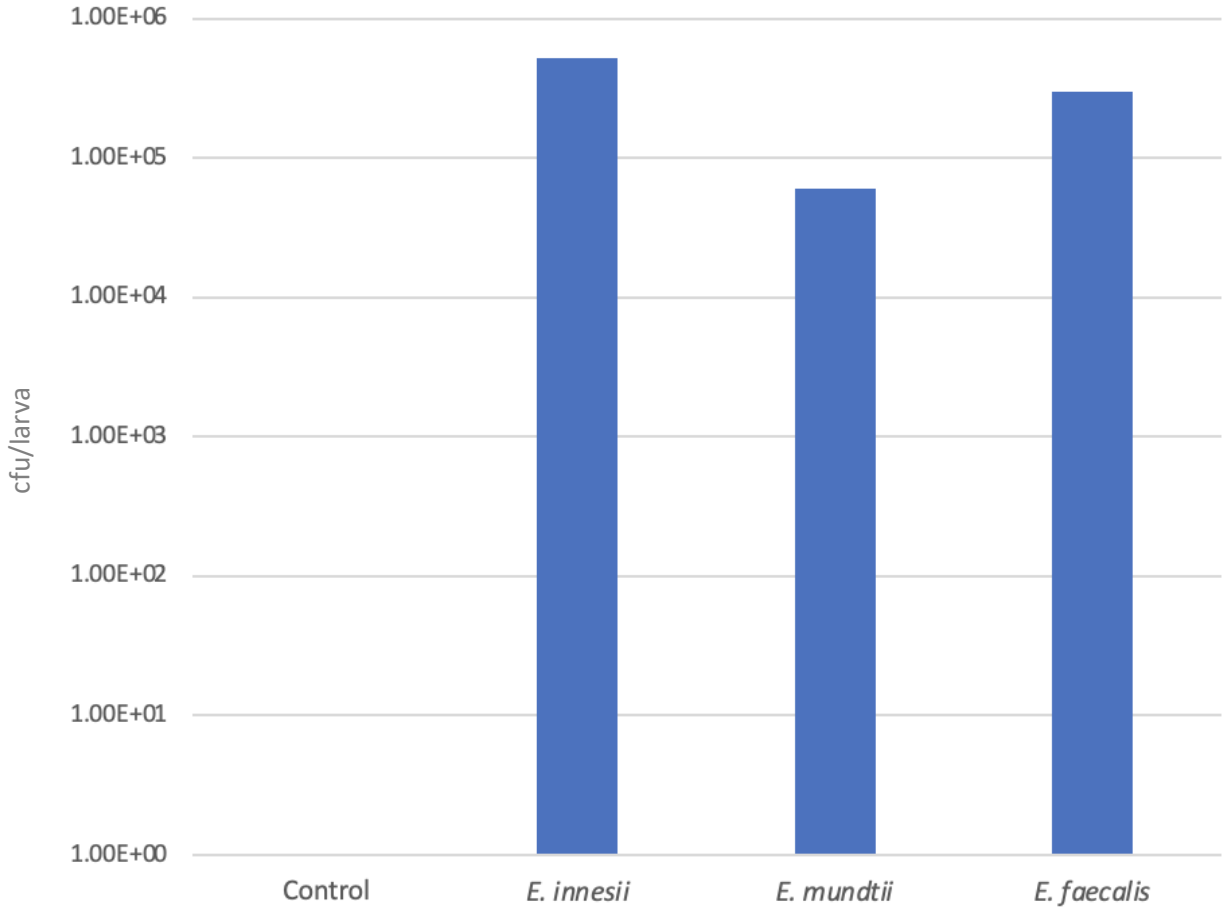


Figure 3.4: Oral colonisation of larvae with bacterial culture. *Antibiotic-treated larvae were fed 1 mL of culture at OD_{600} = 0.5 per 10g of food for 2 days, then homogenised by bead-beating, serially diluted, and plated on BHI agar plates. Plates were incubated at 37℃ for 24 hours and colonies counted. One replicate*

I didn't pursue colonisation through feeding any further than this because I had found that injection was an effective way to colonise *Galleria* with commensal bacteria. Injection allows more precise quantities of bacteria to be delivered, as there is no way of controlling the amount of food a larva will eat in any particular time frame, or whether the bacteria will still be viable at the point at which they are ingested. Injection also allows more precise control over the time at which bacteria are introduced.

3.2.9 Colonisation of *Galleria* through the injection of culture

Injection of bacterial culture is a common method of bacterial infection of *Galleria* using pathogens. I wanted to see if it was also an effective way of colonising *Galleria* with commensal bacteria, which do not have the ability to colonise as aggressively. To do this I used: *E. casseliflavus* and *E. innesii, Galleria* commensals that I had previously isolated; *E. faecalis, S. capitis* and *E. coli* isolates from the infant gut; and *B. cereus,* a soil bacteria strain provided by NCIMB. I treated the larvae with

antibiotics and left them for 2 days as before, then injected them with 10 μL of bacteria suspended in PBS at OD_{600} = 0.5. I used 10 larvae for each group. After 24 and 48 hours I scored the larvae for health using a health scoring index from Champion et al. (Champion, Titball, and Bates 2018). The health index score depends on melanisation, cocoon formation and movement; the highest theoretical score is 10 but the highest realistically achievable score is 9 given the scoring of both movement and cocooning. I then froze the larvae, homogenised them, diluted the homogenate and plated it.

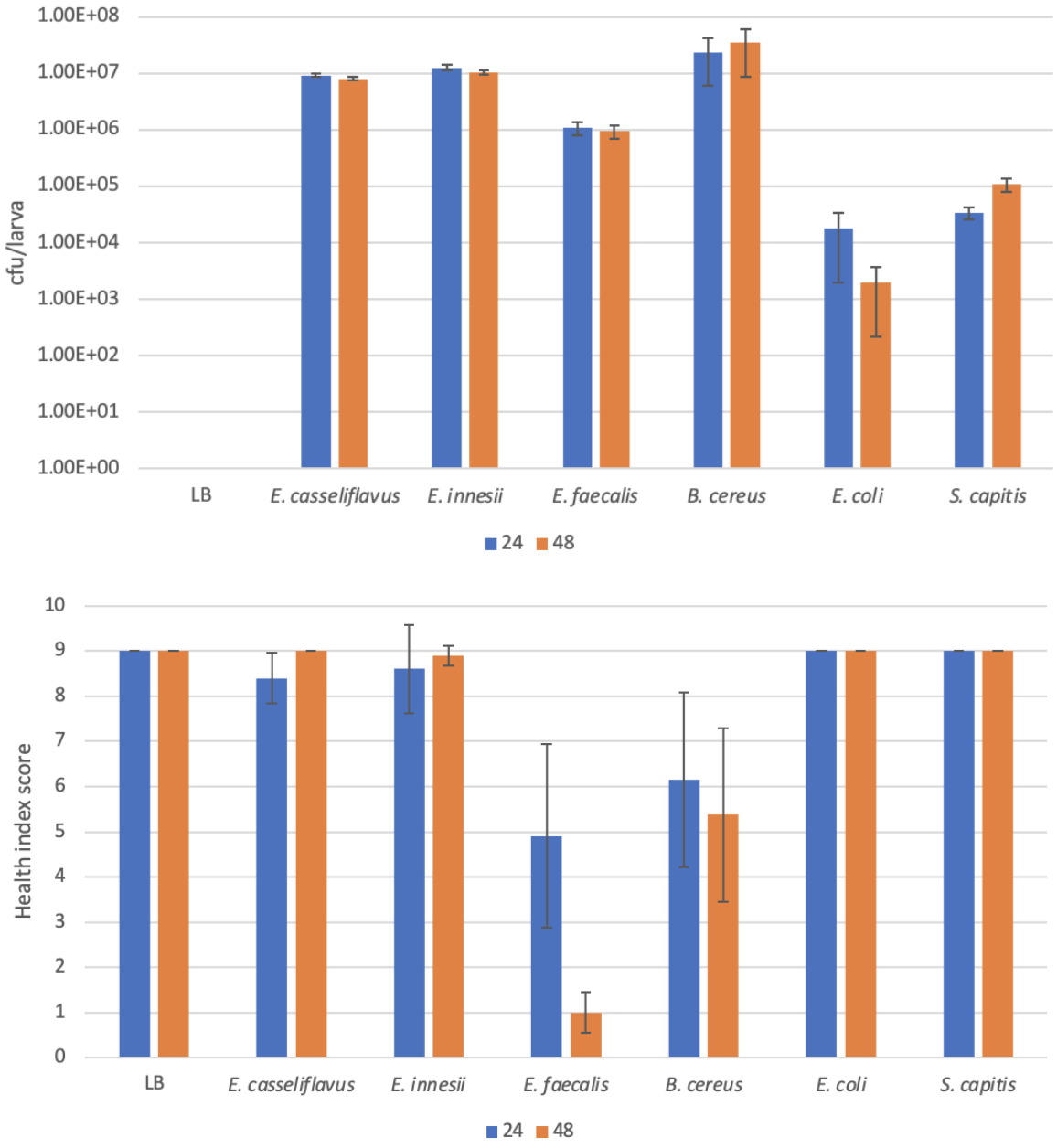


Figure 3.5: Cfu per larva and larval health index following injection of bacterial culture. *Top: cfu per larva following injection with 10 μL bacterial culture at OD_{600} = 0.5, resuspended in PBS and 24 or 48 hours of incubation at 37 ℃. Bottom: health index (as described by Champion et al. (Champion, Titball, and Bates 2018)) of those same larvae*

As seen in **Figure 3.5**, I found that *E. casseliflavus, E. innesii* and *B. cereus* achieved the highest bacterial load in the larvae and that of those, *E. casseliflavus* and *E. innesii* were the least pathogenic. *B. cereus* was moderately pathogenic. *E. faecalis* was highly pathogenic and had moderate bacterial abundance, and *E. coli* and *S. capitis* had low bacterial abundance and had no measurable impact on the health of the larvae. This indicates to me that enterococci are more effective commensal colonisers of *Galleria* than commensals of other genera.

My objective was to replace the *Galleria* microbiome with commensals without causing harmful infection, so knowing which species are more likely to be successful was useful. It is also relevant because my next goal was to use faecal slurry to colonise *Galleria* with a diverse array of different species. If one genus is more capable of thriving in the *Galleria* gut than another, this may make *Galleria* less suitable as a model for this work.

3.3 Discussion

In this chapter I have documented my testing and development of protocols for rearing *Galleria* larvae and for colonising *Galleria* larvae with commensal bacteria from the human gut.

One of the appeals of *Galleria* as a model organism is the relative ease with which they can be reared. I have found this to be true: a *Galleria* colony is easy to maintain when using the correct diet and containment. For my use, a high bran diet has been best, as it maintains integrity upon the addition of water. Watkins and Doncaster lunchboxes have been ideal for all stages of *Galleria* rearing in my experience. Small larvae find it difficult to crawl between the lid and the box, it is large enough that larger larvae do not want for oxygen even in the absence of any ventilation, and adult moths are very happy to lay eggs in the lip between lid and box. Petri dishes, on the other hand, are ideal for experiments, but unsuitable for smaller larvae, which will crawl out and either escape or die attempting to eat masking tape. Adult moths can mate and lay eggs in Petri dishes but the laying pattern is unpredictable and the eggs can be difficult to collect.

I established protocols to isolate and culture bacteria from whole *Galleria* larvae and from the larval gut. Bead beating was the most effective method of extracting bacteria from *Galleria* in my experience and keeping larvae cold while also minimising freeze-thaw cycles was important. When allowed to warm to room temperature in aerobic conditions, *Galleria* homogenates will visibly and rapidly melanise, and this may kill bacteria.

I established protocols for the treatment of *Galleria* using antibiotics to clear the microbiome. The typical antibiotic cocktail for mice is composed of four antibiotics (Bayer et al. 2019) so it's nice that the clearance of *Galleria* can be achieved with fewer antibiotics. Following this treatment, a break of a couple of days must be given to ensure the absence of antibiotic when attempting to colonise larvae. I did not find that colonising larvae through feeding was a useful or efficient method because predictable quantities of bacteria could not be delivered when feeding *ad libitum.* However, this method is more high-throughput than either injection or oral gavage, and therefore may be more attractive for high-throughput assays. Oral gavage is a technique that has been used to introduce bacteria to the *Galleria* gut (Lange, Schäfer, and Frick 2019). I did not try this technique as it is not possible to use oral gavage on larvae any smaller than final instar, which are very close to pupation and therefore do not allow time for any other experimentation.

I found injection of larvae with bacterial culture to be an effective and reliable method of colonising larvae with commensal bacteria. Injection of commensal enterococci results in higher bacterial abundance with lower health costs than injection of other genera. Enterococci are the native residents of the *Galleria* microbiome and therefore may have adaptations that allow them to colonise *Galleria* more effectively, or potentially are more easily tolerated by the *Galleria* immune system.

3.4 Future work

I am aware of at least one group that is working on methods of rearing germ-free *Galleria* larvae by sterilising the eggs using gamma radiation. On a large scale I imagine this would be a practical and effective means of generating germ-free *Galleria* larvae, if the absolute sterility of their conditions could be maintained. Efforts to create standard methods for the use of *Galleria* are ongoing. A standard protocol for generating germ-free *Galleria* would allow the use of *Galleria* for microbiome humanisation studies. It could also increase the comparability of *Galleria* infection and toxicity studies by removing the microbiome as a confounding factor.

It would be interesting to see more work on the oral colonisation of *Galleria* with gut commensals. Thus far the work that has been carried out on this front has almost exclusively been co-infection assays, testing the ability of a probiotic to protect *Galleria* larvae against infection by a pathogen (Köhler 2015; Scalfaro et al. 2017; Vilela et al. 2015). I think *Galleria* would also make a useful model to compare the ability of probiotics to colonise and persist in the gut, and to investigate what genetic factors have an impact there.

Chapter 4

4 Attempts at Humanising the *Galleria* Gut Microbiome

4.1 Introduction

The primary aim of the project was to create a model of the infant human gut microbiome using *Galleria* instead of mice. It is common to humanise the mouse gut microbiome by feeding germ-free mice faecal slurry (Gootenberg and Turnbaugh 2011). *Galleria* larvae have several advantages over murine models: a lower cost, a shorter life cycle and fewer regulations and ethical concerns. Food can be supplemented with antibiotics, or bacteria, or any other substance one might want to test.

4.2 Results

4.2.1 Establishing methods for faecal slurry feeding

I trialled colonising the gut with faecal slurry by initially using 2 individual faecal samples to make a slurry by diluting 1:1 with sterile PBS and mixing well. I mixed this slurry 1:10 with the larval diet and fed it to the larvae for 2 and 4 days before dissecting out and homogenising the guts, pooling 3 to a sample. I serially diluted and plated the gut homogenate on BHI agar (aerobic), MacConkey agar (aerobic), RCA (anaerobic) and cysteine-supplemented MRS agar. Isolates from the aerobic plates were identified as *Proteus mirabilis* and isolates from the anaerobic plates were identified as *Enterococcus faecium.* Both *Proteus mirabilis* and *Enterococcus faecium* are species known to be present in the infant gut, and both were seen when the faecal slurry was plated and cultured prior to feeding to *Galleria.*

I also ground 100 mg of the faecal slurry supplemented food into 500 μL of PBS, serially diluted it, and plated as above. Within 2 days there were <100 cfu bacteria/100 mg food surviving. In the guts, there were more colonies on the 4th day than the 2nd but I did not quantify how many. This suggests that the bacteria in the gut was not just from the food but actively growing in the gut. It is of note that both *Proteus mirabilis* and *Enterococcus faecium* are pathobionts, i.e. commonly present in the gut of healthy hosts, but also capable of causing infection. This may explain why they were more successful colonisers.

4.2.2 Colonising the larval gut with faecal slurry stock

I then tried to colonise the larval gut using faecal slurry. I used faecal slurry that I produced by pooling 50 infant (2-3 months) faecal samples from the Hall lab's BAMBI study (Alcon-Giner et al. 2019) and combining those with 50 mL of sterile PBS, mixing using a vortex mixer until homogenous, and then aliquoting and flash-freezing to store at -80°C. The preparation of the faecal slurry was carried out in an anaerobic cabinet to maximise viability (Papanicolas et al. 2019).

I mixed 1 g of faecal slurry into 10 g of food and fed it to antibiotic-treated larvae for 8 days. I then dissected, homogenised, serially diluted and plated the guts. Again, I plated the homogenate on BHI agar (aerobic), MacConkey agar (aerobic), RCA (anaerobic) and cysteine-supplemented MRS agar. When I plated out the guts I could not see any colonies, indicating that the infant gut bacteria do not manage to survive in the gut for 8 days.

4.2.3 Limited colonisation of the *Galleria* gut can be achieved with faecal slurry

I took antibiotic-treated larvae, did not feed them for the 2 days after treatment to prevent the gut being colonised again from the food. I then fed the larvae faecal slurry mixed into freshly autoclaved food and incubated them for 1, 2, 4 and 8 days, alongside a control fed only sterile food. I dissected out the guts, homogenised them using glass beads in a bead beater, diluted and plated the samples on BHI plates and incubated for 24 hours. I saw no growth on the plates for larvae fed sterile food and many identical-looking colonies on the plates for larvae fed faecal slurry. Using 16S PCR I identified all of these isolates as *E. faecalis.* Due to the poor resolution of 16S amplicon sequencing for differentiating at the species level, there is a chance that this is just a resurgence of the host *Enterococcus,* but I consider this less likely due to the absence of cultures on the control plates.

These initial attempts at colonising the larval gut helped me to refine my protocol in several ways: removing larvae from antibiotics 2 days in advance, not feeding them in between (to prevent contamination) and using larger larvae.

4.2.4 Faecal slurry feeding increases the proportion of *Bifidobacterium* in the gut after 2 days

Following these earlier attempts I then carried out colonisation experiments using this refined protocol followed by 16S amplicon sequencing.

Antibiotic treated larvae were fed food containing either faecal slurry (1 g per 10 g food as before) or PBS (1 mL per 10 g food) for 2 days before sampling. 3 larvae were pooled per sample and guts were

dissected and homogenised. DNA was purified from homogenised larvae underwent 16S amplicon sequencing sequenced by Novogene. As seen in **Figure 4.1**, the larvae fed faecal slurry have a higher proportion of *Bifidobacterium* than both the larvae sampled before the experiment and the larvae not fed faecal slurry.

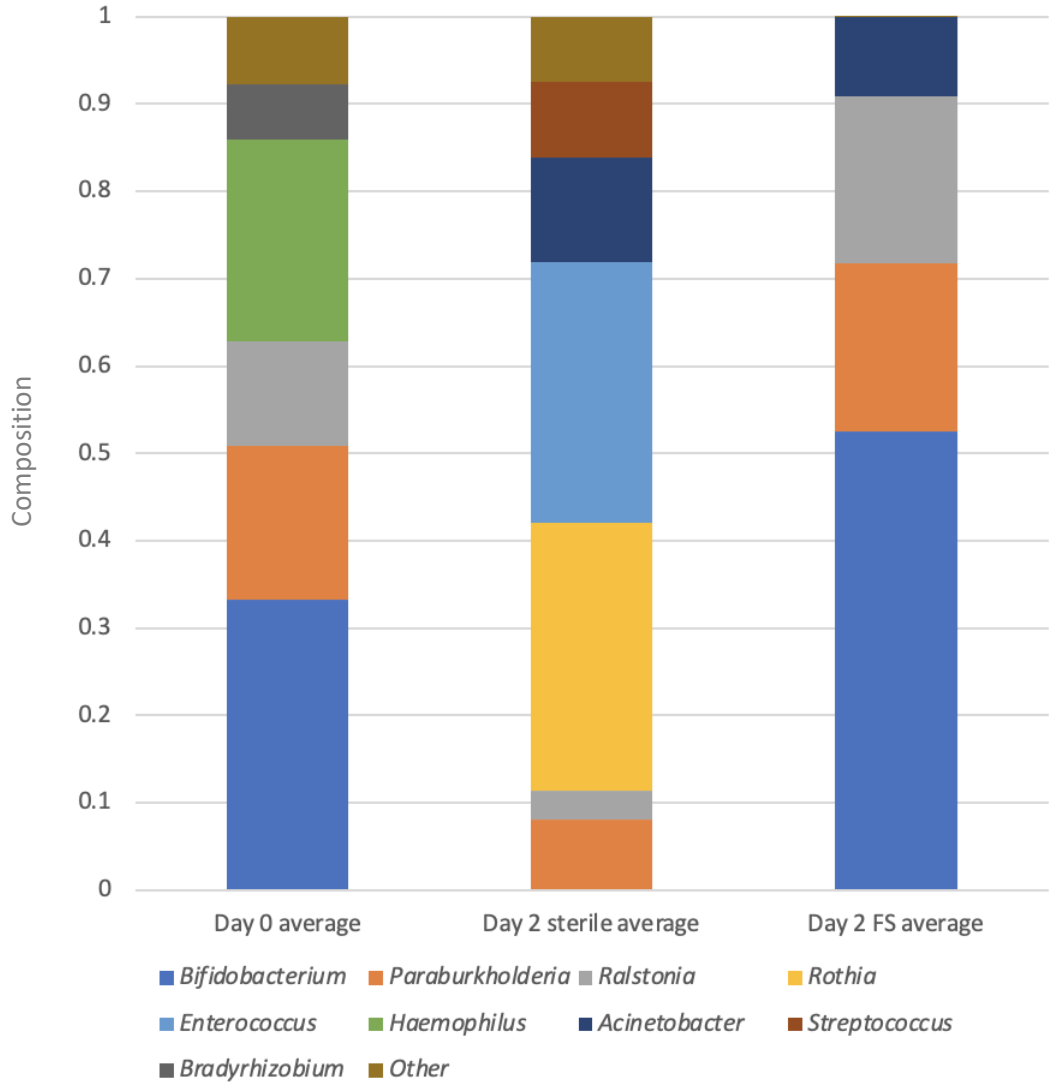


***Figure 4.1: Gut composition by genus following faecal slurry feeding: two days**. Antibiotic-treated larvae were fed faecal slurry for 2 days before they were flash frozen and homogenised. DNA from these samples was purified and subject to 16S amplicon sequencing (V3-V4). Taxa were assigned using Centrifuge (v0.15)*

4.2.5 Faecal slurry colonisation persists over several days

I gave the larvae antibiotic treatment, a 2-day break, and then fed them on faecal slurry. I sampled them at each timepoint by flash-freezing them in liquid nitrogen and homogenising them. I purified the DNA and gave it to David Baker and Rhiannon Evans at QIB to perform 16S amplicon sequencing. I classified the reads using the QITaxon pipeline (Centrifuge v0.15).

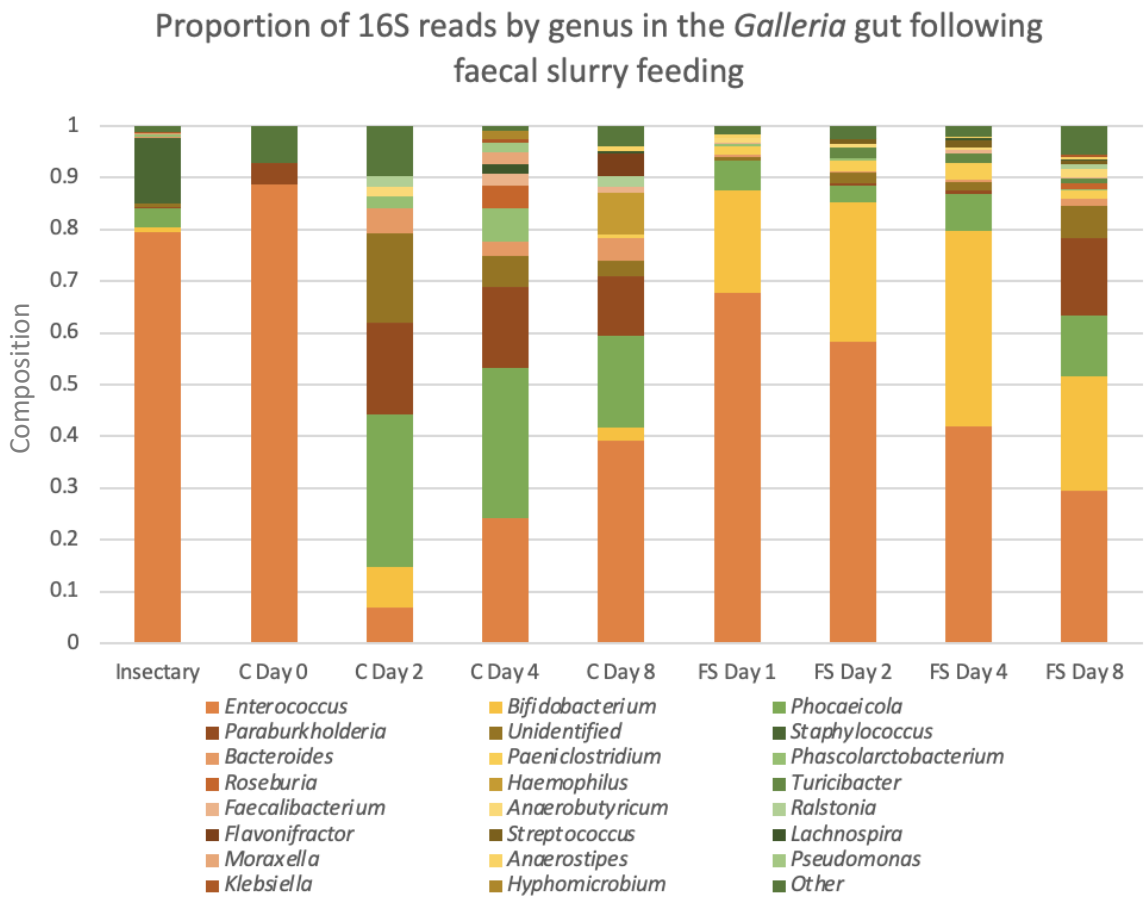


Figure 4.2: Gut composition by genus following faecal slurry feeding: eight days*. Antibiotic-treated larvae were fed faecal slurry (FS) or sterile food (C) for 1, 2, 4 or 8 days before they were flash frozen and homogenised. DNA from these samples was purified and subject to 16S amplicon sequencing (V3-V4). Taxa were assigned using Centrifuge (v 0.15).*

The resulting bacterial composition can be seen in (**Figure 4.2**). The faecal-slurry-fed larvae do have a higher abundance of *Bifidobacterium*, which is very abundant in the infant gut microbiome. Both faecal-slurry-fed larvae and control larvae converge on a similar profile after 8 days. In most of the groups, the most dominant genus remains *Enterococcus,* perhaps reflecting its dominance in the native *Galleria* gut.

4.3 Discussion

Overall it is unclear how successful colonisation of the *Galleria* gut with infant gut bacteria was. Bifidobacteria are the main taxon in the early infant gut (Stewart et al. 2018) so the high proportion of *Bifidobacterium* seen in the larval gut following feeding with faecal slurry is promising. However, 16S rRNA amplification doesn't discriminate between living bacteria and dead ones, so it's unclear

whether they were viable, especially given that I didn't see any bifidobacteria on the plates when I cultured the gut samples.

The high proportion of *Enterococcus* in the faecal-slurry-fed larvae is unsurprising given that it is known to be the dominant species in the native *Galleria* microbiome (Allonsius et al. 2019; Johnston and Rolff 2015) and may therefore have an advantage in the *Galleria* gut.

The composition of the faecal-slurry fed microbiome is not stable: whether or not the infant gut bacteria are viable in the gut, they do not seem to be able to permanently colonise the gut. The composition eventually converges to a similar profile as the other antibiotic-treated larvae. It makes sense that bacteria do not thrive in the larval gut as they would in the human gut, or even if transplanted into a mouse gut, because the structure of the insect gut is fundamentally different. The peritrophic membrane prevents bacteria from having any kind of proximity to the epithelial cells and therefore excludes the human commensals from the mucosal niches they would inhabit in a mammalian gut. The conditions in the lepidopteran gut also differ from the conditions in the mammalian gut in a variety of other ways, such as pH, which is much more alkaline than any part of the human gut (Harrison 2001). Although there is plenty of fibre in the diet I feed the larvae, the lack of access to other sources of nutrition, such as mucus or HMOs from breast milk, may also be limiting the growth of the infant bacteria.

Mortality of the larvae fed faecal slurry was low, which is useful. The larvae did not survive pupation, which is not surprising because most of the larvae that have antibiotic treatment do not, and faecal slurry does have some bacteria that could cause infection.

4.4 Future work

I think before *Galleria* could be used in this way as a model for the human infant gut microbiome, there would need to be evidence that the introduced bacteria are viable in the gut. When I attempted to isolate bacteria out from the gut again, I did not see many of the genera that were seen in the 16S rRNA results.

It might make more sense to colonise the larvae with a simpler community, like a synthetic bacterial community (Mabwi et al. 2021). This could achieve some of the same goals as the faecal slurry feeding but be simpler to achieve.

Chapter 5

5 Characterising the Composition of the Native *Galleria* Gut Microbiome

5.1 Introduction

The section of this chapter describing the discovery and characterisation of *Enterococcus innesii* is adapted from: Gooch HCC, Kiu R, Rudder S, Baker DJ, Hall LJ, Maxwell A. Enterococcus innesii sp. nov., isolated from the wax moth Galleria mellonella. Int J Syst Evol Microbiol. 2021 Dec;71(12):005168. doi: 10.1099/ijsem.0.005168. PMID: 34919037; PMCID: PMC8744253.

The sequencing described in this chapter was carried out by David Baker (QIB) unless otherwise specified. The computational analysis of the genomic data was carried out by Raymond Kiu (QIB) and the computational analysis of the shotgun sequencing data was carried out by Rebecca Ansorge (QIB).

A better understanding of the relationship that Galleria has with its own microbiome residents would be helpful in developing Galleria as a model. Enterococci have long been known to be the dominant residents of the Galleria gut microbiome, since before *Enterococcus* was named as a genus distinct from *Streptococcus* (Jarosz 1975). *Enterococcus mundtii* is most commonly seen as the dominant species in the Galleria microbiome. It is known to produce a bacteriocin, mundticin, that protects Galleria from infection (Johnston and Rolff 2015). I had observed, when working with larvae that I had treated with antibiotics, that they would succumb very quickly to infection if taken out of a sterile environment. This seemed to confirm that the Galleria microbiome was playing a protective role in my larvae too, but I wanted to investigate this further. If the antibiotic-treated larvae are kept in a sterile environment, there is no noticeable decrease in their survival or growth rate. The microbiome may be necessary for other functions that I haven't tested for – particularly given that my larvae are fed an artificial diet - but this implies that the microbiome is not necessary for *Galleria* survival or development in other ways. Investigating the Galleria gut microbiome also offered an opportunity to use different techniques of microbiome analysis on the Galleria gut, providing an opportunity to assess the suitability of these techniques on Galleria and therefore its utility as a model.

The larvae studied in this chapter are not only from the colony in the Maxwell lab, but also wild larvae from Norfolk beehives, and commercial research-grade larvae (Trularv™) from Biosystems Technology. These larvae are sold to researchers for use in assays. They are raised without hormones or antibiotics, unlike the larvae sold by insect feed stores that are often used in studies (Champion, Titball, and Bates 2018). Comparing our larvae to these larvae from other sources ensures that any results are not merely a quirk of my colony.

5.2 Results

5.2.1 Whole genome sequencing of isolates from the *Galleria* gut

I wanted to investigate what bacteria might be present in my larvae and what role they might be playing, so I decided to isolate and sequence some bacteria from their guts. I took larvae from the JIC Insectary colony and from Trularv™, dissected out the guts and plated them. I took 10 isolates from the JIC larvae and four from the Trularv™ larvae. The genomes were sequenced using the Illumina platform and assembled with spADES (Bankevich et al. 2012). Three of the isolates from the insectary larvae could not be assembled because the sequence quality was too low. Based on an initial analysis of the 16S sequences the remaining 11 isolates appeared to be the same species: *E. gallinarum.* However, digital DNA-DNA hybridisation (dDDH, via TYGS) and average nucleotide identity analysis (ANI, via fastANI v1.3), revealed that seven of the isolates – four from the JIC larvae and three from Trularv™ were in fact *E. casseliflavus,* a species very closely related to *E. gallinarum.* And, to our surprise, three of the isolates from the JIC larvae and 1 from the Trularv™ larvae were identified as a novel species. The characterisation of this species is described below: **5.2.6**.

5.2.2 There is no evidence of growth inhibition activity of enterococci native to the *Galleria* gut: bioinformatics

One potential function of the *Galleria* microbiome is colonisation resistance, which has been observed in other studies (Jarosz 1975; Shao et al. 2017). I decided to investigate if the larvae I am using have bacteria expressing any growth inhibition activity and therefore able to play this role in the *Galleria* gut. I used antiSMASH (Blin et al. 2019), which screens for biosynthetic clusters, and BAGEL4 (van Heel et al. 2018), which screens for bacteriocins, to search the genomes of the isolates I had taken from the larval gut for any genes involved in the production of antimicrobials. The results for all the isolates were the same so I have only shown the result for one strain, GAL2, here (**Figure 5.1**). antiSMASH identified two biosynthetic clusters: one producing a terpene and one type 3 polyketide synthase (T3PKS) cluster. Both terpenes and polyketides are large groups of secondary metabolites that could have many potential functions. BAGEL identified a single predicted

bacteriocin. A search using that sequence in PFAM identified it as most likely a bacteriophage-derived holin. Again, although some bacteria have been shown to express holins to supress the growth of other bacteria (Reddy and Saier 2013), this is not definitive evidence of antimicrobial activity.

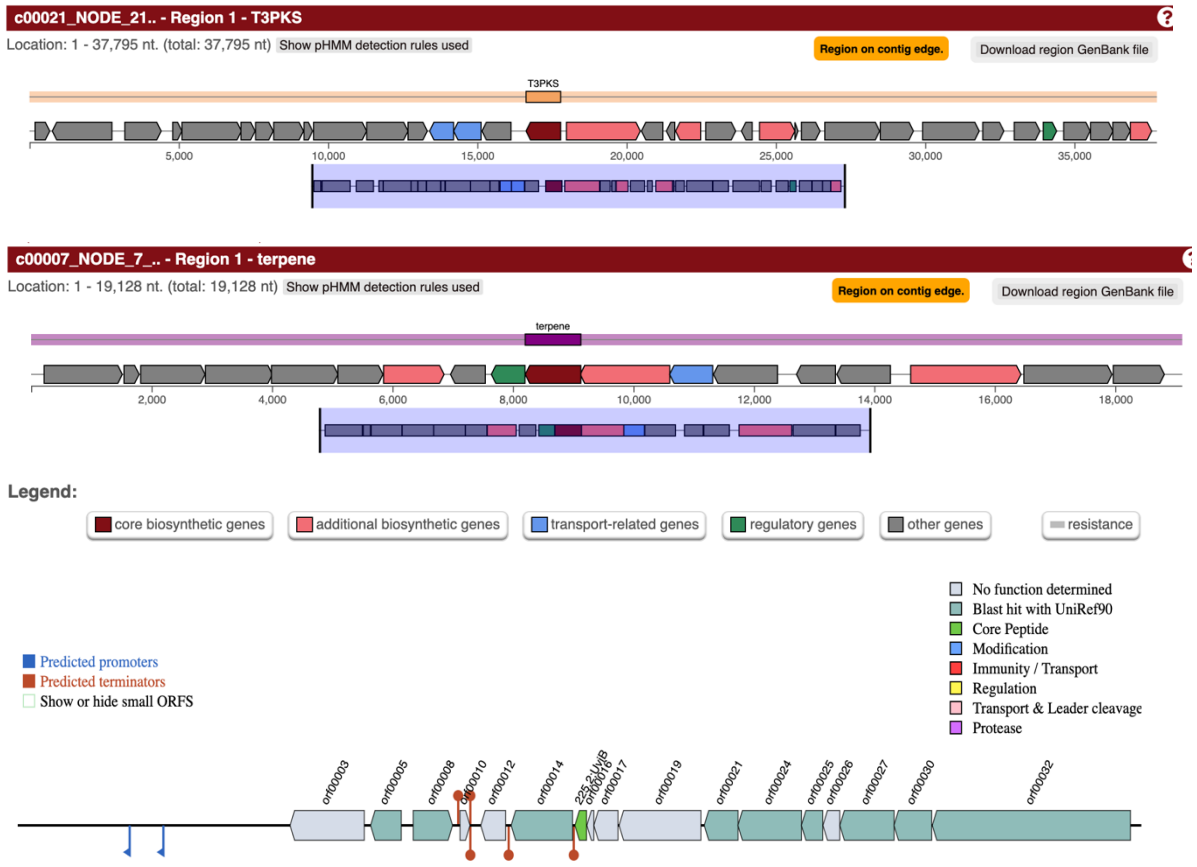


Figure 5.1: Bioinformatic searches for antimicrobials. *From top to bottom: an antiSMASH result showing a terpene biosynthetic cluster in the GAL2 genome; an antiSMASH result showing a T3PKS biosynthetic cluster in the GAL2 genome; a BAGEL4 result showing a predicted bacteriocin in the GAL2 genome*

5.2.3 There is no evidence of growth inhibition activity of enterococci native to the *Galleria* gut: microbiology

To see if I could find more conclusive evidence of my *Enterococcus* isolates having growth inhibition activity, I tried cross-streaking assays. This assay crosses two streaks of bacterial culture over each other to see if one inhibits the growth of another. I used one *E. casseliflavus* strain (GAL2) from the *Galleria* gut, one *E. innesii* strain (GAL7), an *E. coli* strain (MG1655) from the lab as a negative control, and *P. protegens* (PS682) and *P. fluorescens* (SBW25) strains with known activity as positive controls. Additional indicator strains I used were: *S. capitis* and *E. faecalis* (P46G 41), which are of interest to human health; *S. marcescens* (NCIMB 9155) and *B. thuringiensis* (NCIMB 9134), common soil bacteria and insect pathogens with clear colony morphology; and *R. picketii* (NCIMB 13142),

another soil bacterium that I knew to be sometimes present in the *Galleria* gut from my sequencing results. At first, I tried streaking the test strain and then incubating for 24 hours before streaking the indicator strain, due to the slower growth of the *Enterococcus* strains. However, I found that the test strain would grow over the indicator strain and make the result hard to read (**Figure 5.3**). I subsequently tried streaking the indicator strain directly after the test strain and saw no evidence of activity, even from the positive controls (**Figure 5.4**). Given the known antimicrobial activity of the *P. protegens* strain I couldn't make any conclusions about the activity of my isolates until I could see growth suppression from that strain.

Instead I tried a radial streak assay (Coman et al. 2014). In this assay, a spot of culture of the test strain on a plate is incubated for 48 hours at 37°C and then streaks of indicator strain are made towards the centre before incubating for a further 24 hours. I used *E. coli, S. marcescens* and *B. thuringiensis* as the main indicator strains for this assay. On the *P. protegens* plate, a clear zone of inhibition can be seen, while no inhibition can be seen for the other strains (**Figure 5.4**). This means that the test strains were not inhibiting the growth of the indicator strains.

The secretion and activity of antimicrobial products from bacteria can be very species-specific and context-dependent, so these assays do not rule out any activity completely. However, in the absence of strong bioinformatic evidence, I decided that there was nothing to be gained by pursuing this any further.

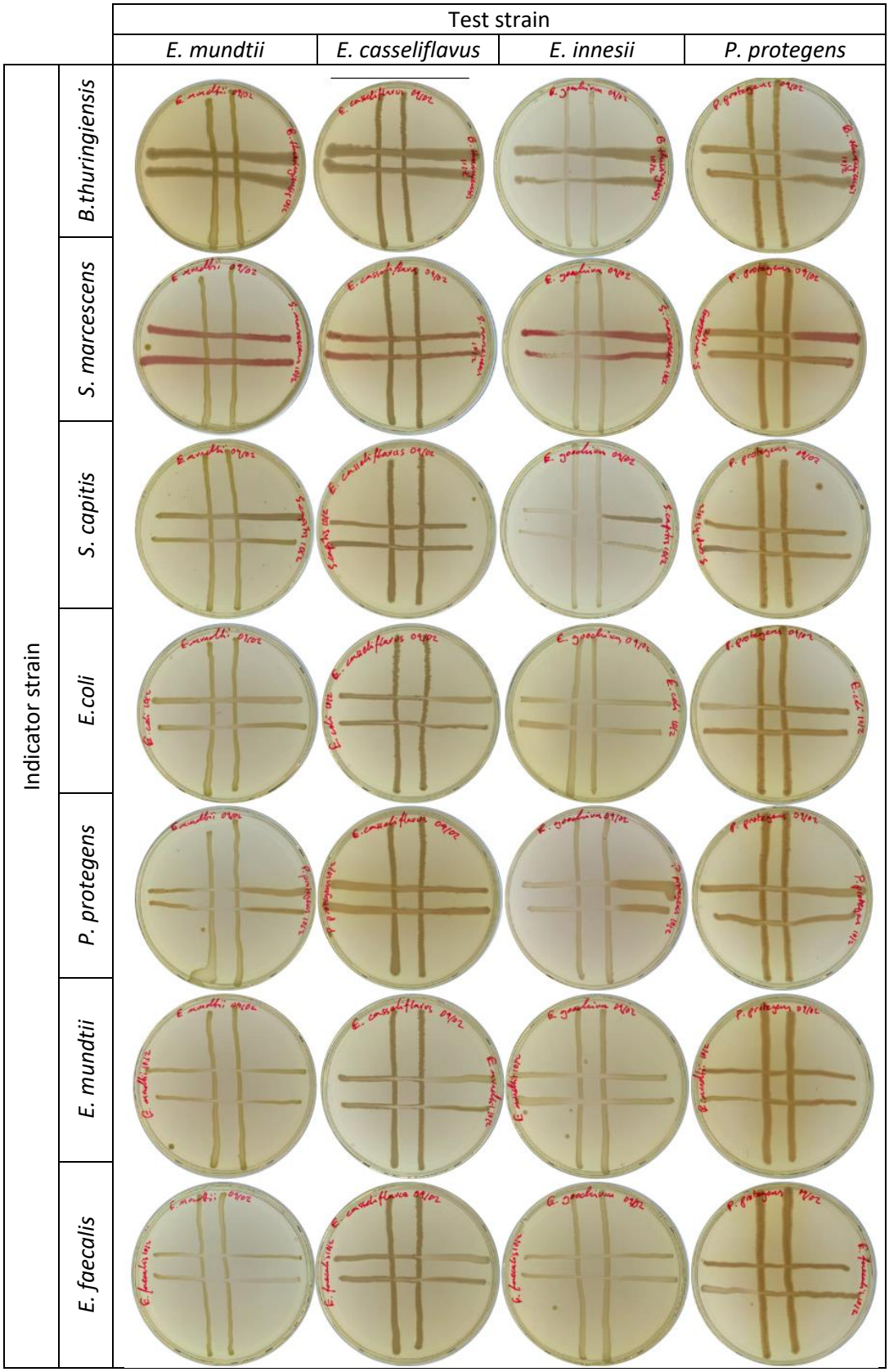


Figure 5.2: Cross-streaking assays of Galleria isolates: preincubated test strains. *Overnight cultures of the test strains and streaked across BHI plates using a toothpick. Plates were incubated at 37℃ for 24 hours and overnight cultures of the indicator strains were streaked across, perpendicular. Plates were incubated for another 24 hours at 37℃ before being photographed.*

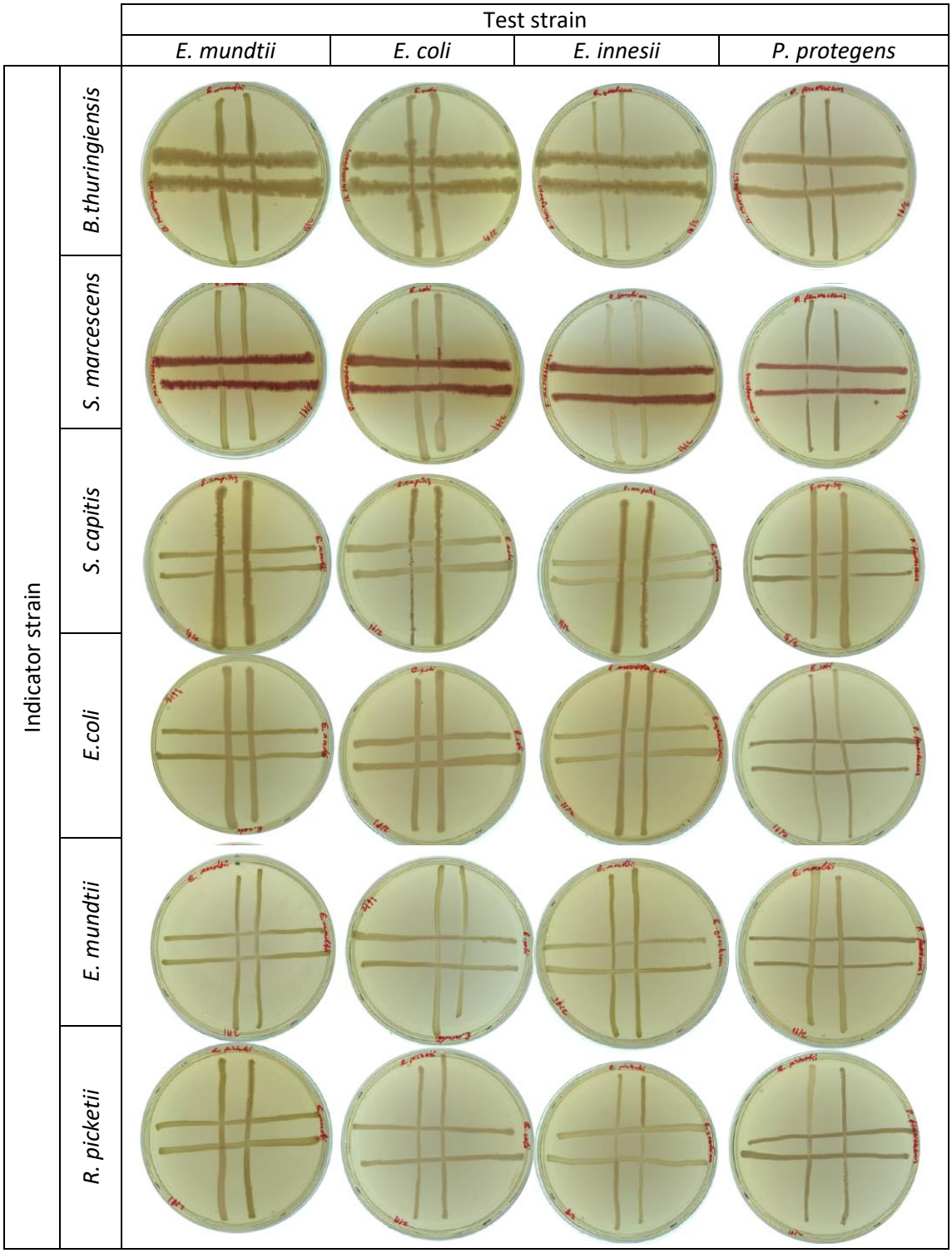


***Figure 5.3: Cross-streaking assays of Galleria isolates: cross-streaked immediately**. Overnight cultures of the test strains and streaked across BHI plates using a toothpick. Overnight cultures of the indicator strains were streaked across, perpendicular. Plates were incubated for 24 hours at 37℃ before being photographed.*

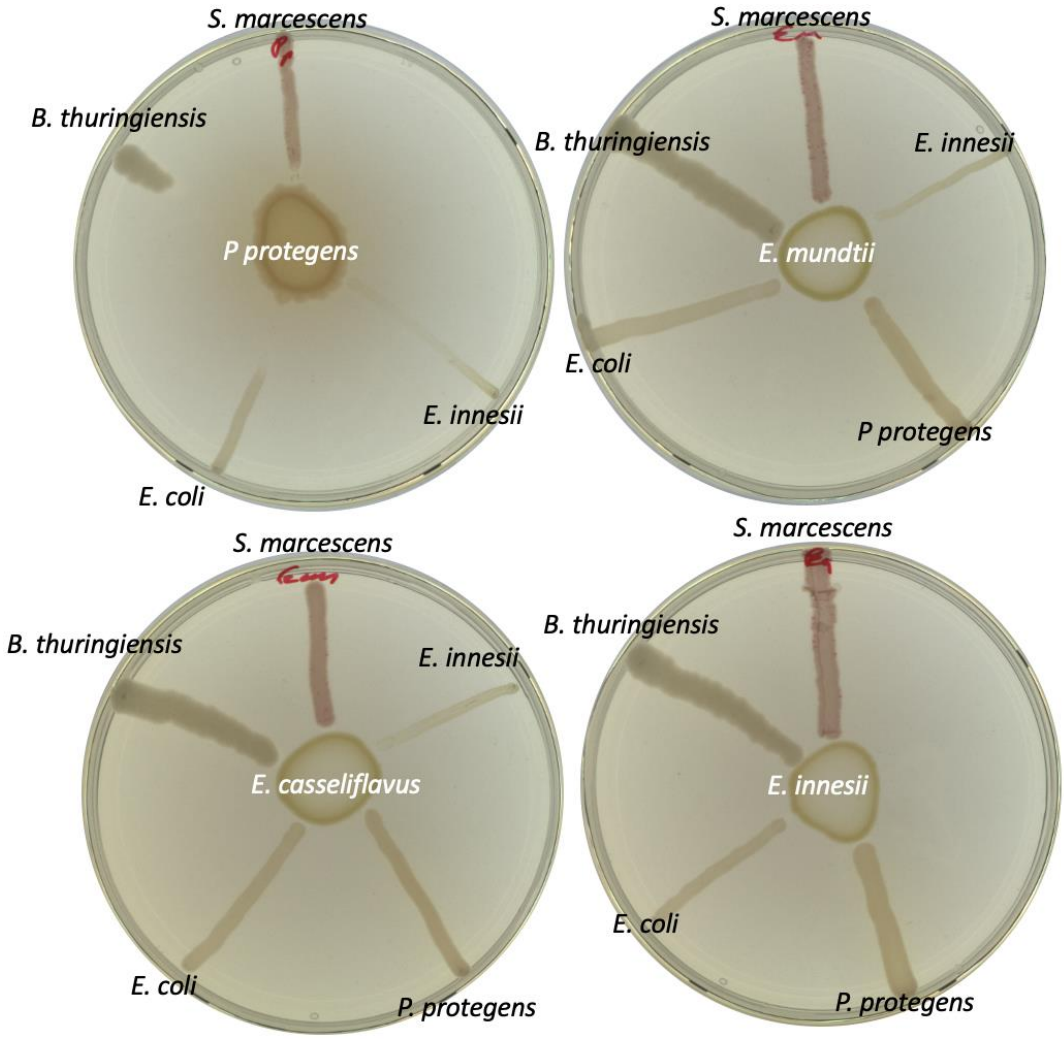


Figure 5.4: Radial streak assay of Galleria isolates. *20 µL of test strain was dropped in the middle of the plate, left to dry, then incubated at 30℃ for 48 hours. Indicator strains were then streaked radially inwards towards the centre with a sterile toothpick, without contacting the centre spot. Plates were incubated for 24 hours at 30℃.*

5.2.4 Shotgun sequencing of the *Galleria* gut microbiome

I wanted to see how the gut microbiome of larvae raised in the lab compared to wild larvae so I advertised in the Norfolk Beekeeper's Newsletter to find sources of wild *Galleria.* Two beekeepers kindly gathered some larvae from their beehives for me to examine. I gathered larvae from four different locations: the JIC insectary; commercial research grade Trularv™; and two wild colonies (from Peter Sunderland and Tom Johnson). I flash-froze and dissected out the guts of these larvae, pooled three guts into each sample, and purified the DNA using the FastDNA™ for Soil kit. I sent the DNA to Novogene for shotgun sequencing (Novaseq, PE150). Rebecca Ansorge from QIB carried out the bioinformatics to analyse this data.

Rebecca Ansorge (QIB) used the Kraken 2 read profiler (Wood, Lu, and Langmead 2019) to assign taxonomic classifications to the reads. These were visualised using Krona **(Figure 5.5)** (Ondov,

Bergman, and Phillippy 2011). As expected, the microbiome of the JIC insectary colony and the Trularv™ larvae is dominated by *Enterococcus,* primarily *E. casseliflavus* and *E. mundtii,* with some soil bacteria also present. This is consistent with the species I had identified from the whole genome sequencing. The Trularv™ samples were generally less diverse than the JIC samples but one had a high proportion of *B. cereus,* which may mean one of the larvae was infected. However, the wild colonies had a more diverse microbiome, dominated by *Bacillus.* They also appeared more melanised than the lab larvae and since they were supplied from old, unmaintained beehives without bee colonies they may not have been healthy. If the larvae were frequently fighting infection, the resulting immune activation, as seen by the melanisation, may have left very few bacteria alive at all.

Unfortunately, there were too many eukaryotic reads from the host and too few bacterial reads to assemble genomes from the reads. In order to quantify the scale of this problem we decided to estimate bacterial reads using genomes of bacteria known to be present in my larvae. Using the *Galleria* genome (Lange et al. 2018) and the genomes of bacteria from the *Galleria* gut that I had previously isolated, Rebecca assigned the reads to either the host or the symbiont and found that there were fewer than 25,000 reads for any of the bacterial genomes (**Figure 5.6**). This is likely the consequence of the large quantity of gut tissue in the samples and the low abundance of bacteria in the *Galleria* gut.

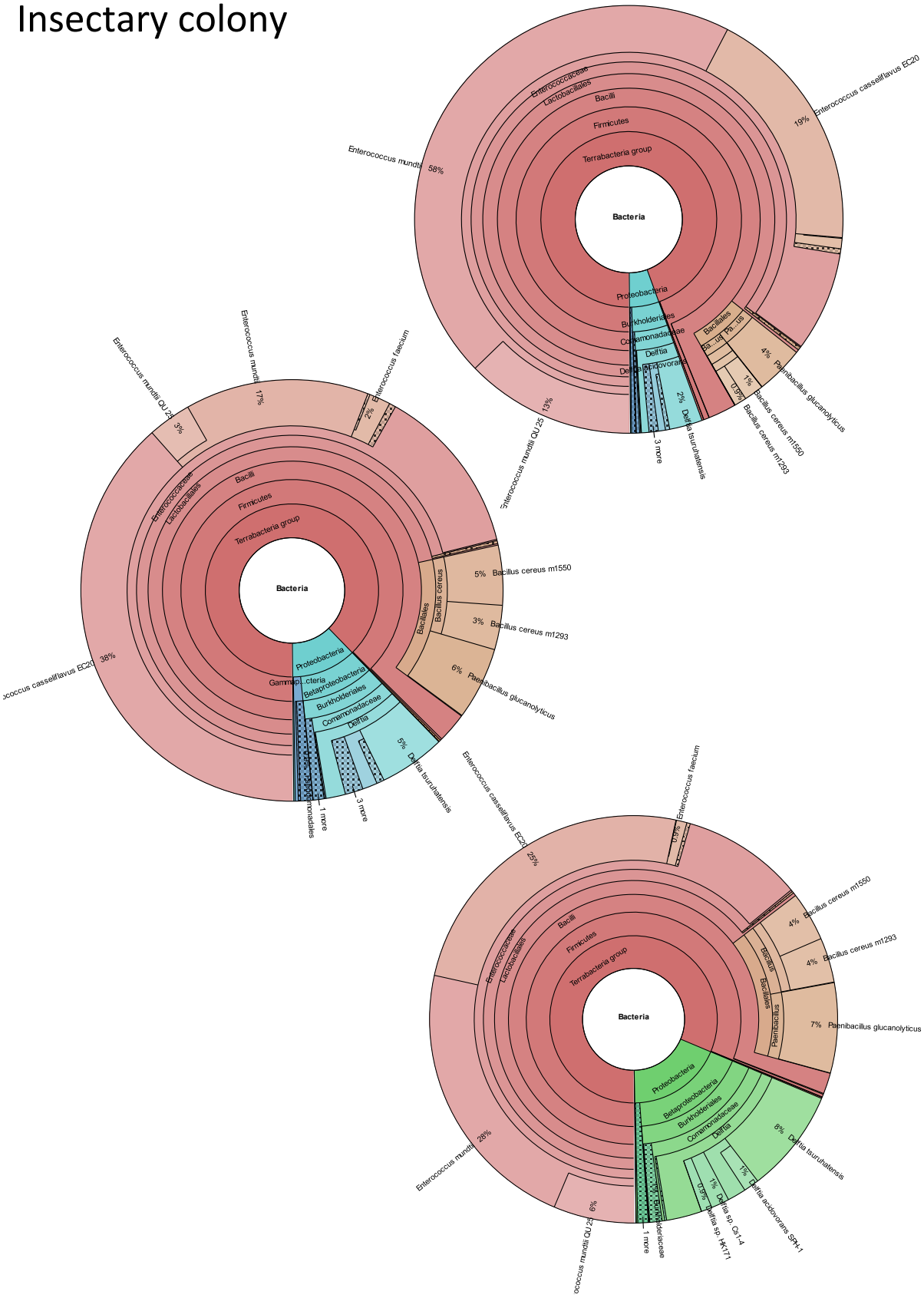


Figure 5.5: Composition of the gut microbial communities of Galleria larvae from different sources*. Larvae were flash-frozen, guts dissected and pooled 3 to a sample, DNA purified and sequenced. Reads were profiled using Kraken 2 (Wood, Lu, and Langmead 2019) and visualised using Krona (Ondov, Bergman, and Phillippy 2011).*

Wild colony (Peter Sunderland)

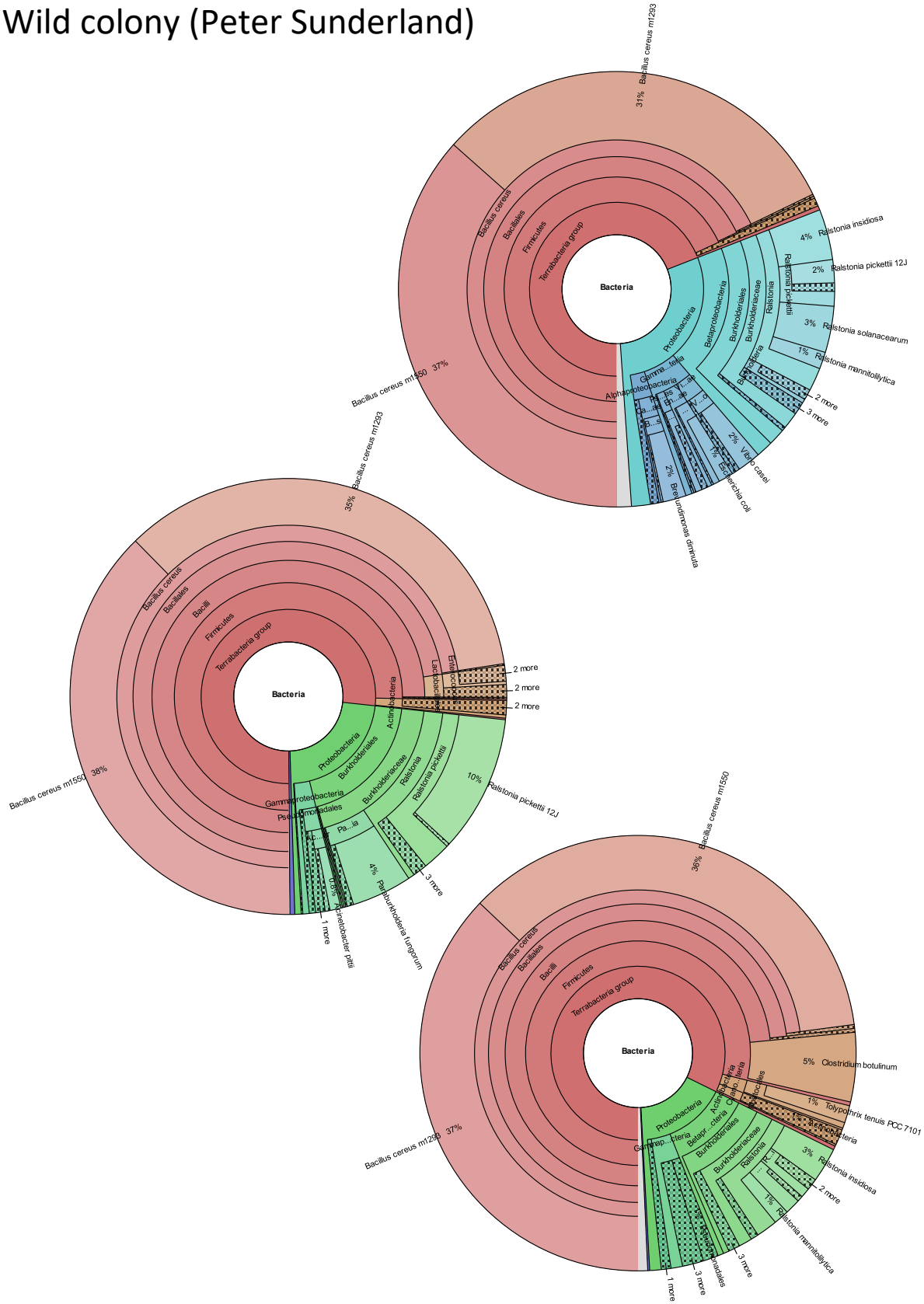
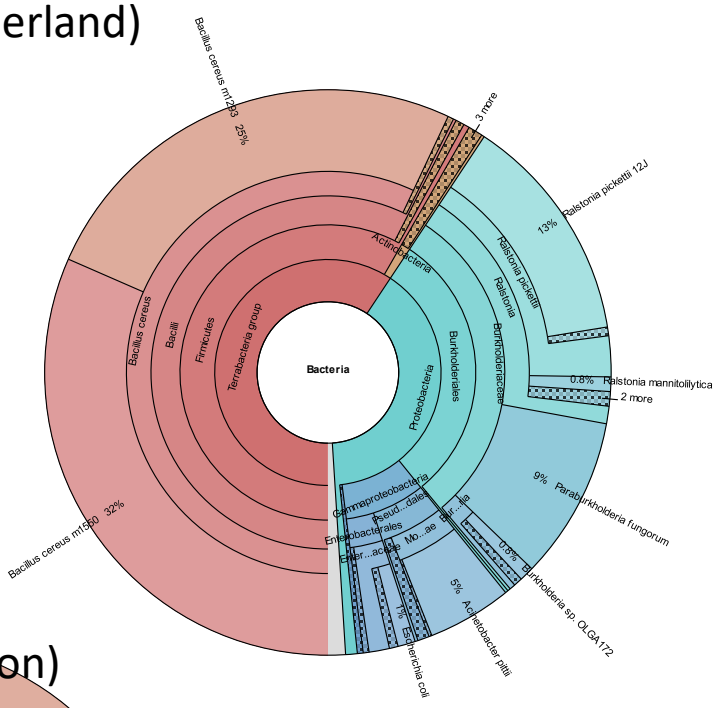


Figure 5.5 *(cont.)*

Wild colony (Peter Sunderland)



Wild colony (Tom Johnson)

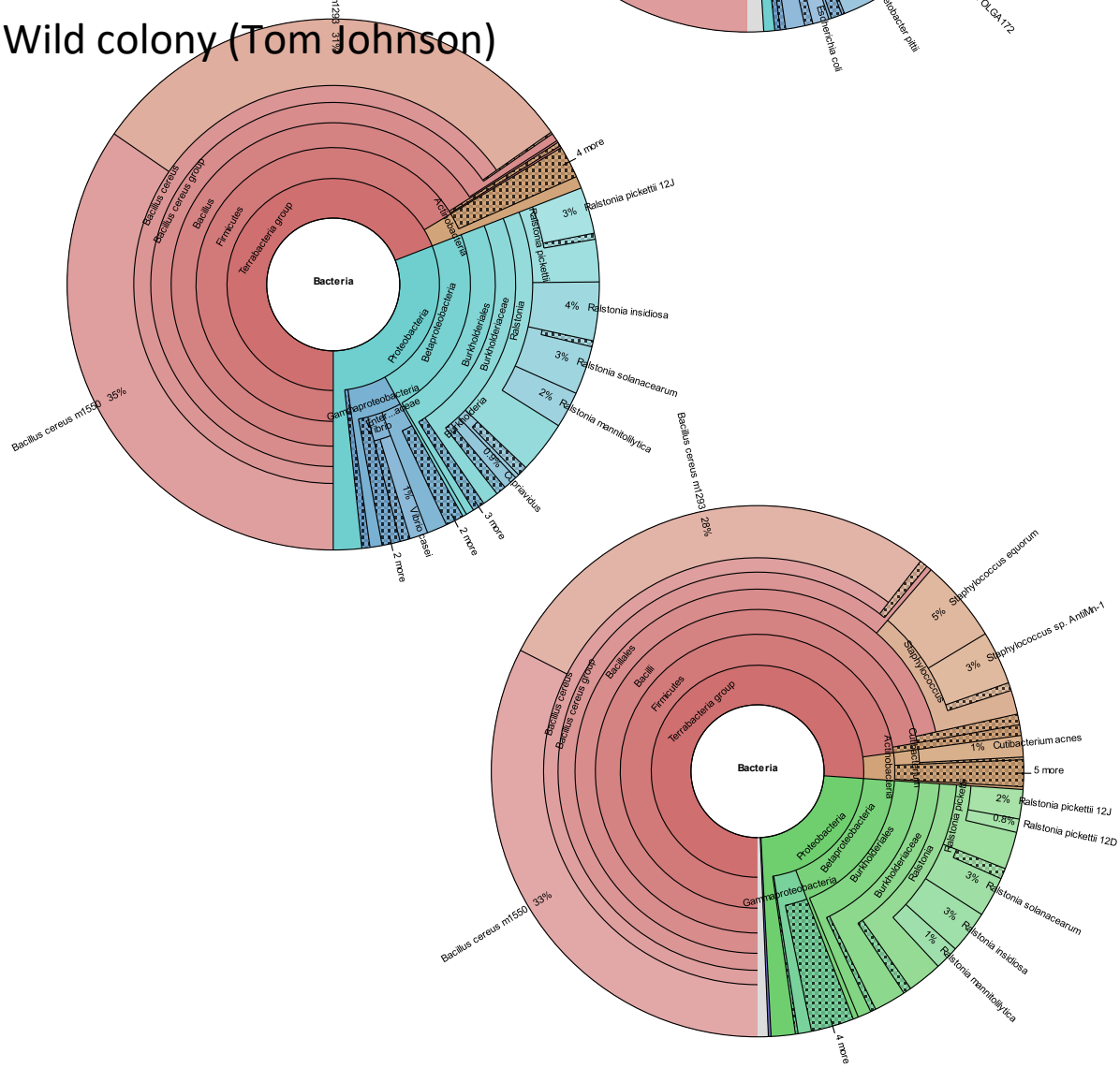


Figure 5.5 *(cont.)*

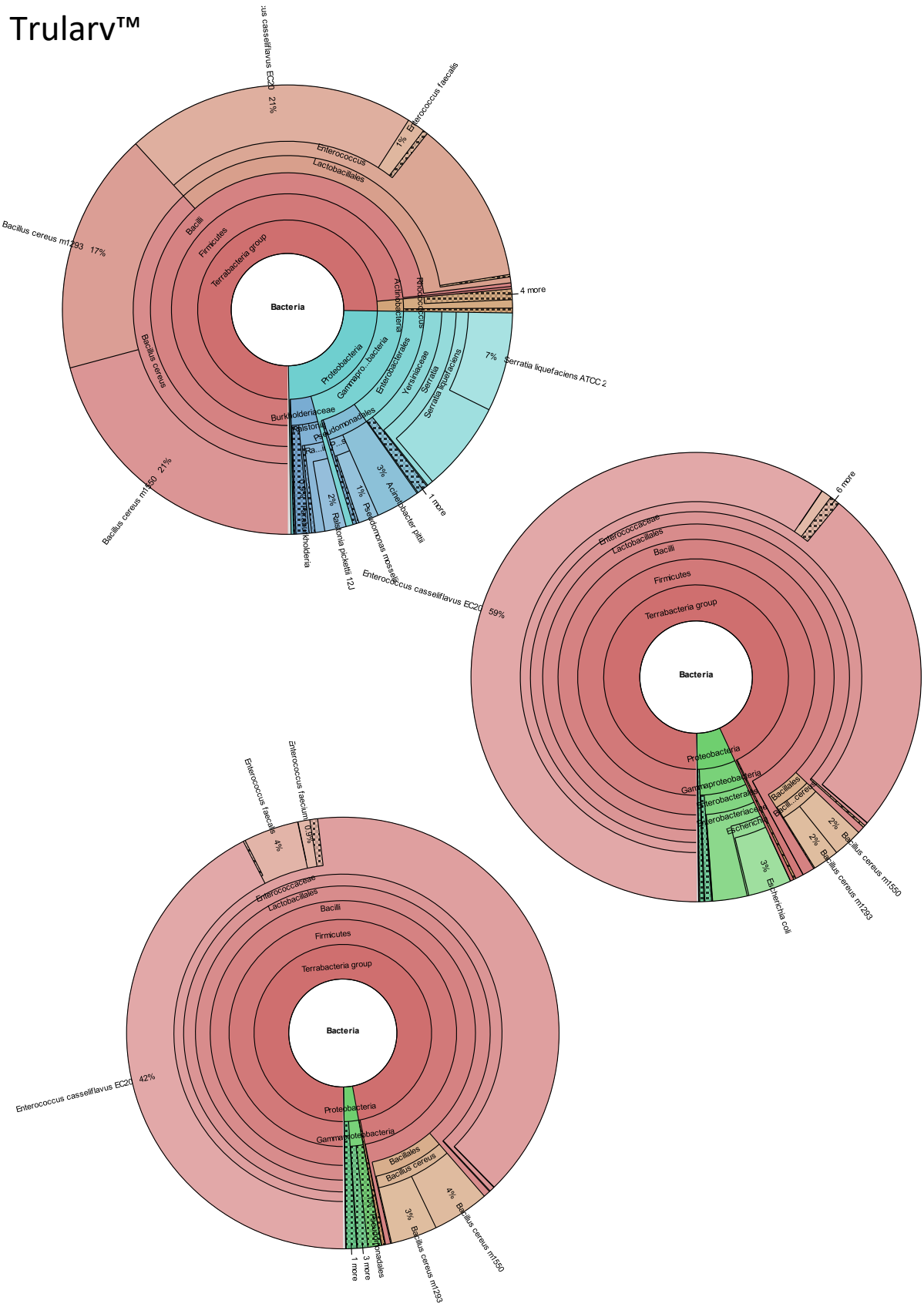


Figure 5.5 *(cont.)*

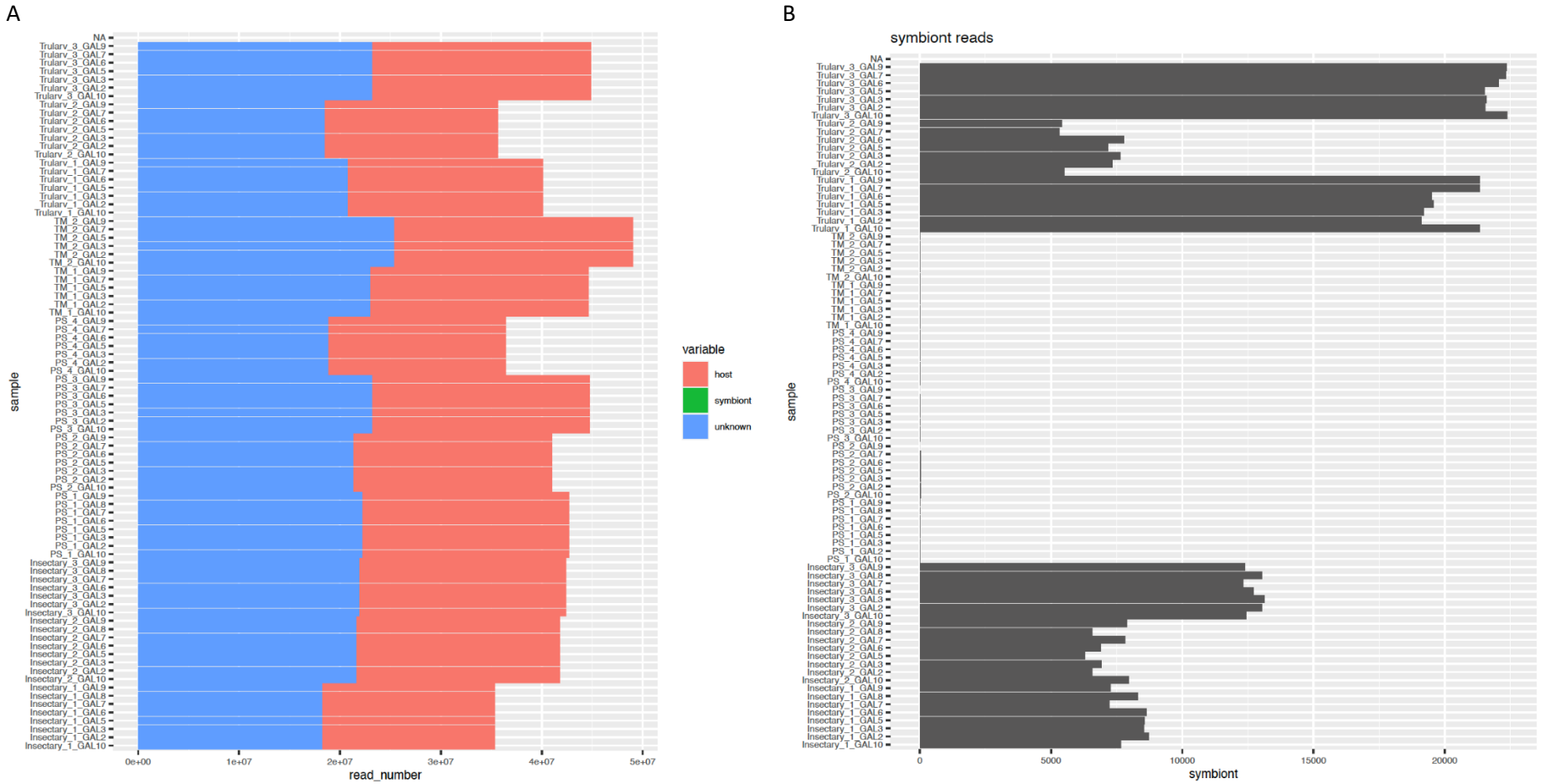


Figure 5.6: Classification of reads from the Galleria gut. *Reads were classified using the published genome of Galleria and the whole genome sequences of the isolates from the Galleria gut. (A) shows all reads of all classifications and (B) shows only reads that were assigned to the genomes of isolates from the Galleria gut.*

5.2.5 16s rRNA amplicon sequencing of the *Galleria* gut

To verify the composition data obtained from the metagenomic sequencing, I sent the remaining samples to David Baker for 16S amplicon sequencing (V3-V4). There were insufficient Tom Johnson larvae to do this so I sent four gut samples from the insectary larvae, five from TruLarv™ and three from Peter Sunderland's colony. Each contained purified DNA from three pooled gut samples. The 16S results (**Figure 5.7**) for the insectary and Trularv™ guts were very similar to the metagenomic result, whereas the wild larvae showed a very different composition. I think this may be due to these samples having a lower bacterial abundance and therefore the sequencing being more vulnerable to contamination during the dissection and extraction. This is a known issue with sequencing of low-biomass samples (Paniagua Voirol et al. 2021).

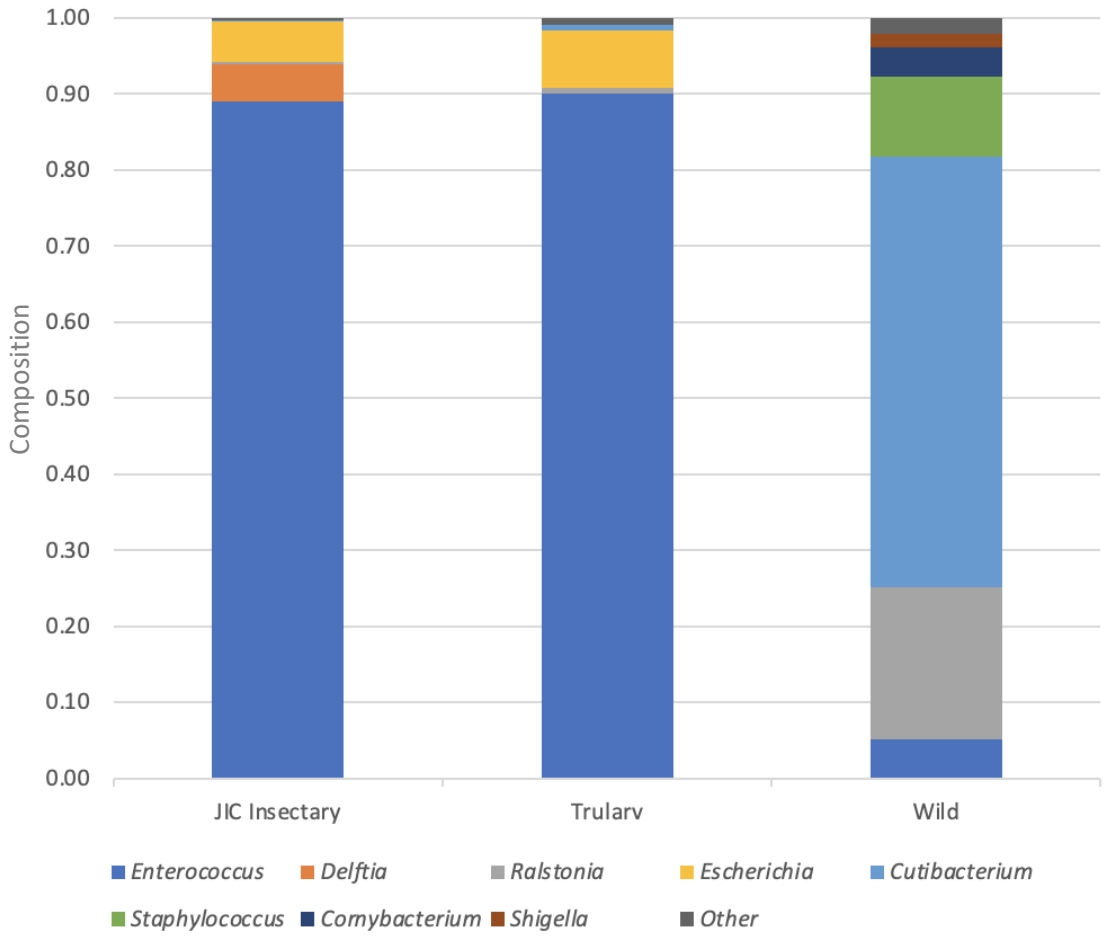


Figure 5.7: Composition of the gut microbial communities of Galleria larvae from different sources, by 16S amplicon sequencing. *Larvae were flash-frozen, guts dissected and pooled to 3 to a sample, DNA purified and sequenced using 16S amplicon sequencing (V3-V4). Reads were classified using Centrifuge v0.15 (Kim et al. 2016). Abundances lower than 0.5% not shown.*

5.2.6 The *Galleria* gut microbiome contains a novel species of *Enterococcus*

I named the novel species discovered in **5.2.1** *Enterococcus innesii* sp. nov. (in.ne´si.i. N.L. gen. n. *innesii*), for the John Innes Centre, after considering a range of more interesting but less appropriate names. The positioning of *E. innesii* in the phylogenetic tree of *Enterococcus* species, based on their 16S rRNA sequences, can be seen in **Figure 5.8**.

Following the identification of the four *Galleria* isolates as a novel species, we decided to resequence the isolates using the Nanopore MinION sequencing platform, for which David used his novel BacteriaHIT method (Baker et al. 2021). These were the sequences used for all subsequent analyses. We chose GAL7 as the type strain. The dDDH was 59.0 % (using TYGS formula d_4) and ANI 94.5 %, when compared to its closest neighbour *E. casseliflavus* NBRC100478^T. Despite the high similarity of 16S rRNA sequences between the two species, both fell below the intra-species thresholds of 70 % dDDH and 95 % ANI (**Figure 5.9**). In contrast, the ANI values among the four *E. innesii* strains were 99.92–99.96 %. The genome statistics of *E. innesii* compared to the published genomes of the type strains of closely related species can be seen in **Table 5.1**.

Next, 10 closest-related *Enterococcus* strains (vs *E. innesii*) identified by TYGS were further examined phylogenetically at a genomic level, with antibiotic resistance genes also screened (using the resfinder database), for the four novel *E. innesii* strains (**Figure 5.9**) (Bortolaia et al. 2020). The pangenome of these 14 strains were investigated using Roary version 3.12.0 (Page et al. 2015) at a blastp threshold of 70% identity for inference of core genes. A total of 15629 genes were present in this pangenome with 564 core genes and 15065 accessory genes. A core-gene alignment was generated and used to build a core-genome maximum-likelihood phylogenetic tree where it showed that *E. casseliflavus* NBRC100478 was genomically distinct from *E. innesii*. This is further supported by single nucleotide polymorphism (SNP) analysis (using snp-dists version 0.7.0) that confirmed the SNP range (8–32 SNPs) among the four *E. innesii* strains, indicating strain distinction yet close genetic relatedness. In contrast, 11538–11540 SNPs were found when comparing the *E. innesii* strains and *E. casseliflavus* NBRC100478^T (**Figure 5.9**) (Seemann 2018a).

Strains	Genome size (bp)	Contigs	G+C (mol%)	rRNA	tRNA	CDS	GenBank accessions
Enterococcus alcedinis CCM8433^T	2 686 367	29	37.59	2	50	2472	GCA_014635985
Enterococcus casseliflavus NBRC100478^T	3 498 264	54	42.35	3	50	3339	GCA_001544095
Enterococcus devriesei DSM22802^T	3 320 653	65	40.22	1	29	3119	GCA_001885905
Enterococcus gallinarum NBRC100675^T	3 774 884	87	39.75	3	49	3600	GCA_001544275
Enterococcus gilvus BAA350^T	4 179 913	5	41.41	21	70	4111	GCA_000407545
Enterococcus innesii GAL10^T	3 678 879	18	42.32	15	69	3868	GCA_018982735
***Enterococcus innesii* GAL7**^{T}	3 692 254	14	42.35	22	67	3866	GCA_018982785
Enterococcus innesii GAL9	3 793 471	13	42.22	18	64	4070	GCA_018982775
Enterococcus innesii TL2	3 806 372	17	42.25	20	63	4075	GCA_018982725
Enterococcus malodoratus ATCC43197^T	4 654 237	10	39.56	16	54	4480	GCA_000407185
Enterococcus massiliensis AM1^T	2 712 841	7	39.64	9	61	2612	GCA_001050095
Enterococcus pseudoavium NBRC100491^T	2 731 874	59	40.06	3	48	2587	GCA_001544295
Enterococcus saccharolyticus ATCC13076^T	2 604 038	2	36.7	6	38	2586	GCA_000407285
Enterococcus viikkiensis LMG26075^T	2 545 311	45	40.26	4	40	2416	GCA_005405345

Table 5.1: Genome statistics comparison between closely related Enterococcus species *(n=10) to E. innesii strains identified by TYGS, including type strain GAL7^T (Meier-Kolthoff and Göker 2019)*

Previously published type strain genomes were retrieved from GenBank for analysis in this study (Benson et al. 2009).Genome assembly statistics were extracted using sequence-stats version 0.1 (Kiu 2020b) while genome annotation was performed using Prokka version 1.13 (Seemann 2014).

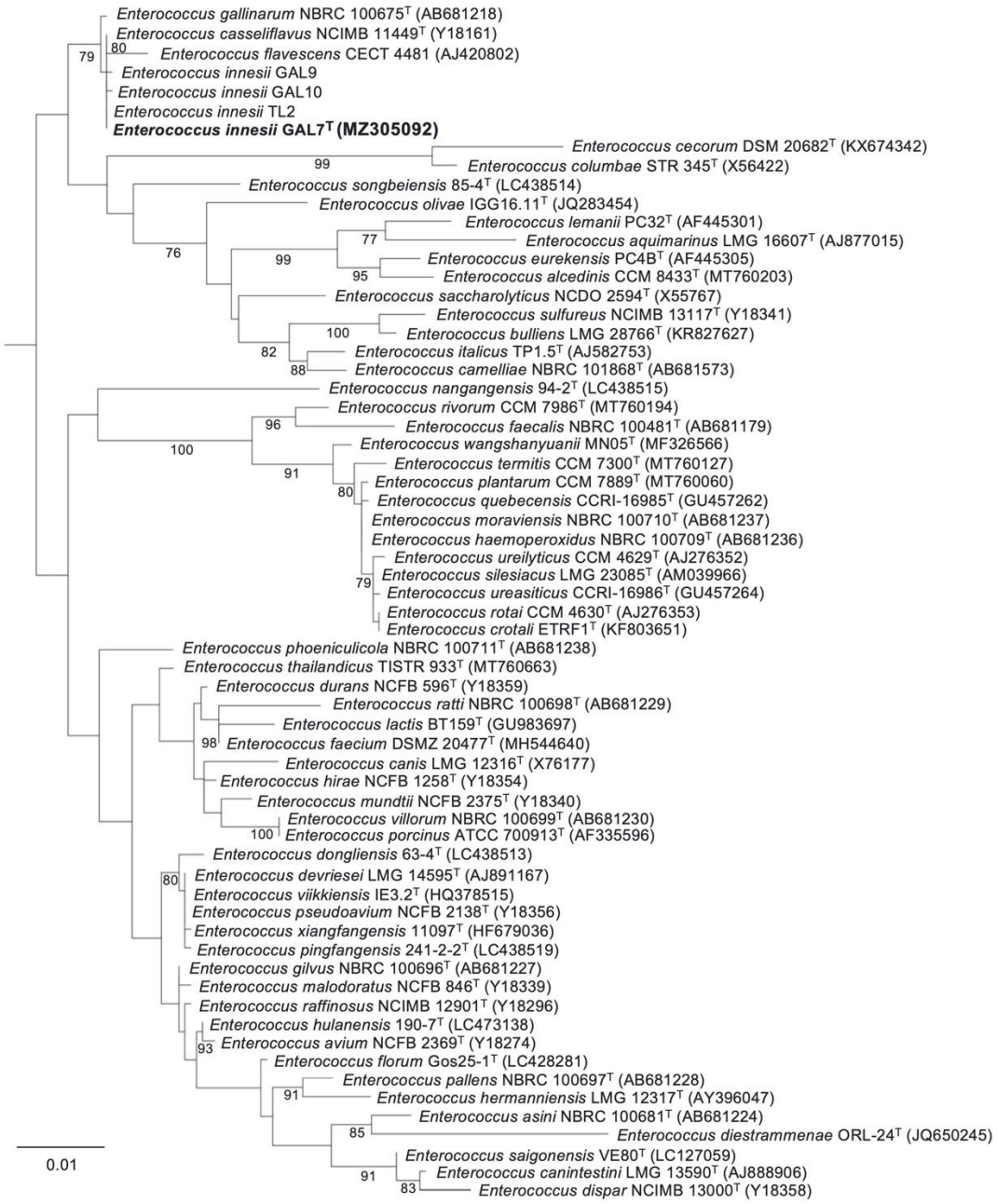


Figure 5.8: Phylogenetic tree of the Enterococcus genus showing Enterococcus innesii*. A mid-point rooted maximum-likelihood phylogenetic tree showing the phylogenetic position of Enterococcus innesii sp. nov. strain GAL7T based on 16S rRNA gene sequences of 61 Enterococcus type strains. Bootstrap values (>70%) based on 1000 replications are listed as percentages at the branches. Bar, 0.01 substitutions per nucleotide base.*

5.2.7 *E. innesii* is mildly resistant to vancomycin

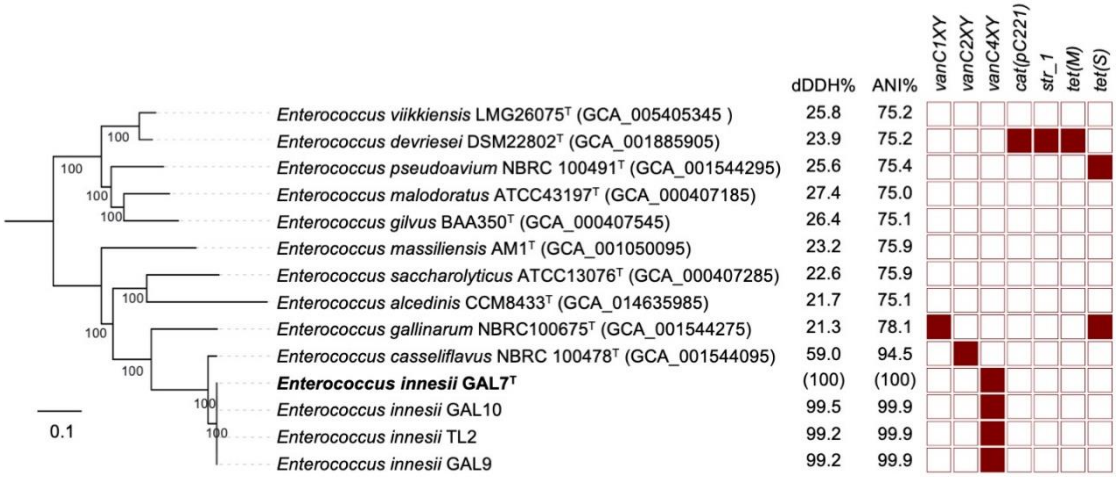


***Figure 5.9: Occurrence of VanC genes in Enterococcus species**. A mid-point rooted maximum-likelihood phylogenetic tree based on 154 826 single nucleotide polymorphisms from 564 core genes, aligned with dDDH (%), ANI (%) and antibiotic resistance gene profiles.*

Raymond Kiu (QIB) carried out a search for virulence and antibiotic resistance genes. The vancomycin-resistance gene *vanC-4* (NCBI accession: EU151752) was uniquely detected (nucleotide sequence identity: 98.52–98.58 % at near 100 % coverage) in all *E. innesii* strains using ABRicate version 1.0.1 with the *resfinder* database, and was not found in any other closely related *Enterococcus* type strains (**Figure 5.9**) (Seemann 2018b; Bortolaia et al. 2020). Notably, we did not detect any other virulence or antibiotic resistance genes in any of the four *E. innesii* strains. Vancomycin resistant determinant *vanC* subtypes had been reported in *E. gallinarum* (*vanC-1*), *E. casseliflavus* (*vanC-2*), and *E. flavescens* (*vanC-3; E. flavescens* has now been re-classified as *E. casseliflavus*), while *vanC-4* has only been reported once previously in *E. casseliflavus*. In this study, the authors described the *vanC-4* encoding clinically associated *E. casseliflavus* isolates as having 'at least two genetic lineages with the distinct *vanC* genes, that is, a single subtype including previously known *vanC-2/C-3*, and a novel subtype *vanC-4*'. We therefore propose that this distinct 'genetic lineage' of *E. casseliflavus* may hypothetically be *E. innesii*, a novel species that uniquely encodes the *vanC-4* gene (Clark et al. 1998; Watanabe 2009). However, as these isolates described in this previous clinical study were not whole genome sequenced, we are unable to determine this conclusively. Furthermore, the *vanC* resistance gene was phenotypically demonstrated in *E. casseliflavus* and *E. gallinarum* as having intrinsic but low-level resistance to vancomycin at a minimum inhibitory concentration (MIC) of 4–32 µg ml^{-1} (Cetinkaya, Falk, and Mayhall 2000) .

Subsequently, we screened through a larger public dataset of *Enterococcus* species via a targeted approach and found that three isolates previously designated as *E. casseliflavus* and *E. gallinarum* appeared to be *E. innesii* based on ANI (however, taxonomy checks on NCBI were inconclusive for these isolates). These include *E. casseliflavus* NCTC4725 (ANI vs *E. casseliflavus* NBRC100478^{T}: 94.88 %; ANI vs *E. innesii* GAL7^{T}: 97.02 %), *E. gallinarum* FDAARGOS163 (ANI vs *E. gallinarum* NBRC100675^{T}: 77.99 %; ANI vs *E. casseliflavus* NBRC100478^{T}: 94.79 %; ANI vs *E. innesii* GAL7^{T}: 95.40 %) and *E. gallinarum* 4928STDY7071463 (ANI vs *E. gallinarum* NBRC100675^{T}: 78.08 %; ANI vs *E. casseliflavus* NBRC100478^{T}: 94.96 %; ANI vs *E. innesii* GAL7^{T}: 95.43%). Importantly, these three isolates NCTC4725 (ATCC27284; GCA_901542395.1), FDAARGOS163 (GCA_001558875.2) and 4928STDY7071463 (GCA_902159265.1) are derived from human sources (Collins, Farrow, and Jones 1986; Sichtig et al. 2019; Shao et al. 2019) These isolates also demonstrated similar genome features as *E. innesii* sp. nov., with genome size range ~3.6–3.7 Mb and G+C ~42 mol%. These data suggest *E. innesii* sp. nov., may also be a clinically important species associated with novel antimicrobial resistance determinants, as *vanC-4* is encoded in all these genomes, and is reported to cause opportunistic human infection.

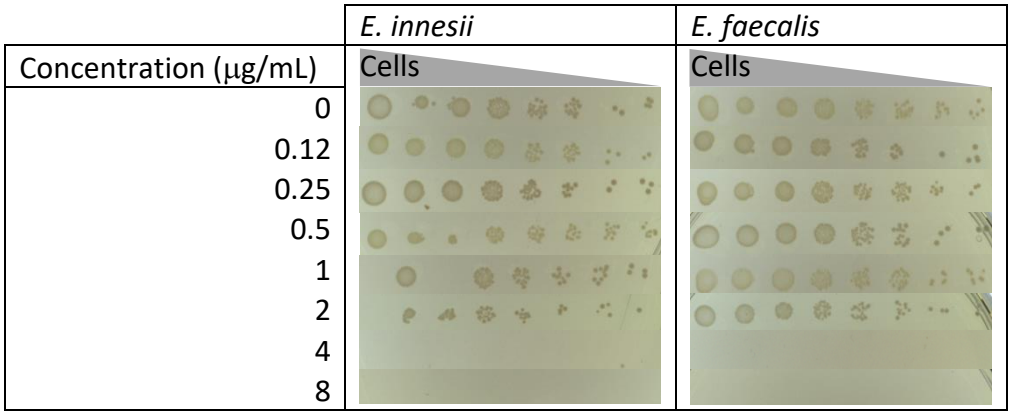


Figure 5.10: MIC assay of E. innesii with vancomycin. *Overnight cultures of E. innesii (GAL7) and E. faecalis (ATCC 29212) were serially diluted and replica plated onto BHI agar plates of increasing vancomycin concentration. Plates were incubated at 37℃ for 24 hours.*

To establish the extent of the resistance conferred by this *vanC-4* resistance gene in *E. innesii* I carried out an MIC assay (**Figure 5.10**) with vancomycin. I used BHI agar plates and *E. faecalis* (ATCC 29212) as a reference. I found that the MIC of *E. innesii* was 4 μg/mL which is the breakpoint (Brown, Wootton, and Howe 2016). This mild resistance is in line with the mild resistance seen in other VanC strains (Cetinkaya, Falk, and Mayhall 2000).

5.2.8 Description of *Enterococcus innesii* sp. nov.

As can be seen in **Figure 5.11**, cells are coccoid shaped, 1.0-1.5 µm in length, and usually occur in pairs or chains, as is typical for enterococci. I found from my own culturing of *E. innesii* that colonies formed on BHI after incubation for 48 h at 37°C are non-pigmented, circular, smooth, shiny, with diameter 1-2 mm and entire margins, and that *E. innesii* is facultatively anaerobic.

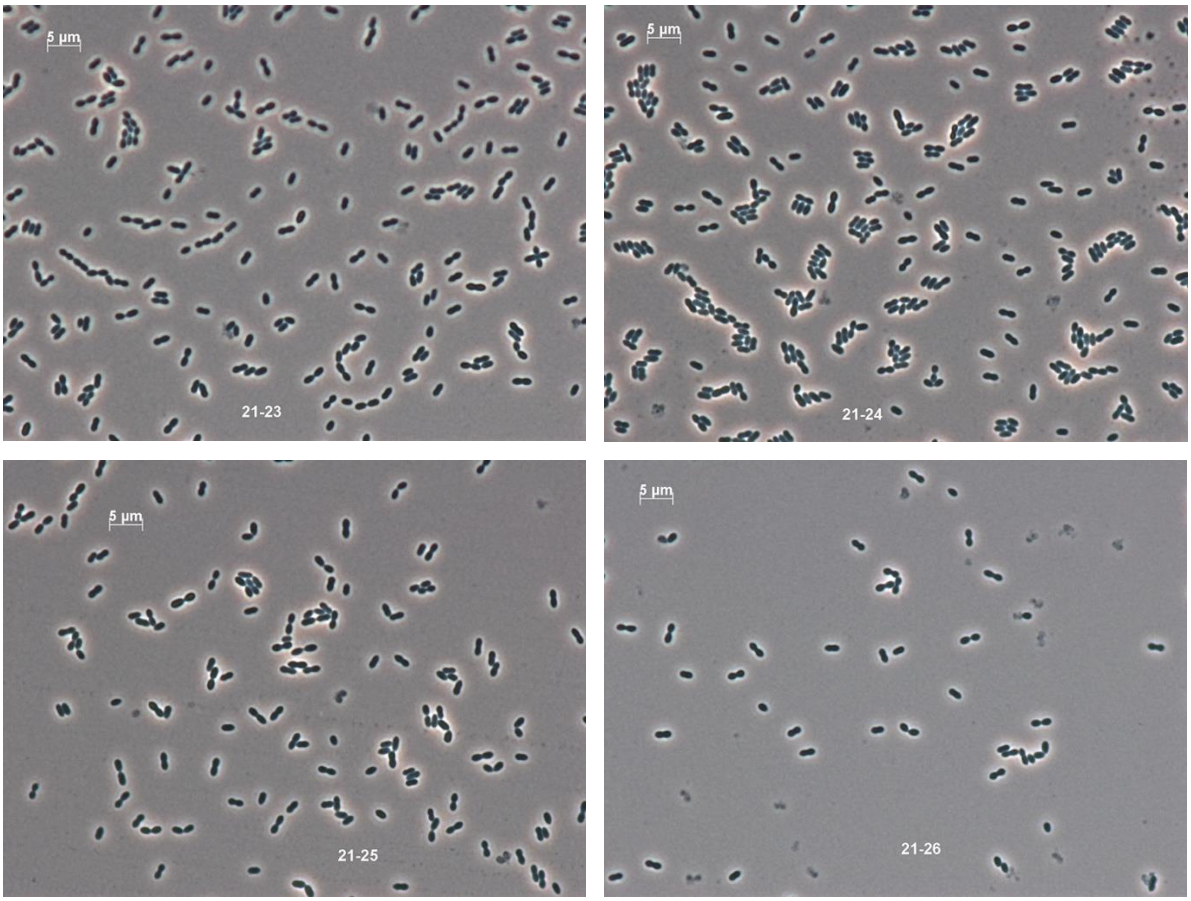


Figure 5.11: Phase-contrast microscopy images of E. innesii *supplied by the Identification Service, Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany*

DSMZ carried out basic biochemical characterisation of *E. innesii* using the API 50CH system on all four strains and the API 32 strep system on GAL7. *E. innesii* was positive for Voges-Proskauer reaction, pyrrolidonyl arylamidase production and hydrolysis of aesculin and arginine dihydrolase. Acid was produced from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, α-Me-D-mannoside, α-Me-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, gentibiose, gluconate, 2-ketogluconate, starch and glycerol. Acid was not produced from erythritol, D-arabinose, L-xylose, adonitol, β-Me-D-xyloside, sorbose, dulcitol, inositol, melizitose, glycogen, xylitol, D-turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 5-ketogluconate. *E. innesii* was negative for urease production, hydrolysis of hippurate, pyruvate utilisation and catalase and

oxidase production. **Table 5.2** lists the phenotypic features that distinguish *E. innesii* from related *Enterococcus* species as well as *E. faecalis* and *E. faecium.*

DSMZ testing also found that *E. innesii* grows at temperatures between 10-45°C (optimum, 30-37°C), and at NaCl concentrations from 0 to 8.0% (optimum, 0-6.5%, at 37°C) in BHI medium. They carried out cellular fatty acid analysis and found that the major fatty acids were $C_{14:0}$, $C_{16:0}$, and $C_{18:1}$ *ω7c*.

5.2.9 *Enterococcus innesii* is motile

I carried out a motility assay on *E. innesii* as part of the characterisation of the species. Most enterococci are not motile, but *E. gallinarum* and *E. casseliflavus,* the two most closely related species to *E. innesii,* are.

I carried out the motility assay using Universal vials filled with 10 mL of Motility Test Media. I used *E. coli* and *E. faecalis* as negative controls and *E. casseliflavus* and *P. fluorescens* as positive controls. I cultured each overnight in BHI broth then used a sterile loop to stab the culture into the test media. I incubated the vials at 37°C for 48 hours before photographing them. This photograph is shown in **Figure 5.12**. Growth along the stab surrounded by clear media indicates non-motility; growth along the stab with turbidity in the surrounding media indicated motility. There is turbidity in the media surrounding the *E. innesii* stab and therefore *E. innesii* is motile.

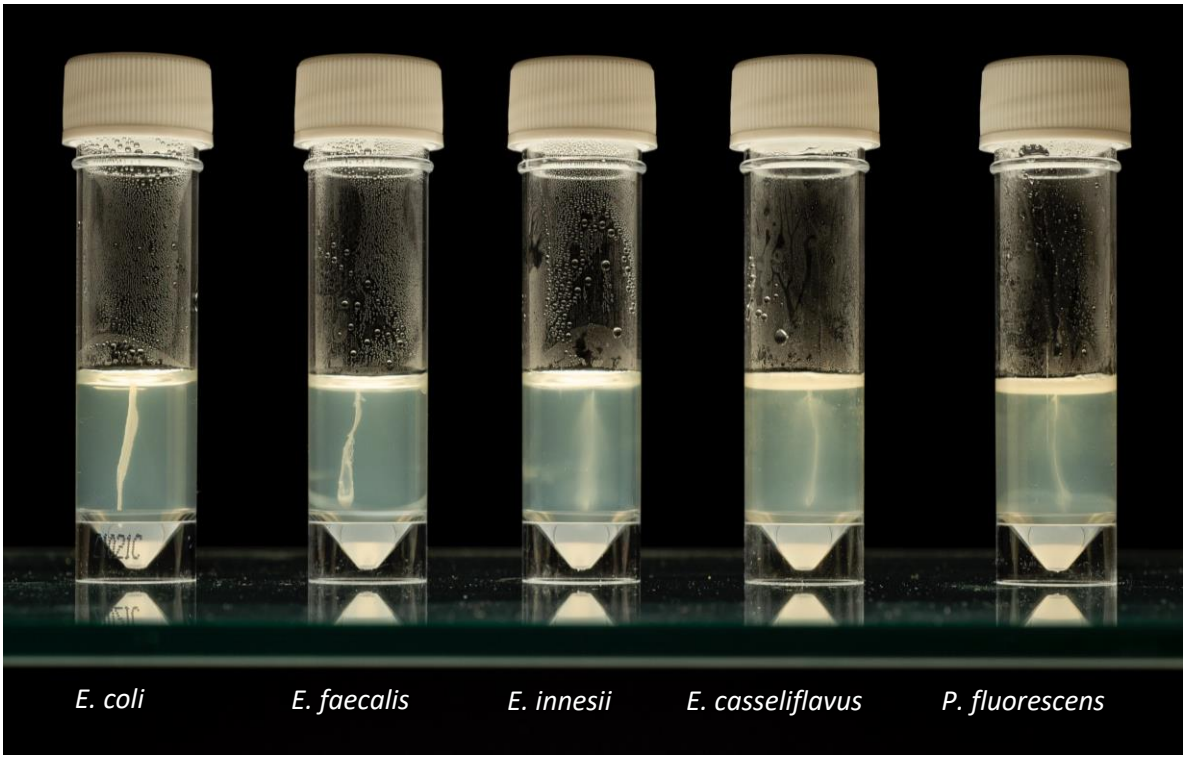


Figure 5.12: Motility assay. *E. coli, E faecalis, E. innesii, E. casseliflavus and P. fluorescens were cultured overnight in BHI broth. A sterile loop was used to stab the culture into 10 mL of Motility Test Medium. Vials were incubated for 48 hours at 37℃ and photographed.*

Characteristics	***E. innesii**** (*n*=4)	*E. casseliflavus*† (*n*=6)	*E. gallinarum*† (*n*=4)	*E. faecalis*† (*n*=6)	*E. faecium*† (*n*=5)
Acid production from:					
d-Xylose	+	+	+	–	–
Sucrose	+	+	+	+	v
Melibiose	+	+	+	–	v
Methyl α-glucoside	+	+	+	–	–
Melizitose	–	–	–	+(–)	–
Mannitol	+	+	+	+	+(–)
Inulin	+	+	+	–	–
Gluconate	+	+	+	+(–)	v
l-Arabinose	+	+	+	–	+
Glycerol	+w‡	–	+	+	+
Rhamnose	+	+(–)	–	v	–
Sorbitol	v	–	+	+(–)	–
Methyl α-d-mannoside	+	+(–)	–	–	–(+)
Raffinose	+	–	+	–	–
Glycogen	–	–	–(+)	–	–
Turanose	–	v	+	–	–
d-Tagatose	–	–	+	+	–
2-Keto-gluconate	+	–	–	v	–
Hydrolysis of:					
Aesculin	+§	+	+	+(–)	+
Hippurate	–§	–	+	+(–)	+
Presence of enzymes:					
Arginine dihydrolase	+§	+(–)	+	+	+
α-Galactosidase	+§	+	+	–	–
β-Galactosidase	+§	+	+	–	+
β-Glucuronidase	+§	–	+	–	–

Table 5.2: Biochemical characterisation of Enterococcus innesii. *Distinctive phenotypic features between E. innesii strains (data from this study) and phylogenetically closely related E. casseliflavus and E. gallinarum strains, also distantly related E. faecalis and E. faecium strains(Collins et al. 1984).*

+, All strains positive; –, all strains negative; +(–), most strains positive; –(+), most strains negative; v, variable; +w, most strains weakly positive, none negative. All strains were positive for ribose, galactose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, trehalose and gentibiose. All strains were negative for erythritol, d-arabinose, l-xylose, adonitol, methyl β-xyloside, sorbose, dulcitol, inositol, xylitol, lyxose, d-fucose, l-fucose, d-arabitol, l-arabitol and 5-keto-gluconate.

**Determined with the API 50CH system.*

†Determined with the API 50CHE system.

‡Shaded area represents distinctive phenotypic features between E. innesii strain(s) and closely related E. casseliflavus and E. gallinarum strains as determined by API systems.

§Determined with API rapid ID32 STREP system on a single strain GAL7T.

5.3 Discussion

Given that *E. mundtii* is the commensal most seen in the *Galleria* microbiome, I had anticipated that it would also be the dominant species in the microbiome of my larvae too. It therefore came as a surprise to me that, both when sequencing the isolates from the *Galleria* gut and when shotgun sequencing the whole gut, *E. casseliflavus* was more dominant in both my larvae and the commercial larvae, Trularv™. It was also surprising that while the microbiome appeared to be playing a protective role for my larvae, none of the isolates I sequenced had any antimicrobial activity that I could find. It is possible that the microbiome merely plays a passive colonisation resistance role by occupying niches and competing for nutrients. When combined with the *Galleria* immune system this may be enough to protect from infection.

Finding a novel species was also unexpected. *E. innesii* does not appear to have any particularly interesting qualities, besides its one mild vancomycin resistance gene. I doubt it will be of major concern to any health authorities, although it may be the cause of occasional opportunistic infections, as is sometimes seen with *E. casseliflavus* and *E. gallinarum* (Monticelli et al. 2018).

I may have been overly optimistic in shotgun sequencing the *Galleria* gut, as shown by quite how few bacterial reads we managed to sequence (**Figure 5.6**). There is no way to sample gut luminal content while excluding the tissue of the gut, as is possible with mice (Gootenberg and Turnbaugh 2011), which is a limitation to the use of *Galleria* as a model for the infant gut microbiome. It is possible to sample *Galleria* faeces but this would have several disadvantages: it would be difficult to collect enough biomass to analyse at a single timepoint; the faeces would be more exposed to the open air than even the gut and therefore underrepresent anaerobic species; the samples would be vulnerable to contamination from the surface of the larvae. For these reasons, the results would not be a true representation of the community in the gut.

It may be that *Galleria* is, in fact, more suited to being a model for symbiotic *Enterococcus* in the microbiome: Enterococci are common commensals of the human microbiome but also important pathogens. Vancomycin resistance in enterococci has long been a concern to health authorities (Cetinkaya, Falk, and Mayhall 2000). *E. faecium,* an ESKAPE pathogen (Mulani et al. 2019; Rice 2008), is a common cause of multidrug resistant nosocomial infections, particularly in neonates. Using *Galleria* to study antibiotic resistance evolution in enterococci is therefore of interest. *Galleria* has been used extensively as an infection model for enterococci (Pereira et al. 2020), but I believe it also has promise to for the study of antibiotic resistance and microbe-host symbiosis in commensal

enterococci, including those from the human microbiome. I have done some preliminary work on this, described in Chapter 6.

5.4 Future work

It has been shown that, as well as the production of mundticin (Shao et al. 2017), *E. mundtii* is adapted to the highly alkaline parts of the Lepidopteran gut through the differential regulation of genes involved in the response to alkaline stress (Mazumdar et al. 2020). I would be interested to see if the *E. casseliflavus* and *E. innesii* isolates from my *Galleria* colony have similar adaptations to this, indicating a similarly deep evolutionary relationship.

There are people working on techniques for the metagenomic sequencing of bacterial communities in samples contaminated with large amounts of eukaryotic DNA. The use of 'Read Until' APIs with Nanopore sequencers allows real-time selective sequencing by ejecting DNA molecules from the pore as they are being sequenced, depending on whether they fit a pre-programmed reference sequence (Kovaka et al. 2021; Payne et al. 2021). As Nanopore sequencers become cheaper and this technology is improved, the issue of host DNA contamination in microbiome samples may become trivial to solve, eliminating it as an obstacle to the use of *Galleria* as a gut microbiome model.

Chapter 6

6 Use of Labelled Bacteria to Track Colonisation of *Galleria*

6.1 Introduction

From the experiments in Chapter 3 and Chapter 4 it was known that the native *Galleria* microbiome could be replaced with various species of enterococci. However, an issue that arose was that the 16S rRNA sequences of different *Enterococcus* species are all very similar to each other and therefore it was hard to distinguish between the bacteria that had been artificially introduced and the bacteria that were present in the larvae prior to antibiotic treatment. This meant it was unclear whether the bacteria isolated from the larvae following attempts to colonise them were truly the ones that had been injected into the larvae or whether they had in fact just grown back following the end of the antibiotic treatment. To address this, strains were labelled using plasmids that conferred both antibiotic resistance and a fluorescence marker that would make them visibly distinct on a plate. The plasmid pMV158$_{mCherry}$ was supplied by Dr Stéphane Mesnage of the University of Sheffield. This plasmid confers tetracycline resistance and carries an mCherry marker, which fluoresces and makes the colonies appear pink to the naked eye. *E. mundtii,* a common *Galleria* commensal, was transformed with the labelled plasmid.

6.2 Results

6.2.1 Loss of the pMV158 plasmid

To test the stability of the pMV158 plasmid in *E. mundtii* I grew the culture up to OD_{600} = 0.5 in selective BHI broth (tetracycline 15 μg/mL) and then added 10 μL of this to 10 mL of non-selective BHI broth and 10 μL to selective broth again (tetracycline 15 μg/mL). I incubated both at 37°C for 8 hours before serially diluting and plating the culture on selective and non-selective BHI agar plates, counting and comparing the number of colonies on each. As seen in **Figure 6.1** the number of colonies on each was broadly the same, and I didn't see any unlabelled colonies, so the plasmid is relatively stable over 8 hours *in vitro*. Of course, it may well be lost more quickly in the more stressful environment of the larva, due to the metabolic burden imposed by plasmids.

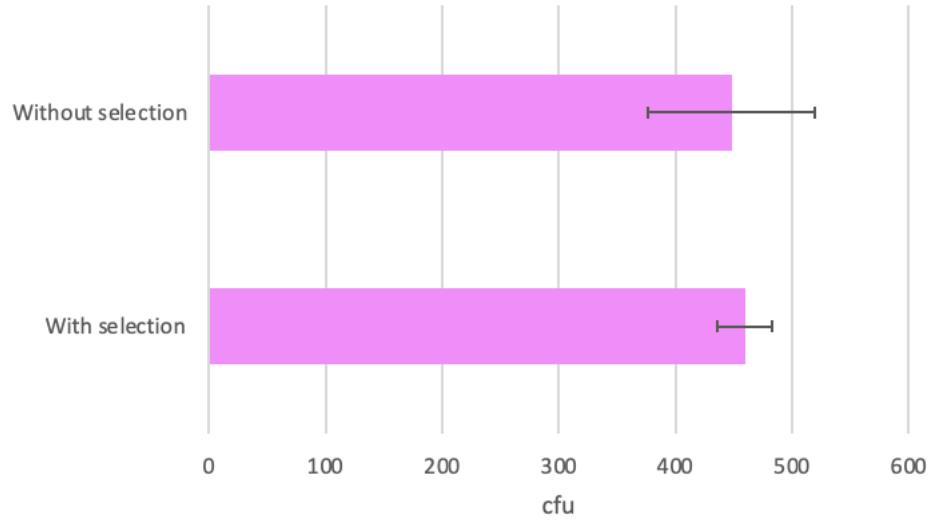


Figure 6.1: Plasmid loss assay. *10 µL of pMV158 E. mundtii culture was added to 10 mL of non-selective BHI broth and cultured for 8 hours at 37°C before serial dilution and plating on BHI agar plates with and without 15 µg/mL tetracycline.*

6.2.2 Passage of labelled *E. mundtii* over generations

I cultured the mCherry-labelled *E. mundtii* to OD_{600} = 0.5 and injected 10 µL into antibiotic-treated and untreated larvae (n=10) before incubating them at 37°C for 2 and 4 days. I then flash-froze the larvae and homogenised through bead-beating, then diluted and plated the homogenate. I counted the number of colonies that were labelled (visibly pink) and those that were not. I also allowed 10 larvae to pupate, eclose and lay eggs. Once the adult moths had died, I collected the unhatched eggs and placed them on sterile food to hatch. I incubated the eggs at 30°C to prevent desiccation and reared them for 4 weeks on sterile food. I then collected 1 g of larvae from each group and homogenised and plated them. The antibiotic treated larvae did not survive pupation. *Galleria* are known to be more vulnerable to infection during pupation without the protection that their native microbiome provides (Johnston and Rolff 2015). Through this project I have also found that larvae are more vulnerable to infection following antibiotic treatment. It is interesting to note that the injected bacteria do not seem to offer protection. Perhaps the strain of *E. mundtii* I have used does not protect as well as strains native to the *Galleria* gut, or perhaps the bacteria have not established themselves enough or in the correct locations to offer protection.

The numbers of bacteria observed is seen in **Figure 6.2**. The native gut bacteria in the antibiotic treated larvae were cleared and successfully replaced with the labelled pMV158 *E. mundtii.* After 2 days the abundance of bacteria is comparable to the numbers in the untreated larvae but the abundance decreases by the 4th day. The numbers of bacteria in the untreated larvae injected with pMV158 *E. mundtii* are comparable – the presence of the native bacteria does not seem to decrease

the number of labelled bacteria that colonise the larvae. Most promising is that the labelled bacteria persist into the second generation of larvae, so are passed from adult to offspring.

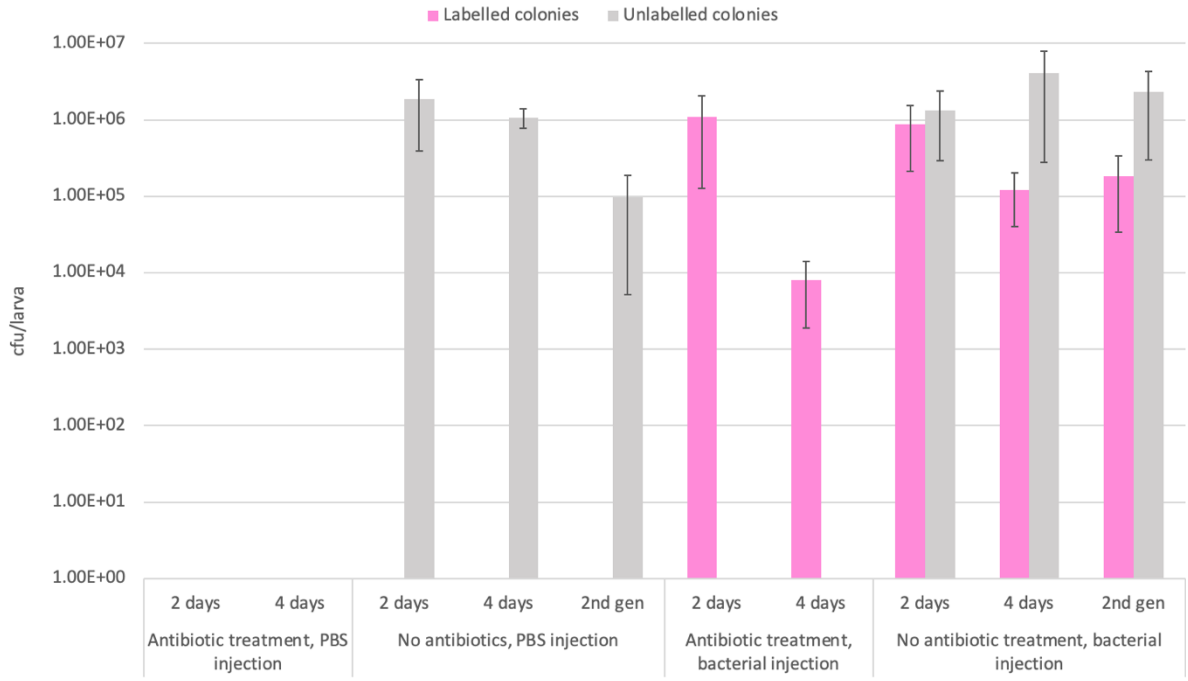


***Figure 6.2: Bacterial abundance of labelled and unlabelled bacteria in Galleria following injection with labelled bacteria**, including passage to the second generation of larvae. Antibiotic-treated and untreated larvae were injected with 10 μL of labelled bacteria at OD_{600} = 0.5 and incubated at 37℃ for 2 and 4 days. Larvae were allowed to pupate and lay eggs, eggs were hatched and larvae sampled again after 4 weeks incubation at 30℃. Larvae were homogenised and the homogenate serially diluted and plated on BHI agar plates.*

6.2.3 Horizontal transfer of the pMV158 plasmid from *E. mundtii* to *E. innesii in vitro*

I was interested in testing to see if the pMV158 *E. mundtii* might be able to transfer the plasmid to the native gut microbiome in the larvae. Initially I tested this *in vitro,* using *E. innesii,* due to its mild vancomycin resistance. *E. mundtii* is not vancomycin resistant and the VanC gene is located on the *E. innesii* chromosome and is therefore unlikely to be transferred to other bacteria. Therefore, following co-culture, any growth on plates containing both tetracycline and vancomycin must be due to horizontal transfer of the pMV158 plasmid from *E. mundtii* to *E. innesii.*

I cultured *E. innesii* and pMV158 *E. mundtii* until OD_{600} = 0.5. I then prepared several co-cultures of volume 5 mL in BHI broth, with no dilution, 100-fold dilution, and 10,000-fold dilution. I cultured these for 8 hours at 37°C. I then serially diluted and plated the cultures onto BHI plates with tetracycline, vancomycin, tetracycline and vancomycin, and no antibiotic. There was growth on the plates containing only a single antibiotic, with all the colonies on the tetracycline plates labelled pink

and all the colonies on the vancomycin plates white, and there was no growth on the plates containing both antibiotics. Both pink and white colonies grew on the non-selective plates. The result for the 10,000-fold dilution co-culture is shown in **Figure 6.3**.

Given that the plasmid did not seem to be transferred *in vitro,* I decided it was not worth attempting the same *in vivo,* particularly given how sparse the *Galleria* microbiome is and the fact that that *E. innesii* makes up a minority of the composition. This makes it unlikely that I would be able to observe any horizontal transfer *in vivo* given that I did not *in vitro.* There may be additional triggers that facilitate horizontal gene transfer *in vivo* that are not present *in vitro.* However, the bacterial density in the *Galleria* larvae that I have colonised with these bacteria is far lower than the bacterial density in the *in vitro* cultures that I did this test with, which may make it difficult to see evidence of horizontal gene transfer in the *Galleria* larva.

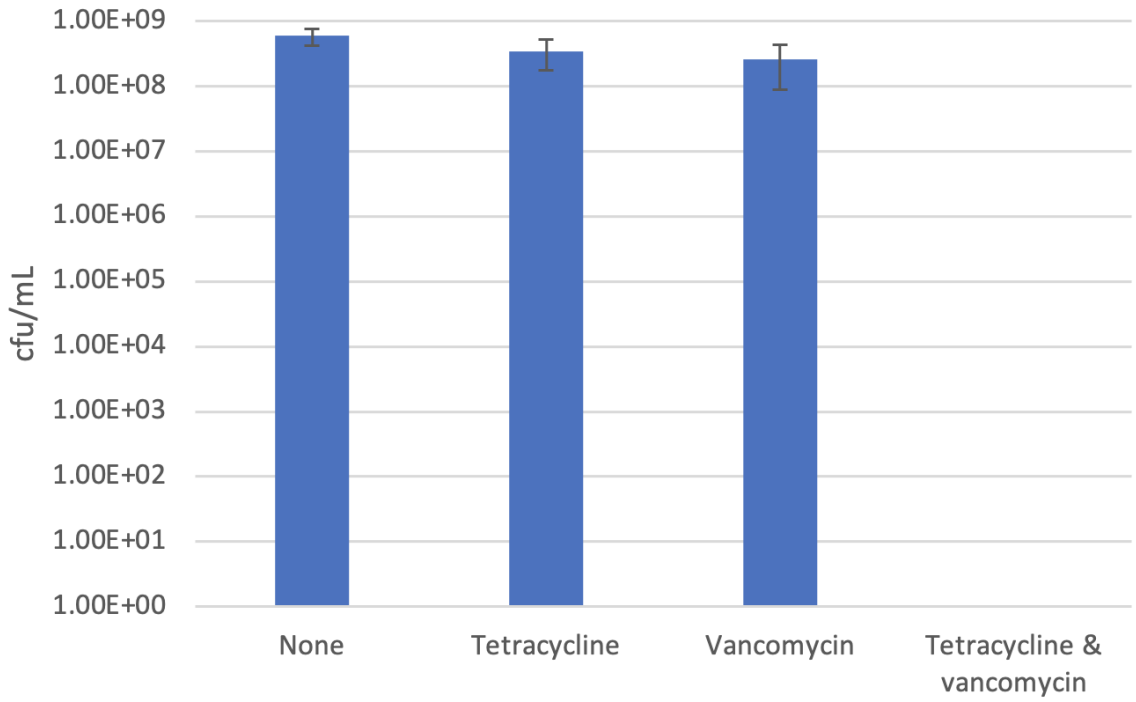


Figure 6.3: Horizontal transfer assay. *E. innesii and pMV158-labelled E. mundtii were cultured to OD_{600} = 0.5. 0.5 µL of both were added to 5 mL of BHI broth and cultured at 37℃ for 8 hours. The co-culture was serially diluted and plated on BHI agar plates containing 15µg/mL tetracycline, 4 µg/mL vancomycin, both tetracycline and vancomycin, and no antibiotic.*

6.3 Discussion

Previously, *Galleria* has only been used for short-term infection assays. I have shown that the *Galleria* native gut microbiome can be replaced with labelled enterococci, and that this persists over generations. It also does not cause any damage to the larvae. Labelling the bacteria allows them to be distinguished from the native gut bacteria and therefore proves that the introduced bacteria are persisting. That the plasmid could not be horizontally transferred to *E. innesii in vitro* is further evidence of this as it shows that the plasmid is still in the original bacterial host.

Conjugation in *Enterococcus* has only been studied in detail in *E. faecalis* and is regulated by a complex system involving 'sex' pheromones (Goessweiner-Mohr et al. 2013). Bacterial conjugation in the gut microbiome is understudied mechanistically and for many bacterial species and plasmids it is unknown what the triggers are which may facilitate horizontal gene transfer (Neil, Allard, and Rodrigue 2021). Whether or not *Galleria* can be used for this research remains to be seen: conjugation often requires high bacterial density, and I'm not sure *Galleria* can sustain that. However, if it is possible, *Galleria* may be a useful model for this research.

6.4 Future work

The fact that antibiotic-treated larvae cannot survive pupation is a limitation to maintaining colonies of *Galleria* with artificially introduced microbiomes. It would be useful to develop some way of managing them that is sterile enough or perhaps involves constant feeding of antibiotics to prevent larvae succumbing to infection during pupation, during which time they are particularly vulnerable. It has been shown that *Galleria* larvae can be protected from infection by bacteria producing bacteriocins (Johnston and Rolff 2015), so maybe this would be a solution.

I have used a known *Galleria* commensal to maximise the likelihood of success: in future I would be interested in seeing human commensals used. This could be used to investigate a wide range of characteristics of human commensals and therefore develop new and better probiotics. What genes are relevant? How do different species interact? How do drugs and nutrients affect different strains? Which strains offer the best protection from infection? I would also be interested in seeing which other genera and species can stably colonise *Galleria*, as I doubt *Enterococcus* is the only one.

Labelled bacteria could be used to develop a better understanding of the dynamics of the *Galleria* microbiome. Fluorescently labelled bacteria could be photographed in the gut to see where they are located. The transfer of bacteria between different larvae could also be tracked.

Chapter 7

7 Use of *Galleria* as a Toxicity and Antimicrobial Efficacy Model

7.1 Introduction

Galleria is very commonly used as an infection and toxicity model (Pereira et al. 2020). *Galleria* is a useful model for toxicity and infection assays because the innate immune system is similar to a human's and can be used in larger numbers than mice. Katarzyna Ignasiak from the Maxwell lab had previously showed that Galleria could be used as a model for acute toxicity testing (Ignasiak and Maxwell 2017b). There were a range of compounds of interest to the Maxwell lab that were promising antimicrobial candidates, the toxicity of which could be tested with *Galleria.*

7.2 Results

7.2.1 *Galleria* can be used for toxicity trials

To learn how to carry out these assays, I repeated some of the assays carried out in Ignasiak et al. (2017b). To determine the LD_{50} of a compound, increasing doses are injected into 5 larvae per stage, in duplicate, until a dose at which half of the larvae are killed. The OECD guidelines can then be used to determine the LD_{50}, as shown in **Figure 7.1**. I incubated the larvae for 48 hours at 30°C before scoring mortality. Doses were 5, 50, 125, 300 and 2000 mg/kg. For ciprofloxacin, glucose, sodium chloride, streptomycin, and tetracycline, I used an additional 5000 mg/kg dose. This was not possible for all compounds because they were not all soluble enough.

My experiment resulted in higher LD_{50} values (**Table 7.1**) than Kat's but are still broadly reflective of the known toxicity of these compounds. The higher LD_{50} may be due to Kat having incubated the larvae for 5 days rather than 2, her initial use of 50% DSMO for some of the compounds, or a difference in injection technique.

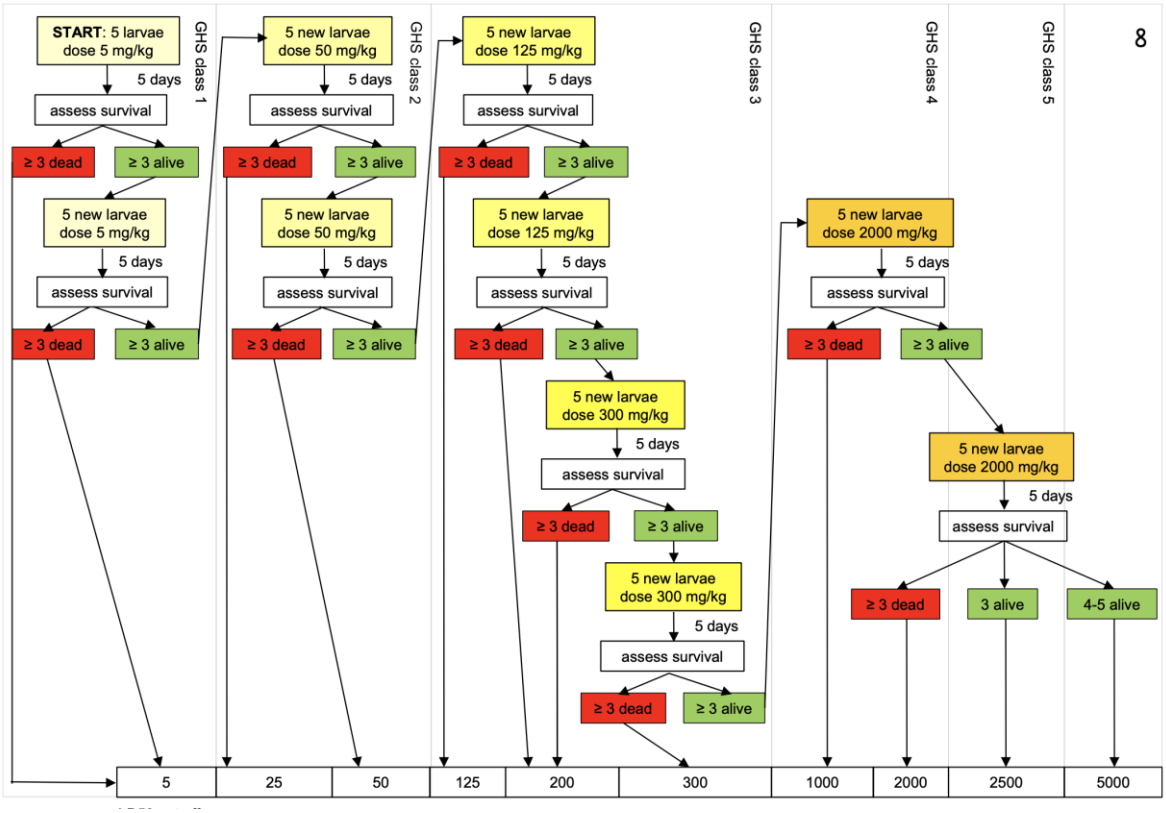


***Figure 7.1: A flowchart representing consecutive steps in the acute toxicity test, according to OECD guidelines (OECD 2002)**. A starting dose of 5 mg/kg body weight was administered and the insects were scored for mortality. If the mortality was over 40%, the compound was assigned the highest toxicity class. If the mortality is below 40%, the dose was re-tested and the testing continued until a toxic dose was established. Taken from Ignasiak and Maxwell 2017b*

Compound	LD_{50} (mg/kg body weight)			
	My assay	Kat's	Rat (oral)	Mouse (oral)
Chloroquine	5000	125	623	500
Ciprofloxacin	>5000	>2000	>2000	>2000
Doxorubicin	2500	5.5		698
Glucose	>5000	>2000	25,800	
Novobiocin	2000	100	3500	962
Sodium chloride	>5000	>2000	3000	4000
Streptomycin	>5000	300	430	430
Tetracycline	>5000	>2000	6443	2759
Ethidium bromide	300	N/A	1503	

***Table 7.1: Toxicity of compounds.** Compounds were assayed for toxicity according to Figure 7.1.*

7.2.2 Martin Goldberg's plant extract is not toxic at doses below 600 mg/kg

Following my trials with compounds of known toxicity, I moved on to testing novel compounds. Martin Goldberg (BCU) is a collaborator who provided a plant extract with antimicrobial activity for toxicity testing. He had found that it had antimicrobial activity against a range of Gram-positive bacterial species *in vitro* and wanted to test the toxicity and antimicrobial activity of the extract *in vivo.* I tested the toxicity of the extract by injecting into *Galleria* larvae and scoring survival over 5 days (**Figure 7.2**). I started by injecting 10 μL of solution up to 2 mg/mL in 25% DMSO. One larva died in the solvent control group and one in the 1.5 mg/mL group but none passed the threshold of 3 larvae dying, so those concentrations were not deemed to be toxic. I then increased the DMSO concentration to 50% to be able to inject up to 15 mg/mL. Many of the solvent control larvae that I just injected with 50% DMSO and PBS died, but not as many as those I injected extract into. This made it difficult to assess the toxicity of the compound so I made a higher concentration stock and injected up to 15 mg/mL in 25% DMSO. The extract doesn't seem to be fully soluble at 15 mg/mL in 25% DMSO as it appeared a lot more opaque than the equivalent concentration in 50% DMSO. Again, only 1 larva died in the 15 mg/mL group, which is below the threshold, so the extract is not toxic when injecting 10 μL at 15 mg/mL. Larvae tested were all between 200 and 250 mg which corresponds to an LD_{50} of at least 600 mg/kg for the extract. The MBC of this extract is 23.4 μg/mL so it is unlikely that the extract will be toxic at therapeutic concentrations, which is promising.

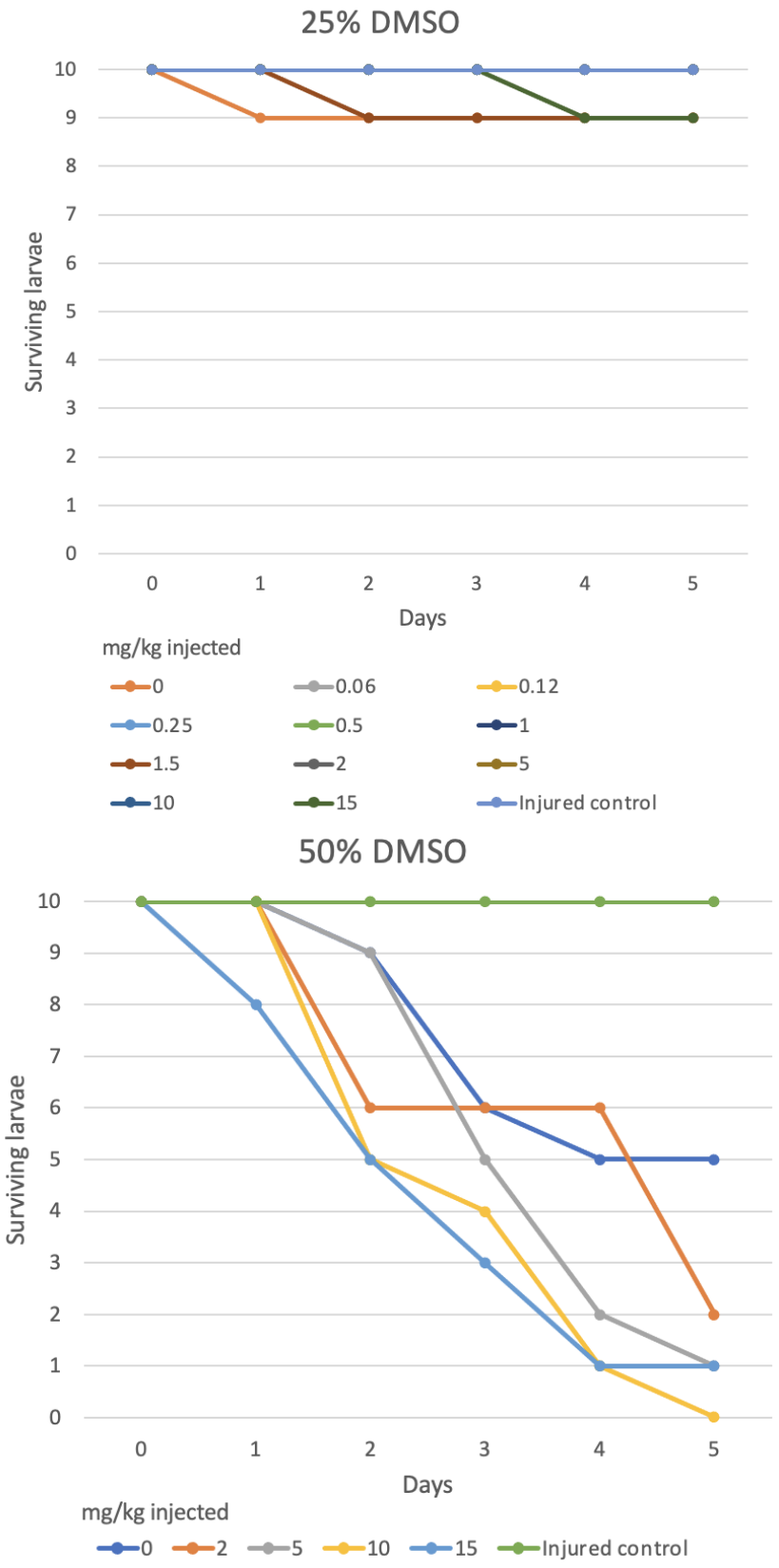


Figure 7.2: Toxicity assay of Martin Goldberg's extract. *Larvae were injected with 10 µL of extract at varying concentrations of extract and DMSO and mortality was scored each day for 5 days.*

7.2.3 Martin Goldberg antimicrobial efficacy assay

After finding that Martin Goldberg's extract wasn't toxic at the MIC, I moved on to carrying out antimicrobial efficacy assays using the extract. The extract is of interest due to its ability to inhibit the growth of a range of Gram-positive bacteria, so I chose to use *Staphylococcus aureus* as the test strain.

I carried out a series of infection assays to determine the infective dose of *S. aureus* (NCIMB 8625) i.e. the dose at which 50% of larvae are dead after 24 hours. I found that the LD_{50} is 15 μL of culture at OD_{600} = 0.5.

I took 10 larvae of mass 180-220 mg, I injected *S. aureus* at the LD_{50}, incubated the larvae for 2 hours at 37°C, and then injected 3.12 mg of compound into each to a final dose in the larvae of 15.6 mg/kg (5x the *in vitro* MIC). I scored for mortality and health index each day for 5 days. I did this three times.

As seen in **Figure 7.3**, I found no significant difference in mortality or health index between the larvae given both the pathogen and the extract and the group and the larvae only given the pathogen without the extract. Therefore the extract has no antimicrobial activity against *S. aureus* at 15.6 mg/kg in *Galleria*.

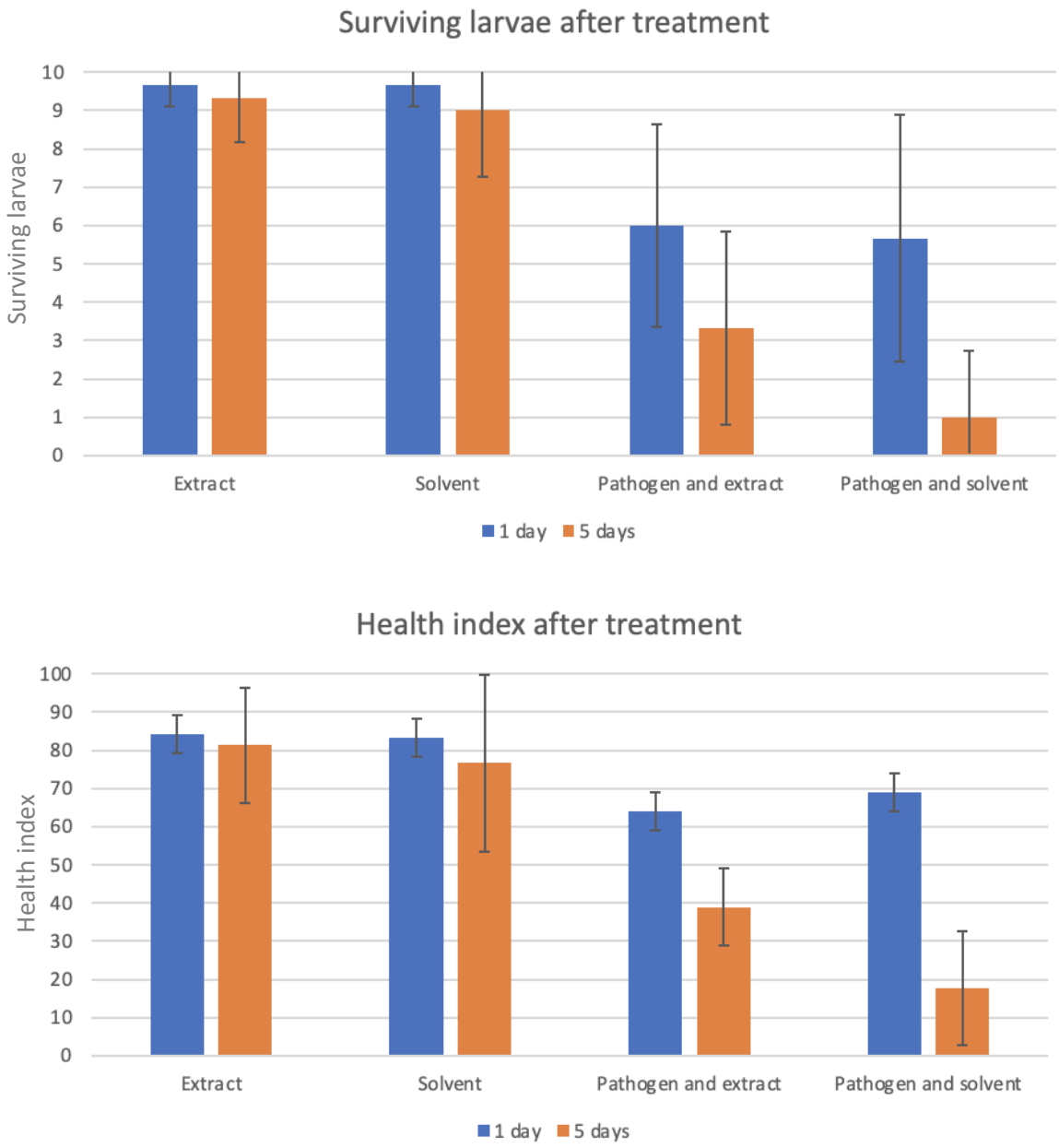


Figure 7.3: Antimicrobial efficacy assay of Martin Goldberg's extract. *Larvae were injected with 10 μL of bacterial in PBS at OD_{600} = 0.5, then with 10 μL of extract at 150 mg/mL and mortality (top) and health index (bottom) (Champion, Titball, and Bates 2018) were scored after 1 day and after 5 days.*

7.2.4 Uppsala compounds

Vicky Baskerville in the Maxwell lab had been working with collaborators at Uppsala University, who had new compounds that were found to kill bacteria and hypothesised to target DNA gyrase. These compounds are synthetic N-olyheterocycles and originated from diversification of dihydroquinazolinones. They have activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii.* To determine if these compounds were toxic, I

carried out toxicity assays in *Galleria* by injecting five larvae with each compound at 10x the MIC and scoring mortality after 5 days (**Figure 7.4**). I carried out this assay three times and at no point did more than 1 larva die after having been injected. Given that no more than 2 larvae died each time, the compounds were determined to be non-toxic to *Galleria* at these concentrations.

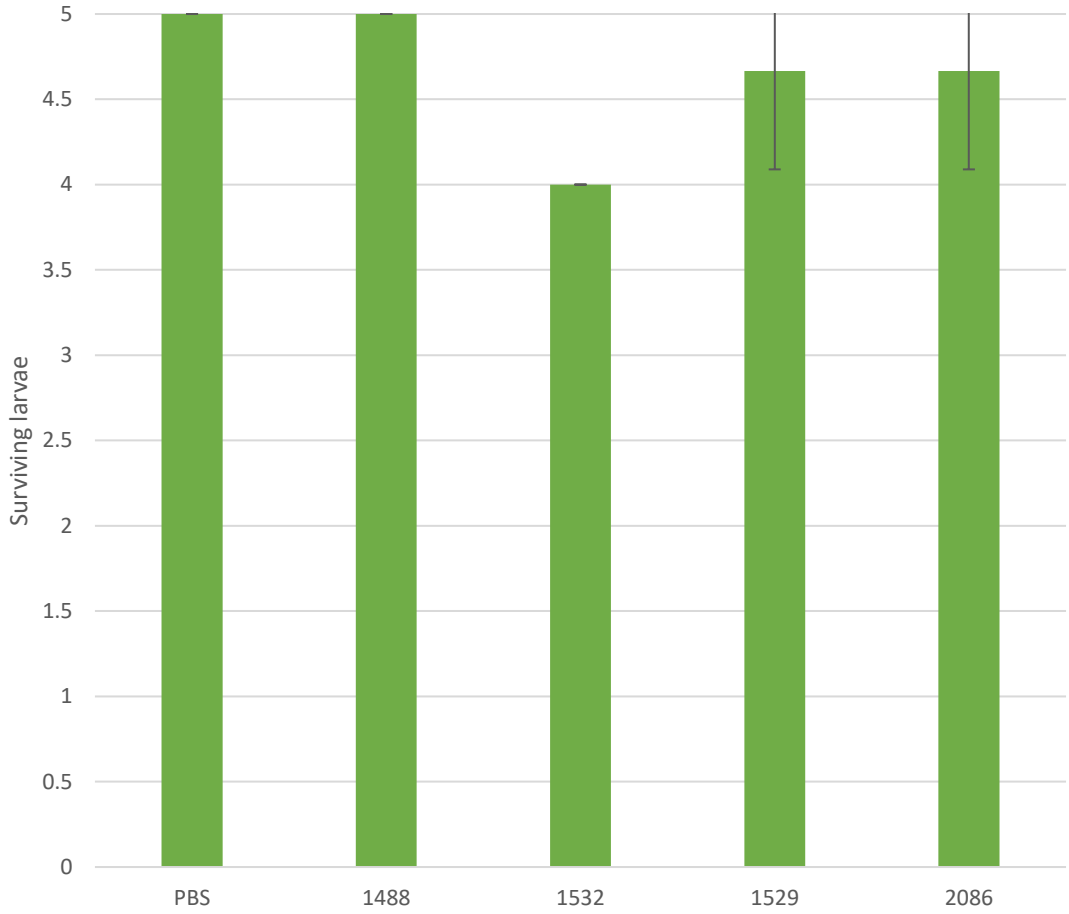


***Figure 7.4: Uppsala compounds toxicity assay**. 5 larvae were injected with compound at 10x the MIC and mortality was scored after 5 days.*

7.3 Discussion

Galleria toxicity assays are useful to determine which compounds among several candidates might be more promising (Ignasiak and Maxwell 2017b). *Galleria* are not similar enough to humans to replace rats or mice for toxicity assays but this assay is very easy and cheap to carry out, so very accessible as an initial screen.

I have found that for infection assays, using the health index scoring described by Champion et al. (Champion, Titball, and Bates 2018) generates more precise results than mortality alone. There is a

stark difference between a healthy larva and a larva ravaged with infection and clinging to life, and the health index score reflects this..

7.4 Future work

Martin Goldberg has found that his extract is active against *Rhodococcus equi,* a bacterial species often used to model *Mycobacterium tuberculosis* due to the pathogenicity of the latter, so intends to also test his extract against *M. tuberculosis.* The extract is very hydrophobic, so he is trying to create a nanoparticle formula.

Galleria still requires a lot of development even as a basic infection model. Many labs rear their own larvae, as I have, but some still purchase larvae from suppliers that sell larvae as animal feed. When using these larvae, very little can be known about the conditions in which they were reared. There are a range of confounding factors that can affect assays using *Galleria,* including: the diet the larvae were fed (Krams et al. 2017); the temperature they were reared at (Mowlds and Kavanagh 2008); the temperature the assay was carried out at ; and the age of the larvae.(Wojda 2017); physical stress (Mowlds, Barron, and Kavanagh 2008); and the age of the larvae. The insect microbiome is known to sometimes play a role in the metabolism of toxins (Siddiqui et al. 2022) and the immune defense of the larva (Johnston and Rolff 2015) so this could also be a confounding factor. Work needs to be done to standardise protocols for the rearing of *Galleria* and for infection assays to ensure consistency across different studies. The main driver of this work has been Biosystems Technology, a company that supplied standardised larvae (TruLarv™) free of hormones and antibiotics for research purposes. They had also been developing a range of products and protocols for high-throughput assays using *Galleria.* Unfortunately, Biosystems Technology has ceased trading, which means researchers will have to rear their own larvae or return to purchasing them from animal feed stores. This is a serious barrier to the widespread use of *Galleria.* Where possible, researchers should try to rear their own larvae according to standard methods (Jorjão et al. 2018).

Chapter 8

8 Discussion

The overall objective of this project was to investigate the use of *Galleria mellonella* as a surrogate system to study the human infant gut microbiome, with the possibility of replacing or reducing the use of rodents in such work, in keeping with the aims of NC3Rs. In order to realise this goal, I needed to carry out a range of experiments to establish *G. mellonella* as a test system and explore various properties of this organism. These experiments in of themselves produced some interesting findings, such as characterising the *G. mellonella* gut microbiome and the consequent discovery of a novel bacterial species.

8.1 Rearing

Although *Galleria* has been used as a model system for several decades (Pereira et al. 2020) and previous to that had been reared for other purposes including as fishing bait and animal feed, I wanted to ensure that the diet and rearing would be suitable for my purposes. To this end, I spent several weeks testing different diets and containment. The rearing conditions I have used do not vary wildly from those that are previously published, but nonetheless it was important to establish rearing conditions that I could use routinely and that would yield consistent results for the ensuing experiments.

8.2 Toxicity and efficacy testing

One of the major uses of *Galleria* as a model is for the testing of toxicity and efficacy of compounds (Ignasiak and Maxwell 2017b). *Galleria* is particularly useful to test compounds that show promise as antimicrobials but are not yet validated enough to justify testing in mouse models, which are expensive. In this project I used *Galleria* to test the toxicity of several compounds of interest to the Maxwell lab. This involved repetition of some previous work (Ignasiak and Maxwell 2017b) but also analysis of some novel compounds. In particular I think the testing of the compounds provided by Uppsala University demonstrates how useful *Galleria* can be for this sort of testing. If a mouse model were being used here, it is likely that only the most promising compound would have been tested, whereas when using *Galleria* it is possible to test a set of similar compounds, rapidly and cheaply. It is also relatively easy to learn to use and does not require specialist facilities to rear. *Galleria* is certainly not a perfect model for toxicity, but it certainly has a use in the testing of compounds. I also

tested a plant extract provided by a collaborator for its antimicrobial efficacy and found that it had no effect, despite being effective *in vitro*.

It was also important for me, as I was feeding the larvae relatively high concentrations of antibiotics, to ensure that these antibiotics were not especially toxic to my larvae. If they had been, this could have caused issues for the experiments I was attempting to carry out.

8.3 Native microbiome

Previously, it was known that *Galleria* had a sparse microbiome (Ignasiak and Maxwell 2018), that this microbiome was usually dominated by *Enterococcus* (Allonsius et al. 2019), and that at least in some studies, these enterococci had been shown to protect the larvae from infection. I wanted to check if the microbiome of my larvae was similar to the microbiome that had been described in other studies.

Initially, I isolated species from the *Galleria* gut that I had dissected out. I isolated strains from both my colony and TruLarv™ larvae. All of the strains I isolated were enterococci; eight were *E. casseliflavus* and three were a novel species, *E. innesii.* I sequenced the whole genomes of these isolates. They were very difficult to tell apart from their 16S rRNA sequences and the species could only be conclusively identified through average nucleotide identity analyses of the whole genome. This is common for enterococci, as they have very similar 16S sequences (Patel et al. 1998). Many studies of the *Galleria* microbiome exclusively use 16S amplicon sequencing to identify bacteria (Krams et al. 2017; Allonsius et al. 2019) and therefore only identify to the genus level. The evidence that *E. mundtii* is the exclusive gut symbiont of *Galleria* comes from a single study (Johnston and Rolff 2015) and, from my work, it seems that this may not be the case. It may be that there are several species of *Enterococcus* capable of thriving in the *Galleria* gut. I also did not find that any of the species I isolated had any antimicrobial activity or genes that would indicate that they might. This contrasts with previous studies that show that *Galleria* gut symbionts protect the larvae from infection through the production of bacteriocin (Johnston and Rolff 2015; Jarosz 1979).

I also carried out both shotgun sequencing and 16S rRNA amplicon sequencing on the guts of several larvae. I investigated the microbiome in the larvae I had, and the microbiome in larvae from TruLarv™ and from wild sources. Both laboratory-reared sources had microbiomes that were similar in composition to that of *Galleria* larvae described previously, i.e. overwhelmingly dominated by

Enterococcus. The wild larvae, in contrast, were more diverse, with a high proportion of either *Bacillus* or *Cutibacterium,* depending on which of the sequencing results you believe.

All previous studies on the *Galleria* microbiome have been carried out on larvae that were reared either for feed or for experimentation and therefore had very stable diets and little bacterial challenge. The wild sources I took larvae from were old hives that no longer had bee colonies in. They were melanised, indicating that they were actively responding to immune challenge. It's hard to tell which of the laboratory-reared larvae or wild larvae is closer to the typical lifestyle of a *Galleria* larva – they do tend to thrive in weak colonies or in hives where the bee colony has collapsed completely, but it may be that these hives were too far gone and consuming the rotting waxcomb had disrupted the microbiome of the larvae. It would be interesting to compare larvae from a variety of hives in a variety of conditions and see what the differences in the microbiomes are. I think a comprehensive study like this would be necessary to describe the 'true' *Galleria* microbiome. A study carried out on the cotton bollworm, *Helicoverpa armigera,* found that while laboratory populations have a microbiome that was relatively simple and dominated by *Enterococcus,* in field populations the microbiome was far more diverse, while still having a large minority of *Enterococcus* (Xiang et al. 2006).

Notwithstanding this, for use as an experimental system, lab-reared *G. mellonella* are desirable as they have a relatively consistent microbiome that can be cleared and manipulated as required. It would therefore be recommended that an in-house *G. mellonella* colony should be established for those working with this model.

8.4 Practical issues with sequencing in Galleria

I found that because of the low bacterial biomass and high amount of eukaryotic tissue, it was difficult to get enough bacterial reads from the *Galleria* gut samples. There are techniques that might solve this problem. Methods for bacterial enrichment prior to sequencing are available but can be labour-intensive and require large numbers of larvae (Chen et al. 2018; Xia et al. 2017). I don't think shotgun metagenomics is necessary to characterise the *Galleria* microbiome, given that it isn't actually very diverse, and many of the less abundant species are soil bacteria that are probably more likely to be incidental than functional. It is probably far easier to just isolate the dominant species and culture and sequence them independently. However, for *Galleria* to be used as a model for the infant gut microbiome, even if it were able to support the kind of diversity that

would make a useful model, shotgun metagenomics would be an important method to use for this investigation, so it is a barrier to the use of *Galleria* as a model.

8.5 *Enterococcus innesii*

In sequencing the isolates from the *Galleria* gut, we discovered a novel species. This species carries a unique *vanC* resistance gene. This vancomycin-resistance gene provides a weak level of resistance to the antibiotic vancomycin. It is very similar to the other *vanC* enterococci, *Enterococcus casseliflavus* and *Enterococcus gallinarum,* both genetically and phenotypically. These species have been known to cause infection in humans (Monticelli et al. 2018), and it is likely that *E. innesii* is also capable of causing infection. Although the level of vancamycin resistance conferred by this *vanC* resistance gene is relatively low, it is still a potential concern in terms of the possibility of transfer to human pathogens.

8.6 The colonisation of the *Galleria* gut using faecal slurry

The main goal of this project was to assess the extent to which *Galleria* could be used as a model for the human infant gut microbiome. Preliminary work done by a previous researcher in the lab, Marjorie Labédan, had shown that antibiotic treatment could clear the *Galleria* gut of bacteria, and that *E. faecalis* and *S. hominis* could be found in the gut following feeding with faecal slurry. I showed that the gut could indeed be cleared using antibiotics, although with a slightly modified protocol to Marjorie's, and then started to try to colonise the gut using faecal slurry.

The most important genus in the infant gut microbiome is *Bifidobacterium* – it is the dominant species in the gut of neonates (Stewart et al. 2018) and it is central to the building of healthy microbiomes (Tamburini et al. 2016). From the 16S amplicon sequencing results it appeared that there was a lot of bifidobacteria in the gut but at no point was I able to culture these bacteria out from the gut again. It may be that they didn't survive the dissection and homogenisation, but this is unclear. The only species I was able to isolate out of the gut after faecal slurry feeding were *E. faecalis* and *P. mirabilis.* If the *Galleria* gut cannot sustain a diversity of bacteria, or key taxa like *Bifidobacterium,* then it cannot be used as a comprehensive model for the infant gut microbiome. It may just be that although the *Galleria* immune system is similar to humans', the *Galleria* gut is too different from the human gut structurally and chemically to be able to host a diversity of human gut bacteria. In some parts of the lepidopteran gut, pH can be as high as 11 or 12 (Dow 1992), whereas the human gut tends to lean slightly acidic (Evans et al. 1988). *E. mundtii,* a common lepidopteran

gut symbiont, is known to respond transcriptionally to this high pH to survive (Mazumdar et al. 2021); an adaption that human gut commensals may not have.

8.7 Using individual species

Prior to attempting to colonise the *Galleria* gut using faecal slurry I attempted to colonise it using bacterial cultures. I had trouble making this work with the protocols I was using at the time, likely due to not giving larvae enough of a break from antibiotic feeding. After the faecal slurry experiments I returned to this, but with a break following the antibiotic treatment, and a focus on using *Enterococcus,* which I knew had successfully colonised the gut following the feeding with faecal slurry. As mentioned above, it is difficult to distinguish species of enterococci from their 16S rRNA sequences, so I labelled the bacteria with both antibiotic resistance and fluorescence

8.8 Using labelled bacteria to track colonisation.

A key question, raised in the previous section, was whether bacteria introduced into *G. mellonella* larvae would thrive and indeed be passed on to subsequent generations. To explore this, I labelled the bacteria with both antibiotic resistance and fluorescence as a way of following their presence. I found that this approach was successful and that the labelled bacteria were indeed found in a subsequent generation. This result holds real promise for using *G. mellonella* as a surrogate system for non-native gut microbiome species, as it allows experimentation over a longer time frame.

8.9 Limitations of this study

Although the faecal slurry was prepared and transported under anaerobic conditions, it was fed to the larvae in aerobic conditions. This limits the ability of obligate anaerobes – of which there are many in the infant gut microbiome – to colonise the larvae. This may be the reason it was difficult to colonise the larval gut with viable bifidobacteria. Faecal slurry was also stored frozen, without the use of a cryoprotectant such as glycerol, which may have reduced viable bacterial counts in the faecal slurry.

Contamination of samples prior to sequencing is a common issue in microbiome research. Contamination can come from many sources, for example the DNA purification kits used to prepare samples. This is known as the ‘kitome’. Samples with low bacterial abundance are particularly vulnerable to having results biased or entirely overwhelmed by the ‘kitome’ (Paniagua Voirol et al. 2021). Therefore this would be a concern when sequencing the relatively sparse *Galleria*

microbiome. This should be considered when looking at some of the results in this study, in particular the sequencing of the microbiomes of the larvae from wild sources.

8.10 Future of the model

I think *Galleria* has a use as a model to investigate the behaviour of human gut commensals, particularly *Enterococcus* species. Enterococci are a health concern – vancomycin-resistant *E. faecium* is in particular a common cause of infection in neonates (Lister et al. 2015). Enterococci are also common human gut commensals. This has led to enterococci, in particular *E. faecalis,* being labelled as a pathobiont: 'a symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host' (Chow and Mazmanian 2010). I think *Galleria* could be useful to study what the environmental conditions may be that cause *E. faecalis* to show either pro-health or pro-pathology behaviour, and how to reduce the possibility of *E. faecalis* causing infection.

Although many bacteria are understood to be 'good' and there are many probiotic products available, there is a limited understanding of what the mechanisms by which these strains promote health (Kleerebezem et al. 2019). *Galleria* could be a useful model to screen strains of bacteria identified as potential probiotics to identify which are likely to cause infection, which are likely to be beneficial, and which are best able to establish themselves in the gut. Already some work has been done using *Galleria* to test the ability of probiotics to protect larvae from infection (Ribeiro et al. 2017).

Although the *Galleria* genome is sequenced, it lacks the genetic tools that make other model organisms, like *Drosophila,* appealing for experimentation. This is an obstacle to its establishment as a model, and although the development of these tools is anticipated (Dinh et al. 2021), it has not yet been reported. Biosystems Technology, the sellers of TruLarv™, were the leading developers of new technologies and uses for *Galleria,* and received a grant to develop genetic tools for *Galleria* in 2017 (NC3Rs 2019), but they no longer operate.

Another useful development would be the establishment of a protocol to rear truly germ-free *Galleria* over generations. I was able to clear the gut microbiome for a brief time using antibiotics but this is not permanent. When I treated the eggs with bleach, I realised it was difficult to rear such small larvae under entirely sterile conditions due to their habit of crawling out of the dishes I was

using. I suspect that with additional effort it will be possible to achieve consistent truly germ-free *Galleria* colonies that would be of significant utility for a variety of experimental programmes.

Abbreviations

BHI	Brain-heart infusion
LB	Luria-Bertani
LD50	Median lethal dose
MIC	Minimum inhibitory concentration
MRS	De Man, Rogosa and Sharpe
PBS	Phosphate-buffered saline
RCA	Reinforced Clostridial Agar
rRNA	Ribosomal RNA
TetR	Tetracycline resistant
OECD	Organisation for Economic Co-operation and Development
ROS	Reactive Oxygen Species

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Appendix I

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Enterococcus innesii sp. nov., isolated from the wax moth *Galleria mellonella*

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Abstract

Four bacterial strains were isolated from two different colony sources of the wax moth *Galleria mellonella*. They were characterized by a polyphasic approach including 16S rRNA gene sequence analysis, core-genome analysis, average nucleotide identity (ANI) analysis, digital DNA–DNA hybridization (dDDH), determination of G+C content, screening of antibiotic resistance genes, and various phenotypic analyses. Initial analysis of 16S rRNA gene sequence identities indicated that strain GAL7^T was potentially very closely related to *Enterococcus casseliflavus* and *Enterococcus gallinarum*, having 99.5–99.9% sequence similarity. However, further analysis of whole genome sequences revealed a genome size of 3.69 Mb, DNA G+C content of 42.35 mol%, and low dDDH and ANI values between the genomes of strain GAL7^T and closest phylogenetic relative *E. casseliflavus* NBRC 100478^T of 59.0 and 94.5%, respectively, indicating identification of a putative new *Enterococcus* species. In addition, all novel strains encoded the atypical vancomycin-resistance gene *vanC-4*. Results of phylogenomic, physiological and phenotypic characterization confirmed that strain GAL7^T represented a novel species within the genus *Enterococcus*, for which the name *Enterococcus innesii* sp. nov. is proposed. The type strain is GAL7^T (=DSM 112306^T=NCTC 14608^T).

INTRODUCTION

Enterococci are Gram-positive facultative anaerobes that are often diplococci, and which belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Enterococcaceae* [1, 2]. They comprise a large genus of lactic acid bacteria that are tolerant to many stress conditions and can be found in a wide range of habitats including water (fresh and marine), soils, and as members of animal, human and plant microbial communities (i.e. microbiomes) [3]. From a clinical perspective, some species, such as *Enterococcus faecalis* and *Enterococcus faecium*, are associated with opportunistic infections, including bacteraemia, endocarditis and urinary tract and catheter infections [4–6]. Crucially, *Enterococcus* species have inherent resistance to many antimicrobial agents including cephaloporins and *β*-lactams [7, 8]. They are also of further concern due to acquisition of multi-drug resistance traits, particularly rising rates of vancomycin-resistant *Enterococcus* strains [9], which are an increasingly common cause of infection in hospitals [10].

As highlighted above, *Enterococcus* species are also common animal microbiota members, and previous work has indicated that the greater wax moth, *Galleria mellonella*, is dominated by Enterococci [11, 12], like many other species of *Lepidoptera* [13]. Although *Galleria* is a pest of honeybee (*Apis mellifera*) hives worldwide [14], in recent years it has gained popularity as a model host for a range of human pathogens. It has the advantages of being inexpensive, easy to use, and able to grow at 37 °C, while not being subject to the same regulations and ethical concerns as mammalian models such as mice [15–17]. It has also been of interest due to the ability of the larvae to metabolize polyethylene [18]. Previous research on endogenous *Galleria* and *Enterococcus* species indicates these bacteria may have a colonization-resistance function, either passively or actively, through the production of antimicrobial bacteriocins [11, 19].

In this study, we isolated four bacterial strains initially identified as *Enterococcus casseliflavus* based on 16S rRNA gene

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Keywords: *Enterococcus*; novel species; *Galleria mellonella*; wax moth; antibiotic resistance; vancomycin resistant.
Abbreviations: ANI, average nucleotide identity; BHI, brain heart infusion; dDDH, digital DNA–DNA hybridization; FAME, fatty acid methyl ester; SNP, single nucleotide polymorphism; TYGS, Type Strain Genome Server.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GAL7^T is MZ305092; for the draft genome sequences (genome assemblies) of strains GAL7^T, GAL9, GAL10 and TL2 they are GCA_018982785.1, GCA_018982775.1, GCA_018982735.1 and GCA_018982725.1, respectively. Strain GAL7^T has been deposited at DSMZ (accession number: 112306) and NCTC (accession number: 14608).
†These authors contributed equally to this work

alignments. However, on further inspection and characterization (genomic and phenotypic) we propose a novel and putative *Enterococcus* species: herein named *Enterococcus innesii* sp. nov. These data expand our knowledge of an important model organism-associated *Enterococcus* species, which encodes atypical vancomycin resistance genes and is therefore also of clinical importance.

ISOLATION AND ECOLOGY

Galleria mellonella larvae were obtained from a colony grown from larvae originally sourced from Livefood UK Ltd and maintained at the John Innes Centre Entomology Facility (Norwich, UK). *Galleria* larvae (TruLarv) were also purchased from BioSystems Technology. Larvae were flash-frozen in liquid nitrogen, and whole guts dissected under sterile conditions (three guts were pooled into each single sample). Each sample was then homogenized in 200 µl PBS, diluted 100-fold in PBS and 50 µl spread-plated on brain heart infusion (BHI) agar plates and incubated at 37 °C for 48 h. Individual bacterial colonies were selected and grown in BHI media. Three pure isolates from distinct single colonies were taken from *Galleria mellonella* larvae from the John Innes Centre Entomology Facility, and one was isolated from TruLarv larvae.

GENOMIC CHARACTERIZATION

The genomes of *E. innesii* GAL7^T, *E. innesii* GAL9, *E. innesii* GAL10 and *E. innesii* TL2 were sequenced using the Nanopore MinION sequencing platform. Prior to this, FastDNA Spin Kit for Soil (MP Biomedicals) was used to extract genomic DNA from each isolate (grown up in BHI media for 48 h) following manufacturer's instructions, with an extended 3 min bead-beating procedure as described previously [20]. The sequencing library was prepared via a modified Illumina Nextera Flex low input tagmentation approach using symmetrical 24 base barcoded primers [21]. Libraries were pooled and stringently size selected on a sageELF 0.75% cassette and fractions from 4 kb and above were pooled and put into a standard Nanopore Ligation reaction using the SQK-LSK109 kit and protocol and loaded onto a MinION following the recommended loading guidelines and run for 48 h. Basecalling was performed using Guppy version 3.6.0 (Oxford Nanopore Technologies) in high accuracy mode (model dna_r9.4.1_450bps_hac). Subsequently, high-quality pure culture genomes (genome size range: 3.6–3.8 Mb) were assembled via Unicycler version 0.4.9 [22] and further polished using Racon version 1.3.1 in the Unicycler pipeline, with a range of 13–18 in contigs and G+C content of ~42 mol% (Table 1). Genomes were further annotated using Prokka version 1.13, with ~3800–4100 CDS predicted for these for *E. innesii* strains.

Initially, the 16S rRNA sequences of 61 validated *Enterococcus* species (60 were *Enterococcus* type strains) were obtained from the web server of List of Prokaryotic names with Standing in Nomenclature (LPSN; May 2021) [23, 24]. Using *in silico* approaches, near-full-length 16S rRNA sequences (~1.5 kb) of *E. innesii* were extracted via bactspeciesID version 1.2 [25],

Table 1. Genome statistics comparison between closely related *Enterococcus* species (*n*=10) to *E. innesii* strains identified by TYGS, including type strain GAL7^T [45]

Previously published type strain genomes were retrieved from GenBank for analysis in this study [46]. Genome assembly statistics were extracted using sequence-stats version 0.1 [47] while genome annotation was performed using Prokka version 1.13 [48].

Strains	Genome size (bp)	Contigs	G+C (mol%)	rRNA	tRNA	CDS	GenBank accessions
Enterococcus alcedinis CCM8433^T	2686367	29	37.59	2	50	2472	GCA_014635985
Enterococcus casseliflavus NBRC100478^T	3498264	54	42.35	3	50	3339	GCA_001544095
Enterococcus devriesei DSM22802^T	3320653	65	40.22	1	29	3119	GCA_001885905
Enterococcus gallinarum NBRC100675^T	3774884	87	39.75	3	49	3600	GCA_001544275
Enterococcus gilvus BAA350^T	4179913	5	41.41	21	70	4111	GCA_000407545
Enterococcus innesii GAL10	3678879	18	42.32	15	69	3868	GCA_018982735
***Enterococcus innesii* GAL7 ^{T}**	3692254	14	42.35	22	67	3866	GCA_018982785
Enterococcus innesii GAL9	3793471	13	42.22	18	64	4070	GCA_018982775
Enterococcus innesii TL2	3806372	17	42.25	20	63	4075	GCA_018982725
Enterococcus malodoratus ATCC43197^T	4654237	10	39.56	16	54	4480	GCA_000407185
Enterococcus massiliensis AM1^T	2712841	7	39.64	9	61	2612	GCA_001050095
Enterococcus pseudoavium NBRC100491^T	2731874	59	40.06	3	48	2587	GCA_001544295
Enterococcus saccharolyticus ATCC13076^T	2604038	2	36.70	6	38	2586	GCA_000407285
Enterococcus viikkiensis LMG26075^T	2545311	45	40.26	4	40	2416	GCA_005405345

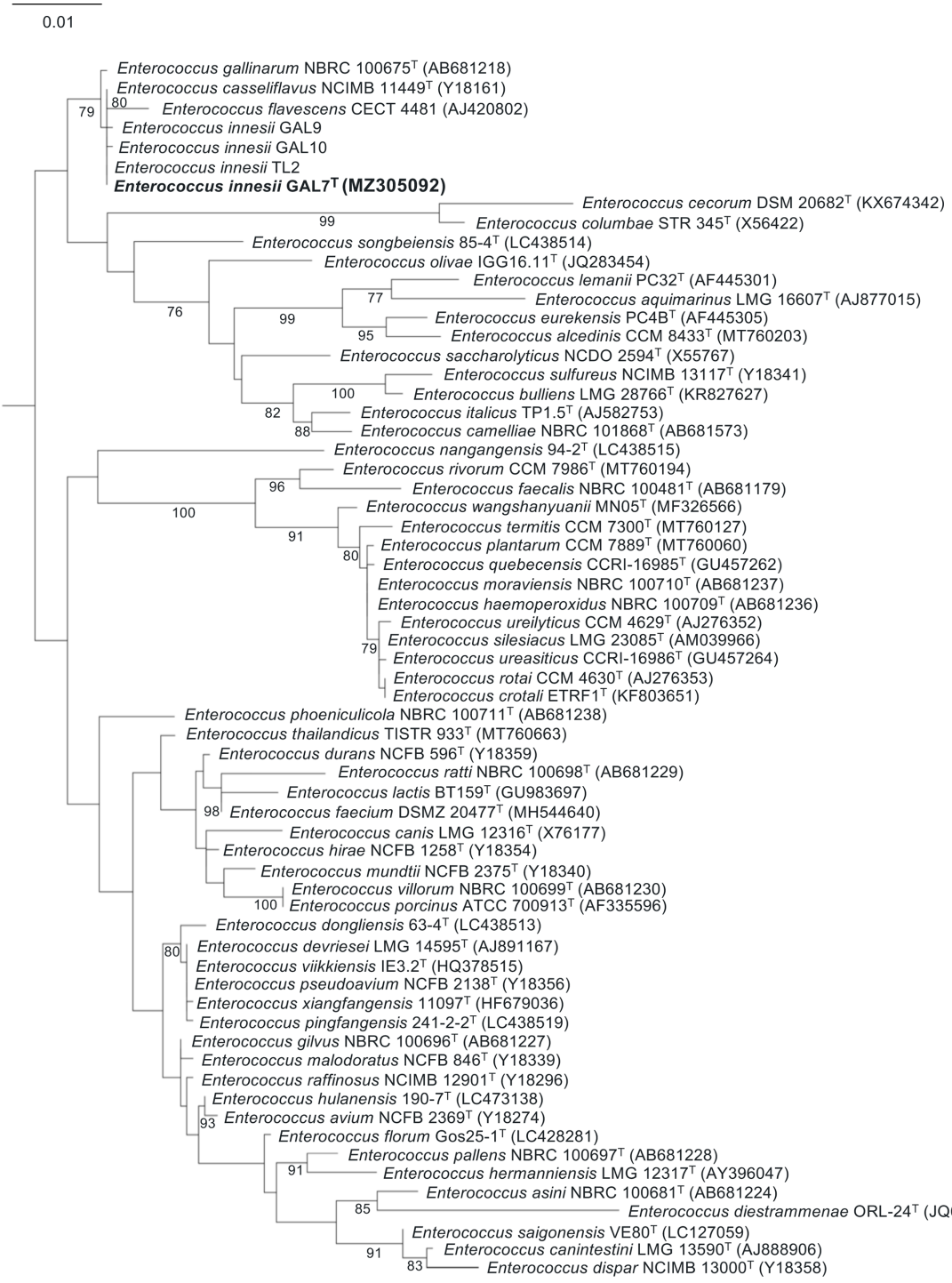

Fig. 1. A mid-point rooted maximum-likelihood phylogenetic tree showing the phylogenetic position of Enterococcus *innesii* sp. nov. strain GAL7^T based on 16S rRNA gene sequences of 61 *Enterococcus* type strains. Bootstrap values (>70%) based on 1000 replications are listed as percentages at the branches. Bar, 0.01 substitutions per nucleotide base.

aligned with 16S rRNA sequences of other 61 public genomes using MUSCLE version 3.8.31 [26], and a 16S rRNA-based maximum-likelihood phylogenetic tree was reconstructed via IQ-TREE version 2.0.5 with the GTR model at 1000 bootstrap replications while visualized with iTOL version 6 (Fig. 1) [27, 28]. *E. innesii* GAL7^T was phylogenetically positioned among *E. casseliflavus*, *E. flavescens* (re-classified as *E. casseliflavus*) and *E. gallinarum* cluster due to its 16S rRNA sequence similarity (99.53–99.93%) [29]. However, when we compared the digital DNA–DNA hybridization (dDDH; via

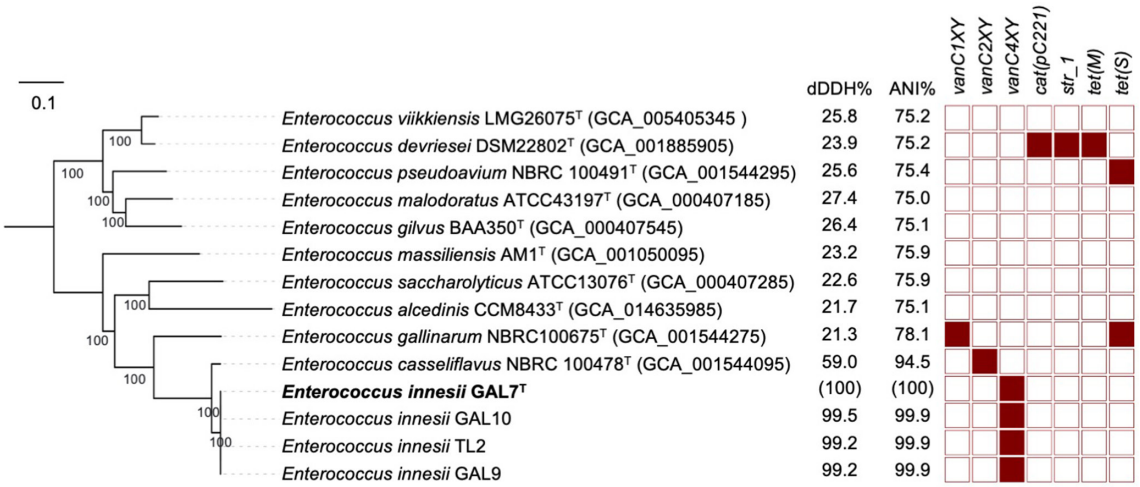

Fig. 2. A mid-point rooted maximum-likelihood phylogenetic tree based on 154826 single nucleotide polymorphisms from 564 core genes, aligned with dDDH (%), ANI (%) and antibiotic resistance gene profiles.

the Type Strain Genome Server, TYGS) and average nucleotide identity (ANI) for genome-based species delineation purposes (via fastANI v1.3), the proposed *E. innesii* sp. nov GAL7^T represented a separate species from *E. casseliflavus* and *E. gallinarum* type strains. The dDDH was 59.0% (using TYGS formula d_4) and ANI 94.5%, when compared to its closest neighbour *E. casseliflavus* NBRC100478^T, despite the high similarity of 16S rRNA sequences between the two species, both fell below the intra-species thresholds of 70% dDDH and 95% ANI (Fig. 2). In contrast, the ANI values among *E. innesii* strains (*n*=4) were 99.92–99.96%.

Next, 10 closest-related *Enterococcus* strains (vs *E. innesii*) identified by TYGS were further examined phylogenetically at a genomic level, with antibiotic resistance genes also screened (using the *resfinder* database), for the four novel *E. innesii* strains (Fig. 2) [30]. The pangenome of these 14 strains were investigated using Roary version 3.12.0 [31] at BLASTp threshold at 70% identity for inference of core genes. A total of 15629 genes were present in this pangenome with 564 core genes and 15065 accessory genes. Next, a core-gene alignment was generated and used to build a core-genome maximum-likelihood phylogenetic tree where it showed that *E. casseliflavus* NBRC100478^T was genomically distinct from *E. innesii*, further supported by single nucleotide polymorphism (SNP) analysis (using snp-dists version 0.7.0) that confirmed the SNP range (8–32 SNPs) among *E. innesii strains* (*n*=4) indicating strain distinction yet close genetic relatedness, while 11538–11540 SNPs were found when comparing *E. innesii* strains (*n*=4) and *E. casseliflavus* NBRC100478^T (Fig. 2) [32].

The vancomycin-resistance gene *vanC-4* (NCBI accession: EU151752) was uniquely detected (nucleotide sequence identity: 98.52–98.58% at near 100% coverage) in all *E. innesii* strains using ABRicate version 1.0.1 with the *resfinder* database, which was not found in any other closely related *Enterococcus* type strains (Fig. 2) [30, 33]. Notably, we did not detect any other virulence or antibiotic resistance genes in any of the four *E. innesii* strains. Vancomycin resistant determinant *vanC* subtypes had been reported in *E. gallinarum*, (*vanC-1*), *E. casseliflavus* (*vanC-2*), and *E. flavescens* (*vanC-3; E. flavescens* has now been re-classified as *E. casseliflavus*), while *vanC-4* has only been reported once previously in *E. casseliflavus*. In this study, the authors described the *vanC-4* encoding clinically associated *E. casseliflavus* isolates as having 'at least two genetic lineages with the distinct *vanC* genes, that is, a single subtype including previously known *vanC-2/C-3*, and a novel subtype *vanC-4*'. We therefore propose that this distinct 'genetic lineage' of *E. casseliflavus* may hypothetically be *E. innesii*, a novel species that uniquely encode *vanC-4* gene [34, 35]. However, as these isolates described in this previous clinical study were not whole genome sequenced, we are unable to determine this conclusively. Furthermore, the *vanC* resistance gene was phenotypically demonstrated in *E. casseliflavus* and *E. gallinarum* as having intrinsic but low-level resistance to vancomycin at a minimum inhibitory concentration (MIC) of 4–32 μg ml^{-1} [36].

Subsequently, we screened through a larger public dataset of *Enterococcus* species via a targeted approach and found that three isolates previously designated as *E. casseliflavus* and *E. gallinarum* appeared to be *E. innesii* based on ANI (however, taxonomy check on NCBI were inconclusive for these isolates). These include *E. casseliflavus* NCTC4725 (ANI vs *E. casseliflavus* NBRC100478^T: 94.88%; ANI vs *E. innesii* GAL7^T: 97.02%), *E. gallinarum* FDAARGOS163 (ANI vs *E. gallinarum* NBRC100675^T: 77.99%; ANI vs *E. casseliflavus* NBRC100478^T: 94.79%; ANI vs *E. innesii* GAL7^T: 95.40%) and *E. gallinarum* 4928STDY7071463 (ANI vs *E. gallinarum* NBRC100675^T: 78.08%; ANI vs *E. casseliflavus* NBRC100478^T: 94.96%; ANI vs *E. innesii* GAL7^T: 95.43%). Importantly, these three isolates NCTC4725 (ATCC27284;

GCA_901542395.1), FDAARGOS163 (GCA_001558875.2) and 4928STDY7071463 (GCA_902159265.1) are derived from human sources [37–39]. These isolates also demonstrated similar genome features as *E. innesii* sp. nov., with genome size range ~3.6–3.7 Mb and G+C ~42 mol%. These data suggest *E. innesii* sp. nov., may also be a clinically important species associated with novel antimicrobial resistance determinants, as *vanC-4* is encoded in all these genomes, and is reported to cause opportunistic human infection.

PHENOTYPIC CHARACTERIZATION

Phenotypic characteristics were also investigated and included cell and colony morphology, motility, Gram-staining reaction, formation of endospores, oxygen relationship, growth at different temperatures, fermentation profiles of carbohydrates, catalase activity, oxidase activity, tolerance to NaCl, Voges–Proskauer reaction, urease production, pyrrolidonyl arylamidase production, hydrolysis of hippurate, deamination of arginine, pyruvate utilization, bile-aesculin tolerance test, haemolysis test, fatty acid analysis and vancomycin susceptibility testing [40]. Motility tests were carried out on *E. innesii* GAL7^T using motility test medium (Merck). Media were prepared according to manufacturer's instructions and outcomes were recorded after culturing for 48 h at 37 °C. The susceptibility of *E. innesii* GAL7^T to antibiotic vancomycin was evaluated using MIC assays on BHI agar plates (carried out in three biological replicates) as described previously [41]. Aside from motility and vancomycin susceptibility tests, all phenotypic analyses were carried out by the Identification Service, Leibniz Institute DSMZ (Germany).

E. innesii cells were coccoid-shaped, 1.0–1.5 µm long, motile and occurred in pairs or in chains under phase-contrast microscopy (Fig. 3). All *E. innesii* strains were Gram-positive, asporogenous, and facultatively anaerobic. Biochemical characteristics were determined using API 50CHE strips for carbohydrate utilization profiles, after incubation for up to 48 h at 37 °C (Table 2). They were capable of growth at 10–45 °C with optimum at 30–37 °C in BHI broth, with only weak growth at 45 °C, and no growth at 5 °C for up to 13 days. Growth was observed at NaCl concentrations from 0 to 8% (w/v), with optimum growth <6.5%. All strains were catalase- and oxidase-negative and showed no haemolytic activity. When compared to the closest related species *E. casseliflavus* (based on 16S rRNA analysis), *E. innesii* strains exhibited a distinctive metabolism in producing acid from glycerol, sorbitol, raffinose and 2-ketoglyconate, while not producing acid from turanose (Table 2). Further phenotypic features were determined using the API rapidID32 STREP system on single strain *E. innesii* GAL7^T where cells were negative for urease production, hydrolysis of hippurate and pyruvate utilization (no detectable growth using sodium pyruvate as sole carbon source in mineral salt medium for 6 days at 37 °C), while positive for Voges–Proskauer reaction, pyrrolidonyl arylamidase production and arginine dihydrolase. GAL7^T cells tested positive for aesculin hydrolysis in complex medium (Bacto-Peptone, 1 g l^{-1} aesculin). Moreover, similar to *E. gallinarum*, GAL7^T cells were positive for *β*-glucuronidase while closest relative *E. casseliflavus*, and related species *E. faecalis* and *E. faecium* were all negative for this enzyme (Table 2).

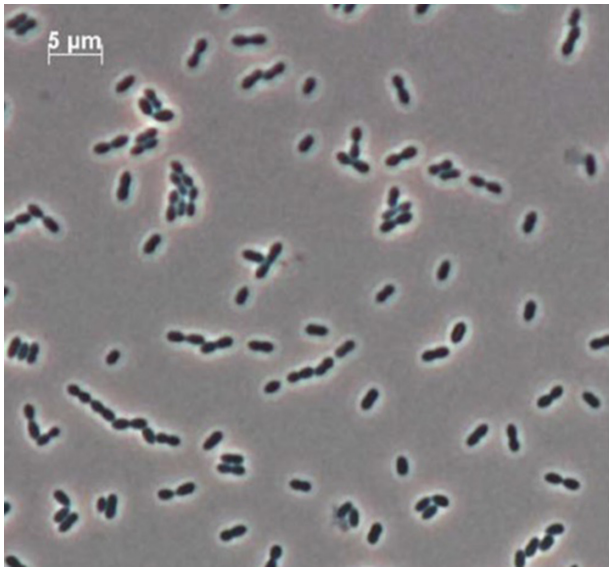

Fig. 3. Phase-contrast microscopy showing *E. innesii* GAL7^T occurring in pairs and in chains.

Cellular fatty acids were analysed after conversion into fatty acid methyl esters (FAMEs) using a modified protocol by Miller [42]. Mixtures of the FAMEs were then separated by gas chromatography and detected by a flame ionization detector using the Sherlock Microbial Identification System (MIDI) based on TSBA6 database. $C_{14:0}$, $C_{16:0}$ and $C_{18:1}$ *ω7c* were the major fatty acids in *E. innesii* GAL7^T. Compared to the closest phylogenetic neighbours *E. casseliflavus* and *E. gallinarum* type strains (JCM8723^T and JCM8728^T, respectively), *E. innesii* GAL7^T cells have a significantly higher $C_{14:0}$ fatty acid content at 26.12%, apparently distinctive from *E. casseliflavus* (7.5%) and *E. gallinarum* (0.2%) as described previously [43].

Importantly, we determined that *E. innesii* GAL7^T, which harboured putative atypical vancomycin resistance gene *vanC-4*, reduced susceptibility to vancomycin at MIC 4 µg ml^{-1} (vancomycin clinical breakpoint for Enterococci is >4 µg ml^{-1}). This is similar to the low-level vancomycin resistance reported previously in *E. casseliflavus* and *E. gallinarum*, strains that encode the *vanC* resistance gene [36, 44].

Based on the results of phylogenomic, physiological and biochemical studies presented above, strain GAL7^T is considered to represent a novel species of the genus *Enterococcus*, for which the name *Enterococcus innesii* sp. nov. is proposed.

Table 2. Distinctive phenotypic features between *E. innesii* strains (data from this study) and phylogenetically closely related *E. casseliflavus* [49] and *E. gallinarum* strains [49], also distantly related *E. faecalis* [49] and *E. faecium* strains [49]

+, All strains positive; –, all strains negative; +(–), most strains positive; –(+), most strains negative; v, variable; +w, most strains weakly positive, none negative. All strains were positive for ribose, galactose, glucose, fructose, mannose, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, trehalose and gentibiose. All strains were negative for erythritol, D-arabinose, L-xylose, adonitol, methyl *β*-xyloside, sorbose, dulcitol, inositol, xylitol, lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol and 5-keto-gluconate.

Characteristics	***E. innesii** (*n*=4)**	**†*E. casseliflavus* (*n*=6)**	**†*E. gallinarum* (*n*=4)**	**†*E. faecalis* (*n*=6)**	**†*E. faecium* (*n*=5)**
Acid production from:					
D-Xylose	+	+	+	–	–
Sucrose	+	+	+	+	v
Melibiose	+	+	+	–	v
Methyl α-glucoside	+	+	+	–	–
Melizitose	–	–	–	+(–)	–
Mannitol	+	+	+	+	+(–)
Inulin	+	+	+	–	–
Gluconate	+	+	+	+(–)	v
L-Arabinose	+	+	+	–	+
Glycerol	+w‡	–	+	+	+
Rhamnose	+	+(–)	–	v	–
Sorbitol	v	–	+	+(–)	–
Methyl α-D-mannoside	+	+(–)	–	–	–(+)
Raffinose	+	–	+	–	–
Glycogen	–	–	–(+)	–	–
Turanose	–	v	+	–	–
D-Tagatose	–	–	+	+	–
2-Keto-gluconate	+	–	–	v	–
Hydrolysis of:					
Aesculin	+§	+	+	+(–)	+
Hippurate	–§	–	+	+(–)	+
Presence of enzymes:					
Arginine dihydrolase	+§	+(–)	+	+	+
α-Galactosidase	+§	+	+	–	–
β-Galactosidase	+§	+	+	–	+
β-Glucuronidase	+§	–	+	–	–

*Determined with the API 50CH system.
†Determined with the API 50CHE system.
‡Shaded area represents distinctive phenotypic features between *E. innesii* strain(s) and closely related *E. casseliflavus* and *E. gallinarum* strains as determined by API systems.
§Determined with API rapid ID32 STREP system on a single strain GAL7^T.

DESCRIPTION OF *ENTEROCOCCUS INNESII* SP. NOV.

Enterococcus innesii (in.ne´si.i. N.L. gen. n. *innesii*, pertaining to British philanthropist John Innes JP and the John Innes Centre, Norwich, UK, where this bacterium was isolated).

Description is based on a single strain. Cells are Gram-positive, facultatively anaerobic, motile, non-haemolytic, asporogenous, coccoid-shaped, 1.0–1.5 µm long and usually occur in pairs or in chains. It grows at temperatures between 10–45 °C (optimum, 30–37 °C), at NaCl concentrations from 0 to 8.0% (optimum, 0–6.5%, at 37 °C) in BHI medium. Colonies formed on BHI after incubation for 48 h at 37 °C are non-pigmented, circular, smooth, shiny, diameter 1–2 mm, with entire margins. Negative for urease production, hydrolysis of hippurate, pyruvate utilization and catalase and oxidase production. Positive for Voges–Proskauer reaction, pyrrolidonyl arylamidase production, hydrolysis of aesculin and arginine dihydrolase. Acid is produced from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, methyl α-D-mannoside, methyl α-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, gentibiose, gluconate, 2-ketogluconate, starch and glycerol. Acid is not produced from erythritol, D-arabinose, L-xylose, adonitol, methyl *β*-D-xyloside, sorbose, dulcitol, inositol, melizitose, glycogen, xylitol, turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 5-ketogluconate. Resistant to 4 µg ml^{-1} vancomycin. The major fatty acids are $C_{14:0}$, $C_{16:0}$ and $C_{18:1}$ *ω7c*.

The type strain, GAL7^T (=DSM 112306^T=NCTC 14608^T), was isolated from the gut of a wax moth *Galleria mellonella* at John Innes Centre (Norwich, UK). The genome of the type strain is characterized by a size of 3.79 Mb and a G+C content of 42.22 mol%.

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Author contributions
Conceptualization, H.C.C.G., R.K., L.J.H and A.M; methodology, H.C.C.G., S.R. and D.J.B.; software, R.K.; validation, R.K., H.C.C.G. and L.J.H.; formal analysis, R.K. and H.C.C.G.; investigation, R.K. and H.C.C.G.; resources, H.C.C.G., S.R. and D.J.B; data curation, R.K.; writing – original draft preparation, R.K., H.C.C.G., D.J.B., A.M. and L.J.H.; writing – review and editing, R.K., H.C.C.G. and L.J.H.; visualization, R.K.; supervision, L.J.H. and A.M.; project administration, H.C.C.G. and R.K.; funding acquisition, L.J.H. and A.M.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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