

# **Investigating the potential of AMR chicken gut strains (*Enterococcus faecium* and *E. coli*) to colonise in vitro chicken gut microbiota and to transfer AMR genes within the resident microbiota**

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## Investigating the potential of AMR chicken gut strains (*Enterococcus faecium* and *Escherichia coli*) to colonize *in vitro* chicken gut microbiota and to transfer AMR genes within the resident microbiota

Antimicrobials (AM), such as antibiotics, are substances used to kill or inhibit microorganisms. WHO categorizes antimicrobials according to their relevance for public health<sup>1</sup>. Antimicrobial resistance (AMR) refers to the ability of a microbe to withstand antimicrobial treatments that are effective against other microbes. The emergence of antimicrobial-resistant bacteria through mutations or the acquisition of genetic material such as resistance plasmids represents a major public health issue. It's been accepted that horizontal gene transfer (HGT) occurs everywhere and plays a critical role in the transmissions of AM genes, also in complex ecosystem. The spread of antibiotic resistance can occur through different routes, one of which is along the food chain. Indeed, drug resistant bacteria can develop during animal production (e.g. chicken), contaminate meat during food processing, and ultimately be transferred to people<sup>2</sup>. Vancomycin-resistant enterococci (VRE) (mostly *Enterococcus faecium*) and extended-spectrum beta lactamase (ESBL)-carrying *Escherichia coli* are of particular interest in that context.

Poultry are naturally adapted to hosting a complex gastrointestinal (GIT) microbial community with hundreds of bacterial species and up to  $10^{11}$  CFU per gram of gut contents<sup>3</sup>. The high cell densities, found in both human colon and chicken cecum, favors genetic exchange between members of the microbiota. Further, the formation of micro-colonies and biofilms favors cell-to-cell contact allowing intra- and interspecies conjugation<sup>4</sup>. Moreover, bacteria from chicken gut are frequently transmitted to humans throughout the food chain via e.g. cross-contamination<sup>5</sup>. This creates an interface for AMR transfer between chicken-derived strains and human commensal or pathogenic strains which can be promoted in the high density environment of the GIT. The enrichment of AMR bacteria in the gut that can be favored when applying antibiotic selection pressure, and the spread and transfer of AMR in the complex microbial communities of the GIT have been little studied.

In this thesis the condition, the focus will be on how different parameters and selective pressure can impact on the potential of AMR chicken gut strains (*Enterococcus* and *E. coli*) to colonize *in vitro* chicken gut microbiota and to transfer AMR genes within the resident microbiota. In particular, in order to better characterize the strains that will be used, phenotypical test will be done to check the resistance and susceptibility of the bacteria. These information will be used to help the screening and the selection of the bacteria in the next step of the thesis. Batch fermentation with different conditions and parameters (e.g. different antibiotics/ different antibiotic-concentrations..) More in details ESBL *E. coli* HV292.1, HV 114.1 and HV 259.1 and VRE 59161 and 59168 will be tested during batch fermentation with complex chicken microbiota using chicken fermentation medium. Fresh and frozen cecum samples will be tested with the following conditions: (i) control, (ii) + antibiotic ii) spiked donor strain (iii) spiked with donor strain + antibiotic. Samples will be taken at different timepoint (with the limit of 24h, as this is the chicken retention time) to study the evolution of the complex microbial community in the presence of the tested donor strain. For each sample, serial dilutions will be plated and CFU will be counted on agar plates containing antibiotic. qPCR for the resistance gene of interest and the unique sequence of donor strains will be performed, thus allowing the quantification of the donor strains in each tested condition as well as the potential identification of new recipient strains (untargeted approach). Colony PCR will be performed on colonies growing on antibiotic-containing medium. The 16S rRNA gene sequences of the new recipient will be sent to sequencing.

16s sequencing will be done to monitor the change in composition of the microbiota and based on the results, metagenomic analysis will be performed.

### **Objectives of the MSc thesis**

- 1) Molecular and phenotypical characterization of *E.coli* and *Enterococcus* donor strains**
  - a. *in silico* characterization of antibiotic resistance genes to confirm phenotypes and investigate resistance to other antibiotics that would be helpful to discriminate donor strains from recipient.
  - b. Antibiotic resistance assays *in vitro*
- 1.1) Confirmation of specificity of unique sequence primers**
  - c. PCR against other MLST strains
  - d. In silico PCR against other *E.coli*
- 2) Investigate different parameters that can affect gene transfer in batch fermentations (eg. Antibiotics, antibiotics concentration, ph, temperature..) and different selection method to isolate new possible recipient ( eg. different selective media)**
- 3) Investigate the response of chicken cecum microbiota to the different treatments**

### **References:**

- 1 World Health Organization. World Health Organization, Geneva, Switzerland, (2016).
- 2 Friedman, M. Journal of agricultural and food chemistry 63, 3805-3822, (2015).
- 3 Barnes EM. e. J Appl Bacteriol 46(3):407 (1979)
- 4 Scott, K. P. Cellular and molecular life sciences : CMLS 59, 2071-2082, (2002).
- 5 MacDonald, E. et al. PLoS One 10, e0139636, (2015).

# Abstract

The emergence of specific antimicrobial resistance (AMR) mechanisms is disturbing the therapeutic use of antibiotics with fatal outcome. It's widely accepted nowadays that the misuse of antibiotics lead to a higher proportion of multi drug resistant (MDR) bacteria communities. The highest non-therapeutical use of antibiotics is used on livestock. Its substantial contribution to the current AMR crisis is under investigation. Here we examined the colonisation *in vitro* of MDR strains in a resident chicken cecum microbiota and the effects of antibiotic treatment within a repeated batch fermentation model. The spread of AMR is mainly driven by horizontal gene transfer (HGT) within bacterial communities. Thus the possibility to quantify the HGT within the chicken microbiota was explored.

Preliminary characterisation of the AMR profile revealed high vancomycin resistance (MIC > 450 µg/ml) in the *Enterococcus faecium* strains and the production of extended spectrum  $\beta$ -lactamase (ESBL) in the *Escherichia coli* strains. The genetic information of both resistances were localised on highly mobile elements (i.e. conjugative plasmids.) The qPCR primers validated *in silico* were able to track the corresponding AMR genes (i.e. *vanA* and *bla<sub>ctx-m1</sub>*) as well as the strain specific sequences in the batch fermentation after 7 h. The concentration [copy number / ml] of the MDR strains remained stable until the end of the experiment on an intermediate level ( $10^5$ - $10^{6.5}$  for *E. coli* and  $10^{4.5}$ - $10^5$  for *E. faecium*.) A significantly higher concentration of the *E. coli* strain ( $10^7$  strains/ml) was found in the antibiotic treated condition, indicating a positive selection of the MDR strain due to the antibiotics. The highest rel. abundance for the amplicon sequence variants (ASVs) which were assigned to the same genus as the MDR strains were found in the antibiotic treated group. Hence, the 16S rRNA community analysis (metabarcoding) strengthened the findings from the qPCR data. The variation of the community was affected significantly ( $P > 0.001$ ) when antibiotic pressure was added. In total, 54% of the overall variation could be explained by the different conditions in the experimental set-up. As AMR genes were found in the resident microbiota, it intrigued the possibility to quantify HGT with the qPCR method only. With metabarcoding, possible HGT in *Escherichia-Shigella* ssp. and *Enterococcus* ssp. could have been discovered. Compared to the initial resistant community, the "new resistant community" fraction was larger when spiked with *E. faecium* (+9.1 %) or with *E. coli* (+18.5 %). the necessary

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*Abstract*

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resolution to track HGT is achieved through metagenomic analysis without loosing the host-plasmid information (Hi-C.)

# Zusammenfassung

Das Auftreten spezifischer antimikrobieller Resistenz (AMR) Mechanismen stört den therapeutischen Einsatz von Antibiotika, z.T. mit tödlichem Ausgang. Es ist allgemein bekannt, dass der Missbrauch von Antibiotika zu einem höheren Anteil an multiresistenten (MR) Bakteriengemeinschaften führt. Der nicht-therapeutische Einsatz von Antibiotika ist bei Nutztieren am höchsten und über dessen Beitrag zur aktuellen AMR-Krise wird stark diskutiert in der wissenschaftlichen Gemeinde. In dieser Arbeit wurde die Besiedelung einer ursprünglichen Hühner-Mikrobiota aus dem Blinddarm (*in vitro*) mit MR-Stämmen untersucht. Die Auswirkungen einer Antibiotika Behandlung auf die Kolonisationsraten wurde mit einem wiederholten "Batch" Fermentationsmodel untersucht. Da der horizontale Gentransfer (HGT) die Hauptursache für die Ausbreitung der AMR Genen innerhalb einer Bakteriengemeinschaft ist, wurde auch untersucht, ob dieser HGT innerhalb der Hühner-Mikrobiota zu quantifizieren ist.

Eine vorläufige Charakterisierung des antimikrobiellen Resistenzprofils ergab eine hohe Vancomycin-Resistenz (MIC > 450 g/ml) im *Enterococcus faecium* Stamm und eine hohe Produktion von  $\beta$ -Lactamase mit erweitertem Spektrum (ESBL) in den *Escherichia coli* Stämmen. Die genetische Information beider Resistzenzen wurde auf hochmobilen Elementen (d.h. konjugativen Plasmiden) lokalisiert. Die *in silico* validierten qPCR-Primer waren in der Lage, die entsprechenden AMR-Gene (d.h. *vanA* und *bla<sub>ctx-m1</sub>*) und die stammspezifischen Sequenzen in der Batch-Fermentation nach 7 h zu quantifizieren. Die Konzentration [Kopienzahl/ml] der MR-Stämme blieb bis zum Ende des Experiments auf mittlerem Niveau stabil ( $10^5\text{--}10^{6.5}$  für *E. coli* und  $10^{4.5}\text{--}10^5$  für *E. faecium*.) Eine signifikant höhere Konzentration des *E. coli* Stammes ( $10^7$  Bakterien/ml) wurde in der mit Antibiotika behandelten Probe gefunden, was auf eine Selektion von dem MR-Stamm hindeutet. Die Analyse der Mikrobiota mit 16S rRNA Sequenzierung (Metabarcoding) bestärkt diese Annahme, da die höchste prozentuelle Häufigkeit für die Amplicon Sequenz Varianten (ASV), die denselben Gattung wie die MR-Stämme zugeordnet wurden, auch in der mit Antibiotika behandelten Gruppe gefunden wurde. Die Variation der Bakteriengemeinschaft war signifikant ( $P > 0,001$ ) beeinflusst, wenn Antibiotika hinzugefügt wurde, und 54% der Gesamtvariation konnten durch die unterschiedlichen Bedingungen (d.h. mit oder ohne Antibiotika) in der Versuchsanordnung erklärt werden. Da AMR-Gene auch schon in der ursprünglichen Mikrobiota gefunden wurden, hat es die Möglichkeit HGT nur

## Zusammenfassung

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mit der qPCR-Methode zu quantifizieren stark beinträchtigt. Mit der Metabarcoding Methode konnte möglicherweise HGT zwischen derselben Art in *Escherichia-Shigella* ssp. und *Enterococcus* ssp. entdeckt werden. Im Vergleich zur anfänglich resistenten Bakteriengemeinschaft war der Anteil der "neuen resistenten Gemeinschaft" größer, wenn die Mikrobiota mit *E. faecium* (+9,1%) oder mit *E. coli* (+18,5%) angereichert wurde. Eine kultivierungsunabhängige Schrotschuss-Sequenzierung ohne Verlust der Wirt-Plasmid-Information (Hi-C Methode) könnte die notwendige Auflösung bringen zur direkten Verfolgung von HGT.

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# Notations

## Abbreviations

Abbreviation	Meaning
ACT	Artemis Comparison Tool
AMR	antimicrobial resistance
ANI	average nucleotide identity
ASV	amplicon sequence variants
BD	Becton Dickinson
BEA	Bile-Esculine agar
BHI	Brain heart infusion
CCUG	Culture Collection University of Gothenburg
CFU	colony forming units
DNA	deoxyribonucleic acid
EARS- Net	Antimicrobial Resistance Surveillance network
ECOFF	epidemiological cut-off values
EEA	European Economoic Area
ESBL	extended $\beta$ -lactamase
EU	European Union
EUCAST	European Commitee on Antimicrobial Susceptibility Testing
GIT	gastro intestinal tract
HGT	horizontal gene transfer
LB	Lysogeny broth
MDR	multidrug resistant
MIC	minimal inhibitory concentration
MPF	mating pair formation complex

## *Notations*

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MSA	Mannitol-Salt agar
NCBI	National Center for Biotechnology Information
oriT	origin of transfer
PCR	Polymerase-Chain-Reaction
PBP	penicillin binding proteins
qPCR	quantitative Polymerase-Chain-Reaction
AST-S	Sensititre Antimicrobial Susceptibility Testing System
SNPs	short nucleotid polymorphismus
SOP	standard operating procedure
SXT/CFX	Sulfamethoxazole and Cefotaxime - antibiotic in 1:1 ration
TEA	Tris-Acetate-EDTA
UTI	urinary tract infection
VRE	vancomycin resistant enterococci
WHO	World Health Organisation
WT	wild type
WGS	whole genome sequencing
XLD	Xylose-Lysin-Desoxycholage

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# 1 Introduction

Antimicrobial resistance (AMR) of human pathogens is a major concern in public health. Recently published numbers by the UK Government and the Wellcome Trust are estimating by 2050, 10 million lives a year and a cumulative 100 billion US dollars of economic output being at risk due to the rise of drug resistant infections worldwide (O'Neill, 2016). Similarly, Cassini et al. (2019) quantified the estimated burden of infections in the European Union (EU) and the European Economic Area (EEA) for one year, using data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2015. Their findings suggested the burden of infections which AMR bacteria caused in 2015 was similar to the cumulative infection load of influenza virus, tuberculosis and human immunodeficiency virus together.

Shortly after the first use of antibiotics in therapy, it expanded to further areas. Especially in the industrialised livestock industry, antibiotics have been used for about 50 years ever since the discovery not only as an antimicrobial agent to treat diseases, but also as a growth-promoting agent or in routinely given subtherapeutic doses to prevent diseases(Van Boeckel et al., 2015, Bagal et al., 2016).The antibiotics mainly effects the gastrointestinal (GIT) by altering their microbial community, leading to decreased competition of nutrients (Costa et al., 2017).

The commercial use of antibiotics in livestock is reported to be the highest compared to other sectors, with yearly increasing consumption rates (Van Boeckel et al., 2015). Simultaneously, the rise of multidrug-resistant (MDR) strains of human pathogens in livestock is well documented. This has lead to greater awareness of its contribution as an antibiotic reservoir for AMR genes (Martínez et al., 2015b, Tong et al., 2017, Mehdi et al., 2018, Ludden et al., 2019). While AMR is a naturally occurring phenomenon in prokaryotes, with a long evolutionary history, its emergence and transmission is shaped by the intensive use of antibiotics by humankind since the first therapeutic use of penicillin in 1940 (Davies and Davies, 2010, Dcosta et al., 2011, Forslund et al., 2013, Tacconelli et al., 2018).

Poultry are naturally adapted to hosting a complex GIT microbial community with hundreds of bacterial species, up to  $10^{10} - 10^{12}$  colony forming units (CFU) per gram of gut contents (Shang et al., 2018). *In vitro* observations show a rapid spread of AMR genes within a bacteria community, when antibiotics are present (Haug et al., 2011a, Zurfluh et al., 2014, Card et al., 2017). As high cell densities are found in chicken

## 1 Introduction

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cecum, it favours cell-to-cell contact. This allows intra- and interspecies conjugation, as a part of horizontal gene transfer (HGT) (Palmer et al., 2010, Ravenhall et al., 2015, Oladeinde et al., 2019). HGT combined with the selective pressure for AMR strains is the main driver for MDR in chicken GIT microbiome (Gogarten et al., 2002, Zurfluh et al., 2014, Card et al., 2017, Yang et al., 2020).

Vancomycin resistant *Enterococcus faecium* (VRE) and extended  $\beta$ -lactamase (ESBL) *Escherichia coli* are of particular interest in that context. Both are representative members of the phyla which are highly abundant in the chicken cecum. *Enterococcus* and *Escherichia coli* represent up to 56 % and 16 % respectively of the GIT microbiome (Shang et al., 2018). Both have been reported to transfer gene elements highly effectively and represent the large majority of the AMR related infections (Martínez et al., 2015b). The World Health Organisation (WHO) classified ESBL *E.coli* as the highest priority (priority 1: "critical") pathogen bacteria and VRE as a high secondary priority pathogen bacteria to combat in terms of global health by developing new antibiotics. (Tacconelli et al., 2018).

In this thesis we examine the HGT potential of different ESBL *E. coli* and VRE strains within a resident chicken gut microbiota *in vitro* and its potential to colonise within it. Further it is hypothesised that the application of antibiotic treatments promote colonisation of AMR strains and transfer of their AMR genes. To mimic real condition, this will be tested *in vitro* during batch fermentation with complex chicken microbiota coming from the inoculum reactor of the polyfermS experiment, described by Zihler Berner et al. (2013).

Antibiotic profiling of the AMR strains will be performed with focus on AMR genes harboured on highly transferable mobile elements. In the batch fermentation, AMR strains are inoculated into the resident microbiota, flanked with- or without antibiotic pressure. Strain specific quantitative Polymerase-chain-reaction (qPCR) primer are designed and will be validated using whole genome assemblies of the strains. A qPCR for the resistance gene of interest and the unique sequence of spiked strains will be performed to track the changes during the batch fermentation under various conditions. By using 16S rRNA analysis Illumina technology, the composition of the community will be identified on a genus specific level.

## 2 Background

### 2.1 Antibiotic century

The discovery of antibiotics more than 70 years ago initiated a period of drug innovation and implementation in human health and animal health and agriculture. The generic term "antibiotic" is used here to denote any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets (Van Hoek et al., 2011). Besides Fleming's discovery of penicillin, a  $\beta$ -lactam antibiotic, there were more classes introduced shortly after; the aminoglycoside in the early 1940s (Schatz and Waksman, 1944), chloramphenicol in 1947 (Ehrlich et al., 1947), glycopeptide in 1955 (McCormick et al., 1955) and quinolone (Lesher et al., 1962) only to name a few.

The huge majority of antibiotics which are used still today were discovered during the 1950-1960s in the so called "golden years" (Davies and Davies, 2010). Since then, only a small number of new antibiotics have reached the market. One reason being the high R&D costs as well as the shrinkage of venture capital funds (O'Neill, 2016). The increasing financial expenditure is offset by a smaller revenue nowadays because new antibiotics are retained as "last-line defense" to treat bacterial infections caused by MDR strains, where initially introduced antibiotics underperform (Forslund et al., 2013).

#### 2.1.1 Antibiotic used as a therapeutical agent

An antibiotic should be effective against the treated pathogenic bacteria but not harm the host. Therefore the perfect target of an antibiotic is a process, which is essential to the prokaryote but absent in the host. Many antibiotics target cell wall synthesis. Other targets are the bacterial ribosomes and enzymatic metabolism of essential compounds and nucleic acid synthesis (Kapoor et al., 2017). Specific antibiotic mechanisms are explained in more detail for the two following antibiotics; cefotaxime and vancomycin.

## Cefotaxime

Like the first known antibiotic penicillin, cefotaxime is a  $\beta$ -lactam antibiotic of the group cephalosporins. Cefotaxime was discovered in 1976, and came into commercial use in 1980 (Dudley and Barriere, 1982). The chemical structure contains a  $\beta$ -lactam ring being the active site of the molecule. This ring binds to penicillin-binding-proteins (PBP). These proteins are enzymes that normally crosslink the peptide side chains of the glycan chains, necessary to build a continuous cell wall. As the  $\beta$ -lactam ring is structurally very similar to the linkage site of the peptide chains, it can bind to the active site of the enzyme, resulting in blocking the enzyme and further hindering the build up of peptidoglycan. The missing cross linkages destabilises the cell wall and due to the high osmotic pressure inside the cell, it bursts (Wencewicz and Walsh, 2016b). The PBPs of enterococci are intrinsically resistant to the effects of the cephalosporins (Chen et al., 2009). Susceptible are *Enterobacteriaceae*, enteric bacilli, staphylococci and non-enterococcal streptococci (Wencewicz and Walsh, 2016a).

## Vancomycin

Vancomycin is an antibiotic of the glycopeptides group. Glycopeptides are molecules built of glycosylated cyclic or polycyclic non-ribosomal peptides. Vancomycin was discovered in 1955, but complex as it is, it is still produced in fermentation with *Amycolatopsis orientalis* (McCormick et al., 1955, Wencewicz and Walsh, 2016a). In contrary to cefotaxime, vancomycin disturbs the assembly of the cell wall by blocking the substrate of the transpeptidase, not the enzyme. Vancomycin has a hollow structure. This covers itself around the fifth and last amino acid of the peptide sidechain in the nascent glycan-strand. This results in a competitive inhibition of the substrate. By weakening the peptidoglycan layer the PBS can no longer access its point of action. Thus killing the bacteria by osmotic lysis. The activity spectrum of vancomycin is limited to gram-positive bacteria. The structure being too large, it can't pass the porins in the outer membrane of gram-negative bacteria (Chen et al., 2009, Wencewicz and Walsh, 2016a). As it can not access the site of action in gram-negative bacteria, such as *Escherichia coli*, all strains of a particular species will exhibit the same modes of innate resistance.

### 2.1.2 Antibiotic used as a growth promoter

Since the 1950's, antibiotics have been fed at low doses to livestock not only to prevent them from disease but to promote higher milk-production, faster growth, and a better feed conversion ratio (Dibner and Richards, 2005, Bagal et al., 2016). This technique is widely applied, with large amounts of antibiotics being fed to animals every year

## *2 Background*

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(Bagal et al., 2016, Costa et al., 2017). Global consumption of antimicrobials in food animal production sector was estimated at 63'151 ( $\pm 1'560$ ) tons by Van Boeckel et al. (2015) in 2010. The trend in antibiotic usage in livestock is still going up. Due to an increased meat consumption, antibiotic consumption will rise. Besides, industrialisation and intensification in animal production in rural regions will also lead to higher demand in antibiotics. With these two factors in mind, it's estimated that antibiotic consumption will rise up to 67% rise (105'000 t) until 2030 (Van Boeckel et al., 2015).

### **Mode of action**

The exact mechanisms leading to that are not known but it is hypothesised to depend on microbial community composition have an influence. Direct effects on the microflora can be used to explain decreased competition for nutrients and reduction in microbial metabolites that depress growth (Dibner and Richards, 2005). Even though microbial structure at a phylum level was not changed by different antibiotics, significant changes in single bacteria abundance were observed (Costa et al., 2017). However by feeding sublethal doses of antibiotics, resistant bacteria in the animal GIT are promoted (Dibner and Richards, 2005, Haug et al., 2011a, Zurfluh et al., 2014, Card et al., 2017).

### **Legislations**

As AMR pathogens turn out to become more and more of a problem in human health, a general concern towards antibiotics in animal feeding was observed. In the EU 1997 avoparcin was forbidden to be used as growth promoter. In 1999 further antibiotics were added to this list (Witte et al., 1999). In 2018, a new EU regulation was enacted. Article 107, paragraph 2 declares that antibiotics are not used as growth-promotor or performance enhancer. Paragraph 3 further rules that prophylactic antibiotic use must only be an exception. This regulation comes into force 28 January 2022 (EU Parliament, 2018).

In Switzerland it is forbidden by law to give antibiotics to animals for growth promotion or as preventive measure (Schweizerischer Bundesrat 2004). Therapeutic use of antibiotics is allowed, but every administration must be authorised and documented by a veterinarian. Providing the farmer with an antibiotics stock exceeding the prescription is not allowed. This will also be the case in EU as of 2022 (EU Parliament, 2018).

Since the introduction of antibiotics, the development of specific resistant mechanisms is disturbing their therapeutic use. Nowadays, it's widely accepted nowadays that the

misuse of antibiotic leads to higher proportion of AMR bacteria communities (Davies and Davies, 2010, Forslund et al., 2013). This was mentioned by Alexander Fleming in his Nobel acceptance speech in 1945; He ended with a warning for future generations: Fleming (1945) (p. 92-93)

"...But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in under dosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant..."

## 2.2 Acquired antibiotic resistance

From an evolutionary perspective a bacterial community uses two major genetic strategies to overcome the antibiotic threat. First, the development of AMR can be the result of spontaneous mutation in chromosomal genes which are passed vertically on descendants. Then subsequently, a natural positive selection for this mutated descendants happens. Or due to rapid horizontal acquisition of foreign Deoxyribonucleic acid (DNA) nucleotide, which code for resistance determinants. AMR mechanisms are shared and the community get resistant. This acquired AMR differs from natural or intrinsic AMR in a way that its temporally or spatially limited, however mechanisms of AMR are identical (Olivares et al., 2013).

### 2.2.1 AMR gene mechanisms

Specific mechanisms of resistance and examples of antibiotics that become ineffective in the face of these circumstances, are listed (Chen et al., 2009, Munita et al., 2016):

- **Drug inactivation.** Bacteria acquire gene-encoding enzymes, such as  $\beta$ -lactamases, that inactivate or destroy antibiotics before they reach their targets. (Example: aminoglycosides)
- **Cell wall changes.** The bacterial outer membrane becomes impermeable to the antibiotic. (Example:  $\beta$ -lactams)

- **Altered targets.** The target site is altered by mutation so that it evades the action or no longer binds the antibiotic. (Example: rifampin)
- **Efflux pump.** The bacteria acquire an efflux pump that expels the antibiotic before it reaches its target. (Example: tetracycline)
- **Bypass targets.** Mutations in bacterial DNA change the target enzyme in a metabolic pathway to bypass the primary target. (Example: trimethoprim)

### Glycopeptide resistances

Vancomycin acts by binding to the terminus of the peptidoglycan precursor as described in subsection 2.1.1, thereby inhibiting cell wall synthesis. Vancomycin resistant organisms modify these peptidoglycan precursor, by replacing the terminal dipeptides. These modified cell walls precursors bind vancomycin with 1'000-fold lower affinity, than do normal precursors (Hollenbeck and Rice, 2012). To create the modified precursors at least seven enzymes are required expressed by eponymous genes and summarised as a *vanA*-gene cluster. The gene cluster is located both on plasmids or on the chromosome of resistant bacteria (Van Hoek et al., 2011). The transfer of a plasmid which encodes vancomycin resistance from an intrinsical resistant organisms was reported in early time of the antibiotic century. The first VRE strain, harbouring the *vanA*-gene cluster on a plasmid, was isolated about 30 years ago (Uttley et al., 1988).

### 3<sup>rd</sup>-generation cephalosporin ( $\beta$ -lactam) resistances

There are several mechanisms of antimicrobial resistance to  $\beta$ -lactam antibiotics. The most common and important mechanism through which bacteria can become resistant against  $\beta$ -lactams is by expressing  $\beta$ -lactamases or ESBL. First reported in Germany in 1980, a new family of plasmid-mediated ESBLs called ctx-m (cefotaxime-hydrolyzing  $\beta$ -lactamase), have risen recently (Bradford, 2001). The ctx-m-ases, which hydrolyse cefotaxime (3<sup>rd</sup>-generation cephalosporin) efficiently are mostly encoded by conjugative plasmids. The gene *bla<sub>ctx-m-1</sub>* has being found predominantly in *Enterobacteriaceae*, most prevalently in the species *E. coli* (Walther-Rasmussen and Høiby, 2004).

#### 2.2.2 Vertical AMR gene transmission

When the first antibiotic Penicillin, was discovered by Alexander Fleming in 1928, it was assumed that the evolution of AMR was unlikely. This was based on the assumption that the frequency of mutations generating resistant bacteria was negligible

(Davies and Davies, 2010). There is growing evidence that besides of HGT, single nucleotide polymorphisms (SNPs) can be the cause for resistances. Mainly against the synthetic antibiotics rifampin, quinolones, sulfonamides, and trimethoprim, resistances due to SNPs were reviewed by Van Hoek et al. (2011). Therefore the PBS from enterococci are a poor target for all kind of celasphorins due to SNPs. A random mutation in the genome of a specific bacteria can affect the activity/effect of the antibiotic, leading to antibiotic resistance. In the presence of an antibiotic pressure, the susceptible population is eliminated. Since there is a lack of competition the resistant bacteria predominates the colony soon. As a result a variety is provided in the genetic information of a bacteria community for successful survival under antibiotic pressure. Explored in (Ravenhall et al., 2015) and reviewed by Zeng and Lin (2017).

### 2.2.3 Horizontal AMR gene transmission

The HGT process enables the transmission of portions of genomic DNA between bacterial cells and subsequent positive selection favours AMR gene harbouring bacteria. AMR genes can be acquired via mobile genetic elements, such as plasmids, prophages, or transposons (Davies and Davies, 2010). Large number of AMR genes are components of natural microbial populations, particularly in environments such as the gut microbiome (Borda-Molina et al., 2018, Sabino et al., 2019).

It has been widely accepted that there are three major forms of HGT: conjugation, natural transformation and transduction (Davies and Davies, 2010, Van Hoek et al., 2011, Jiang et al., 2017). Transformation involves the release of naked DNA followed by uptake and recombination into the bacterial genome. Homologous recombination and DNA-repair processes normally limit this to DNA from similar bacteria (Thomas and Nielsen, 2005). Transduction is the mechanism where DNA is transferred via a bacteriophage. If DNA moves on a mobile element that encode their own transfer and maintenance functions, it has the potential to spread rapidly within a bacterial population, called conjugation.

#### Conjugation

Bacteria carry extrachromosomal, self-replicating genetic elements are called plasmids. A plasmid is defined as a double-stranded, circular DNA molecule capable of autonomous replication (Carattoli, 2009). AMR gene carrying plasmids needs two functions to be considered for conjugation; Firstly a set of mobility genes, such as an origin of transfer (*oriT*), a relaxase and a type 4 coupling protein, secondly a membrane-associated mating pair formation (MPF) complex, which provides the mating channel (Smillie et al., 2010). The *bla<sub>ctx-m-1</sub>*-carrying plasmids , extracted

from ESBL-*E.coli* by Zurfluh et al. (2014), are all subtypes of the Incompatibility group I1 (IncI1) plasmid. They are commonly found in enteric bacteria from food animal sources, and have shown to contain, besides the ESBL genes, a conjugative machinery to spread quickly (Brouwer et al., 2014). As described in Figure 1, grey shaded areas indicate homologies in the plasmid regions. Red boxes mark antibiotic resistance genes. Conjugation-related genes are shown with capital letters in yellow boxes. Blue boxes indicate mobility genes. Green boxes denote maintenance- and stability-related genes. White boxes indicate hypothetical proteins and light blue boxes show the MPF complex. The origin of transfer (oriT) is depicted as a blue circle. The figure is not drawn to scale.

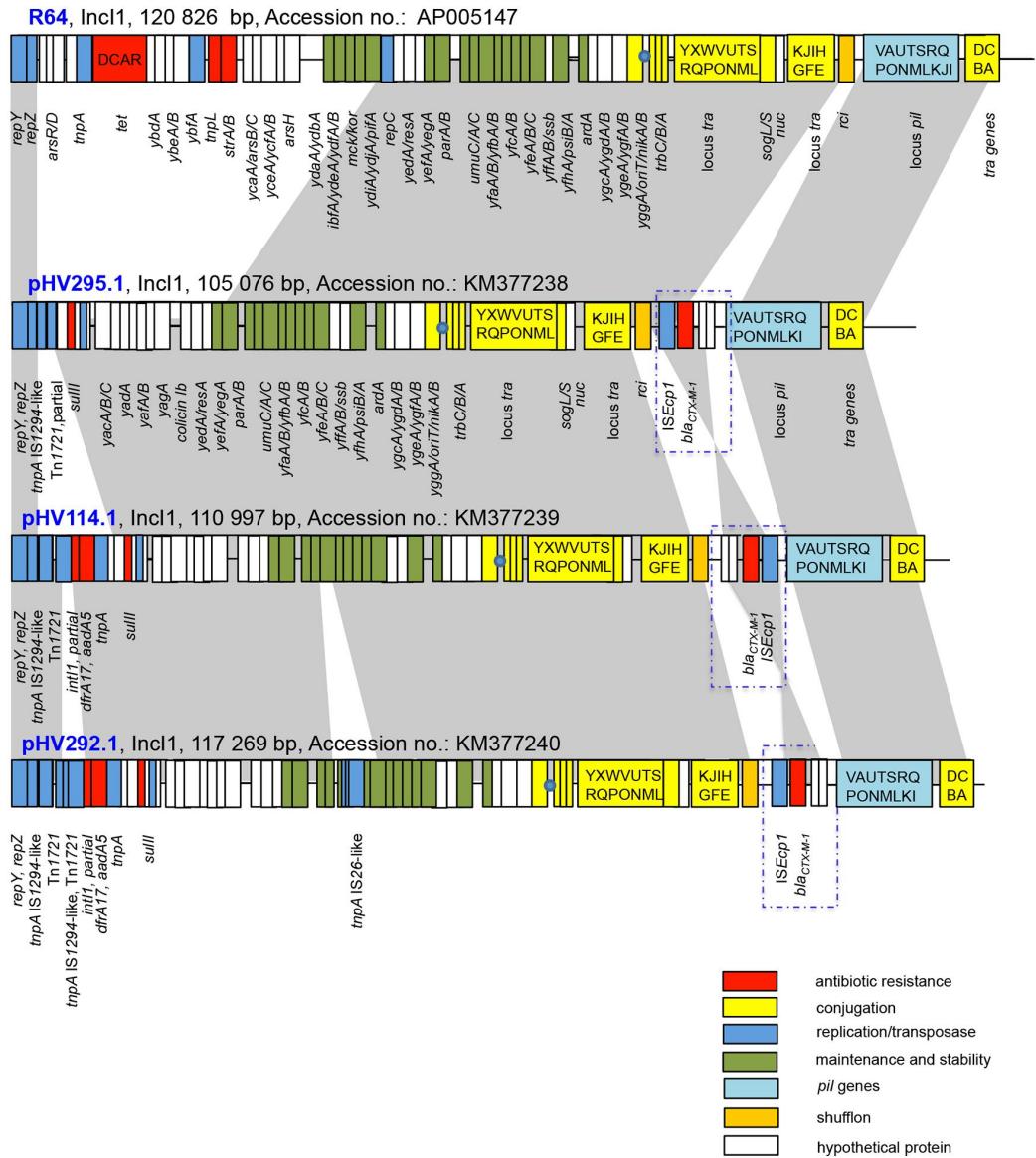
## 2.3 Poultry gut microbiome

With up to  $10^{12}$  CFU/g faeces, the chicken gut is the most densely habitated place in the body. This is not surprising as growing conditions in the gut are optimal. The constant temperature of 37 °C, anaerobic milieu, and an exhaustive food supply allow for such high bacterial densities (Shang et al., 2018). Before a chicken is born, it remains in a fully sterile environment. Soon after the chicken hatches, it comes into contact with thousands of bacteria (Dibner and Richards, 2005). As soon as this happens, the intestinal microbiota starts to build up. Since gut colonisation happens mainly through oral uptake of microbes, the biggest influence on the chicken GIT microbiota is its environment. An empty intestinal tract is quickly and easily colonised as described above. Finally, exogenous amount bacteria arrives in a fully developed chicken gut microbiome. This is likely to be found transiently but they are not able to settle permanently. However when sublethal doses of antibiotics are present, the resident microbiota is compromised, making it easier for an exogenous bacteria to occupy a niche. Highest microbial density and diversity is found in the cecum, and most abundant phylas in the cecum are Firmicutes (44-56 %) and Proteobacteria (16-23 %) (Shang et al., 2018). This includes the species *Enterococcus faecium* and *Escherichia coli*, which both are known to harbour plasmid encoded AMR genes (Uttley et al., 1988, Haug et al., 2011b, Card et al., 2017, Lambrecht et al., 2019). The resistance potential of the two species are explained in further detail.

### 2.3.1 *Escherichia coli*

*E. coli* is a gram-negative bacterium and belongs to the normal gut-flora. *E. coli* has been used as amodel organism for many years leading it to be the best described bacteria today. Most *E. coli* strains are living mutual inhabitants of the gut, but some may become pathogenic when transferred into human GIT. They are responsible for

## 2 Background



**Figure 1** – The sequenced structure of the IncI1-like plasmids by Zurfluh et al. (2014) reveals the MOB genes and MPF complex as well as multiple AMR genes

enteric disease with diarrhoea, infections of the urinary tract as well as sepsis. In Switzerland 5-10 % of the enterohaemorrhagic *E. coli* cases lead to the haemolytic uremic syndrome, which often causes chronic renal damage (Eidgenössisches Departement des Innern (EDI) 2015). Antibiotic resistant *E. coli* strains are a growing problem all over the world. Between 2016 and 2018 in Switzerland, caecum samples of slaughtered chicken were screened for *E. coli* and ESBL *E. coli*. *E. coli* was found in all chicken samples and no strain was resistant to 3<sup>rd</sup> generation cephalosporins (i.e. cefotaxime and ceftazidime). When performing a preliminary selective enrichment step, 52.4% and 30.6% of the flocks were found to harbour ESBL *E. coli* in 2016 and 2018, respectively. These resistant bacteria were further tested for other antibiotic resistances. 94.4% (2016) and 100% (2018) of the bacteria were confirmed to be cefotaxime resistant (Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO, 2018).

### 2.3.2 *Enterococcus faecium*

Enterococci are ubiquitous gram-positive bacteria, that can found in the chicken intestines. In the same experiment as described above, the *enterococcus* ssp. were tested for their AMR profile. In 2016, 3.2% of the *E. faecalis* were resistant to vancomycin. No vancomycin resistant *E. faecium* was detected (Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO, 2018). Enterococci are naturally tolerant of penicillins, and are resistant to cephalosporins, clindamycin, and achievable serum levels of aminoglycosides. Cephalosporin resistance is caused by poor affinity of cephalosporins for enterococcal PBPs (Chen et al., 2009). MDR is also a problematic phenomenon in enterococci. VRE enterococci are not as widespread as ESBL *E. coli*, but they are feared to become also more prevalent. Metagenomic analysis revealed high abundance of AMR genes in fecal microbiota of chicken, among which *vanA* was the most abundant AMR gene, accounting for > 36% of all reads of AMR genes (Tong et al., 2017).

## 2.4 Research aim and study objectives

Livestock provide a perfect reservoir for AMR genes and a potential vector for transmission of plasmid-encoded AMR genes by conjugation to human pathogenic bacteria (Lambrecht et al., 2019). Numerous studies have documented conjugational transfer of AMR genes between bacteria in *vitro* and *in vivo*. High cell densities found in chicken GIT acts as melting point where HGT easily can occur. The enrichment of

AMR bacteria in the gut can be favoured when applying antibiotic selection pressure. To understand better what happens in the chicken GIT we tested the following hypothesis:

- Chicken AMR bacterial strains can colonise and transfer their AMR genes to the resident cecum microbiota
- The application of antibiotic treatments promotes colonisation of AMR strains and transfer of their AMR genes

#### 2.4.1 Research objectives

Primarily, molecular and phenotypical characterisation of the AMR profile of *E.coli* and *Enterococcus faecium* strains should be done using whole genome data and phenotypical antibiotic sensitivity assays. Secondly, the strain-specific tracking method to quantify the strains in the batch fermentation has to be validated by a primer review.

By performing batch fermentation with a representative chicken cecum microbiota, the possibility of the strains to colonise in a resident chicken microbiota is analysed. By using qPCR data with validated primers, HGT quantification has to be explored. Experiments will be repeated to confirm the results.

Finally, variation in the microbial community changes in the experiments has to be observed in context of antibiotic treatment and/or inoculation with the AMR strain. This is achieved by 16S rRNA analysis using Illumina technology to identify the composition at a genus level. Possible recipients of AMR genes from the donor strain in the community should be identified as well.

## 3 Materials and methods

### 3.1 Antimicrobial resistance profiling of selected chicken strains

A pool of AMR chicken strains has been provided from the Culture Collection University of Gothenburg (CCUG) and Zurfluh et al. (2014). The strains from the species *E. coli* were kindly made available by Zurfluh et al. (2014) and were pre-selected based on plasmid characterisation. Only strains with AMR gene on the plasmid were taken into account for further investigations. The selected *E. faecium* strains from the CCUG were all AMR strains but from several different sources and therefore representative of each strain related to poultry additionally one human origin was selected as depicted in Table 1. The AMR gene from the species *E. faecium*, still needed to be located on the plasmid.

All strains from both species underwent molecular and phenotypical AMR characterisation and were further selected for the batch fermentation.

**Table 1** – List of strains with their actual sample origin, inclusive whole genome assembly

Species	Strain	Origin	Source
<i>E. faecium</i>	CCUG59161	Poultry feces	CCUG <sup>1</sup>
<i>E. faecium</i>	CCUG59162	Poultry feces	CCUG <sup>1</sup>
<i>E. faecium</i>	CCUG59167	Human feces, poultry farmer	CCUG <sup>1</sup>
<i>E. faecium</i>	CCUG59168	Poultry feces	CCUG <sup>1</sup>
<i>E. coli</i>	HV114.1	Meconium	Zurfluh et al. (2014)
<i>E. coli</i>	HV292.1	Meconium	Zurfluh et al. (2014)

<sup>1</sup> <https://www.ccug.se/collections> (latest access in November 22th 2020)

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#### 3.1.1 *In silico* characterisation of AMR chicken strains

Whole genome assemblies have been made available and strains were annotated using the tool prokka (prokka version 1.14.6) by Seemann (2014). It produces standards-compliant output files for further analysis. representative strains from each species were taken and used later in a phenotypical antibiotic assay. This is based on the following tests below.

- *In silico* localization of the AMR genes on the plasmid in *E. faecium*
- *In silico* AMR gene characterisation of all strains

##### ***In silico* localisation of the AMR genes on the plasmid in *E. faecium***

The output file GFF3 from prokka annotation contains both sequences and annotations. This was observable through Artemis (Artemis version 18.1.0) for each *E. faecium* strain (Carver et al., 2008). Whole genome visualisation was performed on the CGView Server and saved locally for further specification (Stothard and Wishart, 2005). The position of the AMR genes were evaluated. Relevant gene information was extracted. The whole genome was searched for plasmid related sequences with the tool PlasmidFinder (PlasmidFinder version 2.0) (Carattoli et al., 2014). The threshold for minimum identity and minimum coverage was 95 %. Results were compared to AMR gene sequence position and the plasmid sequences in question were downloaded from NCBI GenBank (Sayers et al., 2019) . Gene sequences were aligned against each other and pairwise comparison was performed to find region of similarity. Comparison file was made using the web based Basic Local Alignment Search Tool (BLAST) by NCBI in Autumn 2020.

Specifically a nucleotide BLAST was performed using the contig40 gene sequence from the strain as a query against known *vanA*-gene complex carrying plasmid pVEF1 (accession number: AM296544.1) as subject sequence with a threshold of 0.05. The plasmid pVEF1 contains the *vanA*-gene cluster and was first isolated in poultry and poultry farmers in Norway by Sletvold et al. (2007). The output was stored locally and alignment was made visual using the Artemis comparison tool (ACT) (ACT version 18.1.0) (Carver et al., 2008).

##### ***In silico* AMR gene characterisation of the strains**

Whole genome mass screening for antimicrobial resistance or virulence genes for the strains, using ABriate by Seemann T. was performed in May 2020 (Seemann T, ABriate, Github). Minimum DNA identity and minimum DNA coverage was chosen at 90 % this is higher than default settings. ABriate comes with the following

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pre-downloaded databases: ARG-ANNOT(Gupta et al., 2014), ResFinder (Zankari et al., 2012), CARD (Alcock et al., 2020) and the National Center for Biotechnology Information (NCBI) Pathogen Detection Reference Gene catalog (Sayers et al., 2019).All genomes were screened against all the databases stated above. The results with highest output (i.e. highest number of detectable AMR genes) were then grouped into antibiotic classes.

- Aminoglycoside - Aminocoumarins - Carbapenems - Cephalosporins - Diaminopyrimidines
- Fluoroquinolons - Fosfomycins - Glycopeptides - Monobactams - Miscellaneous agents - Nitroimidazoles - Penicillins - Polypeptide antibiotics - Sulfomanides - Tetracyclines

#### 3.1.2 Phenotypical antibiotic sensitivity assay

The selected strains were stored at 8 °C on an agar plate, either on a lysogeny broth (LB) (Labolife Sàrl, Pully, Switzerland) plate for *E. coli* or on a brain heart infusion (BHI) broth (Labolife Sàrl, Pully, Switzerland) plate for *E. faecium*. In order to keep the AMR gene information on the plasmid, a new strain suspension was used for every experiment. To achieve the suspension, colony was taken from the plate and inoculated in a suspension with selective antibiotic pressure as described in Table 2 and grown overnight at 37 °C without agitation. All suspensions inoculated with live strains were handled in a BioSafety Level 2 lab.

**Table 2** – The representative strain for each species had specific broth as well as specific antibiotic

strain	suspension	antibiotic [10 µg/ml]
<i>E. faecium</i> CCUG59168	liquid LB	vancomycin
<i>E. coli</i> HV292.1	liquid BHI	SXT/CTX <sup>1</sup>

<sup>1</sup> trimethoprim-sulfamethoxazole and cefotaxime in 1:1 ratio

To validate the *in silico* AMR characterisation of the strains, phenotypical antibiotic sensitivity assays were performed with antibiotics equivalent for all antibiotic classes.

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**Table 3** – Different antibiotic wafers representing various antibiotic classes are used to validate AMR strains as MDR strains

antibiotic name	antibiotic class	dosage [µg/l]	antibiotic name	antibiotic class	dosage [µg/l]
ampicillin	penicillin	10	ciprofloxacin	fluoroquinolones	5
aztreonam	monobactams	30	gentamicin	aminoglycosides	10
chloramphenicol	miscellaneous agents	30	imipenem	carbapenems	10
cefepime	cephalosporins 3 <sup>rd</sup>	30	neomycin	aminoglycosides	10
cefpodoxime	cephalosporins 3 <sup>rd</sup>	10	norfloxacin	fluoroquinolones	10
ceftadizime	cephalosporins 3 <sup>rd</sup>	30	SXT <sup>1</sup>	miscellaneous agents	30
ceftriaxon	cephalosporins 3 <sup>rd</sup>	30	tetracycline	tetracycline	30
cefotetan	cephalosporins 3 <sup>rd</sup>	30	vancomycin	glycopeptides	30
cefoxitin	cephalosporins 2 <sup>nd</sup>	30			

<sup>1</sup> sulfamethoxazole and cefotaxime in 1:1 ratio

#### Disc diffusion test

A corresponding strain from each *Escherichia coli* and *Enterococcus faecium* were used in a disk diffusion test with 17 antibiotics in order to validate the *in silico* AMR gene screening. Wafers containing antibiotics (ThermoFischer, Massachusetts, USA) listed in Table 3 are placed on an agar plate. Casting and inoculation of the plates with 100 µl AMR strain suspension of interest was done previously. The plates were left to incubate at 37 °C overnight. A zone of inhibition is formed by an antibiotic blocking the growth and/or killing the bacteria on the plate. The zones of inhibition around the wafers were calculated by measuring zone diameter, including the wafer in the middle. They were compared with the official zone diameter breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Strains are defined either as susceptible (S), Susceptible with increased exposure (I) or resistant (R).

#### Sensititre Antimicrobial Susceptibility Testing-System (SAST-S

) To test specific antibiotic resistance in more detail an antibiotic liquid assay was performed with the equivalent strains from before. Using a 96-well microtiter plate (ThermoFisher, Massachusetts, USA) containing antibiotics in extended dilution ranges. Specific antibiotic concentrations were mixed with nanopure water by applying a pure antibiotic in a solid state with high potencies, as stated below.

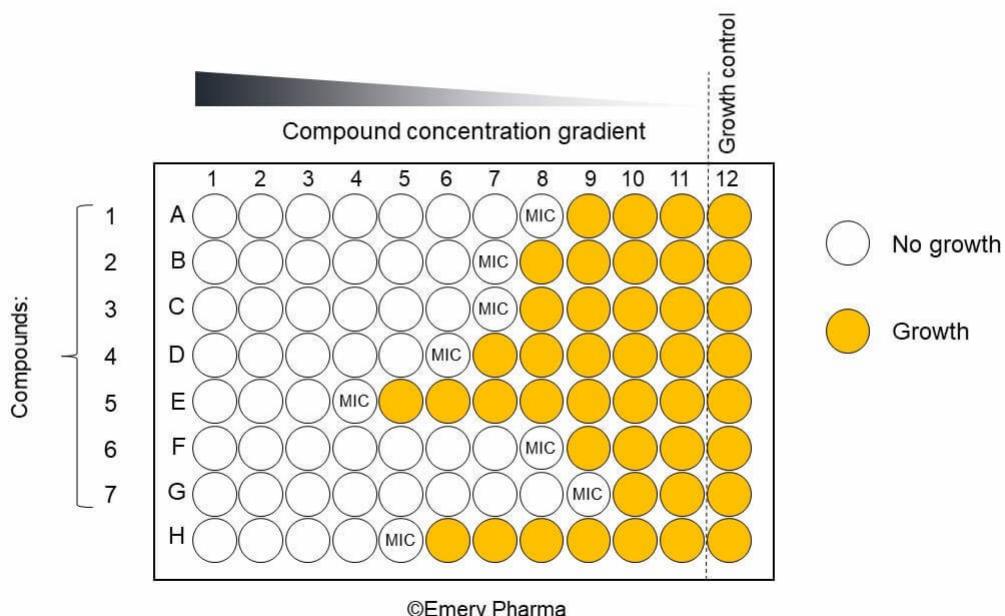
- vancomycin hydrochloride from *Amycolatopsis orientalis*, ≥ 900 µg/mg (Sigma-Aldrich, Missouri, USA )

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- trimethoprim-sulfamethoxazole sodium salt,  $\geq 990 \mu\text{g}/\text{mg}$  (Sigma-Aldrich, Missouri, USA )
- cefotaxime sodium salt,  $\geq 990 \mu\text{g}/\text{mg}$  (Sigma-Aldrich, Missouri, USA )

Antibiotic solutions were diluted with strain specific broth in a series of dilutions. A 96-well plate with each well respectively filled with 200  $\mu\text{L}$  strain specific broth containing defined antibiotic concentration was prepared. Each antibiotic treatment was duplicated. The Figure 2 shows a reading example of an experimental set-up to test the MIC for different antibiotics. To evaluate the Minimal inhibitory concentration (MIC), 2  $\mu\text{L}$  of the AMR strain suspension was added and inoculated at 37 °C for 48 h, according the EUCAST protocol for MIC. The MIC value is the lowest concentration of an antibiotic at which no growth is observed.

#### Interpretation of microdilution MIC results



**Figure 2** – The reading example of a Sensititre Antimicrobial Susceptibility Testing System explains the MIC evaluation. Picture derived from Emery Pharma, <https://emerypharma.com/biology/minimum-inhibitory-concentration/> (last accessed in December 2nd, 2020)

For MICs which were not in the range of screened concentration, MIC was estimated. Estimated MIC was declared to be approximately equal or higher than highest screened concentration ( $\text{MIC} \geq \text{screened conc.}$ ). The MIC of the AMR strains were checked against MIC distributions of wild type (WT) strains from the EUCAST. The

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distribution are based on collated data from a total of more than 30'000 MIC distributions containing more than several million MICs from worldwide sources. The Epidemiological cut-off values (ECOFF) defines the upper limit of MIC values in a WT population. A bacteria is defined as WT bacteria when in absence of acquired and mutational mechanisms of resistance. A bacteria which exceeds the ECOFF value is considered as resistant from an ecological point of view (Olivares et al., 2013).

## 3.2 Design and validation of qPCR primers

The main challenges for the successful development of any quantitative polymerase chain reaction (qPCR) assay is the identification of a specific target sequence and the design of primers that bind exclusively to that target sequence (Dreier et al., 2020). Primers for the AMR genes *bla<sub>ctx-m-1</sub>* and *vanA*, as well as strain specific primers were designed in a preliminary step using the online tool PrimerBlast. All *de novo* designed primer (IDT, Sand Diego, USA) were ordered by the supervisor before the start of the project and primer functionality was tested in a preliminary experiment. All functional *de novo* designed primer pairs which are used in the project are found in the Supplementary material, Table 11.

### 3.2.1 Confirmation of specificity of qPCR primers

Functional primer pairs were validated in a polymerase chain reaction (PCR) against similar strains from the same species. All PCR were conducted by a thermocycler Type T3000 (Biometra, Göttingen, Germany) within 35 cycles including denaturation for 30 sec at 95 °C, followed by an annealing step at 56 °C for 30 sec, and a final extension (72 °C for 30 sec followed by 7 min at 72 °C.) The PCR products were stored at 4 °C until further use. To confirm amplicon length, agarose gel electrophoresis tests were conducted. 1.5% agarose gel in Tris-Acetate-EDTA (TEA) were treated in an electrophoresis chamber with 100 V for 40-70 min. To mark the PCR products the gel was immersed for 20 min in a red gel bath before taking a picture with the Image Lab™ (Version 6.0.0 build 25 Standard Edition).

#### *In silico* qPCR amplification

The results are based on sequence identity. Real-world factors such as melting temperature, annealing time, salt concentration or secondary structure are not accounted for. *In silico* PCR with primer pairs as an input against a reference genome database, using the perl script by Egon A. Ozer with default options input (*in silico*

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PCR, Eonozer, Github). A separate bash script by Vitor C. Piro was used or the reference genome database (Genome updater, Vitor C.,Github). Recent species specific genomes were downloaded from the NCBI genbank (Sayers et al., 2019). Next, the reference strains of *E.coli* HV292.1 and *E.faecium* CCUG 59168 were positively validated using their whole genome assembly.

## 3.3 Batch fermentation

From preliminary findings in section 3.1, *E. coli* strain HV292.1 and *E. faecium* strain CCUG59168 was used in the batch fermentation. Strains were inoculated in suspension as well as specific antibiotic pressure (Table 2 and cultured overnight. In order to determine the concentration, 100  $\mu$ l of culture was diluted in 900  $\mu$ l sterile water. The light absorbance was then measured with OD600 DiluPhotmeter(IMPLEMENT, München, Germany). 1 optical density  $\approx 10^8$  cfu/ml, according to SOP. A Series of dilution was prepared and  $\approx 10^4$  bacterias were added anaerobically using a syringe filled with  $CO_2$ . The concentration of both antibiotics were 20  $\mu$ g/ml for vancomycin and 100  $\mu$ g/ml for SXT/CTX. These were prepared as described in section 3.1.2 and added to the flasks.

### 3.3.1 Batch fermentation media

Batch fermentation medium (Table 10) was prepared, according to SOP. Instead of autoclaving, the batch fermentation medium was boiled for 15 min on a heating stirrer to remove  $O_2$  from the liquid. The steam was steadily cooled with a condenser. Afterwards, the condenser was removed and the flask was covered with aluminium foil. The head space was then flushed with  $CO_2$  and the medium was left to cool down to 50 °C before FOS and the vitamins were added, as described in SOP. With the  $CO_2$ -flush ongoing, the medium was stirred at 50 °C for another 15 min. 19.8 mL of the medium was filled in pre- $CO_2$ -flushed Hungate tubes. Finally, the Hungate tubes were sealed and autoclaved.

### 3.3.2 Representative gut microbial community

The microbial community was derived from an inoculum PolyFermS reactor used for continuous fermentation. Continuous fermentation was held at 41 °C under anaerobic conditions (head space was constantly flushed with a gas mix of 5%  $H_2$ , 10%  $CO_2$ , and 85%  $N_2$ .) pH was set to 6.0 and automatically adjusted with 2.5 M HCL and 2.5 M NaOH. The reactor was stirred at 180 rpm, containing glass beads with immobilised

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microbiome from a cecum of a broiler and chicken gut fermentation medium. Fresh hicken gut fermentation medium was fed at a rate of 8.3 mL/h with an average retention time of 24 h. For the representative microbial community used in this project 2 mL were taken from the inoculum reactor and collected in an anaerobic Hungate tube filled with 18 mL anaerobic 0.1% peptone water. Peptone water was prepared according to SOP in an anaerobic chamber.

#### 3.3.3 Treatment and enrichment step

A gut microbial community as well as an AMR strain is incubated in a fermentation media for a maximum of 48 h under static batch conditions. The microbial community was derived from an inoculum PolyFermS reactor. Incubations were set-up for several consecutive time points between 0 h - 48 h to assess the kinetics of community growth and changes. All suspensions inoculated with live intestinal bacteria were handled in BioSafety Level 2 labs. An adapted version of the inline standard operating procedure (SOP) for batch fermentation with chicken gut microbial community succeeded. 19.8 ml batch fermentation medium as well as 0.2 ml gut microbial community were added into brown (20 ml)-serum flasks. Batch fermentation was triplicated and treatments were added as explained hereunder.

- Untreated: No added strain or antibiotic
- Antibiotic added: 2000 µg vancomycin or 400 µg SXT/CTX, depending on batch fermentation
- Strain added:  $10^4$  bacteria from either *E. coli* or *E. faecium* strain, depending on batch fermentation
- Antibiotic/Strain added: Antibiotic and strain was added simultaneously

1% of the fermentation slurry was reinoculated into a new, fresh and sterile batch fermentation media at each sampling point, this ran for 24 h. Hence for each condition fermentation was conducted two times, by taking the end of the first fermentation as the starting point for the second one. This was defined as an "Enrichment step". The aim of the enrichment step was to select the resistant community fraction.

#### 3.3.4 Sampling

To begin with all 4 different conditions were sampled at timepoints 0 h, 7 h, 24 h and 48 h, 3 mL of medium was taken per condition and per time point accordingly with a sterile syringe and filled into three Eppendorf tubes. One sample was placed in a dilution series in preparation for plating. The two remaining samples were centrifuged for 10 min at 4 °C and 14'000 rcf in a centrifuge 5417 R (Eppendorf,

### 3 Materials and methods

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Hamburg, Germany). After centrifugation, the supernatant was removed and the pellets were stored at -18 °C. One centrifuged sample remained frozen as a backup while the other was used for DNA extraction. For all samples their enrichment steps were carried out respectively at time points 7 h and 24 h. The Hungate tube's gaskets were punctured and inoculated with a syringe for each respective sample.

#### Selective plating

To assess the batch fermentation and to explore new resistant strains, a plating experiment was done. 100 µL of a sample-dilution ( $10^0 - 10^4$ ) was plated on an antibiotic enriched (20 µg/mL) agar plate with selective media:

- Becton Dickinson (BD) Endo Agar; selective media for gram-negative bacilli, (e.g. *E. coli*)
- (BD) Mannitol Salt Agar (MSA); selective differentiation medium for staphylococci
- (BD) Bile Esculin Agar (BEA); selective differentiation medium for *enterococcus* ssp.
- (BD) Xylose-Lysin-Desoxycholate- Agar (XLD); moderately selective differentiation medium for gram-negative enteric pathogens (*Salmonella* ssp. and *Shigella* ssp.)

According to the inline guidelines selected colonies are confirmed as spiked strains by colony-PCR, as depicted from the BEA and Endo agar. A single colony was taken and dissolved in 20 mL peptone water. 2 µL was taken as DNA template for PCR. The colony-PCR was performed in triplicate. All plates were screened for new recipients in a explorative way.

#### DNA extraction

The pellet of 1 mL fermentation sample was used to extract the DNA. FastDNA® Spin Kit for Soil (MP Biomedicals, Illkirch, France) was used for the extraction following the corresponding protocol. To destruct the bacterial cells during DNA extraction, the FastPrep-24™ 5G (MP Biomedicals, Illkirch, France) was used with the following settings: speed: 6 m/sec, adapter: QuickPrep, time: 40 sec, 1 cycle. Final DNA-concentration was measured with the ND-1000 Spectrophotometer (NanoDrop). Lastly the DNA samples were diluted to 20 ng/µL for further experiments.

## 3.4 Tracking method using qPCR

A qPCR was performed to quantify the number of resistance genes sequences as well as unique sequences of the spiked strains in the batch fermentation. The samples were generally measured in triplicates or at least duplicates. Standard curves were run once. One dilution of the standard was included per plate to calibrate to the standard curve. LightCycler® 480 was used to measure as well as it's in-house software (LightCycler® version 1.5.1.62). 9 µL master mix (SensiFAST™ Probe NO-ROX mix 2x) per master mix, 0.5 µL forward and reverse primer respectively(10 µM) 3 µL molecular grade water, and 1 µL DNA-dilution (20 ng/µL) were used with the following scheme in the qPCR: Pre-incubation step for 3 min at 95 °C. The denaturation, annealing and extension step were at 95 °C and 60 °C for 30 sec respectively. This was repeated for 40 cycles. Fluorescence signal and melting curve detection started at 65 °C to 97 °C (0.11 °C/sec). The PCR products were cooled down to 40 °C for 30 sec.

Melting curves of each sample were compared with the standardised melting curves and only samples with same melting temperature profile were taken into account for further analysis. Concentrations are derived from the standard curve. Samples were set to 0 if they had no signal upon 35 cycles. Mean of experimental replicates were calculated. Post-processing and visualisation was done by using the tidyverse wrapper function in R(gplot2 v.3.3.2; purrr v.0.3.4; tibble v.3.0.4; dplyr v.1.0.2; tidyr v.1.1.2; stringr v.1.4.0; readr v.1.4.0;forcats v.0.5.0.)

### 3.4.1 qPCR standard

A bacterial strain containing the plasmid of interest was extracted from a liquid culture using GeneJet Plasmid Miniprep Kit (ThermoFisher, Massachusetts, USA). The copy number was calculated after measuring the concentration with nanoDrop®. with the following formula:

$$\text{Gene copy number } \left[ \frac{\text{copy number}}{\mu\text{L}} \right] = \frac{\text{plasmid } [\frac{\text{ng}}{\mu\text{L}}] * 6.022 * 10^{23}}{\text{plasmid} + \text{insert length } [\text{bp}] * 10^9 * 650}$$

The plasmids were linearised using the restriction enzyme PST-1. Due to dilution during linearisation, the final concentration of the plasmid is then 1/ 10 of the previously calculated gene copy number.

### 3.4.2 Measuring HGT by measuring ratio of AMR gene over strain specific bacteria

Monitored higher ratio in a pure culture is hypothesised to detect higher plasmid replication than expected from bacteria growth. This is a novel qPCR method to detect HGT by quantifying changes of ratio over time. Ratio of AMR gene concentration over specific strain sequence concentration in a pure culture was determined in a preliminary experiment. In the growth phase of this experiment ratio remains stable at 1.12 in the pure *E. coli* strain HV292.1 culture and 117 in the pure *E. faecium* strain CCUG 59168 culture respectively. A similar approach was only found in a paper from Wan et al. (2011), where they confirmed a plasmid/chromosome ratio of 1:1 in pure *E. coli* culture throughout batch growth as well.

## 3.5 Metabarcoding

Using a tag-encoded 16S rRNA gene MiSeq-based (Illumina, San Diego, USA) high throughput sequencing the bacterial community composition was determined. The V4 region of the 16S rRNA gene was amplified with the primers listed below:

- 806R (5-GGACTACHVGGGTWTCTAAT-3)
- 515F (5-GTGCCAGCMGCCGCGGTAA-3)

The PCR amplification step, which selectively enriches library fragments that have primer adapters ligated on both ends, was performed according to inline SOP for 16S amplicon sequencing. The size of the 16S rRNA gene PCR products were confirmed by electrophoresis on a 1.5% agarose gel. The PCR products were then used as a template for Index PCR to attach dual indices. PCR was done using Thermocycler Type 3000 (Biometra, Göttingen, Germany) polymerase in 13 cycles (denaturing at 95 °C for 15 s; annealing at 55 °C for 20 s; elongation at 68 °C for 20 s) with a final extension at 68 °C for 5 min. The index PCR products were cleaned with 80% ethanol and the final DNA concentration was measured using Qubit fluorometer (Thermo Scientific, Darmstadt, Germany). Each sample of the final DNA concentration was adjusted to 4 nM. Concentration in  $\mu$ L of diluted DNA was calculated for each sample and were mixed for pooling the libraries with unique indices. Microbial profiling by 16S rRNA gene amplicon sequencing was performed in collaboration with the Genetic Diversity Center (GDC, ETH Zürich, Switzerland).

### 3.5.1 Postprocessing

Raw data obtained from 16S rRNA sequencing were processed using Cutadapt (Marcel, 2011) and DADA2 (Callahan et al., 2016) pipeline to obtain amplicon sequence variants (ASV). Taxonomy was assigned using SILVA database (SILVA version 138). Community was analysed according to inline protocol for community analysis (Laboratory of Food biotechnology, ETH Zürich, Switzerland)

A mock community (ATCC® MSA-1000™) with 10 even predefined strain mixtures were used as a positive control. ATCC® microbiome standards are mock microbial communities that mimic mixed metagenomic samples. It comprises genomic DNA prepared from fully sequenced, characterised, and authenticated ATCC genuine cultures including:

- 10% *Bacillus cereus* (ATCC 10987)
- 10% *Bifidobacterium adolescentis* (ATCC 15703)
- 10% *Clostridium beijerinckii* (ATCC 35702)
- 10% *Deinococcus radiodurans* (ATCC BAA-816)
- 10% *Enterococcus faecalis* (ATCC 47077)
- 10% *Escherichia coli* (ATCC 700926)
- 10% *Lactobacillus gasseri* (ATCC 33323)
- 10% *Rhodobacter sphaeroides* (ATCC 17029)
- 10% *Staphylococcus epidermidis* (ATCC 12228)
- 10% *Streptococcus mutans* (ATCC 700610)

The mock community whole genomes were downloaded from the NCBI database (Sayers et al., 2019). 16S sequences were extracted using the barrnap tool (barrnap version .0.9.), a rapid ribosomal RNA prediction bioinformatic pipeline from T. Seemann (Seemann T, barrnap, Github). 16S sequences were imported and checked for identical sequences. To get the V4 region of the 16S rRNA sequences, the 515f/806r primer pair as defined above was taken. A perl script from Egon. A. Ozer was used to conduct a *in silico* PCR as described in section 3.2.1.

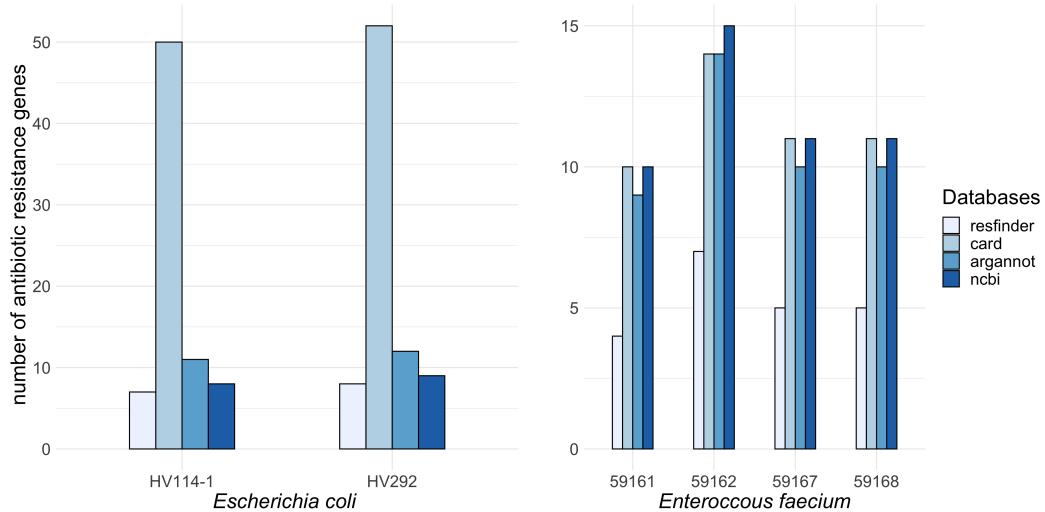
### **3.6 Statistical Analysis**

All statistical analysis were performed using R (R version 4.0.3) and graphical interface RStudio (RStudio version 1.3.947). Significant differences in copy numbers were identified by means of Analysis of Variance (ANOVA) using anova function (rstatix v.0.6.0). Significant differences in-between communities were identified by means of Permutational Multivariate Analysis of Variance (PERMANOVA) using the adonis function (vegan v.2.5-2).

# 4 Results

## 4.1 Antimicrobial resistance profiling of selected chicken strains

The ABriate pipeline revealed numerous AMR genes in all screened strains. No matter what species, the lowest reported output was found with the Resfinder database, shown in Figure 3. The different AMR databases had a large impact on the reported output. The greater difference was found in *E. coli* strains ( $\pm 43$  AMR genes.) The discrepancy in the results is possible because "hypothetical genes" are handled differently within the databases. If focused on complete and exhaustive search, database with highest reported output should be used.



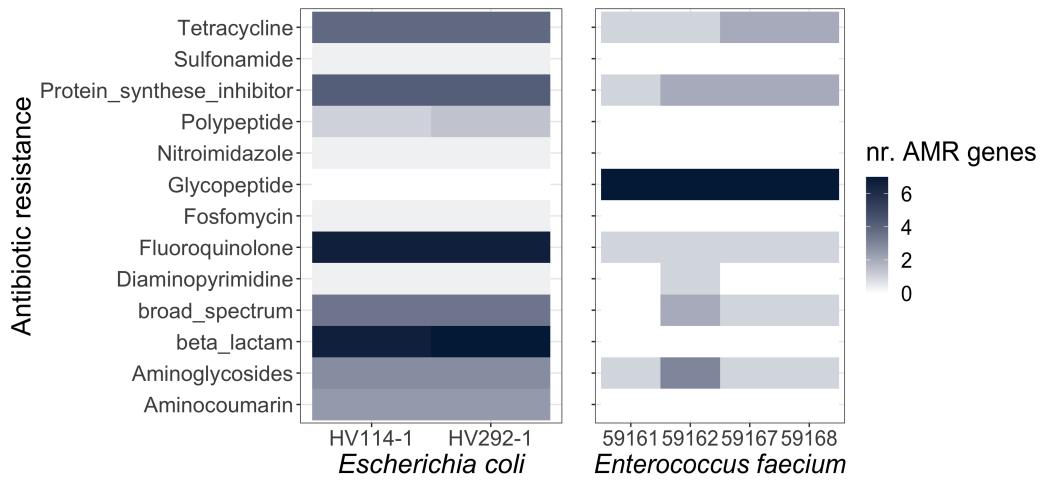
**Figure 3** – Depending on the choice of database, unequal number of AMR genes were reported

The NCBI database resulted with highest output for *E. faecium* strains, however the CARD database showed almost (-1 AMR gene) same performance as the NCBI database. In addition CARD database resulted with highest number of AMR gene annotations in *E. coli* strains. This was the reason that it was chosen to be worked

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with. The AMR genes were then manually screened and divided into corresponding classes. The results were visualised in a heatmap (Figure 4). It revealed a clear clustering of genes encoding for glycopeptide resistance in *E. faecium* strains, whereas *E. coli* had a higher variability of AMR genes. A increased number of genes confer resistance against  $\beta$ -lactam antibiotics, which is expected for (ESBL) *E. coli* strains.



**Figure 4** – Numerous AMR genes were found in CARD database, encoding for multiple antibiotic classes. Similarities were detected in-between strains from the same species

Previous investigations for the *E. coli* strain HV292.1 and the *E. faecium* strain CCUG 59168 showed superior results in filter mating experiments as well as growth tests in chicken effluent. Resistance profiles do not differ from the other strains as depicted in Figure 4. Therefore phenotypical antibiotic tests were done with only one representative strain for each species. From now on, unless explicitly stated otherwise, when *E. coli* or *E. faecium* are mentioned in the project, the strains: *E. coli* strain HV292.1 and *E. faecium* strain CCUG 59168 are implied.

To validate the *in silico* AMR profiling from above, disk diffusion test was conducted with the representative bacteria strains. The results are summarised in Table 12 and Table 13 (See Supplementary material). The AMR genes *tet(M)* and *tet(O)*, which encode for tetracycline resistance are found in both species. The result from the disk diffusion test for tetracycline should also be positive. As this was the case, it's an example for true positive correlation between AMR genes and phenotypical AMR. No AMR genes were reported against  $\beta$ -lactam antibiotic for *E. faecium*. These results matched the diffusion test results as *E. coli* was susceptible to ampicillin, but not the *E. faecium*. Multiple AMR genes were found in both species against cephalosporins (in Figure 4 classified as syntheses inhibitors). The *E. coli* strain found resistant

against cefpodoxime, ceftriaxon and cefepime in the disk diffusion test. Interestingly, all cephalosporins were unsuitable for the *E. faecium* strain. It must be mentioned that they strain already covers a natural resistance. Several AMR genes against fluoroquinolone were found in the *E.coli* genome (*acraA*, *ermB*,..) The corresponding antibiotics ciprofloxacin and norfloxacin both had a reduced effect (I = Susceptible with increased antibiotic exposure) on the disk diffusion test.

### 4.1.1 Sensititre Antimicrobial Susceptibility Testing-System in a 96-well titre plate

In order to address the amplitude of AMR, 5 different antibiotics were determined for MIC. As depicted in Table 4, MIC or estimated MIC in *E. faecium* for vancomycin and tetracycline was found to be higher than their corresponding ECOFFs. For *E.coli* same results were found for SXT, cefotaxime and ampicillin. For antibiotics where species confer an intrinsic resistance the ECOFFs were not derivable. As described in subsection 2.1.1, *E. coli* is intrinsically resistant against vancomycin. Secondly, Enterococci, including *E. faecium* can absorb folic acid from the environment, bypassing the effects of SXT. This was first described by Bushby and Hitchings (1968). Due to the fact that the agar media contained folic acid, the susceptible testing was arbitrary. Chloramphenicol was found to be susceptible for both species. Finally, in consensus with the disk diffusion test and the *in silico* screening no high-level gentamicin resistance were found in *E. faecium*.

As the phenotypical AMR tests and the *in silico* AMR profiling are in agreement, *E. faecium* is trusted to be a VRE, a common MDR bacteria. The *E. coli* which is highly resistant against cefotaxime showed equivalent results. Thus, it is an (ESBL) *E. coli*. Both strains are from now on stated as MDR strains.

### 4.1.2 Localisation of the AMR genes in MDR *E. faecium*

The circular visualisation of the whole genome of *E. faecium* (annotation by prokka, see Supplementary material, Figure 14) revealed that the *vanA*-gene complex is found on one continuous DNA strand (contig40.) In this section, a 1500 bp sequence was found to be identical to the pVEF1 plasmid with PlasmidFinder. The BLAST search against the pVEF1 plasmid confirmed the exact position of the *vanA*-gene complex in the two sequences (Figure 5). The regions with undifferentiated (percentage of identical bases between query and subject: < 99 %) sequences are visualised in red, including the vancomycin resistance. Differences between the two aligned sequences are depicted as white spaces. The blue block link a region that is inverted with respect to the other sequence. The reverse inserted region encodes for a replication initiation

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**Table 4** – AMR strains being highly AMR in SAST-S for at least 2 different antibiotic. For the underlined antibiotics, their AMR genes were found on a conjugative plasmid (Zurfluh et al., 2014)

antibiotic [ $\mu$ g/ml]	screened conc. [ $\mu$ g/ml]	MIC [ $\mu$ g/ml]	estimated MIC [ $\mu$ g/ml]	ECOFF
<b><i>Enterococcus faecium</i></b>				
vancomycin	1 - 450	-	>450	4
SXT <sup>1</sup>	4 - 128	-	>128	ND <sup>2</sup>
tetracycline	0.5 - 32	-	>32	4
chloramphenicol	8 - 256	8	-	32
gentamycin	0.2 - 12.5	6.25	-	32
<b><i>Escherichia coli</i></b>				
<u>SXT</u>	0.05 - 120	-	>120	64
<u>CTX</u>	0.05 - 120	-	>120	0.25
AMP	2 - 300	-	>300	8
chloramphenicol	4 - 128	4	-	16
vancomycin	0.2 - 12	-	>12	ND <sup>2</sup>

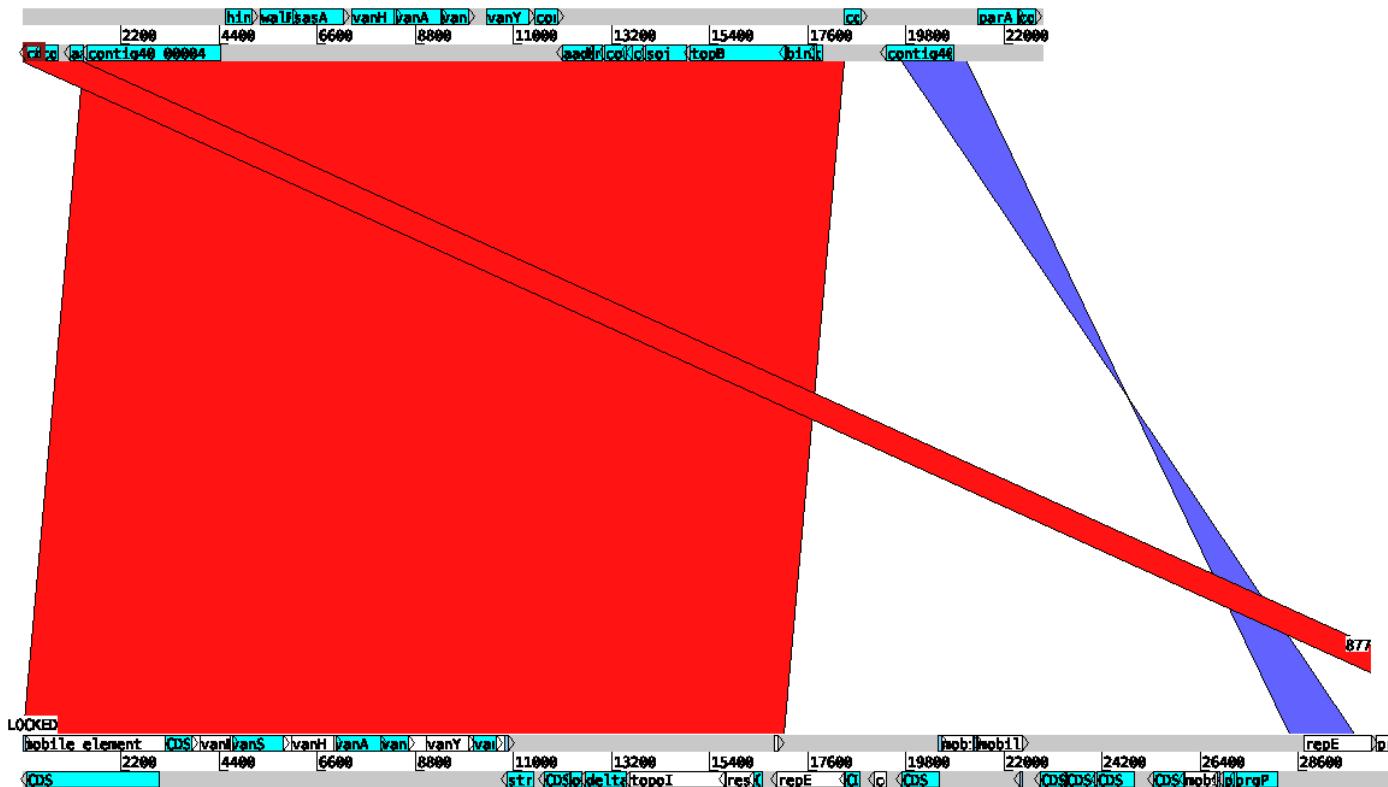
<sup>1</sup> trimethoprim-sulfamethoxazole

<sup>2</sup> not derivable (i.e. intrinsic resistance)

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protein (*repE*), a self replication machinery. This is necessary in order to transcript a conjugative plasmid.



**Figure 5** – The BLAST search of predefined gene sequence from the MDR *E. faecium* against well-known *vanA*-gene complex harbouring plasmid pVEF1 in *Enterococci* found highly similar regions

## 4.2 *In silico* verification of qPCR primers

To confirm specificity of the qPCR primers, the *in silico* PCR pipeline ran all the primer pairs against related sequences from the same species. In total, there were 4'234 sequences for *E. faecium* and 1'267 sequences for *E. coli* from the NCBI database, accessed in Summer 2020 (Table 5). The screening included all kinds of genomes; From whole genome assemblies to small contigs. As all qPCR primers did not amplify for any other strain, it strengthened the assumption that they are truly unique (i.e. strain-specific).

**Table 5** – Strain-specific sequences were validated *in silico* on a species level, using reference genomes of different levels

genomes	number	type <sup>1</sup>	nr. amplification	accesing date
<i>Enterococcus faecium</i>	4'235	<i>all</i>	0	July 14th, 2020
<i>Escherichia coli</i>	1'267	<i>all</i>	0	June 8th, 2020

<sup>1</sup> Assembly levels were in this order: Complete Genome, Chromosome, Scaffold, Contigs

### 4.2.1 Positive selection of qPCR primers

Positive amplification with whole genomes of the MDR strains were carried out successfully, except for one primer pair in *E. coli*. Since maximal amplicon length in the tool was limited to 600 bp, the expected length for the 48Contig61-P2 primer was oversized (714 bp). Thus, the tool excluded the amplification and made it undetectable. As the amplicon length was measured in a PCR (see Table 11 in Supplementary material) the length values *in silico*, which are depicted in Table 6, are comparable. If exactly 40bp were removed from the *in silico* results, the estimated values *in vitro* would be equal. The pattern can be explained through the fact that in the theoretical PCR set-up, the primer pair (each 20bp long) are not included. Therefore, it is logical that the amplification length *in vitro* must be longer than *in silico*. This results in functional and well designed primers for the MDR strains.

### 4.2.2 PCR with related strains

PCR was carried out with the *in silico* tested primers in combination with their related strains, as described in section 3.1. Additionally, three AMR *E. faecium*

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**Table 6** – Strain specific primers were positive amplified *in silico* with strain of interest, using default setting and limited to 600 bp as the maximum amplicon length.

Primers	amplification	contig	position	length
<b><i>Enterococcus faecium</i></b>				
348_contig61-P1	<b><i>yes</i></b>	contig60	7016	432
348_contig61-P2	<b><i>no</i></b>			
348_contig61-2-P1	<b><i>yes</i></b>	contig60	1869	439
<b><i>Escherichia coli</i></b>				
292_contig27-P1	<b><i>yes</i></b>	contig00027	20077	142
292_contig50a-P1	<b><i>yes</i></b>	contig00050	4315	433
292_contig50a-P2	<b><i>yes</i></b>	contig00050	4756	141
292_contig50b-P1	<b><i>yes</i></b>	contig00050	9475	279

strains from the lab, the strains 37-18, 38-18, 68-17, were chosen to cross-validate the results. Figure 6 shows the colorised gel with each column is DNA specific for different strains and amplified with the primers shown on top of the figure.

For a strain specific primer, only 348\_contig61-P2 and 348\_contig61-2-P1 are reasonable, because they amplified thoroughly as indicated with the white stripes on the far left.

To test the primer pairs for MDR *E. coli* strains, PCR was carried out with in a total of five *E. coli* strains, including the strain HV292.1 and HV114.1 (Figure 7) and a negative control (water). As the strain of interest being: HV292.1, it is labelled here as positive control. Primer pair 292\_contig50b-P1 is not strain specific, due to the fact that stripes also appeared for another strain. The primer pair 292\_contig27-P1 did not amplify at all.

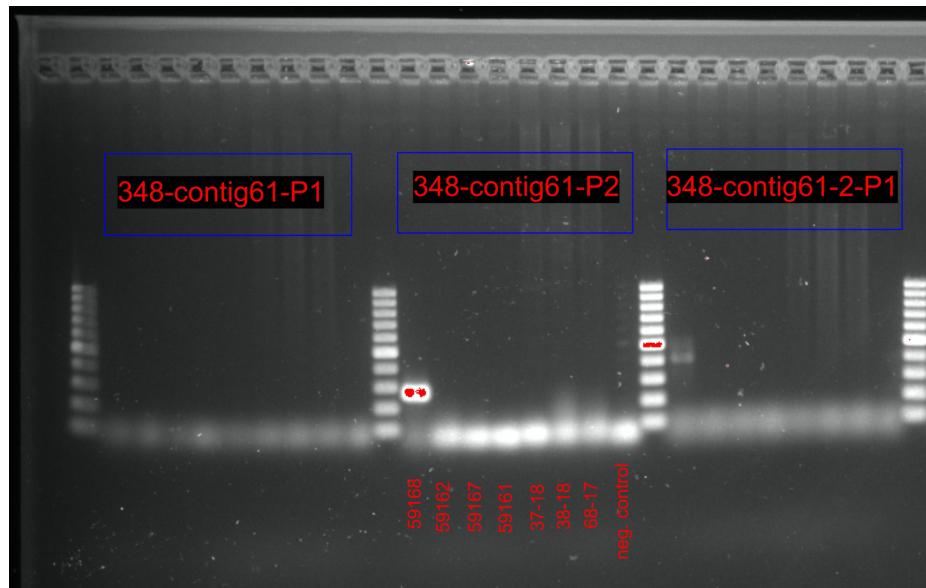
Therefore to quantify the strain specific sequence in the batch fermentation, primer pair 348\_contig61-2-P1 was chosen for the MDR *E. faecium* strain CCUG59168 and 292\_contig50a-P1 for MDR *E. coli* strain HV292.1 (See Supplementary material, Table 11.)

To track the resistance genes *vanA* and *bla<sub>ctx-m-1</sub>*, the following primers were used:

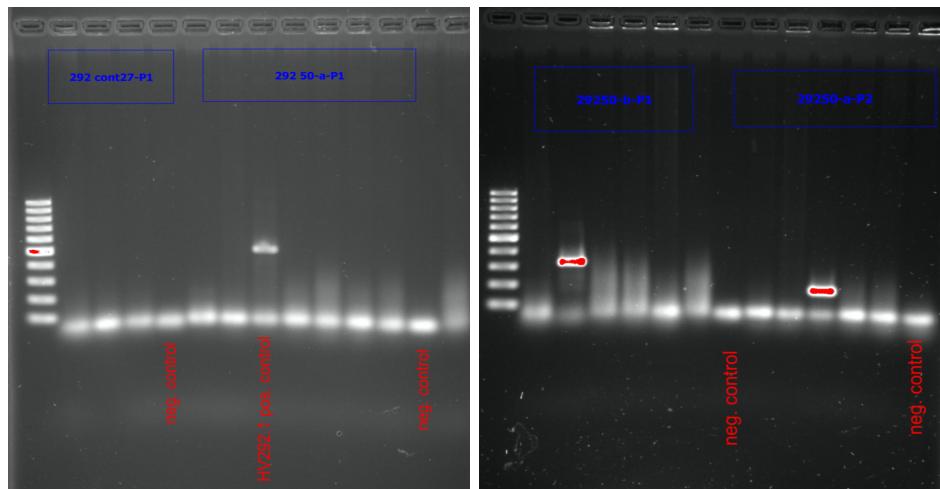
- 57C-608nt-CTX (5'->3'):

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**Figure 6** – All tested primer seems to be specific only for the *E. faecium* strain CCUG 59168, but primer pair 348\_contig61-P1 did not amplify at all.



**Figure 7** – The primer pair 292\_contig50a-P1 seems the most reasonable candidate for a strain specific primer. The primer pair 292-50b-P1 seems not only specific for the *E. coli* strain HV292.1

- F : GTGAAAGCGAACCGAATCTG; R : GTTGGTGACGATTTAGCCG
- VanA (5'->3'):
  - F: GTACAATGCGCCGTAA; R : GGGAAAACGACAATTGC

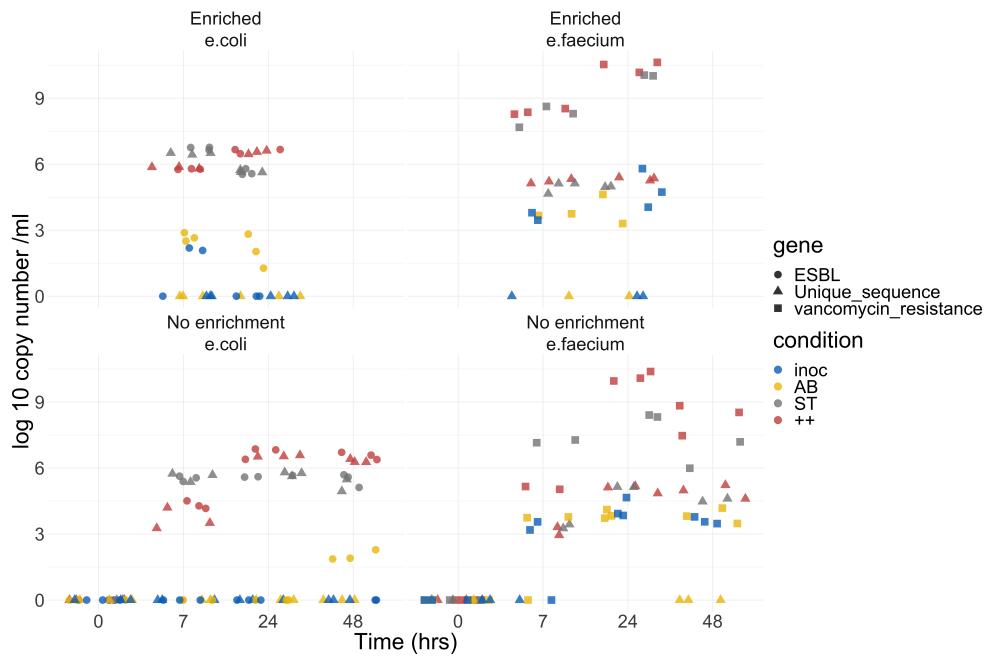
## 4.3 Batch fermentation

A dilution series of the bacteria community was prepared for each sampling point and cultivated on selective agar plates. On the selected agar plates for the control batch fermentation with the chicken cecum microbiota, no visible growth was detected. Red colonies with a green metallic shimmer as is characteristic for *E. coli* species were recognised after 24 h on endo agar plates (See Supplementary material, Figure 15). Enterococci were able to grow after 24 h on selective BEA media plates. When fermentation medium was plated on MSA, which is selective for staphylococci, no growth was detected. Finally bacteria from all 4 conditions were cultivated on XLD media plates and were detected after 24 h. Therefore XLD plates were not further used to characterise the colonies by morphology.

### 4.3.1 qPCR tracking AMR gene and spiked MDR strain

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**Figure 8** – qPCR data from unenriched samples (bottom graphs) reveals that AMR genes are detected in resident chicken cecum microbiota. The copy number of strain specific sequence per ml seem to reach a plateau after 24h. Enriched conditions (top graphs) give no additional information to overall gene sequence distribution over time

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The results of the qPCR are depicted in Figure 8. The graphs are subdivided on spiked MDR strains as well as the dependency of enrichment step. On the y-axis the log10 concentration of copy numbers per ml is shown. The x axis shows the duration with 4 time points (time point 0 as baseline and sampling points 7 h, 24 h and 48 h.) No AMR genes or strain specific sequences were detected in the control batch fermentation (inoc). While the microbiota was not spiked with MDR strains, they remained undetected (blue and yellow triangular points). Even without antibiotic pressure (grey triangular points) when the strains were added, strain specific sequences were traceable after 7 h where the strains had been added. AMR genes were traceable in both batch fermentations. The *vanA* gene complex (square points) and the AMR gene *bla<sub>ctx-m1</sub>* (round points) were present after 7 h. Surprisingly, it was detectable even when no strain was added (inoculum plus antibiotic [yellow] or inoculum [blue]) after 7 h, 24 h and 48 h. It seems that the AMR genes were already present in low numbers within the resident microbiota and reached the detectable limit of the system during the batch fermentation. Spiked MDR strains could be traced after 7 h and seemed to remain a permanently high level copy number/ml ( $\approx 10^6$ ), irrelevantly whether or not antibiotic pressure was added to the microbiota. It appeared that with antibiotic pressure the concentration of strain specific sequence was higher. This was an indication for positive selection of the spiked MDR strain, either for *E. coli* or *E. faecium*. To test significant differences in concentration between the conditions; only the strain (ST) and the strain with the antibiotic (++) was performed. An unpaired two-sided t-test comparing both condition for each time point. The results of the statistics are presented in Table 7 and the copy number of strain specific sequences per ml were significantly larger when antibiotic pressure was amplified after 24 h and 48 h respectively for *E. coli* but not for *E. faecium*.

**Table 7** – Statistical table from t-test, comparing spiked strain (ST) condition with spiked strain and antibiotic (++) condition, results in significant differences in copy number/ml.

Time	strain	c(+ST) <sup>1</sup>	c(++) <sup>1</sup>	statistic	df	p.adj.	signif.
7	<i>E.coli</i>	$10^6$	$10^5$	2.25	5.69	0.27	ns
24	<i>E.coli</i>	$10^{5.5}$	$10^7$	-16.52	5.45	0.001	***
48	<i>E.coli</i>	$10^5$	$10^{6.5}$	-7.27	2.68	0.04	*
7	<i>E.faecium</i>	$10^{4.5}$	$10^5$	-0.75	7.08	0.48	ns
24	<i>E.faecium</i>	$10^5$	$10^5$	-1.78	6.87	0.36	ns
48	<i>E.faecium</i>	$10^{4.5}$	$10^5$	-1.78	2.08	0.43	ns

<sup>1</sup> Values are approximate to real numbers

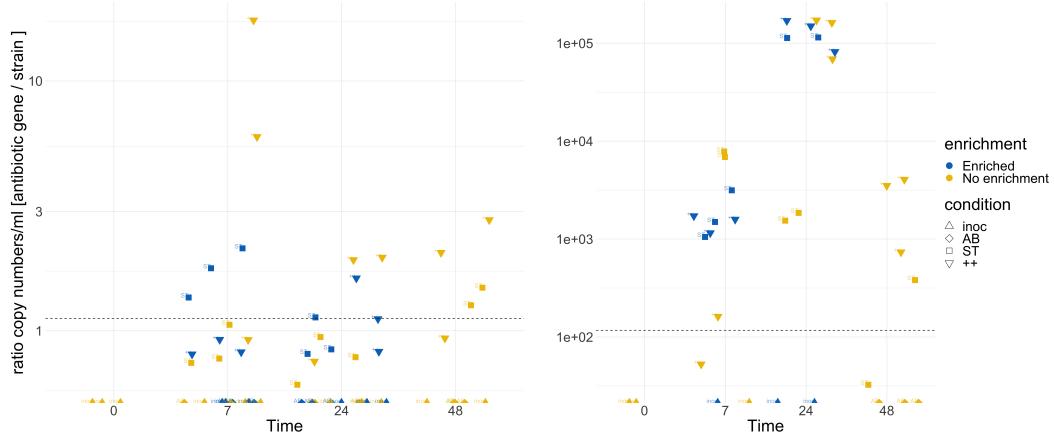
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### Ratio of AMR gene over strain specific sequence

The ratio, as described in subsection 3.4.2, are shown in Figure 9. The dashed line represents the ratio for a pure culture. The plasmid carrying the *vanA*-gene complex is a high copy number plasmid, therefore the plasmid to strain specific sequence ratio of 117:1 seems reasonable. For the *bla<sub>ctx-m1</sub>* plasmid, the ratio is  $\approx 1:1$  (1.12). For the conditions without MDR strains ((inoc) and (AB)) no values were detected (under the detection limit), resulting in no ratio. Whereas for the two other conditions there were higher ratios than would be expected in a pure culture. Since AMR gene sequence concentration was found in the resident microbiota (subsection 4.3.1) results may be intrigued.

Enriched samples, depicted with blue colour, has a different effects depending on time points and condition equalling without clear pattern. Interestingly for MDR *E.coli* (right picture), enrichment had a negative effect on ratio rate. One can imagine that the dilution effect during the enrichment step could be to great which results in a lower ratio than expected. In conclusion higher ratios indicated that possible HGT could have happened when spiking with *E.coli* or *E.faecium*, irrelevant wether antibiotic pressure is added.



**Figure 9** – AMR gene concentration over strain specific sequence concentration for *E.coli* (left) and *E.faecium* (right) indicates possible HGT in the condition were the MDR strains were added.

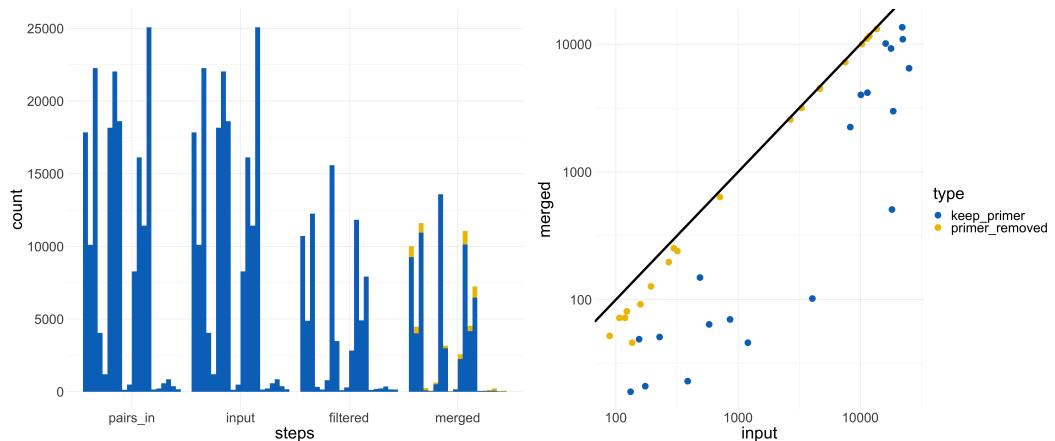
### 4.4 Metabarcoding

The sequence depth (i.e. read size of the sequence) of different DNA samples were evaluated. When sorted by size, a certain gap in read size between sample 11 with

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625 dp and sample 12 with 2574 dp was discovered. Up to and with sample 11 are categorised as low read size samples. As it is important to address low read size samples, a different pipeline was used to process the rawdata. The trimming algorithm in this pipeline had been removed. As removing the primer before proccesing results in a lower read size, this allowed the pipeline to process low read size samples. The two pipelines were compared and the read size was checked. The read size distribution in each step of the pipeline is depicted in the left graph in Figure 10. Samples were processed from left to right accordingly. The right graph compares the read sizes of samples before going into a pipeline and afterwards. The line shows hypothetical samples without loss of read size during the process. The samples closer to the line have previously been processed by the trimming algorithms (yellow points). The sequences may be larger with the primer to begin with, but they are certainly smaller after all processing steps (blue points.)

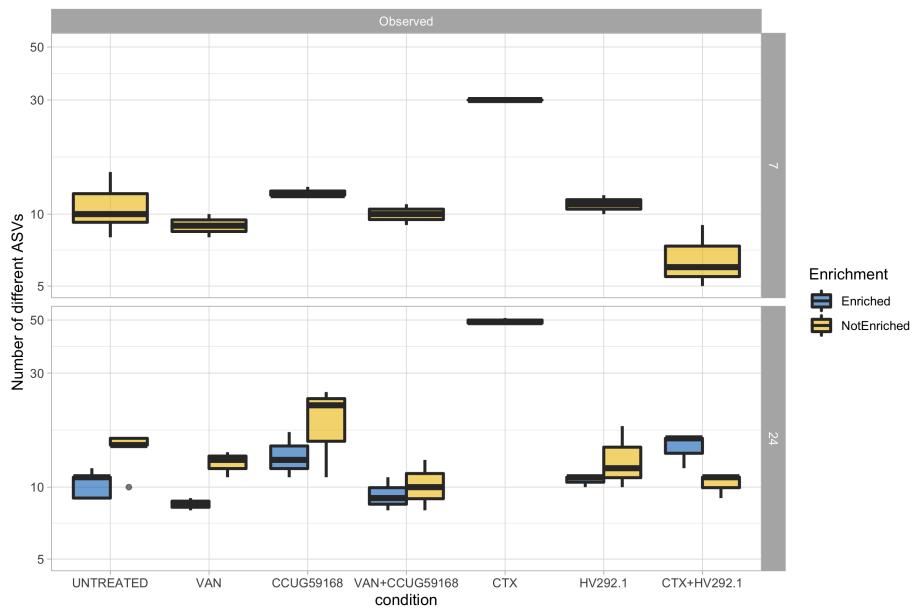


**Figure 10** – More reads are passed without trimming the primers (left), but when primers are trimmed after the filtering and merging step a higher percentage of reads are passed to downstream analysis. Most of the samples are smaller in size when the pipeline is used without the primmer trimming algorithm (right)

Number of observed ASVs and the different size of the reads were compared with each other by examination of their rarefaction curves (not shown.) As the curve hits the horizontal, a larger read size did not offer anymore information in form of additional ASVs. Thus all samples can be reduced to the same size in order to carry out further investigations. Before, un-rarefied samples were confronted with rarefied samples to find outliner of the general assumption which imply that same information is extracted from both sample types. Since this was not the case, all samples were then rarefied to read size 2'574 and passed downstream for further analysis

#### 4.4.1 Diversity analysis in the community

Observed alpha-diversity ( $\alpha$ -diversity) is the mean species diversity in the samples. It is calculated by a weighted generalised mean of the ASVs proportional abundances. Despite the overall value being low as shown in Figure 11 for enriched and non enriched samples, all  $\alpha$ -diversity indices differed significantly (See supplementary material, Table 14) between the conditions. The reason for enrichment was to give the new recipients of AMR genes more time and space in order to overgrow the resident microbiota. Therefore a lower variation in community structure was feasible. This was detectable in most conditions. The community diversity was slightly higher after 24 h than in the beginning of the batch fermentation. Highest observed diversity was found within the antibiotic CTX treated group with 50 different ASVs. Nevertheless the observed diversity at time point 0 h (not shown) was significantly higher in all conditions with 60 different observable ASVs. Therefore diversity dropped after the batch fermentation started and was unable to recover.



**Figure 11** – Lowest observed  $\alpha$ -diversity of different ASVs was found if CTX and the MDR *E. coli* was added. When only CTX was applied to the microbiota, highest diversity was found. This indicated an intrinsic resistance against CTX in the resident community

For the beta-diversity ( $\beta$ -diversity) which explains the difference between the communities, an ordination plot was created in order to visualise the variation (Figure 16 in Supplementary material.) Each dot is a sample and the shape of the dots encodes the time. Seemingly with the start of batch fermentation a different community struc-

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ture was soon established. This is in consent with the findings from the  $\alpha$ -diversity. Table 8 shows the different possible influencing factors. The residuals represent the non-explained variations by the factors. This was 18 % for the given statistical setup. The different conditions were responsible for 72 % of the overall variation, whereas time points only had a small effect. The combination of both factors were significant ( $P < 0.01$ ) meaning that each treatment had a different effect on depending it's time point.

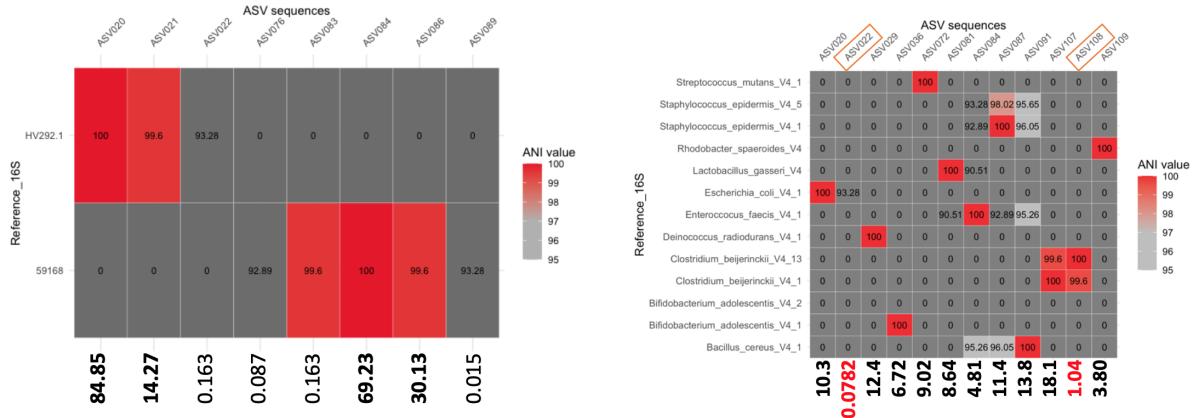
**Table 8** – The factors "condition" and "time points" had a highly significant influence ( $P < 0.001$ ) on variation. Together they were able to explain 71 % of all variations. Significant differences were identified using PERMANOVA

factor	df	sums of sqs	mean sqs	R <sup>2</sup>	pr.f.
condition	6	3,568.82	594.80	0.54	< 0.001
time points	2	1,148.35	574.17	0.17	< 0.001
Treatment:time points	6	367.27	61.21	0.06	0.002
Residuals	52	1,501.57	28.88	0.23	
Total	66	6,586.01		1	

In a next step, ASVs were examined in more detail. In order to validate the functionality of the DADA2 pipeline, a positive control was sequenced and assigned as well. The mock community ATCC® MSA-1000™ with evenly distributed strain abundances were used as the mock community. From those strains, the V4 regions were extracted and used as a reference. In the 16S rRNA sequences, the majority of the V4 regions were identical for each strain. Two unique V4 regions were found particularly in: *Clostridium beijerinckii* (ATCC 35702), *Staphylococcus epidermidis* (ATCC 12228) and *Bifidobacterium adolescentis* (ATCC 15703). With an adapted R script by Philipp v. Bieberstein, sequences were aligned and the average percent identity (ANI) between the ASVs from the pipeline and the V4 regions of the reference sequences were calculated (Figure 12). A unique ASV (ANI coverage 100 %) for each V4 region was detected. For the second V4 region of *Bifidobacterium adolescentis* no ASV sequence was found and therefore excluded. The second V4 region of *Staphylococcus epidermidis* was assigned to the same ASV as the first V4 region, thus it was excluded as well. The two ASVs which could not be assigned for only one V4 region were excluded (orange boxes) and not used for further analysis. In the same manner, two unique ASVs for the spiked MDR strains were defined (left figure). The chosen strain specific ASVs for MDR *E. faecium* and MDR *E. coli* are in relative abundance of 1:3 and  $\approx 1:9$ , respectively. The ASV083 is strain specific in the same magnitude

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as the ASV086 but was in such low abundance that it had no biological meaning. Therefore ASV083 was not used for further strain specific evaluations.



**Figure 12** – The ANI coverage higher than 90% are displayed for the MDR strains (left) and for the mock community (right). Relative abundances are in letters under the heatmap. The excluded ASV are highlighted (red.) Unique ASV are in bold black letters

The unique ASVs were able to reflect the relative abundance of the different strains in the mock community. Less than half the expected value (4.81 % and 3.80 % than 10 % each) of relative abundance was found for ASV084 (unique for *Enterococcus faecalis*) and ASV109 (unique for *Rhodobacter sphaeroides*.) This issue was not further discussed.

### 4.4.2 Characterisation of the changes of chicken bacterial community

The shift of the relative ASV abundances in the community during the batch fermentation was investigated and associated with the different treatments. The full data is visualised on two heatmaps. All samples were shown in order to draw conclusion that they are reproducible (Supplementary material, Figures in section 8.) The heatmaps are sorted by relative abundance size in a descending order and grouped by condition and time points. Most abundant ASV was the ASV020, which is assigned to *Escherichia-Shigella* ssp.. When the batch fermentation started, a reduced variability of ASVs were found, irrelevant of the condition. If the community was treated with CTX, a reverse shift to more variability was observed. Compared to the resident microbiota, members of the *Ruminococcaceae* family showed higher relative abundances (+ 11.5 %) and the ASV022 (*Proteus* ssp.) was more abundant (+18 %.) It seemed that with antibiotic treatment space or resources were cleared for other variants to

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grow, resulting in higher ASV diversity. Most common ASVs had the highest relative abundance measured in the condition where microbiota was treated with CTX and spiked with the MDR strain. This favours the assumption of selective pressure by the antibiotic and subsequent domination of the MDR *E. coli* strain over the community. The most abundant ASV, assigned as *Enterococcus* ssp., declined after vancomycin was added. Thus it seemed that vancomycin was highly effective against *Enterococcus* ssp.. When the MDR *E. faecium* strain was added to the vancomycin treated group, the relative abundance of the strain specific ASVs (ASV084 and ASV086) recovered. The behaviour of the strain-specific ASVs was further investigated.

### Exploration of possible interspecies HGT in *Enterococcus* ssp. and *Escherichia-Shigella* ssp.

If there's a higher relative ASV abundance in the antibiotic treated condition in which the MDR strain was spiked compared to all other conditions, it is hypothesised to be an indicator of possible HGT from the MDR strain to the resident microbiota. This pattern was observable only in the ASVs which seemed to be strain-specific (see figures in section 8). To enlarge the resolution, the second strain specific ASV, as described in Figure 12, was taken into account (i.e. ASV021 and ASV086). Since the second ASV appeared only if the MDR strain was actually added, it can be argued that the rel. ASV abundance of the second ASV is directly proportional to the rel. MDR strain abundance. Table 9 shows the average relative abundance of both strain specific ASVs at time point 24 h for the condition; inoculum (UNTREATED), antibiotic added and antibiotic + strain added. In order to quantify possible HGT, the author proposed the following steps; Average relative abundance of the second ASV is subtracted from the first ASV and adjusted by the fraction present in the condition where only the antibiotic was added. With the experimental limitations taken into account, this represents the resistant community without the MDR spiked strain. The resulting fraction is called the "new resistant community" in Table 9. When compared to the intrinsic resistant community (i.e. community treated with antibiotic only) the "new resistant community fraction" is larger in *E. faecium* (+9.1 %) and in *E. coli* (+18.5 %.) This implied a possible HGT from the spiked MDR strain into the ASV020 group (*Enterococcus* ssp.) as well as into the ASV084 group (*Escherichia-Shigella* ssp.)

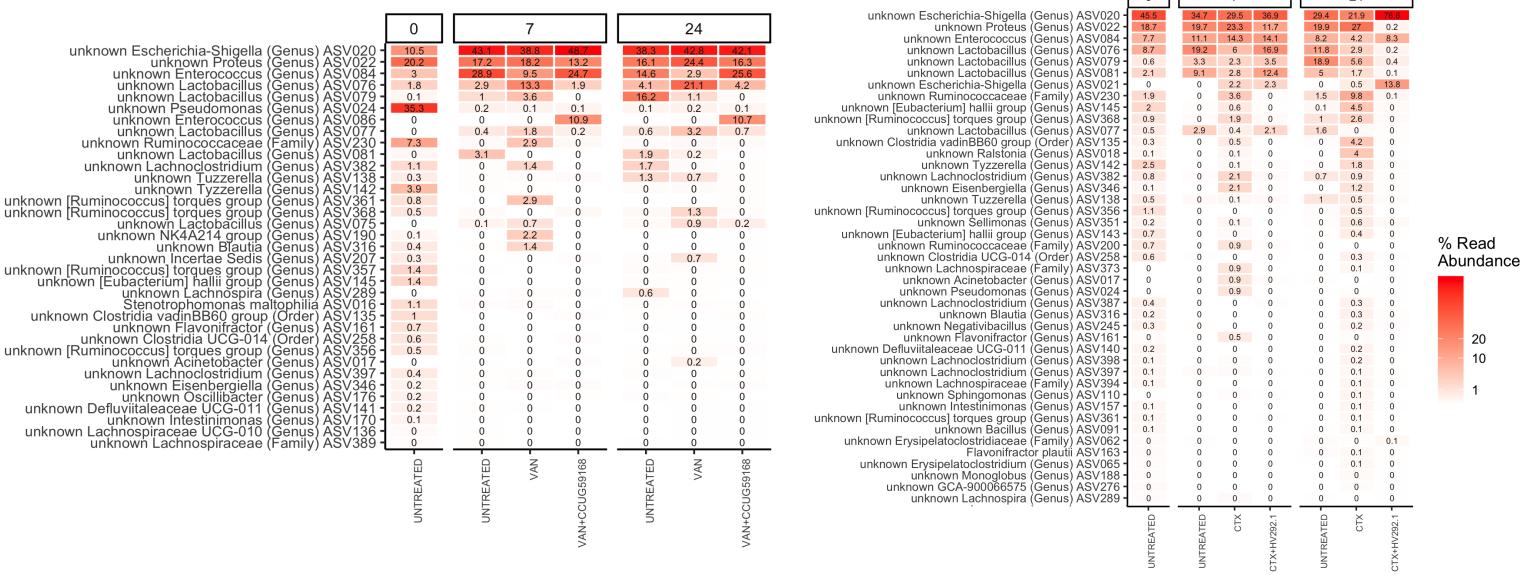
The numbers used for the calculation are depicted in Figure 13. As it remained unclear whether the second strain specific ASV would not only be an artefact from the sequencing without biological meaning or that other biases between the conditions would be present during batch fermentation, the author refused to confirm any conclusions derived from the "new resistant community". The possibility to quantify HGT is described and discussed further in the next chapter.

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**Table 9** – The "new resistant community" after 24 h is derived by the first specific ASV minus the second specific ASV and any relative abundances in the condition which is treated with antibiotic only (+ antibiotic.) The baseline (inoc) shows no value in the second strain specific ASV, strengthening the assumption of MDR strain specificity

ASV name	average rel. abundance [%]		
	inoc	+ antibiotic	+ (antibiotic + strain)
<i>Enterococcus</i> ssp. ASV084	14.6	2.9	25.6
<i>Enterococcus</i> ssp. ASV086	0	0	10.7
"new resistant community"	<b>12.0</b>		
<i>Escherichia-Shigella</i> ssp. ASV020	29.4	21.9	76.6
<i>Escherichia-Shigella</i> ssp. ASV021	0	0.5	13.8
"new resistant community"	<b>40.4</b>		



**Figure 13** – The batch fermentation favoured the fast growers (i.e. *Escherichia-Shigella* ssp. ASV020) after 24 h. When vancomycin was added *Enterococcus* ssp. (ASV084) was reduced (left picture). Treatment with cefotaxime (CTX) was less effective on its target community (*Escherichia-Shigella* ssp. ASV020). Overall taxonomic richness is highest in the baseline (UNTREATED) group

## 5 Discussion

AMR in human pathogens is a threat to modern health care and, thus representing one of the main social challenges for mankind. Some estimations suggest that around 700'000 deaths per year are associated with AMR bacteria at present, while there are predictions of up to 10 million deaths per year by 2050 (O'Neill, 2016). With the decreasing cost per sequenced base, together with the increasing availability of sequencing platforms and bioinformatics pipelines the picture becomes even more threatening because AMR genes are ubiquitous present in a range of environments (Tong et al., 2017, Shang et al., 2018, Sabino et al., 2019, Baumgartner et al., 2020). Highly transferable AMR genes from MDR bacteria, such as VRE and ESBL *E. coli*, are rapidly circulating within the chicken GIT microbiota. The rise of MDR strains in livestock is coupled with the increasing consumption of antibiotic in sub-lethal doses for disease prevention. Nevertheless, there is a lacking understanding of the spread of the AMR genes within the environments and the potential effect of selective pressure by the antibiotic treatments. Here, the spread of AMR genes within a commensal chicken microbiota was examined *in vitro* using a representative microbiota derived from a chicken cecum and two characterised MDR strains harbouring their AMR genes on mobile genetic elements. With the extracted genomic data, the colonisation of the MDR strains and the dissemination of their AMR genes were tracked *in vitro* in a repeated batch fermentation experiment.

### MDR strains harbouring their AMR genes on conjugative plasmids

The molecular and phenotypical characterisation of the antimicrobial resistance profile before starting the batch fermentation had its own importance. It's crucial to define MIC of AMR strains to distinguish between WT population covering several antibiotic resistances and clinical relevant human pathogens. The latter causing severe infections and needs to be monitored in order to react rapidly in case of an outbreak (Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO, 2018, Cassini et al., 2019). Using the whole genome information, it's possible to discover novel resistance variants and can serve as a basis for predicting resistant phenotypes. However, it must be mentioned that firstly AMR genes only do not represent the full potential of a bacteria to combat antibiotics. For example, bacteria usually lack the ability to absorb folate from the environment and

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as such require *de novo* folate synthesis in order to produce nucleic acids. The antibiotic combination trimethoprim-sulfamethoxazole inhibits two sequential steps in the tetrahydrofolate synthesis pathway, thereby inhibiting folate synthesis and synergistically killing a broad spectrum of bacterial species. Enterococci are unusual in that they can absorb folic acid from the environment, bypassing the effects of trimethoprim-sulfamethoxazole (Hollenbeck and Rice, 2012). Other intrinsic resistances are the cephalosporin resistance in *E. faecium* which is caused by poor affinity of cephalosporins for enterococcal PBPs. Or vancomycin resistance in all gram-negative bacteria due to the sterical hindrance of the target by the peptidoglycan layer (Chen et al., 2009, Wencewicz and Walsh, 2016a). Secondly, the detection of a homologous sequence to a known AMR gene does not necessarily imply an equivalent phenotypical resistance (i.e. false positive annotation) (Martínez et al., 2015b, Alcock et al., 2020). Currently, the gold standard for detecting AMR is via culturing (Feldgarden et al., 2019). For this reason, disk diffusion tests and SAST-S were performed for a representative strain and compared with the *in silico* AMR gene screening. As both methods were consistent by expressing high vancomycin resistances in the *E. faecium* strain and high 3<sup>rd</sup>-generation celaphorin resistances in the *E. coli* strain, both are truly MDR strains. The VRE and the ESBL *E. coli* are relevant human pathogens, challenges modern health care systems (O'Neill, 2016, Tacconelli et al., 2018). Since the project its main emphasis is on the spread of AMR genes, the highly mobile genetic elements in MDR strains were from particular interest. Conjugative plasmids are, along with phages, the key vectors of HGT (Smillie et al., 2010). They replicate autonomously from the bacterial chromosome and often encode for genes involved in detoxication, virulence, ecological interactions, and antibiotic resistance (Carattoli, 2009, Smillie et al., 2010, Melnyk et al., 2015). Whole genome assemblies of the MDR *E. faecium* strains were screened for plasmids using BLAST homologous alignment and rapid genome annotation tools (Seemann, 2014, Carver et al., 2008). Highly (< 99%) similar regions were found between the conjugative plasmid pVEF1 and a contig (contig40) of the MDR *E. faecium* strain. The ESBL encoding gene *bla<sub>ctx-m1</sub>* was found on a Incl1-like plasmid, which is also known as a highly persistent conjugative plasmid (Adamczyk and Jagura-Burdzy, 2003, Zurfluh et al., 2014). Therefore, tracking of the AMR was limited to the AMR genes which were located on mobile genetic elements. In this context the *vanA*-gene complex in VRE and the *bla<sub>ctx-m1</sub>* in ESBL *E. coli*. In the latter MDR strain, Zurfluh et al. (2014) found also the *sul1* gene on the same plasmid. The gene encodes for a sulfonamide resistance (i.e. sulphafurazole). The *sul1* gene was not tracked by qPCR method, though sulphafurazole-resistant strains are common in urinary tract infection (UTI) (Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO, 2018). Since the WHO stated the VRE and ESBL *E. coli* as the highest threat to human health, the focus in this project was on genes which causes those resistances in the MDR strains only (Tacconelli et al., 2018).

### Tracking the performance of MDR strains and their relevant AMR genes in the batch fermentation by qPCR

The qPCR data showed robust results over time, thus were able to track AMR genes and the spiked MDR strains. The strains were tracked soon after they were added in the batch fermentation as well as their corresponding AMR genes. Interestingly, the *vanA*-gene complex as well as the *bla<sub>ctx-m1</sub>* gene, though the latter later in the experiment, was traceable also when no strain was added. Thus, it could have been present in the resident microbiota from the beginning. This finding is consistent with the state of knowledge, as AMR genes are commonly detected in chicken GIT microbiota (Card et al., 2017, Oladeinde et al., 2019, Álvarez-Molina et al., 2021). Since the plasmid in VRE is a high copy number plasmid, it is present in multiple copies in one bacteria. Thus they are detected in high numbers and may have reached the detection limit of the qPCR approach earlier than the *bla<sub>ctx-m1</sub>* harbouring plasmid. It was hypothesised that by tracking the overall ratio of AMR gene concentration over bacteria concentration, HGT could be quantified. A ratio derived from the batch fermentation which is higher than from a pure culture of the respective strain, demonstrates more AMR genes in the microbiota than expected. It's hypothesised that more AMR genes are produced because the plasmid which harbours the AMR gene was spread in the microbiota through HGT. A higher ratio was seen in the condition where the MDR strains were added into the resident microbiota. As the AMR gene were present from the beginning on, it questions the possibility of tracking HGT using the qPCR data. Thus, it remains unclear if the higher ratio is due to HGT (i.e. new recipients producing the AMR gene) or other reasons, such as a positive selection for resistant bacteria or over-expression due to antibiotic pressure.

The MDR strains were traceable by qPCR after a short time in the resident microbiota and remained at high level ( $10^6$  copy numbers/ml) until the end of the experiment (48 h). MDR strains were detected in a highly competitive environment even without selective antibiotic pressure. The results suggest that the MDR strains were able to colonise without antibiotic pressure in a resident chicken GIT microbiota *in vitro*. In a continuous fermentation experiment with the PolyFermS model (not published), same representative chicken cecum microbiota and MDR strains were used and their findings were consistent. Therefore it can be assumed that carrying a conjugative plasmid encoding for AMR does not have negative effect on overall fitness and MDR strains can persist in the chicken GIT microbiota. The same finding is pointed out by Zurfluh et al. (2014), which studied the same MDR *E. coli* strain (HV292.1) intensively. In their project, harboring the *bla<sub>ctx-m1</sub>* gene showed no or negligible fitness cost and the plasmids also persisted in the absence of antibiotic pressure. This is supported by a systematic review of Melnyk et al. (2015) which have evaluated overall growth and AMR genes. They concluded that there's no negative relationship between expressing AMR in an environment without antibiotic pressure and fitness.

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Theres evidence that in a highly competitive environment, such as the chicken GIT, plasmid-encoded elements are beneficial to the Enterobacteriaceae for invasion and adhesion (Carattoli, 2009). The qPCR data revealed that the concentration of the MDR strain *E. coli* (copy number/ ml) was significantly higher when microbiota was treated with 20 µg/ml SXT/cefotaxime. This finding suggest that positive selection for the MDR strain occurred only for *E. coli* but not for *E. faecium*. As stated in subsection 2.3.1, *E. coli* species isolated from swiss flocks were commonly resistant against the chosen antibiotic combination cefotaxime and sulfametaxotzahol (Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO, 2018). The positive selection of MDR *E. faecium* may have happened but could not be observed in the qPCR data. Therefore, whole community analysis with the metabarcoding approach has to be considered as well.

### Investigation of microbial community changes with 16S rRNA analysis

The result of the 16S rRNA analysis (metabarcoding approach), has to be evaluated before interpretation can occur. The raw amplicon sequences from the metabarcoding approach have to be analysed into meaningful biological information (i.e. tables of ASVs counts). Bioinformatic pipelines, such as the DADA2 (used in this project), attempt to reconstruct the exact biological sequences present in the sample and thus have per definition biological meaning (Callahan et al., 2016). In a recently published study by Prodan et al. (2020), the DADA2 pipeline showed the best sensitivity and resolution for studies focused on differentiating closely related strains. However, since the microbial profiling of samples is done by amplification and pyrosequencing of the 16S rRNA , it has some limits when resolution on species- or even strain-level is required. The distinctions between highly related species, *E. coli* and *Shigella* spp. are sharing almost identical 16S rRNA gene sequences, remains a difficult undertaking (Stanley et al., 2013). Cultivation-independent metagenomic approaches overcome this issue by fragmentation and subsequent sequencing, assembly and annotation of the total genomic DNA isolated from a given sample (e.g., a food or a clinical origin). Also known as shotgun metagenomics, it allows information on its entire (prokaryotic and eukaryotic) gene content to be elucidated. This approach is able to reconstruct strain- or species-level information from mixed bacterial cultures (Álvarez-Molina et al., 2021). Thus, to monitor changes in the microbiota of a given environment on strain-level, shotgun metagenomics should be to be carried out. Nevertheless, conclusions on a genus level can be drawn from the metabarcoding data. When batch fermentation started, an impressive shift of the community structure was detected. It can be argued that the dilutive effect, only 1% of microbiota was spiked into sterile batch fermentation media, offered space and reduced competition. Since it favoured the "fast growers" (mainly *Escherichia-shigella* ssp.) in the bacteria kingdom, the dilutive effect may have been the main driver for the observed complexity

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losses. The loss of taxonomic richness was also monitored when antibiotic pressure was applied to the resident microbiota and it did not recovered during the batch fermentation. This indicated a selection for dominant strains. This finding is consistent with the result from the qPCR data, which imply a selective selection for the MDR strains. The metabarcoding data strengthened the results from above. As the ASVs for *Escherichia-Shigella* ssp. are more abundant when the antibiotic cefotaxime (abbreviated with CTX in the heatmaps, see Figure 13 or section 8 ) is applied, positive selection for MDR strain could happened. The positive selection for *Enterococcus* ssp. group can be observed as well. Interestingly, in the cefotaxime treated group the diversity is higher than in the corresponding untreated group. Especially members of the *Enterobacteriaceae* family (i.e *Escherichia-Shigella* ssp., *Proteus* ssp.) showed higher rel. abundances (See Supplementary material, section 8). As being resistant to one or more  $\beta$ -lactam antibiotic is evolutionary widely distributed (i.e. non-target PBPs,  $\beta$ -lactamase or ESBL), this could explain that other ASVs appears at lower rel. abundances (Bradford, 2001, Davies and Davies, 2010, Hollenbeck and Rice, 2012, Van Hoek et al., 2011, Olivares et al., 2013).

If addressing the quantification of HGT, it adds a layer of complexity. In the case of conjugation it needs a transfer of a conjugative plasmid harbouring the AMR gene. Therefore, to prove HGT the host-plasmid association has to be detectable. Since metabarcoding or metagenomic approach disconnects plasmid and chromosomes DNA, this is not possible. Recently, proximity-ligation methods, such as Hi-C method, overcome this issue by linkage the DNA molecules with their original bacteria cell within a microbial community (Stalder et al., 2019). Nevertheless, it is impressive to state that with the metabarcoding approach, possible HGT was explored. It was done by comparing the communities of the different conditions in the batch fermentation. When the resident microbiota was spiked with the MDR strains, new ASVs were detected. This ASVs were in the same group as the MDR strains and were not detected in the metabarcoding approach when no MDR strain was added. It can be hypothesised that the ASVs were strain-specific. The ANI coverage calculation of the strain specific ASVs candidates with the 16S rRNA sequence of the MDR strains revealed high similarities (ASV021 for *E. coli* and ASV086 for *E. faecium* with each 99.6 % coverage). This strengthened the assumption it is strain specific. When the rel. abundance of the *E. faecium* strain specific ASV was subtracted from the relative abundance of the second *E. faecium* ssp. assigned ASV, the remaining fraction is an additional group of *E. faecium* ssp. Since the bacteria in the group were able to grow with antibiotic pressure applied, this group was called "new resistant community" by the author. It is hypothesised that they were able to grow due to acquisition of the *vanA*-gene complex by conjugation. Since metabarcoding approach is not practicable for strain resolution findings, this pushes the application to its limit. Further, it is questionable whether the bacteria higher growth rate has its origin in the HGT of the AMR gene harbouring plasmid. The causal relationship between higher relative abundance of the ASVs and HGT has not been validated

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yet. Therefore a cultivation-independent metagenomic approach without loosing the host-plasmid information (Hi-C) must be carried. This allows the identification HGT from a MDR strain to new recipients. Thus, it is a more suitable approach to monitor HGT directly in a chicken GIT microbiota rather than the metabarcoding only.

### **Chicken gut microbiome and its role as vector for transmission of plasmid-encoded AMR genes to human pathogenic bacteria**

An opinion article by Martínez et al. (2015b) placed the request to be more cautious by the assessment of risks when dealing with AMR genes. They proposed bottleneck effects (ecological connectivity, founder effect and fitness costs) drastically limits the transfer of AMR genes from environmental samples to clinic relevant human pathogens. As biological relevance of fitness costs due to acquisition of AMR genes is at least questionable, it led to a stimulated discussion (Bengtsson-Palme and Larsson, 2015, Martínez et al., 2015a). It has to be stated that if MDR strain is able to grow in an *in vitro* chicken cecum microbiota, it does not imply directly a health threat to humans. Nevertheless, the proposed ranking system classifies the resistance genes investigated in this study on the highest rank anyway (Martínez et al., 2015b). In a study by Ludden et al. (2019) whole genome assemblies from different origins were compared and in conclusion there was limited evidence that AMR pathogens associated with serious human infection had originated from livestock. Based on core genome and accessory genome analyses, the bacterial population were distinct. Identical AMR genes were found in livestock and human isolates, but there was limited overlap in the mobile elements carrying these genes. On the other hand, a large majority of studies showed experimental evidence of HGT between different environments (Smillie et al., 2010, Davies and Davies, 2010, Haug et al., 2011a, Stecher et al., 2012, Abgottspón et al., 2014, Jiang et al., 2017, Lambrecht et al., 2019, Álvarez-Molina et al., 2021). What the findings imply is that with antibiotic pressure a larger fraction of the microbiome got resistant, which favours the possibility of HGT.

The overall findings from above pointing out the importance of a healthy and balanced gut microbiome. It has been identified as a key protective factor against AMR development (Kelly et al., 2020). Since ecological niches are occupied by commensal bacteria, AMR strains are more transient and temporarily present and thus HGT is reduced. As the results from this project imply, this can be significantly affected by antibiotic treatment. Adding a certain antibiotic pressure resulted in a reduction of taxonomic richness. Antibiotic mediated changes in the fecal microbiome of chickens were observed by Xiong et al. (2018) as well. Avoiding antibiotic exposure of the gut microbiota may help to reduce the enrichment for AMR genes and subsequently the emergence of AMR in chicken microbiome. Therefore, strategies of reducing the overall antibiotic use in clinical and agriculture setup has to be implemented to reduce

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further selection of MDR bacterial communities.

## 6 Conclusions

In conclusion, treatment with high antibiotic concentration in this experimental set up seems to promotes the colonisation of MDR bacteria in the resident cecum microbiota. The quantitative PCR showed significantly higher ( $10^7$  bacteria/ml) *E. coli* strain HV292.1 concentrations in the condition with antibiotic compared to the concentration in the condition without antibiotic ( $10^{5.5}$  bacteria/ml). When antibiotic pressure is applied, 16S rRNA analysis revealed that the relative abundances of the strain specific ASVs are highest in the batch fermentation after 24 h. Even no significant higher growth was measured in real time PCR for the MDR *E. faecium* strain, the metabarcoding analysis strengthen the assumption of positive selection when antibiotic is applied for both strains. As batch fermentation has its limitations, a continuous fermentation with the PolyFermS model was carried out with same microbial composition. The findings were consistent with the results found in this project. Overall as the projects studied effects of antibiotic treatment on resident chicken cecum microbiota *in vitro*, the next step may be a Randomised Controlled Trial with living organisms.

The investigations to quantify HGT in the batch fermentation have shown no conclusive evidence. First, as AMR genes were found *in vitro* in the resident chicken cecum microbiota, it questions the possibility to quantify HGT using the qPCR method. Next, as the microbiota profiling was done by 16S rRNA analysis, its resolution was too low to explore HGT directly. Remarkably, when different condition were compared, a strain-specific ASV may was found. Based on this assumption, possible interspecies HGT in *Escherichia-Shigella* ssp. and *Enterococcus* ssp. was discovered. A cultivation-independent metagenomic approach without loosing the host-plasmid information (Hi-C) must be carried out in order to allow the direct tracking of HGT in a complex chicken gut microbiota.

## 7 Acknowledgements

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# References

- Abgottspö, H., Stephan, R., Bagutti, C., Brodmann, P., Hächler, H., and Zurfluh, K. (2014). Characteristics of extended-spectrum cephalosporin-resistant *Escherichia coli* isolated from Swiss and imported poultry meat. *Journal of Food Protection*, 77(1):112–115.
- Adamczyk, M. and Jagura-Burdzy, G. (2003). Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochimica Polonica*, 50(2):425–453.
- Alcock, B. P., Raphenya, A. R., Lau, T. T., Tsang, K. K., Bouchard, M., Edalatmand, A., Huynh, W., Nguyen, A. L. V., Cheng, A. A., Liu, S., Min, S. Y., Miroshnichenko, A., Tran, H. K., Werfalli, R. E., Nasir, J. A., Oloni, M., Speicher, D. J., Florescu, A., Singh, B., Faltyn, M., Hernandez-Koutoucheva, A., Sharma, A. N., Bordeleau, E., Pawlowski, A. C., Zubyk, H. L., Dooley, D., Griffiths, E., Maguire, F., Winsor, G. L., Beiko, R. G., Brinkman, F. S., Hsiao, W. W., Domseelaar, G. V., and McArthur, A. G. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic acids research*, 48(D1):D517–D525.
- Álvarez-Molina, A., de Toro, M., Alexa, E. A., and Álvarez-Ordóñez, A. (2021). Applying Genomics to Track Antimicrobial Resistance in the Food Chain. *Comprehensive Foodomics*, 1:188–211.
- Bagal, V., Khatta, V., Tewatia, B., and Raut, S. (2016). Relative efficacy of organic acids and antibiotics as growth promoters in broiler chicken. *Veterinary World*, 9:377–382.
- Baumgartner, M., Bayer, F., Pfrunder-Cardozo, K. R., Buckling, A., and Hall, A. R. (2020). Resident microbial communities inhibit growth and antibiotic-resistance evolution of *Escherichia coli* in human gut microbiome samples. *PLoS Biology*, 18(4):1–30.
- Bengtsson-Palme, J. and Larsson, D. G. (2015). Antibiotic resistance genes in the environment: Prioritizing risks. *Nature Reviews Microbiology*, 13(6):396.
- Borda-Molina, D., Seifert, J., and Camarinha-Silva, A. (2018). Current Perspectives of the Chicken Gastrointestinal Tract and Its Microbiome. *Computational and Structural Biotechnology Journal*, 16:131–139.

## References

---

- Bradford, P. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical microbiology reviews*, 14(4):933–951.
- Brouwer, M. S. M., Bossers, A., Harders, F., van Essen-Zandbergen, A., Mevius, D. J., and Smith, H. E. (2014). Complete Genome Sequences of IncI1 Plasmids Carrying Extended-Spectrum  $\beta$ -Lactamase Genes. *Genome announcements*, 2(4):e00859–14.
- Bushby, S. R. and Hitchings, G. H. (1968). Trimethoprim, a sulphonamide potentiator. *British journal of pharmacology and chemotherapy*, 33(1):72–90.
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7):581–583.
- Carattoli, A. (2009). Resistance plasmid families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy*, 53(6):2227–2238.
- Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., and Hasman, H. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial agents and chemotherapy*, 58(7):3895–3903.
- Card, R. M., Cawthraw, S. A., Nunez-Garcia, J., Ellis, R. J., Kay, G., Pallen, M. J., Woodward, M. J., and Anjum, M. F. (2017). An in Vitro chicken gut model demonstrates transfer of a multidrug resistance plasmid from *Salmonella* to commensal *Escherichia coli*. *mBio*, 8(4):1–15.
- Carver, T., Berriman, M., Tivey, A., Patel, C., Böhme, U., Barrell, B. G., Parkhill, J., and Rajandream, M.-A. (2008). Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. *Bioinformatics*, 24(23):2672–2676.
- Cassini, A., Höglberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., Strauss, R., Mertens, K., Struyf, T., Catry, B., Latour, K., Ivanov, I. N., Dobrevá, E. G., Tambic Andraševic, A., Soprek, S., Budimir, A., Paphitou, N., Žemlicková, H., Schytte Olsen, S., Wolff Sönksen, U., Märtin, P., Ivanova, M., Lyytikäinen, O., Jalava, J., Coignard, B., Eckmanns, T., Abu Sin, M., Haller, S., Daikos, G. L., Gikas, A., Tsiodras, S., Kontopidou, F., Tóth, Á., Hajdu, Á., Guólaugsson, Ó., Kristinsson, K. G., Murchan, S., Burns, K., Pezzotti, P., Gagliotti, C., Dumpis, U., Liuimiene, A., Perrin, M., Borg, M. A., de Greeff, S. C., Monen, J. C., Koek, M. B., Elstrøm, P., Zabicka, D., Deptula, A., Hryniewicz, W., Caniça, M., Nogueira, P. J., Fernandes, P. A., Manageiro, V., Popescu, G. A., Serban, R. I., Schréterová, E., Litovová, S., Štefkovicová, M., Kolman, J., Klavs, I., Korošec, A., Aracil, B., Asensio,

## References

---

- A., Pérez-Vázquez, M., Billström, H., Larsson, S., Reilly, J. S., Johnson, A., and Hopkins, S. (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet Infectious Diseases*, 19(1):56–66.
- Chen, L. F., Chopra, T., and Kaye, K. S. (2009). Pathogens Resistant to Antibacterial Agents. *Infectious Disease Clinics of North America*, 23(4):817–845.
- Costa, M., Bessegatto, J. A., Alfieri, A., Weese, J., Barbosa Filho, J., and Oba, A. (2017). Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. *PLOS ONE*, 12:e0171642.
- Davies, J. and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, 74(3):417–433.
- Dcosta, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., and Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature*, 477(7365):457–461.
- Dibner, J. J. and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, 84(4):634–643.
- Dreier, M., Berthoud, H., Shani, N., Wechsler, D., and Junier, P. (2020). SpecieSPrimer: a bioinformatics pipeline dedicated to the design of qPCR primers for the quantification of bacterial species. *PeerJ*, 8:e8544.
- Dudley, M. N. and Barriere, S. L. (1982). Cefotaxime: microbiology, pharmacology, and clinical use. *Clinical pharmacy*, 1(2):114–124.
- Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R. (1947). Chloromycetin, a New Antibiotic From a Soil Actinomycete. *Science*, 106(2757):417 LP – 417.
- EU Parlament (2018). Verordnung (EU) 2019/ des Europäischen Parlaments und des Rates vom 11. Dezember 2018 über Tierärzneimittel und zur Aufhebung der Richtlinie.
- Federal Office of Public Health FOPH and Federal Food Safety and Veterinary Office FSVO (2018). Swiss Antibiotic Resistance Report 2018. Usage of antibiotics and occurrence of antibiotic resistance in bacteria from humans and animals in Switzerland. Technical report, Bundesamt für Gesundheit.
- Feldgarden, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., Tyson, G. H., Zhao, S., Hsu, C. H., McDermott, P. F., Tadesse, D. A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster, J. P., and Klimke, W. (2019). Validating the AMRFINDER tool and resistance gene database by using

## References

---

- antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrobial Agents and Chemotherapy*, 63(11):1–20.
- Fleming, A. (1945). Penicillin- Nobel Lecture.
- Forslund, K., Sunagawa, S., Kultima, J. R., Mende, D. R., Arumugam, M., Typas, A., and Bork, P. (2013). Country-specific antibiotic use practices impact the human gut resistome. *Genome Research*, 23(7):1163–1169.
- Gogarten, J. P., Doolittle, W. F., and Lawrence, J. G. (2002). Prokaryotic evolution in light of gene transfer. *Molecular Biology and Evolution*, 19(12):2226–2238.
- Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L., and Rolain, J.-M. (2014). ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrobial agents and chemotherapy*, 58(1):212–220.
- Haug, M. C., Tanner, S. A., Lacroix, C., Stevens, M. J., and Meile, L. (2011a). Monitoring horizontal antibiotic resistance gene transfer in a colonic fermentation model. *FEMS Microbiology Ecology*, 78(2):210–219.
- Haug, M. C., Tanner, S. A., Lacroix, C., Stevens, M. J., and Meile, L. (2011b). Monitoring horizontal antibiotic resistance gene transfer in a colonic fermentation model. *FEMS Microbiology Ecology*, 78(2):210–219.
- Hollenbeck, B. L. and Rice, L. B. (2012). Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*, 3(5):421–569.
- Hooper, L. V., Xu, J., Falk, P. G., Midtvedt, T., and Gordon, J. I. (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proceedings of the National Academy of Sciences*, 96(17):9833–9838.
- Jiang, X., Ellabaan, M. M., Charusanti, P., Munck, C., Blin, K., Tong, Y., Weber, T., Sommer, M. O., and Lee, S. Y. (2017). Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nature Communications*, 8:1–7.
- Kapoor, G., Saigal, S., and Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of anaesthesiology, clinical pharmacology*, 33(3):300–305.
- Kelly, S. A., Rodgers, A. M., O'Brien, S. C., Donnelly, R. F., and Gilmore, B. F. (2020). Gut Check Time: Antibiotic Delivery Strategies to Reduce Antimicrobial Resistance. *Trends in Biotechnology*, 38(4):447–462.

## References

---

- Lambrecht, E., Van Coillie, E., Van Meervenne, E., Boon, N., Heyndrickx, M., and Van de Wiele, T. (2019). Commensal *E. coli* rapidly transfer antibiotic resistance genes to human intestinal microbiota in the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME). *International Journal of Food Microbiology*, 311.
- Lesher, G. Y., Froelich, E. J., Gruett, M. D., Bailey, J. H., and Brundage, R. P. (1962). 1,8-Naphthyridine Derivatives. A New Class of Chemotherapeutic Agents. *Journal of Medicinal and Pharmaceutical Chemistry*, 5(5):1063–1065.
- Ludden, C., Raven, K. E., Jamrozy, D., Gouliouris, T., Blane, B., Coll, F., de Goffau, M., Naydenova, P., Horner, C., Hernandez-Garcia, J., Wood, P., Hadjirin, N., Radakovic, M., Brown, N. M., Holmes, M., Parkhill, J., and Peacock, S. J. (2019). One health genomic surveillance of *Escherichia coli* demonstrates distinct lineages and mobile genetic elements in isolates from humans versus livestock. *mBio*, 10(1).
- Marcel, M. (2011). Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. *EMBnet.journal*, 17(1):10–12.
- Martínez, J. L., Coque, T. M., and Baquero, F. (2015a). Prioritizing risks of antibiotic resistance genes in all metagenomes. *Nature Reviews Microbiology*, 13(6):396.
- Martínez, J. L., Coque, T. M., and Baquero, F. (2015b). What is a resistance gene? Ranking risk in resistomes. *Nature Reviews Microbiology*, 13(2):116–123.
- Mccormick, M. H., M, M. J., Pittenger, G. E., PIttenger, R. C., and Stark, W. M. (1955). Vancomycin, a new antibiotic. I. Chemical and biologic properties. *Antibiotics annual*, 3:606–611.
- Mehdi, Y., Létourneau-Montminy, M. P., Gaucher, M. L., Chorfi, Y., Suresh, G., Rouissi, T., Brar, S. K., Côté, C., Ramirez, A. A., and Godbout, S. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. *Animal Nutrition*, 4(2):170–178.
- Melnyk, A. H., Wong, A., and Kassen, R. (2015). The fitness costs of antibiotic resistance mutations. *Evolutionary Applications*, 8(3):273–283.
- Miller, W. R., Munita, J. M., and Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert Review of Anti-Infective Therapy*, 12(10):1221–1236.
- Munita, J. M., Arias, C. A., Unit, A. R., and Santiago, A. D. (2016). HHS Public Access Mechanisms of Antibiotic Resistance. *Microbiol Spectr.*, 4(2):1–37.
- Oladeinde, A., Cook, K., Lakin, S. M., Woyda, R., Abdo, Z., Looft, T., Herrington, K., Zock, G., Lawrence, J. P., Thomas, J. C., Beaudry, M. S., and Glenn, T. (2019). Horizontal Gene Transfer and Acquired Antibiotic Resistance in *Salmonella*

## References

---

- enterica Serovar Heidelberg following In Vitro Incubation in Broiler Ceca. *Applied and environmental microbiology*, 85(22).
- Olivares, J., Bernardini, A., Garcia-Leon, G., Corona, F., Sanchez, M. B., and Martinez, J. L. (2013). The intrinsic resistome of bacterial pathogens. *Frontiers in Microbiology*, 4(APR):1–15.
- O'Neill, J. (2016). Tackling drug-resistant infections globally. Technical report, Wellcome Trust, UK.
- Palmer, K. L., Kos, V. N., and Gilmore, M. S. (2010). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Current Opinion in Microbiology*, 13(5):632–639.
- Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A. H., Nieuwdorp, M., and Levin, E. (2020). Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS ONE*, 15(1):1–19.
- Ravenhall, M., Škunca, N., Lassalle, F., and Dessimoz, C. (2015). Inferring Horizontal Gene Transfer. *PLoS Computational Biology*, 11(5):1–16.
- Sabino, Y. N. V., Santana, M. F., Oyama, L. B., Santos, F. G., Moreira, A. J. S., Huws, S. A., and Mantovani, H. C. (2019). Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nature Communications*, 10(1).
- Sayers, E. W., Agarwala, R., Bolton, E. E., Brister, J. R., Canese, K., Clark, K., Connor, R., Fiorini, N., Funk, K., Hefferon, T., Holmes, J. B., Kim, S., Kimchi, A., Kitts, P. A., Lathrop, S., Lu, Z., Madden, T. L., Marchler-Bauer, A., Phan, L., Schneider, V. A., Schoch, C. L., Pruitt, K. D., and Ostell, J. (2019). Database resources of the National Center for Biotechnology Information. *Nucleic acids research*, 47(D1):D23–D28.
- Schatz, A. and Waksman, S. A. (1944). Effect of Streptomycin and Other Antibiotic Substances upon Mycobacterium tuberculosis and Related Organisms,. *Proceedings of the Society for Experimental Biology and Medicine*, 57(2):244–248.
- Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)*, 30(14):2068–2069.
- Shang, Y., Kumar, S., Oakley, B., and Kim, W. K. (2018). Chicken gut microbiota: Importance and detection technology. *Frontiers in Veterinary Science*, 5(254).
- Sletvold, H., Johnsen, P. J., Simonsen, G. S., Aasnæs, B., Sundsfjord, A., and Nielsen, K. M. (2007). Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway. *Antimicrobial Agents and Chemotherapy*, 51(2):736–739.

## References

---

- Smillie, C., Garcillan-Barcia, M. P., Francia, M. V., Rocha, E. P. C., and de la Cruz, F. (2010). Mobility of Plasmids. *Microbiology and Molecular Biology Reviews*, 74(3):434–452.
- Stalder, T., Press, M. O., Sullivan, S., Liachko, I., and Top, E. M. (2019). Linking the resistome and plasmidome to the microbiome. *ISME Journal*, 13(10):2437–2446.
- Stanley, D., Geier, M. S., Hughes, R. J., Denman, S. E., and Moore, R. J. (2013). Highly variable microbiota development in the chicken gastrointestinal tract. *PLoS ONE*, 8(12):6–12.
- Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M. J., Pickard, D. J., Barthel, M., Westendorf, A. M., Krogfelt, K. A., Walker, A. W., Ackermann, M., Dobrindt, U., Thomson, N. R., and Hardt, W. D. (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences of the United States of America*, 109(4):1269–1274.
- Stothard, P. and Wishart, D. S. (2005). Circular genome visualization and exploration using CGView. *Bioinformatics (Oxford, England)*, 21(4):537–539.
- Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, N., Theuretzbacher, U., Magrini, N., Aboderin, A. O., Al-Abri, S. S., Awang Jalil, N., Benzonana, N., Bhattacharya, S., Brink, A. J., Burkert, F. R., Cars, O., Cornaglia, G., Dyar, O. J., Friedrich, A. W., Gales, A. C., Gandra, S., Giske, C. G., Goff, D. A., Goossens, H., Gottlieb, T., Guzman Blanco, M., Hryniwicz, W., Kattula, D., Jinks, T., Kanj, S. S., Kerr, L., Kieny, M. P., Kim, Y. S., Kozlov, R. S., Labarca, J., Laxminarayan, R., Leder, K., Leibovici, L., Levy-Hara, G., Littman, J., Malhotra-Kumar, S., Manchanda, V., Moja, L., Ndoye, B., Pan, A., Paterson, D. L., Paul, M., Qiu, H., Ramon-Pardo, P., Rodríguez-Baño, J., Sanguinetti, M., Sengupta, S., Sharland, M., Si-Mehand, M., Silver, L. L., Song, W., Steinbakk, M., Thomsen, J., Thwaites, G. E., van der Meer, J. W., Van Kinh, N., Vega, S., Villegas, M. V., Wechsler-Fördös, A., Wertheim, H. F. L., Wesangula, E., Woodford, N., Yilmaz, F. O., and Zorzet, A. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, 18(3):318–327.
- Thomas, C. M. and Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology*, 3(9):711–721.
- Tong, P., Ji, X., Chen, L., Liu, J., Xu, L., Zhu, L., Zhou, W., Liu, G., Wang, S., Guo, X., Feng, S., and Sun, Y. (2017). Metagenome analysis of antibiotic resistance genes in fecal microbiota of chickens. *Agri Gene*, 5:1–6.

## References

---

- Uttley, A. H. C., Collins, C., Naidoo, J., and George, R. C. (1988). Vancomycin-resistant Enterococci. *The Lancet*, 331(8575-8576):57.
- Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., and Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America*, 112(18):5649–5654.
- Van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., and Aarts, H. J. (2011). Acquired antibiotic resistance genes: An overview. *Frontiers in Microbiology*, 2(SEP):1–27.
- Walther-Rasmussen, J. and Høiby, N. (2004). Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum  $\beta$ -lactamases. *Canadian Journal of Microbiology*, 50(3):137–165.
- Wan, Z., Varshavsky, J., Teegala, S., McLawrence, J., and Goddard, N. L. (2011). Measuring the rate of conjugal plasmid transfer in a bacterial population using quantitative PCR. *Biophysical Journal*, 101(1):237–244.
- Wencewicz, T. and Walsh, C. (2016a). Antibiotics That Block Peptidoglycan Assembly and Integrity. In *Antibiotics: Challenges, Mechanisms, Opportunities*, pages 68–100. American Society of Microbiology, Washington, DC.
- Wencewicz, T. and Walsh, C. (2016b). Assembly of the Peptidoglycan Layer of Bacterial Cell Walls. In *Antibiotics: Challenges, Mechanisms, Opportunities*, pages 36–67. American Society of Microbiology, Washington, DC.
- Witte, W., Klare, I., and Werner, G. (1999). Selective pressure by antibiotics as feed additives. *Infection*, 27(2):S35–S38.
- Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., Li, A., Zeng, Z., and Zhang, T. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. *Microbiome*, 6(1):1–11.
- Yang, Y., Xie, X., Tang, M., Liu, J., Tuo, H., Gu, J., Tang, Y., Lei, C., Wang, H., and Zhang, A. (2020). Exploring the profile of antimicrobial resistance genes harboring by bacteriophage in chicken feces. *Science of the Total Environment*, 700.
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M., and Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *The Journal of antimicrobial chemotherapy*, 67(11):2640–2644.
- Zeng, X. and Lin, J. (2017). Factors influencing horizontal gene transfer in the intestine. *Animal Health Research Reviews*, 18(2):153–159.

## References

---

- Zihler Berner, A., Fuentes, S., Dostal, A., Payne, A. N., Vazquez Gutierrez, P., Chassard, C., Grattepanche, F., de Vos, W. M., and Lacroix, C. (2013). Novel Polyfermentor intestinal model (PolyFermS) for controlled ecological studies: validation and effect of pH. *PLoS one*, 8(10):2–11.
- Zurfluh, K., Wang, J., Klumpp, J., Nüesch-Inderbinen, M., Fanning, S., and Stephan, R. (2014). Vertical transmission of highly similar blaCTX-M-1-harbouring IncI1 plasmids in Escherichia coli with different MLST types in the poultry production pyramid. *Frontiers in Microbiology*, 5(SEP):1–7.

# 8 Supplementary material

## Appendix 1: Macfarlane medium composition

**Table 10** – A adapted Macfarlane medium was used as chicken fermentation medium

components	in 2L	supplier
<b>Proteins</b>		
Yeast extract	10 g	Sigma-Aldrich / Merck
Beef extracts	4.8 g	Sigma-Aldrich
Tryptose	20 g	Sigma-Aldrich
L-Cysteine HCl	1.6 g	Sigma-Aldrich
Uric acid	1.4 g	Sigma
<b>Carbohydrates</b>		
Maltodextrin	5 g	Aldrich
Pectin from citrus peel	5 g	Sigma
Mucin from porcine stomach	4 g	Sigma
<b>Lipids</b>		
Bile salts	0.8 g	Oxoid
<b>Minerals</b>		
KH <sub>2</sub> PO <sub>4</sub>	1 g	VWR chemicals
NaHCO <sub>3</sub>	3 g	Fisher Scientific
NaCl	10 g	Sigma-Aldrich
KCl	9 g	Avantor performance materials
MgSO <sub>4</sub>	1.21 g	Sigma-Aldrich
CaCl <sub>2</sub> • 2 H <sub>2</sub> O	0.2 g	VWR chemicals
MnCl <sub>2</sub> • 4 H <sub>2</sub> O	0.4 g	Fluka
FeSO <sub>4</sub> • 7 H <sub>2</sub> O	0.001 g	Sigma-Aldrich
Tween 80	2 mL	Sigma-Aldrich
Hemin solution	2 mL	unknown
Fibrulose F97 chicory root fibre	5 g	Cosucra
Vitamin solution	2 mL	unknown

## Appendix 2: Designed primer pairs

**Table 11** – Primers for all specific strains, designed by PrimerBlast. Amplicon length was validated running PCR for each primer pair at 60 °C

Name	amplicon length [bp]	F/R primer (5' -> 3')
<b><i>E. coli</i> HV114.1</b>		
114N27-P1	325	F: TATAACCTCATCCGGGAGCG R: TGGTTAGCTGTTGCCGGTT
114N27-P2	330	F: AATCTATAGGCAGGAAGGC R: GGTGTCTTCGTGACCACTCTT
114N54-P2-F	579	F: GTCAGCGACAGTGAGTTGC R: CGGTGTAGCGGAAGATGACT
<b><i>E. coli</i> HV292.1</b>		
202Contig27-P1	473	F: CAGAAAAGCGTAAGGAGCCG R: CTCCAAGCATTGCTCGAT
<b><i>E. faecium</i> CCUG 59161</b>		
59161UN1-P1	473	F: ACGCTAACGCTGGTAAATGGA R: CAGGCCAACGTACAGGGTG
59161UN2-P1	360	F: ATGCCTCCGAATACCTAGAACTC R: CCCAGTTTGAGGACTTTTCCTG
59161UN4-P1	389	F: TGTTTAGCCCATTCACTTCGC R: GCTGACGCACAATTGGCAAG
<b><i>E. faecium</i> CCUG 59168</b>		
348Contig61-P1	472	F: AGTACGGAGACTGCTCGGAT R: GGTAGCCCGCAAAAGTCGTA
348Contig61-P2	714	F: GTTACGCCACGTTGTGTTGG R: GCCCGCAAAAGTCGTAACAT
348C61-2-P1	479	F: TAGCATTCCCTCCGTGCGCTTG R: TCCCCAATCATCATGTGCGT

### **Appendix 3: Disc Diffusion Test**

To validate the result of the *in silico* AMR gene screening using the CARD database, disc diffusion test was performed, using one representative strain for *E.coli* and *E.faecium*, respectively.

*Escherichia coli*

*Enterococcus faecium*

**Table 12** – *E.coli* strain HV292.1 found to be resistant against multiple antibiotic classes resulting in a MDR strain

antibiotic	EUCAST breakpoints		zone diameter [mm]	result
	S ≥	R <		
<b>Penicillins:</b>				
ampicillin	14	14	0	R
<b>Cephalosporins:</b>				
2nd generation				
cefoxitin	19	19	20	S
3rd generation				
cefotetan	20	17	27	S
cefpodoxime	21	21	0	R
ceftadizime	22	21	24	S
ceftriaxon	25	22	0	R
4th generation				
cefepime	27	24	22	R
<b>Carbapenems:</b>				
imipenem	50	17	30	I
<b>Monobactams:</b>				
aztreonam	26	21	18	R
<b>Fluoroquinolones:</b>				
ciprofloxacin	25	22	38	S
norfloxacin	24	22	32	S
<b>Aminoglycosides:</b>				
gentamicin	20	16	19	I
neomycin	21	12	14	I
<b>Glycopeptides:</b>				
vancomycin	-	-	0	R <sup>1</sup>
<b>Tetracycline:</b>				
tetracycline	18	18	1	R
<b>Miscellaneous agents:</b>				
chloramphenicol	17	17	25	S
SXT <sup>2</sup>	14	11	0	R

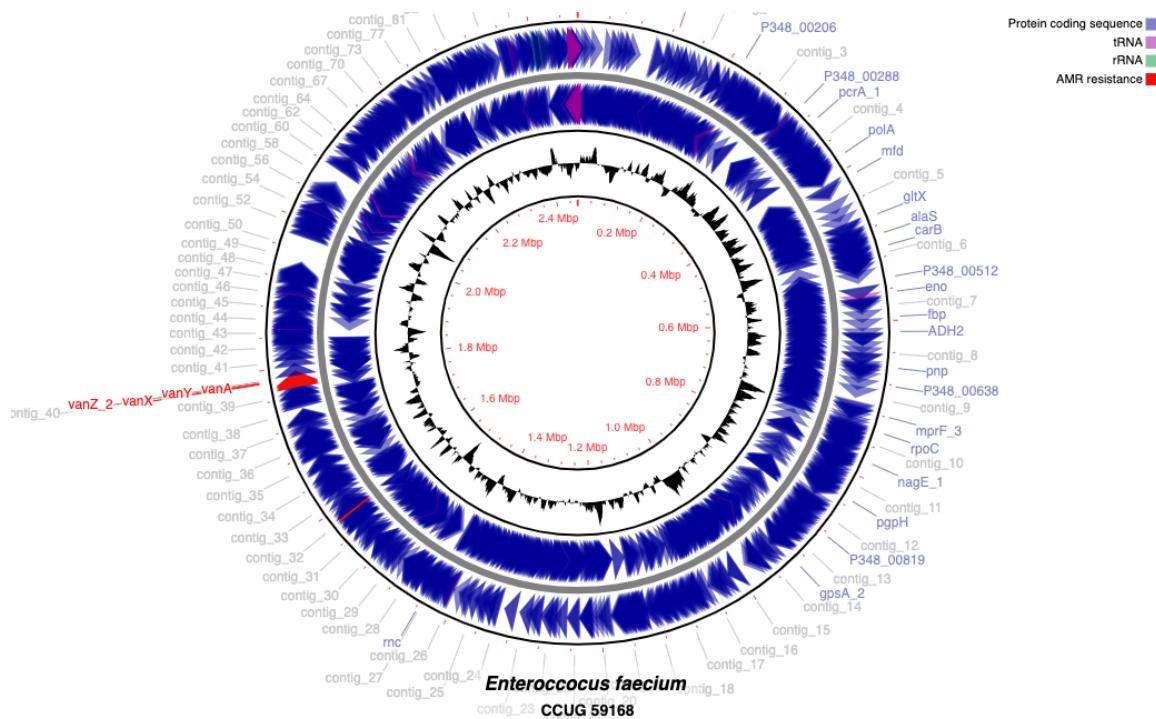
<sup>1</sup> Tested species is poor target for therapy with the antibiotic (i.e. intrinsically resistant)<sup>2</sup> trimethoprim-sulfamethoxazole

**Table 13** – *E.faecium* strain 56189 found to be resistant against multiple antibiotic classes, inclusive vancomycin and trimethoprim-sulfamethoxazole, results in a MDR strain

antibiotic	EUCAST breakpoints		zone diameter [mm]	result
	S ≥	R <		
<b>Penicillins:</b>				
ampicillin	10	8	23	S
<b>Cephalosporins:</b>				
2nd generation				
cefoxitin	-	-	8	R <sup>1</sup>
3rd generation				
cefotetan	-	-	0	R <sup>1</sup>
cefpodoxime	-	-	0	R <sup>1</sup>
ceftadizime	-	-	0	R <sup>1</sup>
ceftriaxon	-	-	24	R <sup>1</sup>
4th generation				
cefepime	-	-	22	R <sup>1</sup>
<b>Carbapenems:</b>				
imipenem	50	21	26	I
<b>Monobactams:</b>				
aztreonam	-	-	0	R <sup>1</sup>
<b>Fluoroquinolones:</b>				
ciprofloxacin	15	15	21	S
norfloxacin	12	12	20	S
<b>Aminoglycosides:</b>				
gentamicin	8	8	12	S
neomycin	-	-	0	R <sup>1</sup>
<b>Glycopeptides:</b>				
vancomycin	12	12	0	R
<b>Tetracycline:</b>				
tetracycline	-	-	0	R <sup>1</sup>
<b>Miscellaneous agents:</b>				
chloramphenicol	-	-	22	R <sup>1</sup>
SXT <sup>2</sup>	(23)	(23) <sup>3</sup>	0	R

<sup>1</sup> Tested species is poor target for therapy with the antibiotic (e.g. intrinsically resistant)<sup>2</sup> trimethoprim-sulfamethoxazole<sup>3</sup> *E.faecium* can absorb folic acid from the environment, bypassing the effects of SXT. When present, poor target for therapy with SXT

### Appendix 4: Whole genome of *Enterococcus faecium* strain 59168



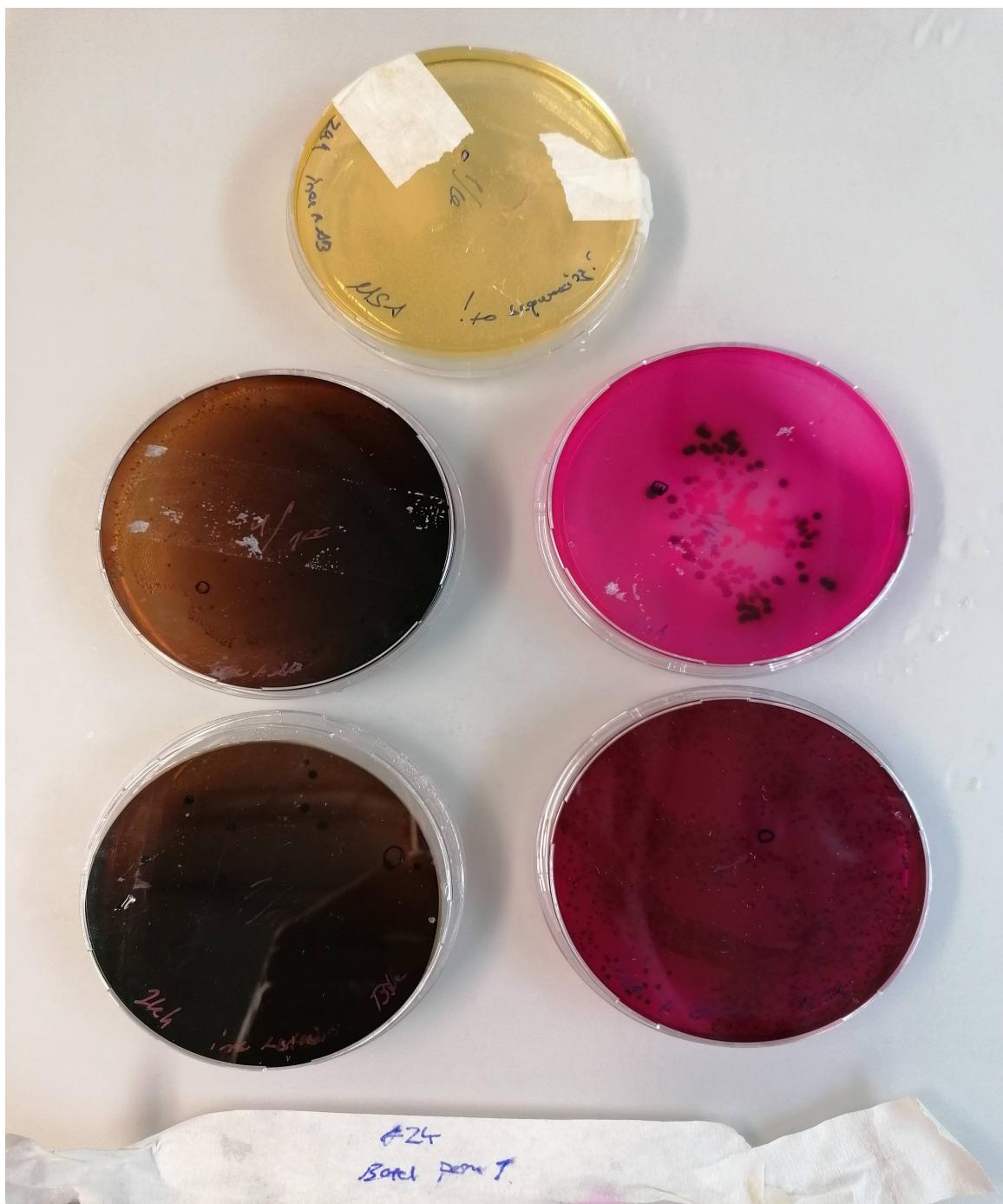
**Figure 14** – On the whole genome between 1.78 Mbp and 1.786 Mbp, the *vanA*-complex is encoded. The contig 40 of the genome is hypothesised to be a plasmid

### Appendix 5: Plating experiments with selective agar (next page)

### Appendix 6: Significant different $\alpha$ -diversity for each condition

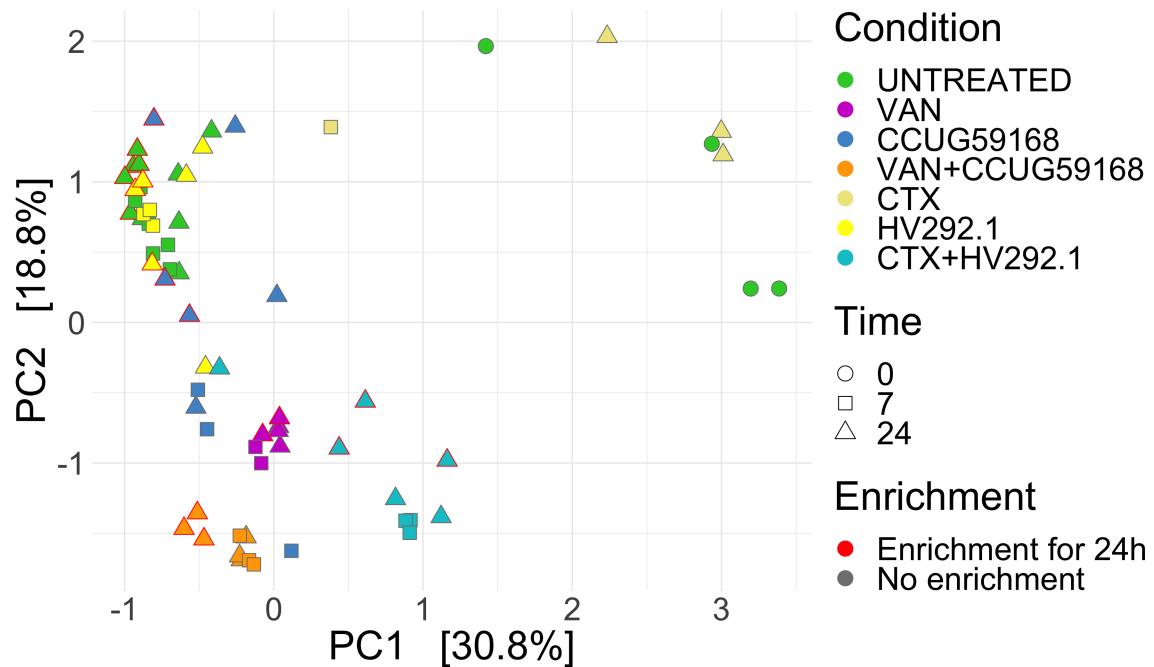
**Table 14** – Alpha-diversity is significant different between conditions in all diversity indices. Differences are calculated using ANOVA

alphadiversity	.y.	p	p.adj	p.format	p.signif
observed	value	<1e-16	0	<2e-16	****
diversity shannon	value	<1e-16	0	<2e-16	****
evenness pielou	value	1.10e-14	0	1.1e-14	****
diversity inverse simpson	value	1.67e-13	0	1.7e-13	****



**Figure 15** – The two red agar plates on the right are the Endo agar plates, on the left the two brownish coloured plates are with BEA and on the top it is a MSA plate

**Appendix 7: Beta-diversity ordination plot**



**Figure 16** – Biggest two components explaining 30.8 % and 18.8 % of the variation in taxa between communities. From the baseline (round points) to time point 24 (triangular point) theres a shift in community structure. Different condition are clustered together, irrelevant of enrichment step and time

**Appendix 8-9: Heat-map of abundance of reads assigned to taxa ID, spiked with *E. faecium* or *E. coli* (next pages)**

