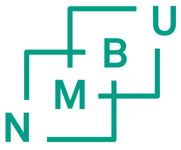


Acknowledgements

This thesis was performed at the Institute of Chemistry, Biotechnology and Food Sciences, at the Norwegian University of Life Sciences, with Professor Knut Rudi as supervisor and PhD student Jane Ludvigsen as secondary supervisor.

First of all, I would like to thank my supervisors, Knut Rudi and Jane Ludvigsen, for always staying positive throughout the whole process of the work with this thesis, and also for always answering all of my thousand questions. You have encouraged me, and thought me how I should always look at things from a positive side. A special thanks to Jane, for all the help in the practical lab work. A big thanks also to Professor Gro Amdam and her research group Aging and Nutritional Physiology in Animal Models, for letting me work in their lab and use their bees for this research. I'm especially grateful for the help from technician Claus D. Kreibich and PhD student Eva Hystad for helping me with the cages and sampling of bees from the hive.

Also thanks to the master students, Krister Lundgård, Helene Gårdsvold and Eva Lena Estensmo for good company in the lab, and the rest of MiDiv group for keeping a high spirit throughout the year. You always made me feel welcome.

Also, I would like to thank all the bees for always treating me kindly. You sure have amazed me by being remarkable living organisms, constructed perfectly to fulfill your important tasks in the environment.

Last but not least, thanks to all my friends and family, for always believing in me and supporting me in all my years of studies.

Ås, August 2015

Inga Leena Angell

Abbreviations

AMS	Treatment with American strain of *G. apicola* and sugar
AMT	Treatment with American strain of *G. apicola* and tetracycline
ANOVA	Analysis of Variance
bp	Base pair
CFU	Colony Forming Unit
ddNTP	Dideoxynucleotides
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
ds	Double stranded
ESBL	Extended spectrum beta lactamase
GI	Gastro intestinal
HGT	Horizontal gene transfer
MANOVA	Multivariate analysis of variance
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant staphylococcus aureus
NOS	Treatment with Norwegian strain *G. apicola* and sugar
NOT	Treatment with Norwegian strain *G. apicola* and tetracycline
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of the mean
S.T.A.R	Stool Transport and Recovery buffer
SBS	Sequencing by Synthesis
tRNA	Transfer ribosomal ribonucleic acid

Abstract

Honey bees, along with other wild type bees such as bumble bees, are the world's most important pollinators. Their existence provide humans and animals important food supply, and also contribute to maintenance of the ecological biodiversity. By their pollination services they cover large spatial areas and their high interaction with the environments lead to spread of bacteria, thus also, presumably gene fragments, between them and the environment. The merging spread of antibiotic resistance is of major concern, and the environment play an important role, were spread of such genes, are being exchanged and transferred between and within different habitats. The role insects play in such spread of resistance is not well studied, and this thesis direct focus on potential role of the commensal bacteria *Gilliamella apicola* in the gastro intestinal tract of the honey bee *Apis mellifera,* as vectors for transfer of the antibiotic resistance gene *tetB.* In addition we aimed to look at bacterial compositions of the honey bee gut, after exposure to the broad-spectrum antibiotic tetracycline, and compare the microbiota of the two different gut compartments midgut and hindgut.

By in vivo study, using 1080 caged honeybees, feeding trials were performed, by providing the honeybees sugar solutions with supplements in the form of tetracycline, and/or bacterial cultures of *G. apicola.* Methods used in the study involved both qualitative and quantitative PCR, 16S rRNA metagenome sequencing, and Sanger sequencing. A total of 267 midgut and 267 hindgut samples were analyzed.

Our findings show high prevalence of *tetB* in Norwegian honeybees, with presence of the gene in 44% of the honeybees. No effects on abundance of the gene was observed with treatment with tetracycline, however a significant effect of treatment with *G. apicola*, and presence of the gene was observed, although evidence for gene transfer could not be confirmed. Bacterial composition comparison showed an increased abundance of *Lactobacillus* spp., associated to tetracycline treatment. In addition, differences in bacterial compositions in the midgut and hindguts were observed, where midgut microbiota showed to harbor a more unstable microbiota, compared to hindgut microbiota. Big differences were also observed in the midgut microbiota of bees from the colony versus caged bees.

Sammendrag

Honningbier, sammen med andre villtype bier som humler, er verdens viktigste pollinatorer. Deres eksistens gir mennesker og dyr viktig matforsyning, og de bidrar også til vedlikehold av økologisk biologisk mangfold. Med deres pollinerings tjenester, dekker de store områder og ved å interagere med omgivelsene i stor grad, bidrar de muligens til spredning av bakterier, derav også trolig gen-fragmenter, mellom dem og miljøet. Den økende forekomsten av spredning av antibiotika resistens er av stor bekymring, og miljøet spiller trolig en viktig rolle i forbindelse med slik spredning. Hvilken rolle insekter spiller i spredning av resistens er ikke kjent, og denne oppgaven retter fokus mot *Gilliamella apicola,* en tarmbakterie funnet i honningbien *Apis mellifera*, som potensiell vektor i forbindelse med overføring av antibiotika resistensgenet *tetB*. I tillegg var det ønskelig å undersøke bakterie komposisjonen i tarmen hos bie, etter eksponering for det bredspektrede antibiotikumet tetrasyklin, samt sammenligne bakterieflora i midtmage og endetarm.

I et in vivo forsøk, med 1080 honningbier i bur, ble det utført fôringsforsøk ved å gi sukkerløsninger med supplementer i form av tetrasyklin, og/eller bakteriekulturer med *G. apicola*. Metoder som ble brukt i forbindelse med studien involverte både kvalitativ og kvantitativ PCR, 16S rRNA metagenom sekvensering, og Sanger-sekvensering. Totalt ble 267 midtmage og 267 endetarms prøver analysert.

Våre funn viser høy forekomst av *tetB* i norske honningbier, med tilstedeværelse av genet i 44% av biene. Behandling med tetrasyklin ga ingen effekt på antall bier positive for *tetB*, men derimot ble det observert effekt av behandling med *G. apicola,* selv om det ikke ble funnet noen bevis for genoverføring. Ved behandling med tetrasyklin, økte mengden av arter tilhørende *Lactobacillus*. I tillegg ble det observert forskjeller i bakteriesammensetninger i midtmage og endetarm, hvor mikrobiotaen i midtmage viste seg å være mer ustabil, enn mikrobiota i endetarm. Det ble også observert store forskjeller i midtmage mikrobiota mellom bier i kube og bier i bur.

Table of Contents

1. Introduction 1

1.1 Apis mellifera gut microbiota *2*

1.1.1 The gastrointestinal tract of the honeybee........ 2

1.1.2 Honeybee gut commensals........ 3

1.1.3 *Gilliamella apicola*........ 5

1.2 Antibiotics and antibiotic resistance........ *5*

1.2.1 Tetracycline........ 6

1.2.2 Development and mechanisms of resistance........ 6

1.2.3 Mobile genetic elements 7

1.2.4 Horizontal gene transfer........ 8

1.2.5 Commensals as a source of resistance........ 8

1.2.6 Antibiotic resistance in the honeybee gastrointestinal tract 10

1.3 Nucleic acid based approaches to study microbiota *11*

1.3.1 Qualitative polymerase chain reaction 11

1.3.2 Quantitative polymerase reaction........ 11

1.3.3 First generation sequencing........ 12

1.3.4 Next generation sequencing 13

1.3.4 Bacterial community studies 14

1.4 Aim of thesis........ *15*

2. Material and Methods........ 17

2.1 Design of Experiments and experimental conditions........ *17*

2.1.1 Experiments 18

2.1.1.1 Pilot study September........ 18

2.1.1.2 Pilot study October 18

2.1.1.3 Main experiment........ 18

2.1.2 Caging conditions........ 20

2.1.3 Feed compositions........ 21

2.1.4 Sampling procedures........ 22

2.1.5 Gut dissection........ 22

2.2 DNA extraction *23*

2.3 Polymerase chain reactions *24*

2.3.1 Qualitative PCR 24

2.3.2 Illumina adapter PCR 25

2.3.3 Quantitative PCR 26

2.3.4 Purification of PCR products by Ampure 27

2.4 Sequencing *29*

2.4.1 Sanger sequencing 29

2.4.2 Illumina sequencing 29

2.5 DNA quantity and quality control *30*

2.5.1 Qubit measurements 30

2.5.2 Gel electrophoresis 30

2.5.3 High resolution melting point analysis 30

2.6 Culture dependent methods *31*

2.6.1 *G. apicola* growth conditions 31

2.7 Data Analysis *31*

2.7.1 Absolute quantification by the standard curve method 31

2.7.2 Scoring procedures for amplicon detection 32

2.7.3 Analyses of sequenced data 33

2.7.4 Statistical analyses 33

3. Results 35

3.1 qPCR screening results *35*

3.1.1 Quantification of total amount of bacteria and *G. apicola* 35

3.1.2 Presence of *tetB* 36

3.1.3 Detection of *G. apicola* strains given in sugar solution 38

3.2 Metagenome analyses of the 16S rRNA gene *40*

3.2.1 Dominating OTUs in gut samples 40

3.2.2 Comparison of relative abundance of bacteria in the different groups 42

3.2.3 Effect of treatment determined by MANOVA 44

3.2.4 Comparative analyses of the main bacterial groups 44

3.2.5 α-diversity 48

3.3 Sanger sequencing of the tetB gene *49*

3.4 Primer optimization *50*

4. Discussion 51

4.1 Presence and origins of the tetB gene *51*

4.2 Detection of G. apicola given in sugar solution *52*

4.3 Differences in bacterial compositions in midgut and hindgut *52*

4.4 Effects of tetracycline on gut microbiota *54*

4.5 Methodological considerations *55*

4.6 Future work *56*

Conclusion 57

References 58

Appendix 65

APPENDIX A: Feed compositions in the different experiments *65*

APPENDIX B: Preparations of sugar solutions *66*

APPENDIX C: PRK Illumina primer sequences for index PCR *67*

APPENDIX D: Sequence alignment of tetB *69*

APPENDIX E: Gel electrophoresis of positive strains with strain specific primers *71*

APPENDIX F: Controls and standard curves *73*

APPENDIX G: Copy numbers of the tetB gene in midgut and hindgut *74*

APPENDIX H: Copy numbers of American strain of G. apicola *76*

1. Introduction

Antibiotic resistance is of major concern worldwide, and a big threat towards human health. The role of the dense populated gastrointestinal (GI) tract serving as an area for gene trafficking, thereby also trafficking of antibiotic resistance genes, have recently been given a lot of attention. Spread of antibiotic resistance in this habitat have been studied in a variety of organisms including both vertebrates, such as humans (Broaders et al. 2013; Huddleston 2014; Marshall et al. 2009; Salyers et al. 2004), and invertebrates, e.g. insects such as houseflies (Zurek & Ghosh 2014), gypsy moth larval (Allen et al. 2009), beetles (Channaiah et al. 2010), fruit flies (Kuzina et al. 2001), oil flies (Kadavy et al. 2000), cockroaches (Tetteh-Quarcoo et al. 2013; Wannigama et al. 2013), and bed bugs (Lowe & Romney 2011).

Insects are important pollinators, and one third of our food is dependent on the pollination of fruits, nuts and vegetables provided by insects (Li et al. 2012; Wallberg et al. 2014). In addition to food crops in agriculture, also wild plant species are highly dependent by pollinations services done by insects and their existence contribute to maintenance of ecological biodiversity. Estimations done in Norway, show that probably around 80% of wild Norwegian plant species are highly dependent of insects for their maintenance (Totland et al. 2013).

Honey bees (species belonging to the genus *Apis*) are assumed to be the most economically valuable pollinators in the world, where mainly the species *Apis mellifera,* often referred to as the Western or European honey bee, play a key role in agriculture (Klein et al. 2007; McGregor et al. 1976). They are estimated to contribute to 70 % of all pollination of fruits and vegetables for human and animal consumption, and their pollination services alone, is valued at >$200 billion per year worldwide (Gallai et al. 2009).

In spite of the small size, honeybees foraging flights makes them able to cover large spatial areas, where they collect pollen, nectar, water and other compounds for the colony, e.g. to produce honey. A study done by Beekman and Ratnieks in Sheffield, UK, in 1996, showed that the foraging ranges of the honey bee could exceed a distance of more than 9,5 km, where <50% of the forages had a mean foraging distance of 6 km (Beekman & Ratnieks 2000). It is also claimed that one foraging bee in average visits 1 500 flowers per day, and that the production of about four liters of honey, requires a colony to collect pollen and nectar from around 500 million flowers (Benjamin & McCallum 2009; Schacker 2008).

In their foraging, honeybees are continuously in close interaction with the environments. They contribute to exchange of e.g. bacteria, minerals, thereby also highly likely gene fragments, thus also make bacteria associated with them potential vectors for i.e. antibiotic resistance genes.

To understand more of the environmental microbiota associated antimicrobial resistance patterns, focus often have been directed toward pathogenic bacteria, and the role of commensal bacteria have, until recent years, been underestimated. The inhabitants of the healthy gut microbiota are given much more attention because they are seen to be of bigger importance for spread of resistance than first assumed (Broaders et al. 2013; Marshall et al. 2009). New methods, developed during the last few years, such as next generations sequencing, along with other nucleic acid based methods available, such as qPCR, makes it possibly to study microbial communities without the need of culturing, and provides a good platform to uncover unknown knowledge of major importance.

1.1 *Apis mellifera* gut microbiota

1.1.1 The gastrointestinal tract of the honeybee

The digestive system of the honeybee consists of different compartments, the esophagus, salivary glands, crop, midgut, malpighian tubules, ileum and rectum (figure 1). Food enters to the latter parts of the GI tract trough the esophagus, into the crop, also called the honey stomach. The crop serves as a storage for nectar, or water, when being collected outside the hive, before provided to the colony. Food particles from the crop enters the midgut, through the proventriculus, located below the crop and consists of muscles and valves, avoiding foreign particles to enter the midgut. Most of the digestion and absorption occurs in the midgut, also called ventriculus, true stomach, or cylindrical sac. This compartment makes up the largest part of the GI tract, where digestive enzymes found in the epithelial layer break down sugars, fats, and proteins, and motoric movements occur by both circular and longitudinal muscles on the outside of the epithelial layer. Due to a peritrophic membrane located along the whole side of the midgut, food particles are not in directly contact with the epithelial layer, but still enzymes are allowed to pass through. From the midgut, the residues from the digestive process are passed into the rectum, entering through the pylorus, and ileum, where the waste is stored until the bee leaves the hive and passes the waste (Davis 2004; Santos & Serrão 2006). Nitrogenous waste,

in form of uric acid, are removed by the malphigian tubules, located between the midgut and the ileum, but this compartment is not technically a part of the honeybee digestive system. A valve called pylorus is located in the intercept between the midgut and ileum (Dade 2009; Davis 2004; Kačániová et al. 2004; Snodgrass 1910). The different gut compartments are divided into foregut, midgut and hindgut, where the foregut consists of the pharynx (not shown in figure), crop, and proventriculus, the midgut corresponds to the gut part with the same name, and hindgut correspond to ileum and rectum.

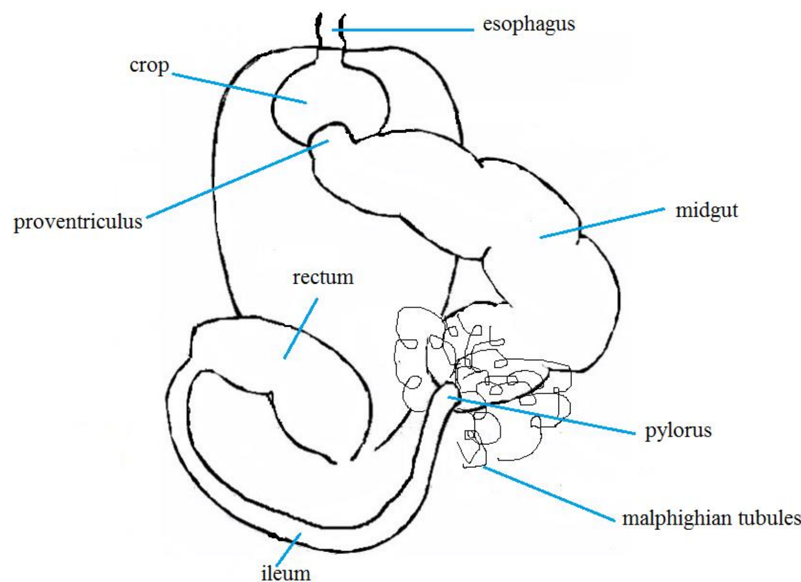


Figure 1.1: The digestive tract of the honey bee. Shows the different compartments of the gut where food enters the crop through esophagus, before it is further digested in the midgut and transported to the ileum and rectum, respectively. Redrawn and modified from Davis (2004).

1.1.2 Honeybee gut commensals

The intestinal microbiota of the honeybee is essential to the host and play an important role in e.g. pathogen defense, regulation of immune responses and nutritional uptake (Dillon & Dillon 2004; Martinson et al. 2012). In bumble bees (*Bombus* spp.), which also contain similar bacteria as *A. mellifera*, some of the symbionts are shown to protect against parasitic protozoans (Koch & Schmid-Hempel 2011)

Compared to humans and other animals (both vertebrates and invertebrates), the gut microbiota of honeybees seem to be simple and less complex. Only termites have shown to harbor a similar

distinctive microbiota between individuals, but in contrast to honeybees, termites harbor far more phylotypes in their gut (Dillon & Dillon 2004; Engel & Moran 2013).

Several studies has shown that the honeybee microbiota consists of eight distinctive bacterial phylotypes: two Alphaproteobacteria*;* Alpha1 and Alpha2/Acetobacteraceae*,* two Gammaproteobacteria*;* Gamma 1, recently identified as *Gilliamella apicola* (Kwong & Moran 2013)*,* Gamma 2, recently identified as *Frischella perrara (Engel, P. et al. 2013)*, two *Lactobacillus*; Firm4 and Firm5, one Betaproteobacteria, identified as *Snodgrassella alvi (Kwong & Moran 2013)*, and one *Bifidobacterium*; Bifido (Cox-Foster et al. 2007; Engel, P. et al. 2013; Jeyaprakash et al. 2003; Koch et al. 2013; Kwong & Moran 2013; Martinson et al. 2011; Moran et al. 2012; Vásquez & Olofsson 2009). Many of the phylotypes found in the gut of the honeybee are closely related to bacteria found in other insects, but the three phylotypes *G. apicola*, *F. perrara*, and *S. alvi*, are so far only found in honeybees, and bumblebees. However, *G. apicola* and *F. perrara* are nested within a larger clade that has been recovered from guts of other insects (Moran et al. 2012).

Although many studies are based on pooled samples from guts dissected from several bees, the same phylotypes are also found despite different geographical areas, and different bee species across the world (Mohr & Tebbe 2006), and within individuals (Engel et al. 2012; Martinson et al. 2011; Moran et al. 2012).

Despite the findings of few phylotypes making up a core microbiota, the species within the phylotypes have revealed a quite high prevalence of strain variation. Especially the two species, *G. apicola* (belonging to Gammaproteobacteria: Orbales) and *S. alvi* (belonging to Betaproteobacteria: Nesseriales), have revealed large strain variations within the species (Engel et al. 2014). The same occurrence have been observed in honeybee associated *Lactobacilli* and *Bifidobacterium* spp. (Ellegaard et al. 2015). The consistent presence of the same phylotypes, despite different individuals, colonies and localizations throughout the world, suggest that these bacteria are essential for the honey bee health and have central functions in bees (Martinson et al. 2011). Strain variations between the different phylotypes could also have different functionalities, thus play an important role (Engel et al. 2014).

Studies done on community composition and colonization patterns have so far revealed that honeybee GI tract seem to lack bacteria until the age of 4-6 days within the hive (Guo et al. 2015; Martinson et al. 2012; Powell et al. 2014). Also early culture-based studies noted that

bees removed from frames as pupae could remain free of gut bacteria through adulthood (Gilliam 1971). Potential inoculation routes for young workers are contact with the environments such as direct contact with the bee bread and comb, and interactions with older bees in the colony (Anderson et al. 2013; Powell et al. 2014). Also, different communities are found in different gut compartments, where the crop and midgut contain very few bacteria, respectively around 10^4 and 10^6, whereas ileum and rectum, making up the hindgut, harbor a large community with characteristic compositional profiles with total bacterial numbers of around 10^7 and 10^8 respectively (Anderson et al. 2013; Martinson et al. 2012).

1.1.3 *Gilliamella apicola*

Gilliamella apicola, named after Martha A. Gilliam, a famous bee researcher who contributed greatly to honeybee research, and apicola meaning bee-dweller, has been reported as one of the most abundant species in the honeybee gut, compromising between 10-30 % of total amount of bacteria (Anderson et al. 2013; Moran et al. 2012). The bacterium is a gram negative, rod shaped, non-motile bacterium and do occasionally form filament chains. Growing on agar, it forms round, white and smooth colonies, approximately 2.5 mm in diameter, however strains vary in morphology. The species is negative for nitrate reductase, oxidase and catalase (Engel, Philipp et al. 2013; Kwong & Moran 2013). The species was first classified into the *Pasteurellaceae* family, but are now belonging to the family *Orbaceae.* The bacteria have in earlier studies shown signs of clumping behavior, and formation of dense aggregates, which is thought to be due to short hair-like structures on the surface of cells (Kwong & Moran 2013). It has been suggested that *G. apicola* indirectly adhere to the gut wall, by adhering to *S. alvi*, which serve as a basis forming a biofilm for other bacteria to adhere to (Martinson et al. 2012).

1.2 Antibiotics and antibiotic resistance

The discovery of Penicillin, in 1928, by Alexander Fleming, and the further development for an effective and large-scale production of the drug during the years after until it finally was succeed, and could be used during world war II, in 1943, was a breakthrough in medical science, leading to a tremendous decrease in number of deaths in the world caused by bacteria

(Blair et al. 2015). Since the discovery, a large number of antibiotics[1] and antimicrobial[2] agents have become available on the market. The prerequisite properties of an antibiotic or antimicrobial agent is that it must have selective toxicity, where it only kill or inhibit the microbial pathogen while damaging the host as little as possible. Different antibiotics act in different ways, and generally the mode of action falls within one of four different mechanisms. Three of them involves inhibition or regulation of enzymes involved in either cell wall biosynthesis, nucleic acid metabolism and repair, or protein synthesis, and the fourth mechanisms involves disruption of membrane structure (http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=14837959)

1.2.1 Tetracycline

Tetracycline is a broad-spectrum antibiotic, belonging to the family tetracyclines, which were discovered in the late 1940s. The antibiotic acts against a wide range of both gram-positive and gram-negative bacteria, by inhibition of protein synthesis. The mode of action involves passively diffusion through protein channels in the cell membrane and binding to both the small 30S subunit and the larger 50S subunit of the prokaryotic 70S ribosomes. By binding to the small subunit, it inhibits protein synthesis by preventing access of aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex. The binding to the larger subunit leads to altering of the membrane and leakage of intracellular compounds. The antibiotic, along with other members of the family have been extensively used, to treat infections in both humans and animals and have also been used at sub therapeutic levels in animal feeds as growth promoters, due to few side effects and the inexpensive cost of the antibiotic. (Chopra & Roberts 2001)

1.2.2 Development and mechanisms of resistance

Not surprisingly, already a year after penicillin came to the market, resistant bacteria towards the antibiotic was detected. Bacteria and their ability to adapt to the environment, thus also antimicrobial therapy, have led to a competition, where humans have been able to compete against resistance within disease-causing bacteria, by introducing new antimicrobials to the market, whenever others have failed to be effective. However, discovery of new antibiotics has

[1] From the Greek words anti (against), and biotikos (concerning life). Refers to substances produced by microorganisms that act against other microorganisms by killing or inhibiting them (Varley et al. 2009)
[2] Derived from the Greek words anti (against), mikros (little), and bios (life). Refers to all agents that act against microbial organisms such as bacteria, viruses, fungi, and protozoa.

now turned to a point when the development have become highly expensive and not economically favorable for pharmaceutical companies, thus leading to a serious decline in number of new antibiotics on the market. We are now dealing with bacteria that have become multi resistant to antibiotics available, such as the multi resistant *Staphylococcus Aureus* (MRSA) and the extended spectrum beta lactamases (ESBL) producing bacteria, which both are examples of causes of a steadily increasing amount of deaths throughout the world today (Steinbakk et al. 2014).

Mainly, bacterial resistance against antimicrobial agents either can be innate (also called intrinsic), or acquired. Innate resistance refers to natural insensitivity (no genetic alteration) and is inherited, whereas acquired resistance is resistance development of the bacteria, and mainly happen in one of two ways; either by horizontal gene transfer (HGT), involving mobile genetic elements, or by spontaneous mutations. Spontaneous mutations in the DNA can be caused by various different reasons, such as errors in DNA replication, spontaneous lesions or transposable genetic elements (Blair et al. 2015). In addition, exposure to antibiotics has indicated to promote to spontaneous mutations (Blázquez et al. 2012).

Mechanisms of resistance can be caused by various reasons, e.g. production of enzymes that inactivate the drug exposed, such as β-lactamases[3], inaccessibility of the drug into the bacterial cell due to molecular aspects of membrane spanning proteins, or simply lack of affinity of the bacterial target and the drug compound. In addition, the bacterial cell membrane can be provided with efflux pumps, which are transporters made up by proteins, acquiring chemical energy to function. These pumps works against the introduced drug molecules by pumping the drug continuously out of the cell before it reaches its target (Blair et al. 2015; Huddleston 2014).

1.2.3 Mobile genetic elements

Mobile genetic elements (MGE), first described by Barbara McClintock in the maize genome (McClintock 1950), are pieces of DNA that can move around within the genome (Frost et al. 2005). They include transposons, or transposable elements, plasmids, bacteriophage elements, and two groups of introns (group I and II). Plasmids do not usually integrate into the main genomic DNA, and are not essential for the normal growth of the host, but can be of advantage e.g. if the bacteria is exposed to external stress. Plasmids code for synthesis of a few proteins not coded for by the bacterial chromosome. Transposons, are often referred to as "jumping

[3] Enzymes disrupting the β-lactam ring of β-lactam antibiotics e.g. penicillin derivates, cephalosporins and carbapenems.

genes", and the name is suitable for the properties of the gene element to integrate into genomic DNA, due to incorporated enzymes that enable the transposon to move from one DNA location to another. Transposons can also be found in plasmids (Rankin et al. 2011).

1.2.4 Horizontal gene transfer

HGT, also called lateral gene transfer, lateral meaning "coming from the side", is defined as movement of genes between different, or same species. HTG is also shown to occur across broad taxonomic categories, and even across different kingdoms (Keeling & Palmer 2008).

Mechanisms of HGT are transduction, transformation and conjugation. Transduction is carried out by bacteriophages, and involves transfer of genes from one bacterium into another by the use of the virus as a vector. In addition, genes can directly come from the virus itself. (Huddleston 2014). Transformation is a process were bacterial cells take up naked DNA fragments, e.g. from dead bacteria, from the environment. Uptake of small fragments often occur in by transformation. In contrast to transduction and transformation, conjugation acquire direct contact between the donating and the host cell. By this mechanism, DNA fragments are exchanged between bacteria through a pilus, and in this way, also large genetic elements, with various number of genes, such as plasmids, can be transferred. Plasmid-mediated transmission is the most common mechanisms of HTG (Norman et al. 2009)

1.2.5 Commensals as a source of resistance

In studies done to understand more of the mechanisms and spread of resistance, much focus have been directed towards disease causing bacteria. However, in a typical microbial community, commensals[4] in most cases outnumber pathogenic bacteria (both true pathogens and opportunistic pathogens). The high number of inhabitants belonging to the normal, or core microbiota, including transient colonizers found in microbial communities, suggests that these bacteria in large extent contribute in e.g. gene trafficking, thus also trafficking of resistance genes. Relationships between these bacteria are illustrated in figure 1.2, which presents the amount of true pathogens as a small fraction of the total microbial community, in addition to a much smaller amount of so called pathogenic commensals, when compared to the proportions

[4] From Latin, directly transferred as "eating at the same table", and is defined as a host-microbial relationship when one of the organisms benefits from the other, while the other is not harmed. Despite of the definition, the word is often used for bacteria belonging to a normal microbiota, and the word is also often used when the symbiotic relationship is beneficial for both organisms (Casadevall & Pirofski 2000)

of core and transient colonizers, in addition to environmental commensals found in e.g. soil, sludge and water. The illustration suggest that bacteria belonging to the non-pathogenic bacteria are the major source of resistance genes.

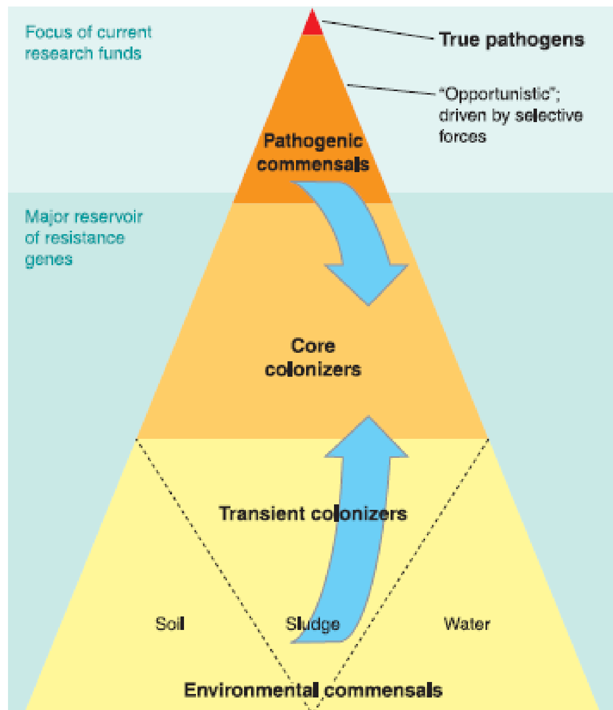


Figure 1.2: Illustration of a typical microbial community found e.g. in the GI tract, where the relationships between transient colonizers (according to abundance) between core colonizers, pathogenic commensals, and true pathogens is illustrated. Figure is reprinted from Marshall et al. (2009). In addition environmental commensals are included in the figure.

In commensals found in human GI tract, antibiotic resistance genes have been found to be highly present. A study done by Liu et al. (2012), where they did whole genome searches in 300 different gut microbes commonly found in the human GI tract, revealed a large number of resistance genes. Many of the genes were considered as high confidence HGT genes.

In studies of resistance development concerning resistance toward tetracycline, high accuracy of tetracycline resistant gene transfer have been revealed among *Bacteroides* spp., among *Bacteroides* and other genera commonly in human colon (Shoemaker et al. 2001), and among bacteria commonly found in the human GI tract and bacteria commonly found in livestock (Nikolich et al. 1994). The role commensal bacteria play in development of resistance is probably of major importance.

1.2.6 Antibiotic resistance in the honeybee gastrointestinal tract

Since the 1950's American honey bees have been treated with tetracycline to avoid the diseases nosemosis, and American and European foulbrood caused by *Nosema apis* or *N. ceranae, Paenibacillus larvae,* and *Melissococcus pluton,* respectively (Reybroeck et al. 2012; Tian et al. 2012). The causative agents of foulbrood affect bees at the larvae stage. The disease cause massive death in bee colonies and can, within short time, wipe out an entire colony. In Europe, and most parts of the world, treatment with antibiotics have been strictly restricted, and in most cases not allowed, which is also the case for Norwegian beekeepers (Reybroeck et al. 2012).

A study done by Tian et al. (2012), including honeybees from the U.S, Switzerland, Czech Republic and New Zealand, revealed a higher frequency of tetracycline resistance genes in the gut commensals of American honey bees, which had been treated with the antibiotic, than in honey bees from the other countries who had not received antibiotic treatment. In addition to frequency, also a higher number of different genes was seen in the American bees treated with the antibiotic. Compared to the eight different tetracycline resistant loci, found in American bees (*tetB, tetC, tetD, tetH, tetL* and *tetY, tetM* and *tetW),* only three of them (*tetB, tetC* or t*etW*) were found in the other countries, as well as in American honeybees who had not received antibiotic treatment during the last 25 years (Tian et al. 2012). The three last mentioned genes were also found in wild American bumblebees.

Six of the loci (*tetB, tetC, tetD, tetH, tetL* and *tetY*), encoded efflux pump genes, whereas *tetM* and *tetW* encoded so called ribosome protection genes.

The found resistance genes were also shown to be attributable for known resistance loci for which nucleotide sequences and flanking mobility genes were nearly identical for those of human pathogens and from bacteria associated with farm animals (Tian et al. 2012).

1.3 Nucleic acid based approaches to study microbiota

Today, culture-independent methods for study of bacteria are widely used. Methods such as polymerase chain reactions, and sequencing technology, have made it a lot easier to study bacteria, where no need of culturing is necessary, are probably the most used methods in today's microbial studies, and are explained in detail in the sections below.

1.3.1 Qualitative polymerase chain reaction

Qualitative, or conventional polymerase chain reaction (PCR), first described in 1985 (Saiki et al. 1985), makes detection of a DNA fragment possible by amplifying the fragment of interest from a sample by introducing it to a mix of reagents, including primers specially designed for the fragment of interest, a DNA polymerase, buffers, salts, and deoxyribose nucleotide triphosphates (dNTPs). By performing multiple cycles of three different steps (i.e. denaturation, annealing and elongation/extension) at different temperatures, the DNA polymerase extends the 3'OH end of the DNA template by adding the complimentary dNTPs, and in this way multiplying the fragment of interest exponentially, leading to a large number of copies of the fragment. In this way, detection of a fragment of interest can be detected in just a scarce sample of DNA (Evans et al. 2013).

1.3.2 Quantitative polymerase reaction

The basic principles for PCR and quantitative PCR (qPCR) are the same, but in contrast to PCR, where the product only is detected at an end-point, leading to no information about initial amount of target nucleotide sequence, qPCR combines both amplification and detection in one single step and measures the amount of amplified fragment in real-time. The measuring is done by using fluorescent dyes that either are unspecific, and bind to double stranded DNA (e.g. SYBR® Green I or EvaGreen®), or specific, by using hydrolysis probes (e.g. TaqMan® probes) containing fluorescent labels that only will emit fluorescence when bound to specific sequences (Brankatschk et al. 2012).

Increased fluorescence is proportional to increased amount of PCR product, leading to a non-exponential plateau phase, where the reagents become limiting. A qPCR plot is generated, where cycle numbers are plotted against the fluorescence measure and a quantification cycle (Cq) value represent the cycle number where the fluorescent signal reaches a threshold line,

where the background noise levels are outcome, thus representing the initial amount of fragment of interest in a sample (figure 1.3).

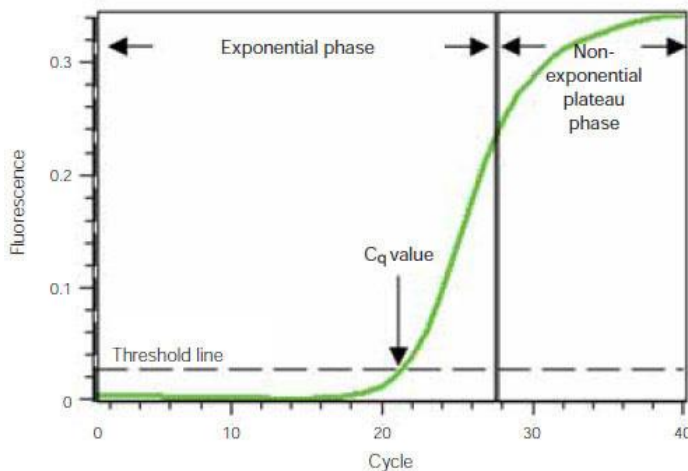


Figure 1.3: qPCR plot, showing the response curve for a qPCR reaction, where the measured C_q value corresponds to the cycle number where the measured amplicon reaches beyond the noise signal. Figure from (http://www.bio-rad.com/en-no/applications-technologies/qpcr-real-time-pcr).

The technique is useful to detect and quantify DNA fragments due to its high accuracy, high sensitivity, reproducibility and low cost compared to use of e.g. sequencing.

By using a dilution series of standards with known template concentrations in the qPCR assay, detection of the DNA fragment in the sample can be made at copy number level. This is done by creating a linear plot where the standard concentrations are plotted against corresponding Cq values, and the linear relation is used for calculations of template concentrations in the sample, assuming the efficiency is the same for both standards and sample.

1.3.3 First generation sequencing

Sanger sequencing, also called the chain termination method, developed by Fredrick Sanger and colleagues in the end of the 70s, lead to a big breakthrough in science and has been the most used sequencing method for the years after. The method is based on incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication, and requires a single template DNA strand, deoxynucleotides (dNTPs), ddNTPs, a DNA primer and a DNA polymerase. The ddNTPs lacks an OH group on the 3’ end, leading to a termination of the formed chain of bases, due to the absence of the phosphodiester bond which is required for to nucleotides to bind together. By fluorescently, or in other ways marking of the ddNTPs, it is possible to detect the presence of the incorporated base (Sanger et al. 1977)

1.3.4 Next generation sequencing

Next generation sequencing (NGS), also known as high-throughput sequencing, or massively parallel sequencing is a core technology for genomic studies and have revolutionized the study of genomics and molecular biology, since its arrival ten years ago. The technology makes it possible to sequence DNA and RNA faster and less expensive than previously used sequencing technologies (i.e. Sanger sequencing).

Since the first technology to be released, the pyrosequencing method by 454 Life Sciences (now Roche), several other NGS sequencing platforms have been developed. Illumina is one of them and was introduced to the market a year after, in 2006. The Illumina method of sequencing (figure 1.4) is based on the sequencing by synthesis (SBS) technology, where reversible dye-terminators enable identification of single bases as they are introduced into DNA strands.

The first step, includes sample preparation of extracted and purified DNA, where adapters are added to the DNA insert. These adapters contain sequencing primer binding sites, regions complementary to oligonucleotides on the flow cell, and also, unique barcodes, on both sites, to discriminate between sequences from different samples after sequencing. The second step, involves clustering where each fragment is isothermally amplified. The flow cell contains channels, where each channel has two types of nucleotides attached to the slide, where one of the types are complementary to the adapter region of the DNA insert, thus leading to hybridization of the DNA fragment to the slide. The attached fragment is then copied, making a reverse strand, before the double-stranded DNA molecules is denatured and the original template washed away. The remaining reverse strand "bends over" and hybridize to the second oligonucleotide-type on the slide, making a bridge formation. A complementary strand is then generated by a polymerase, forming a double-stranded bridge (bridge-amplification). The double-stranded bridge is further denatured, resulting in two single-stranded copies, one forward and one reverse strand, and the process is repeated over and over and occur simultaneously for millions of clusters, resulting in clonal amplification of all the fragments. After the bridge-amplification, the reverse strains are cleaved and washed off, leaving only forward strands, and the 3' ends are blocked to prevent unwanted priming.

The third step involves the sequencing process and begins with extension at the sequencing primer. Four fluorescently tagged nucleotides compete for addition to the growing chain, where only one nucleotide is added at a time, and simultaneously measured by a light source and a characteristic fluorescent signal. For a given cluster, all identical strands are read

simultaneously and hundreds of millions clusters are sequenced in a massively parallel process (Buermans & den Dunnen 2014; Shendure & Ji 2008)

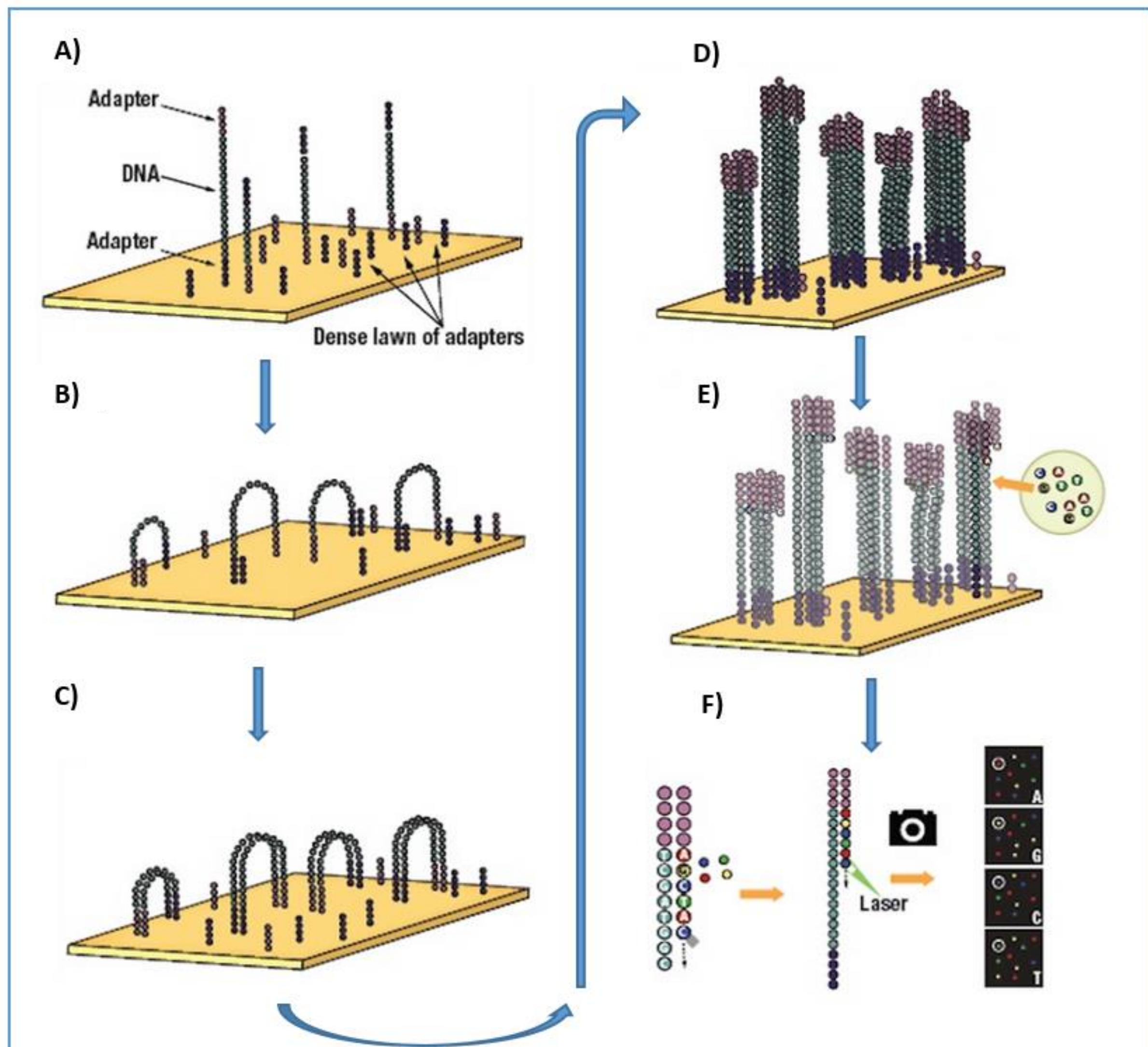


Figure 1.4: Illumina sequencing process. Shows the process in which the adapter sequences binds to the complementary sequences on the flow cell (A), free DNA ends binds to complementary primer to form a bridge (B), amplification of bridge (C), dense cluster forming of each single stranded DNA (D), initiation of first sequencing cycle by adding all four labeled reversible terminators and DNA polymerase (E), and incorporation of correct base and capture of signal via camera (F). Figure is redrawn and modified from (http://openwetware.org/images/7/7a/DOE_JGI_Illumina_HiSeq_handout.pdf)

1.3.4 Bacterial community studies

The study of bacteria and bacterial communities, was revolutionized, due to the discovery of the prokaryotic 16S rRNA gene, first in the 1960s, by Dubnau et al. (Dubnau et al. 1965), and then introduced to be used for taxonomy assignment by classical work done in the 1980s (Fox

et al. 1977). The gene discovery contributed to the classification of living organisms into the three domains of life, Bacteria, Archaea and Eukarya.(Woese et al. 1990).

The gene, with the size of about 1500 bp, codes for a fragment of the 30S small subunit of prokaryotic ribosomes, and contains both variable and highly conserved regions, which makes the gene suitable for both identification and phylogenetic studies of bacteria and archaea (Woo et al. 2008). By designing primers, targeting the conserved regions, amplification of the gene, or parts of the gene, by doing PCR is possible, and the gene can be further sequenced, by the high throughput sequencing technologies available today.

For bacterial identification sequences are searched against type strain databases, such as Greengenes, RDP and SILVA, after the raw sequences have been processed in platforms such as QIIME (Caporaso et al. 2010), for quality filtering and operational taxonomic units (OTU) generation. The OTU definition is the operational definition of a species or group of species, and generation of OTUs involves clustering of identical sequences, often at 97-99 % identity (Blaxter et al. 2005).

1.4 Aim of thesis

The emerging spread of antibiotic resistance is of major concern worldwide, and to be able to develop alternative antimicrobial therapy to fight against disease causing bacteria and their merging development of resistance, it is highly necessary to understand more of the environmental and commensal antimicrobial resistance patterns. (Broaders et al. 2013; Marshall et al. 2009).

The GI tract, harboring high number of bacteria, presents an ideal arena for bacterial communication, thereby also potential spread of antibiotic resistance genes, but studies done on the role gut commensals play in spread of resistance are lacking. In addition highly complex gut microbiota found in most vertebrates and invertebrates, makes these habitats challenging to use for in vivo study.

In contrast, the honeybee presents a good model for such studies, due to a rather simple core microbiota and relatively easy management in lab. In addition, the insect have not been exposed to a vast array of antimicrobials, such as humans, and the prevalence of antibiotic resistance loci, seem to mainly involve resistance towards tetracycline. Furthermore the honeybee gut

commensal *G. apicola* have been shown to harbor resistant genes toward tetracycline, which makes this bacteria suitable for these kind of studies.

Therefore, **the aim of this thesis was to study the potential role of the honeybee gut commensal *G. apicola* as a vector for transfer of the tetracycline gene *tetB* in the midgut and hindgut of the honeybee** by introducing a previously isolated *G. apicola* strain (from a healthy honeybee gut microbiota) containing *tetB* to the honeybees through sugar meals, and exposing honey bees to the broad-spectrum antibiotic tetracycline, to create a selection pressure. To address the aim for the study, the following sub goals were included:

- Design of strain specific qPCR assay for detection of specific strain of *G. apicola*
- Detection of prevalence of *tetB* before and after tetracycline treatment

The following sub goals were also included to compare the gut microbiota:

- In midgut and hindgut treated and not-treated with tetracycline
- In midgut and hindgut in colony versus caged bees

Approaches used to achieve the goals for this study included qPCR, Sanger sequencing and metagenome Illumina sequencing of 16S rRNA.

Potential gene transfer was explored by using qPCR and strain specific primers designed for the specific strain of *G. apicola*, together with primers for detecting the *tetB* gene. To evaluate the origin of the gene, Sanger sequencing was used. To study composition of microbiota, Furthermore, sequencing of 16S rRNA it was used to study the composition of the microbiota, in addition to qPCR to detect total number of bacteria.

2. Material and Methods

2.1 Design of Experiments and experimental conditions

Two pilot studies were conducted prior to the main experiment. A schematic view of all three experiments is shown in figure 2.1. Caging conditions, sampling procedures, and gut dissection were the same for all experiments as explained in section 2.1.2 to 2.1.5.

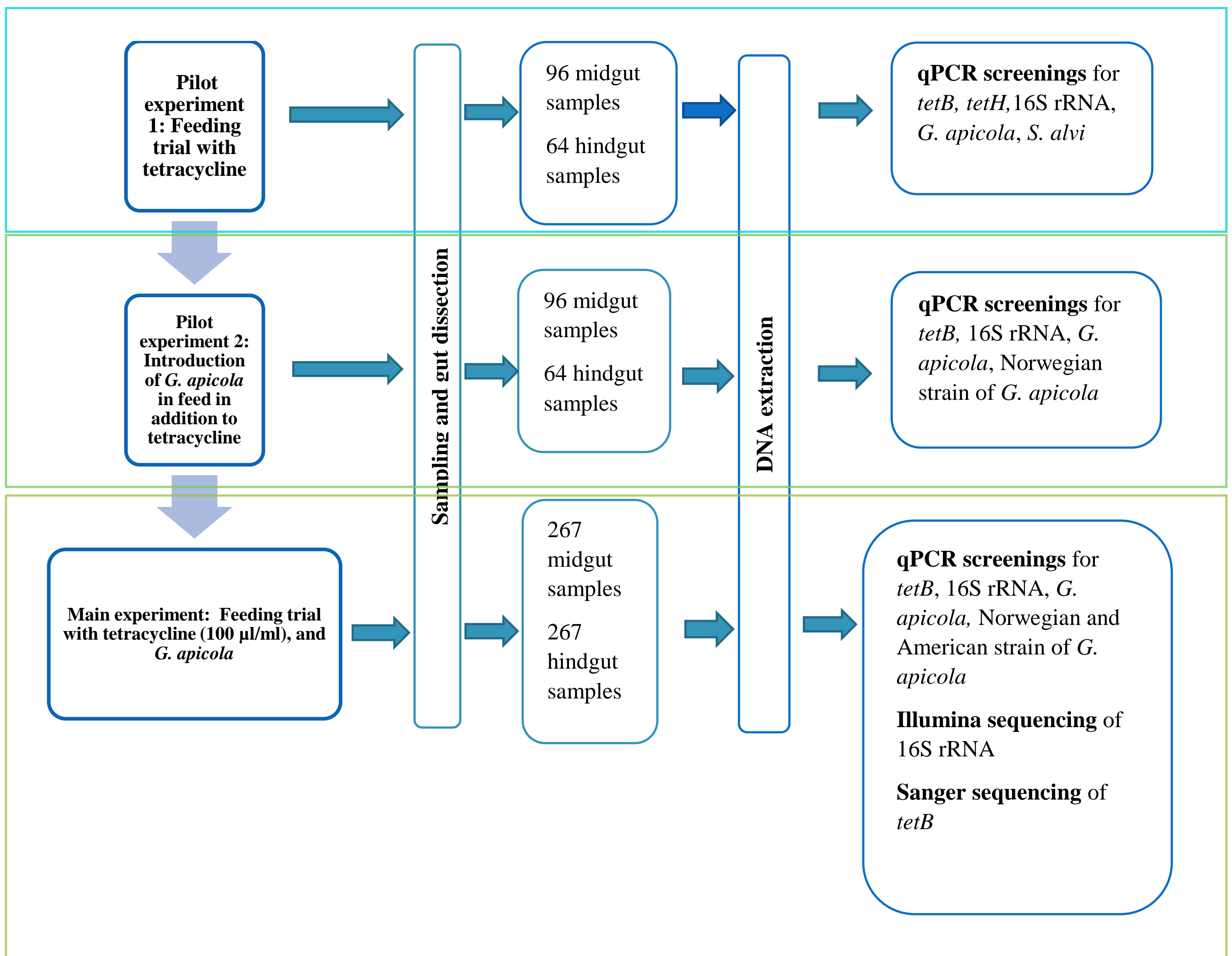


Figure 2.1: Flowchart of the experimental set up. Three experiments were performed in total, where two of them were pilot small-scale experiments, conducted before the main experiment. QPCR screenings were done for all three experiments. In addition, 16S rRNA sequencing and sequencing of *tetB* was done for the main experiment.

2.1.1 Experiments

Tables of the feed compositions corresponding to the different treatments in all three experiments are given in appendix A.

2.1.1.1 Pilot study September

To test different concentrations of tetracycline, given in sugar solution, a pilot experiment was set up, including in total 240 adult summer bees in four cages with approximately 60 bees per cage. Bees were sampled from the same colony in Ås, Akershus, in September 2014. The different concentrations of tetracycline supplemented in the sugar feed was 0.25 µl/ml, 12 µl/ml and 100 µl/ml. The concentrations were chosen according to minimal inhibitory concentrations (MICs) found from cultivation experiments previously done on the two different strains of *G. apicola* used later in the experiments. A control cage were bees were given only sugar solution, without supplements was included. Sampling was done twice the first day and once a day the following 8 days. QPCR screenings were done for *S. alvi, G. apicola, tetB, tetH,* and 16S rRNA.

2.1.1.2 Pilot study October

A second pilot study was conducted to check whether the bacterial strain, given in sugar solution, was possible to detect by qPCR from the gut sample, by using primers designed to target the strain. Number of bees and cages were the same as in the first study, and bees were sampled from the same colony, in Ås, Akershus, in October 2014. A Norwegian strain of *G. apicola,* was chosen for the experiment, and given in the sugar solution in a concentration of 10^7 CFU/ml. Details on preparations of the bacterial culture are given in appendix B. Two different concentrations, 6 µl/ml and 100 µl/ml tetracycline was included in the study. These concentrations were chosen based on results from the first pilot experiment. Three of the four different treatments in total consisted of a supply of bacterial culture in sugar solution, while the fourth treatment was a control where bees were given sugar solution without supplements. Details on feeding procedure is described in section 2.1.3. Sampling was done once a day for 9 days. QPCR screenings were done for same genes as in the first experiment, with exceptions of *tetH* and *S. alvi.*

2.1.1.3 Main experiment

A schematic representation of the main experiment is given in figure 2.2.

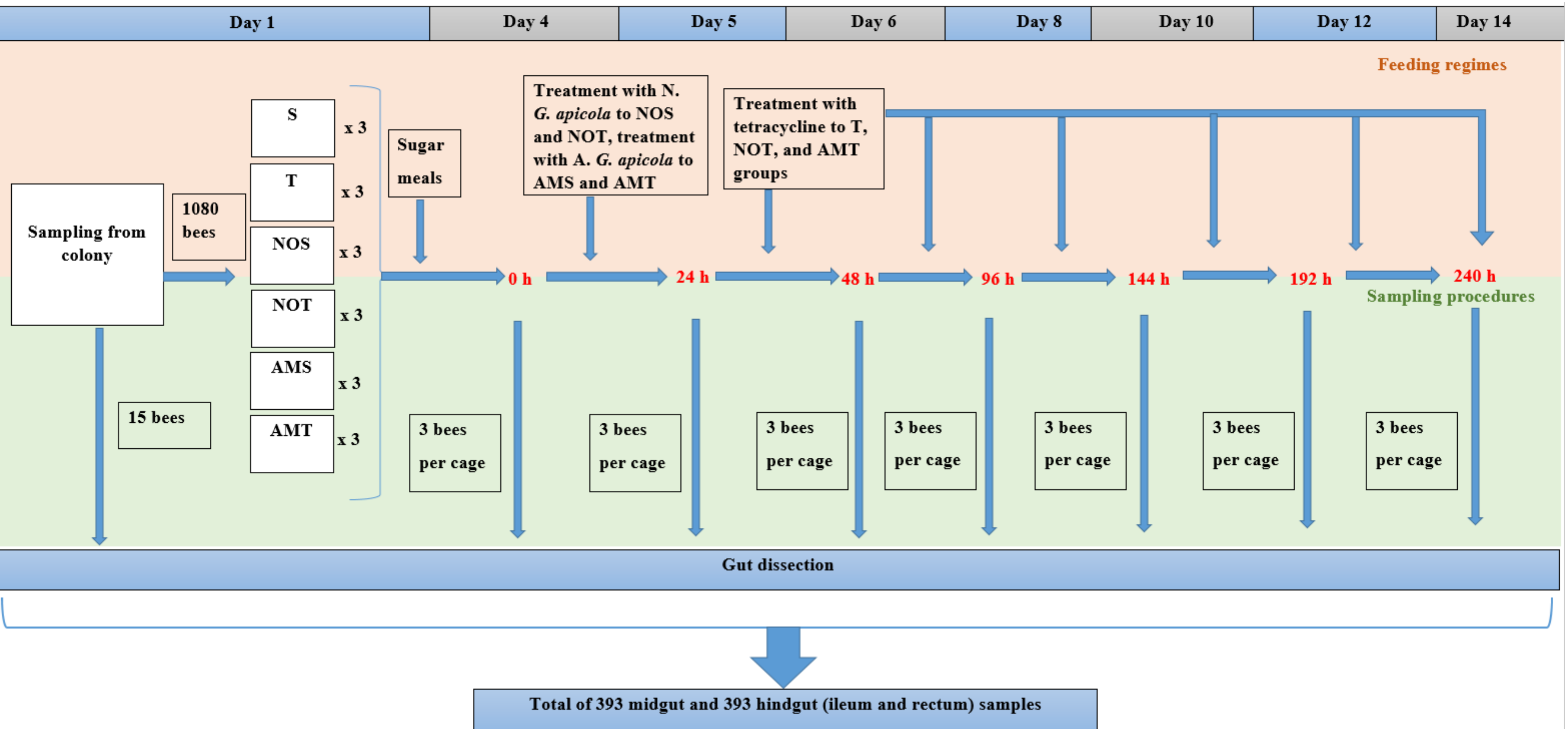


Figure 2.2: Schematic view of the design of main experiment. In total 18 cages were maintained throughout the experiment, corresponding to six different treatment groups where each treatment group had 3 replicate cages. S=treatment with sugar, T=treatment with tetracycline, NOS= treatment with Norwegian *G. apicola,* NOT= treatment with Norwegian *G. apicola* and tetracycline AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline. Concentrations of bacteria and tetracycline were respectively 10^7 CFU/ml, and 100 µg/ml. Sampling was done at time points 0h, 24h, 48h, 96h, 144h and 240h, and guts were dissected from three bees from each cage at each sampling point.

For the main experiment, a total number of 1080 healthy, long-lived adult winter bees of an undefined age were collected, from the same colony in Ås, Akershus in November 2014. Bees were randomly put into cages, and given six different treatments, with three replicate cages of each treatment, giving a total of 18 different cages with 60 bees per cage. In addition, 15 bees were collected for gut sampling the same day, for comparison of gut samples from caged bees versus bees from the colony.

Prior to the start of the experiment, the bees received only sugar solution without any supplements for the first three days before sampling, mainly to ensure healthy bees, and to establish a stable, non-stressful environment before the start of the experiment.

In addition to the Norwegian strain, also an American strain of *G. apicola* was included in the experiment. The concentration of tetracycline, added in the sugar solution in groups receiving the antibiotic, was chosen to be 100 µl/ml.

Sampling was done at time points 0h, 24h, 48h, 96h, 144h, 192h, and 240h, according to figure 2.2, and four bees where removed, from each of the cage at every time point, to ensure successful gut sampling from three bees.

2.1.2 Caging conditions

The caging conditions were the same for all three experiments. Cages were made of plastic material and had a size of 500 cm^3 (figure 2.3). In all experiments, a total number of approximately 60 bees were hold in each cage. Each cage was provided with wire mesh on two of the walls of the cage to ensure proper ventilation, and a wax foundation of 8 x 6 cm was included in each cage to mimic more realistic conditions. In addition, a mesh floor was constructed about four cm from the cage floor, surrounded by a stripping foam, to avoid contamination of bees in case of e.g. leakage from the feeders. (Williams et al. 2013)

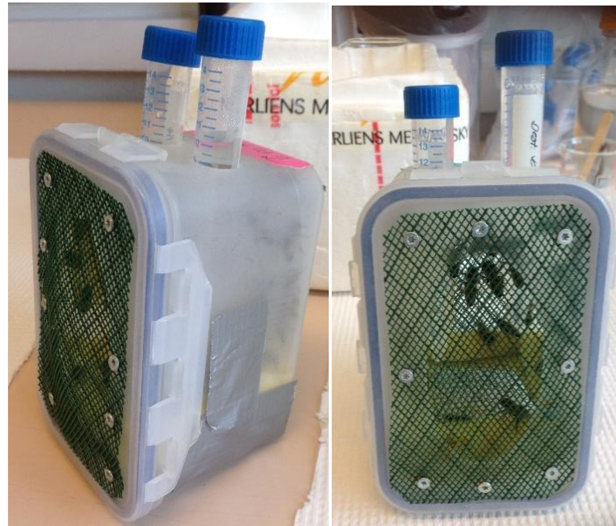


Figure 2.3: Plastic cages used in the experiments. The plastic tubes on top of the cages represents the feeders for both water and sugar solution. Wire mesh was applied on two of the walls, in addition to a mesh floor seen in the lower part of the cage.

To be able to collect bees, holes with the size of 2 cm in diameter were drilled into the side of the cages. In addition, two holes of the same dimension was drilled on the top of all of the cages, to enclose 15 ml plastic tube feeders (Greiner Bio-One, Austria) containing provided sugar solution and water supply, respectively. To ensure proper food and water supply from the plastic tubes, four holes (1 mm in diameter) were drilled into the lower part of the plastic tubes (not seen in figure 2.3). By turning the plastic feeders up and down a couple of times, after filling them with sugar solution, a vacuum was obtained inside the tube, allowing the bees to get the solution out of the tube without spilling.

The cages were stored dark in an incubator cabinet, where the temperature was kept at 26-27 °C, and relative humidity at 50%, throughout the whole experiment. Cages were removed from the incubator, one at a time, only when sampling was done.

2.1.3 Feed compositions

The sugar solution given in the feeders consisted of~40 % sugar, and was prepared by mixing a 50% v/v of Bifor® (Nordic Sugar, Denmark) sugar solution in tap water. Bifor® is a sugar mixture of inverted sugars with no need for break down in the bee intestine. Details on preparations of sugar solution with tetracycline and viable *G. apicola* are listed in appendix B.

The two bacterial strains of *G. apicola* were isolated from a Norwegian honeybee gut, previously sampled from a colony in Ås, Akershus, and an American honeybee gut, previously

sampled from a colony in Arizona, USA, respectively (Jane Ludvigsen, unpublished work). The stains were isolated in 2013 and had been stored at -80 ° C in Hart Infusion Broth (DIFCO Laboratories, USA), containing 10% glycerol (Merck KGaA, Germany).

Bees receiving bacterial cultures in their feed were exposed to feed with viable *G. apicola* for 24 hours from time 0h, before the feed was removed and replaced by either sugar solution without supplements, or sugar solution supplemented with tetracycline throughout the rest of the ongoing experiment.

Both sugar solution (with or without tetracycline) and water feeders were replaced with new ones every 24 hours, to ensure fresh supply.

2.1.4 Sampling procedures

All bees were randomly collected directly from the frames inside the hive, by forceps, one by one, and randomly placed into different cages, corresponding to different treatments. The technique of removal of live bees from the cages, at each sampling point, involved the use of a transparent plastic tube and full covering of the cage, making the cage dark, so that bees, because they are attracted to light, would seek the light in the plastic tube, thereby removed out of the cage in a controllable manner.

2.1.5 Gut dissection

Bees were immobilized on ice by chilling at 0 °C before the guts were aseptically removed. The procedure was done by pulling out the intestine by the sting of the bee, by using forceps. In this way, the intestine would be separated apart between the crop and the midgut, where the crop would stay inside the body of the bee, while the midgut and hindgut compartments would follow the sting and be separated from the body. The gut was further cut into two different parts, giving two different samples, where the first part consisted of the midgut and pylorus, and the second, the hindgut containing both ileum and rectum (figure 2.4).

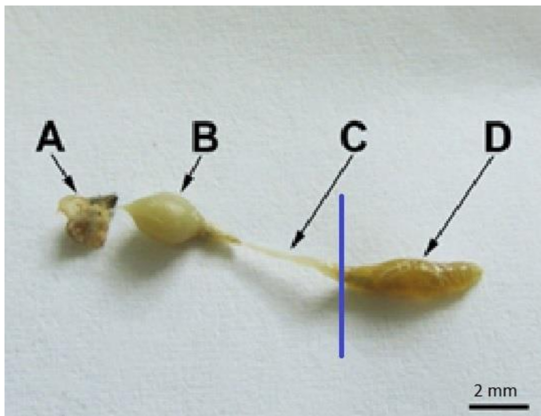


Figure 2.4: Posterior section of the GI tract from adult worker honeybee. A; shows the sting apparatus, B; rectum, C; ileum or small intestine, and D; midgut/ventriculus. The blue line shows the area where a cut was done, by small scissors, to divide the lower part of the GI tract into the two different compartments, the rectum and ileum, and the midgut and pylorus, respectively. The sting apparatus was not included to the rectum and ileum sample. Figure is modified and reprinted from Dade (2009).

Each gut compartment were added into a 2 ml sample tube (Sarstedt, Germany) containing 300 µl stool transport and recovery (S.T.A.R) buffer (Roche, Germany), to ensure inactivation of infectious organisms, minimal degradation of nucleic acids, and enhancing the binding of nucleic acids to magnetic beads in isolation of DNA (Espy et al. 2006). Samples were stored at -20 °C before further processing. The sample tubes were also pre-filled with 0.15-0.20 gram acid-washed glass beads (<106 µm) (Sigma-Aldrich, Germany).

2.2 DNA extraction

To isolate DNA from the cells from the gut samples, the cell walls were disrupted both mechanically and chemically, by using a modified extraction protocol, developed for stool samples.

Thawed sample tubes (Sarstedt, Germany) were processed twice in MagNaLyser (Roche, Germany) at 6500 rpm for 20 seconds where the samples were kept cold for 1-minute rests between runs. In this step, the glass beads already present in the sample tubes contributed to mechanical crushing of the sample, thus also disrupting cell walls. After the mechanical crushing, the genomic DNA was extracted by using the MagLGC™ Total Nucleic Isolation kit (LGC Genomic, Germany) for blood samples.

All extraction steps were performed in a KingFisher Flex robot (Thermo Scientific, USA).

The extraction method involves centrifugation of the sample tubes at 13000 rpm for 5 min, before a lysis step is performed, were 5 µl proteinase and 50 µl lysis buffer is added to 50 µl supernatant before incubation at 55 °C for 10 minutes. The further steps involves binding of DNA to paramagnetic beads, binding negatively charged DNA from the supernatant, and three different washing steps, removing contaminants by salt and alcohol based buffers, before extracted DNA is released in elution buffer.
Extracted DNA was stored at -20 °C before further processing.

2.3 Polymerase chain reactions

Detailed information about the primers and the thermal conditions used for all PCR reactions are listed in table 2.1. Concentrations of genomic DNA in the samples used in PCR and qPCR assays are given in appendix F.

2.3.1 Qualitative PCR

All qualitative PCRs consisted of the reaction mixture described below.

Each reaction contained 1,25 U HotFirePol® DNA polymerase, 1x HotFirePol® buffer B2 , 2,5 mM magnesium chloride ($MgCl_2$), 200µM dNTP (Solis BioDyne, Estonia), 200 µM of both forward and reverse primer (according to table 2.1) (Invitrogen™, Thermo Fischer Scientific, USA) and 1 µl template DNA. Initial denaturation was set at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, an annealing step for 30 sec (temperatures and details shown in table 2.1), elongation at 72°C for 30 sec[5], and a final elongation at 72 °C for 7 min before cooling at 4 °C ∞. All reactions were performed on a 2720 Thermal Cycler (Applied Biosystems, USA). A control, was included in all reactions, consisting of nuclease free water (Amresco, USA).

Gradient PCR

The thermal conditions for the gradient PCRs, where the same as described in 2.3.1.

[5] In the PCR reaction of *tetB* amplicons for sequencing, elongation was prolonged to 60 min.

Annealing temperature was set to 52 °C, with a 5°C gradient, giving an annealing temperature interval between 47-57 °C for the primers targeting the Norwegian strain of *G. apicola*. For primers targeting the American strain of *G. apicola*, the annealing temperature was set to 55 °C, with a 5 °C gradient, giving an annealing temperature interval between 50-60°C. All gradient PCRs were performed on a Mastercycler® (Eppendorf, USA).

2.3.2 Illumina adapter PCR

Regions of the 16S rRNA (variable regions V3 and V4) was sequenced by Illumina sequencing, from the samples of midgut and hindgut, in order to study bacterial composition. A nested PCR was performed, involving two steps, before samples were ready for sequencing.

The first PCR included the same reaction mixture as previously described in 2.3.1., with the following thermal conditions; initial denaturation at 95 °C for 15 min, followed by 25 cycles of 95 °C for 30 sec, annealing at 50 °C for 30 sec and elongation at 72 °C for 45 sec. The final elongation step was set to 72 °C for 7 min. A cleaning step of the PCR products, by using AMPure XP beads was preformed, as described in section 2.3.4. Correct size of the amplicons were checked on 1 % agarose gel, for some of the samples.

For the second PCR, Illumina-specific adapters were added to the fragments by using modified Illumina-indexed PRK primers. The 3' end of the modified PRK primers contained the gene specific region, while the 5' ends contained a colony amplification region for attachment to complement oligonucleotide strands on the flow cell of the Illumina sequencing platform, along with an Illumina sequencing region and a unique primer tag sequence. By using different combinations of modified primers (16 different forward, and 36 different reverse primers, listed in appendix C), different primer combinations were possible (in total 576 different primer combinations). In this way, each sample would have specific primer tags, making it possible to distinguish amplicons from different samples from each other. Each reaction contained the same as previously mentioned, with the exception of all samples containing different combinations of primers. The thermal conditions were the following; 95 °C for 15 min, followed by 12 cycles of 95 °C for 30 sec, 50 °C for 1 min and 72 °C for 45 sec, with a final elongation step at 72 °C for 7 min.

Quantification of the PCR products, done by qPCR and standard curve method by using hydrolysis probes (TaqMan), is described in 2.3.3.

2.3.3 Quantitative PCR

QPCR assays using EvaGreen® dye

Detection and quantification of the presence and abundance of total amount of bacteria (i.e. 16S rRNA), *G. apicola*, *S. alvi, tetB*, *tetH,* and the two different strains of *G. apicola* (Norwegian and American), was done by qPCR, using the DNA-binding dye EvaGreen®.

The reaction volumes were 20 µl and contained 1x HotFirePol® EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia), nuclease free water (Amresco, USA), and 200 nM both forward and reverse primers (Life Technologies™, USA). Templates were added in 2 µl volume per reaction. Triplicates of no-template controls, containing nuclease free water, were included in each run.

The thermal conditions were the same for all reactions, except for different annealing temperatures, according to the different primers used (table 2.1). Initial denaturation was set at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, an annealing step for 30 sec (temperatures shown in table 2.1), and elongation at 72 °C for 30 sec, where fluorescence was measured after each cycle. All reactions were performed on 96 well LightCycler qPCR plates (Roche, Germany), with a Light Cycler 480 II (Roche, Germany). After each run, a high resolution melting (HRM) curve analysis was performed to verify the presence of the desired amplicon.

Standard curves were included on each qPCR plate, according to the fragment of interest. Fragments to be used in the standard curves were obtained by doing qualitative PCR, and were serially 10-folds diluted in nuclease free water. DNA concentrations was measured by qubit™ fluorometer, described in 2.5.1.

TaqMan qPCR for quantification of Illumina adapter PCR products

PCR products from the Illumina adapter PCR were diluted 1:200 in nuclease free water prior to the quantification. Each reaction contained 1x HotFirePol probe qPCR mix (Solis BioDyne, Estonia), 0,2 µM of each forward and reverse colony primers (Yu et al. 2005) , 0,1 µM TaqMan probe (Milinovich et al. 2008) (Life Technologies™, USA) and 1 µl DNA template. The thermal conditions were the following; 95 °C for 15 min followed by 40 cycles of denaturation

at 95 °C and a combined annealing and elongation step at 60 °C for 1 min. Fluorescence was measured after each cycle and the qPCR was performed by a Light Cycler 480 II (Roche, Germany).

A standard curve was included to the qPCR, for copy number calculations to determine number of copies in each PCR product.

2.3.4 Purification of PCR products by Ampure

To obtain a pure PCR product before sequencing, a purification step, involving AMPure® XP beads (Beckman Coulter, USA) was used. By purifying the PCR product, a higher and purer yield of the product is obtained and possible and contaminators, such as primer dimers, salts, polymerase and nucleotides, are removed, leaving the purified PCR product free of contaminants. AMPure® XP beads are paramagnetic beads, that become magnetic only when a magnet is present. First, DNA binds to the beads and is separated from the contaminants in the solution by a magnet. Secondly, beads with the PCR amplicons attached, are washed twice in freshly prepared 70-80% ethanol to remove further contaminants, and thirdly a last and final step involves elution of the purified amplicons from the beads (by e.g. distilled water). The purification was automatically done by a Biomek® robot (Beckman Coulter, USA) with a bead to PCR product volume of 1:1.

Table 2.1: Primers and thermal conditions used in PCR.

Target gene	Primers	Amplicon length (bp)	Annealing temp (° C)	Annealing time (sec)	Used for	Primer sequences F/R (5'-3')	References
16 S rRNA (V3 and V4 region)	PRK341F PRK806 R	450	60	60[1]	d/qPCR	TCCTACGGGAGGCAGCAGT/ GGACTACCAGGGTATCTAATCCTGT	(Nadkarni et al. 2002)
G. apicola	Gamma1-F Gamma1-R	210	55	30	d/qPCR	GTATCTAATAGGTGCATCAATT/ TCCTCTACAATACTCTAGT	(Martinson et al. 2011)
TetB	TetB-QF TetB-QR	206	61	30	d/qPCR	ATACAGCATCCAAAGCGCAC	(Aminov et al. 2002)
Norwegian *G. apicola*[3]		179	52	30	d/qPCR	GACACAGCAAGAATAACAAC/ CCCAATGAAGCTGATTTAC	This work
American *G. apicola*[3]		330	59	30	d/qPCR	TGTCTACGCCGCTATAAT/ CGGCGATTGATACCGTTT	This work
16 S rRNA	PRKi F PRKi R	466	50	30	Illumina PCR[2]	CCTACGGGCBGCASCAG/ GGACTACYVGGGTATCTAAT	(Yu et al. 2005)
tetB	TetB-F TetB-R	659	60		PCR for Sanger sequencing	TTGGTTAGGGGCAAGTTTTG/ GTAATGGGCCAATAACACCG	(Fan et al. 2007)
S. alvi	Beta-F Beta-R	128	55	30	d/qPCR	CTTAGAGATAGGAGAGTG/ TAATGATGGCAACTAATGACAA	(Martinson et al. 2012)
tetH	TetH-F TetH-R	407	60	30	d/qPCR	CCAGAACCGCCAAAGACATACC/ GTGATGTGACTCCCGCTAAAAAT	(Fan et al. 2007)

[1] Combined annealing and elongations step at 60 °C for 1 minute.
[2] Illumina primers, used for the index PCR are listed in Appendix C.
[3] Primer design and optimization is listed in section 3.4

2.4 Sequencing

2.4.1 Sanger sequencing

Labelling of the *tetB* amplicons before sequencing was done by BigDye® Terminator v1.1 Cycle sequencing kit (Applied Biosystems, USA) in a 2720 ThermalCycler (Applied Biosystems, USA). The following thermal conditions were used; activation at 95 °C for 1 min, followed by 25 cycles of denaturation at 96 °C for 15 sec, annealing at 50 °C for 5 sec, and an annealing/elongation step at 60 °C for 4 min. Final step was set to 10 °C for ∞.

Both forward and reverse primers were run separately on all samples, creating complimentary 3'end labeled sequences.

Extension products were purified by ethanol precipitation (conducted by Professor Knut Rudi). The purification involves addition of salt and ethanol to the PCR product solution, which leads to precipitation of nucleic acids out of the solution. The nucleic acids can then be separated from the rest of the solution by centrifugation, before the pellets are washed in ethanol, and further centrifuged, before drying and resuspension of the pellet in a buffer.

2.4.2 Illumina sequencing

The Illumina index PCR products, quantified by TaqMan qPCR and the standard curve method, as described in 2.3.3, was normalized and pooled using a Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter, USA). The pooled sample was purified by AMPure® XP beads, described in 2.3.4, and the DNA concentration was quantified by using the Perfecta® NGS library quantification kit for Illumina sequencing platforms (Quanta Biosciences, USA). The amplicon library was loaded on the flow cell in a concentration of 4 μM, following the Illumina protocol for 16S rRNA sequencing, before loading on the MiSeq® system (Illumina, USA).

2.5 DNA quantity and quality control

2.5.1 Qubit measurements

DNA concentrations of both genomic DNA, and PCR products, were calculated by using a Qubit™ fluorometer (Life Technologies, USA). Assay tubes were prepared according to the manufacturers recommendations, were 2 µl were added to 198 µl working solution, containing Quant-iT™ reagent in a volume of 1:200 in Quant-iT™ buffer.

2.5.2 Gel electrophoresis

PCR products were controlled for correct amplicon size by gel electrophoresis on a 1% agarose gel, prepared by dissolving agarose (Sigma-Aldrich, Germany) in 1x tris-acetate EDTA (TAE) buffer. Agarose is a sugar compound consisting of repeated L- and D- galactose subunits, forming a non-covalently bound network in gel, where the amount of agarose added, determines pore size, thus molecular filtering properties.

The negatively charged DNA fragments are determined by size due their migration abilities from a positively charged, to a negatively charged electrode, where fragments of small sized diffuses faster in the gel complex.

Voltage and time was set between 80-90 V and 30-45 min, respectively. Two different staining methods were used during the study, Gel red™ (Biotium, USA) and PeqGreen (Peqlab, Germany), where both bind to DNA, but the Gel red is added directly to the PCR product, and ladder, while PeqGreen is only added to the gel solution before gelification.

As a size marker 100bp DNA ladder (Solis BioDyne, Estonia) was used, and The Molecular Imager® Gel Doc™ XR Imaging system with Quantity One 1-D analysis software v.4.6.7 (Bio-Rad, USA) was used for visualization of the DNA bands, by using UV light.

2.5.3 High resolution melting point analysis

In addition, PCR products from the qPCRs were quality checked by performing a high-resolution melting point (HRM) analysis at the end of each of the qPCR reaction. The analysis was performed on the LightCyler after amplification and was performed by a precise warming of the amplified product from 50 °C to 95 °C. Due to the fact of different amount of GC, different amplicons can be detected from each other, since different amounts of CG give rise to different melting temperatures, this being the temperature when the DNA strand is 50 %

denatured. Depending on amount of GC in the product, double stranded DNA melts apart with different melting points, where a melting point of double stranded DNA is defined at the time where the two strands are 50 % denatured. By using fluorescence targeting double stranded DNA, the melting point is possible to detect.

2.6 Culture dependent methods

2.6.1 *G. apicola* growth conditions

G. apicola were cultivated on both tryptic soy agar (TSA) and blood agar (TSA plates infused with 5 % horse blood), at 37 °C in a CO_2- enriched atmosphere for 72 hours. The CO_2- enriched atmosphere (producing 5% CO_2) was provided by a GasPack™ EZ CO_2 sachet (BD, USA) in an airtight container.

2.7 Data Analysis

2.7.1 Absolute quantification by the standard curve method

Copy number calculations of each gene was done by the standard curve method were standard curves were obtained by using serial dilutions of PCR products of each of the gene of interest. Equation 1 was used to calculate the number of genes in the used standards, where the concentration of DNA in the PCR products was obtained by Qubit.

$$number\ of\ copies\ (molecules) = \frac{amount\ of\ amplicon\ (ng)\times 6{,}0221\times 10^{23}\ molecules/mole}{\left(length\ of\ amplicon\ (bp)\times 660\frac{g}{mole}\right)\times 1\times 10^{9}\ ng/g} \quad (1)$$

Copy number estimates assume an average molecular weight of 660 g/mole for a base pair in double stranded DNA, and one gene copy number per genome.

Standards were then plotted by template starting concentration in log scale versus quantification cycle (Cq), giving equation 2.

$$C_q = -slope \times \log copy\ number + Ct\ intercept \qquad (2)$$

To find log copy number in sample, equation 2 was rearranged to equation 3.

$$\log copy\ number = \frac{Ct\ sample - Ct\ intercept}{-\ slope} \qquad (3)$$

Amplification efficiencies were calculated by equation 4.

$$Efficiency\ E = 10^{\frac{-1}{slope}} - 1 \qquad (4)$$

The standard curves used for copy number calculations for each of the genes are given in Appendix F.

2.7.2 Scoring procedures for amplicon detection

Due to large variation in the samples in copy numbers of *tetB* and the American *G. apicola* strain, a scoring procedure was performed to determine non-presence, or presence of the gene fragments.

Minimum detection limits were chosen based on Cq values in the negative controls on the qPCR plates, and scoring was performed by giving all samples a score of either 0 or 1, corresponding to either presence or non-presence of amplicon of interest respectively, where copy numbers lower than the determined detection limit gave a score of 0, while copy numbers equal or higher was given a score of 1. From these scorings, means from each of the treatments at each time point was calculated, and given that there were sampled three bees from each cage, with three replicates per cage at each sampling point, a mean score of three would apply to all samples from the same treatment being positive for the amplicon, at given time point.

In addition, HMR curves were checked for all positives, to reassure correct amplicon.

2.7.3 Analyses of sequenced data

The sequences obtained from the Sanger sequencing were visualized and analyzed using the CLC Main Workbench v.7.6 software (Qiagen, Germany). Consensus sequences were assembled from the complementary sequences and alignments were done from all consensus sequences, including the *tetB* sequence from the American *G. apicola*.

The 16S rRNA sequences, obtained from the Illumina sequencing, were analyzed by using Usearch (Edgar 2010) and processed in the bioinformatics pipeline Qualitative Insight In Microbial Ecology (QIIME) (Caporaso et al. 2010) for quality filtering and OTU clustering. The quality filtering included removal of reads with an average score <Q25, reads shorter than 200 bp and reads with mismatches in the barcode region. Clustering of OTUs, to obtain an OTU table, was done by a 99 % homology threshold, and taxonomy assignment was done by using the Greengenes database (McDonald et al. 2012).

For phylogenetic analysis of the dominating OTUs, multiple sequence alignment was performed by ClustalW in BioEdit (Hall 1999), and phylogenetic tree was constructed in MEGA6 (Tamura et al. 2013) by using the distance based method Neighbor-Joining and tested for robustness by bootstrap statistical tests with 1000 iterations of bootstrapping.

2.7.4 Statistical analyses

Statistical analysis was performed by multivariate analysis of variance (MANOVA) (conducted by Professor Knut Rudi). Further post-hoc testing was done by analysis of variance (ANOVA), following by Bonferroni tests. In addition two-tailed t-tests assuming equal variance, was done for some of the analysis. The different statistical methods are presented briefly as follows, where all test are done by comparing variances (σ^2) of mean values (corresponding to response values, or independent variables) obtained from a population, or group:

In short t-tests are used when comparing only two means, such as two treatment groups, while statistical analysis by ANOVA, allows for comparison of more than two groups. In addition

ANOVA analysis can be used for statistical testing by including only one (one-way ANOVA), or two treatments (two-way ANOVA).

When comparing more than two different treatments, a MANOVA can be performed. MANOVA is an extension of ANOVA and is used to test simultaneously the relationship between several categorical variables (e.g. treatments) and two or more metric dependent variables.

All statistical tests performed in this thesis were done at 95% confidence levels (α=0.05).

3. Results

3.1 qPCR screening results

3.1.1 Quantification of total amount of bacteria and *G. apicola*

Total number of copies of 16S rRNA (A) and *G. apicola* (B) in the samples are shown in figure 3.1, where data from the midguts and hindguts are presented in (a) and (b) respectively. The total copy number in the midguts ranged between 10^4 and 10^6, while the hindgut showed copy numbers between 10^6 and 10^8. *G. apicola* copy numbers ranged around 10^3 and 10^4 in the midguts, and 10^5 and 10^6 in the hindguts. In the midguts, both 16S rRNA, and total amount of *G. apicola,* tend to decrease, when bees were treated with tetracycline, whereas the hindguts did not show the same tendency.

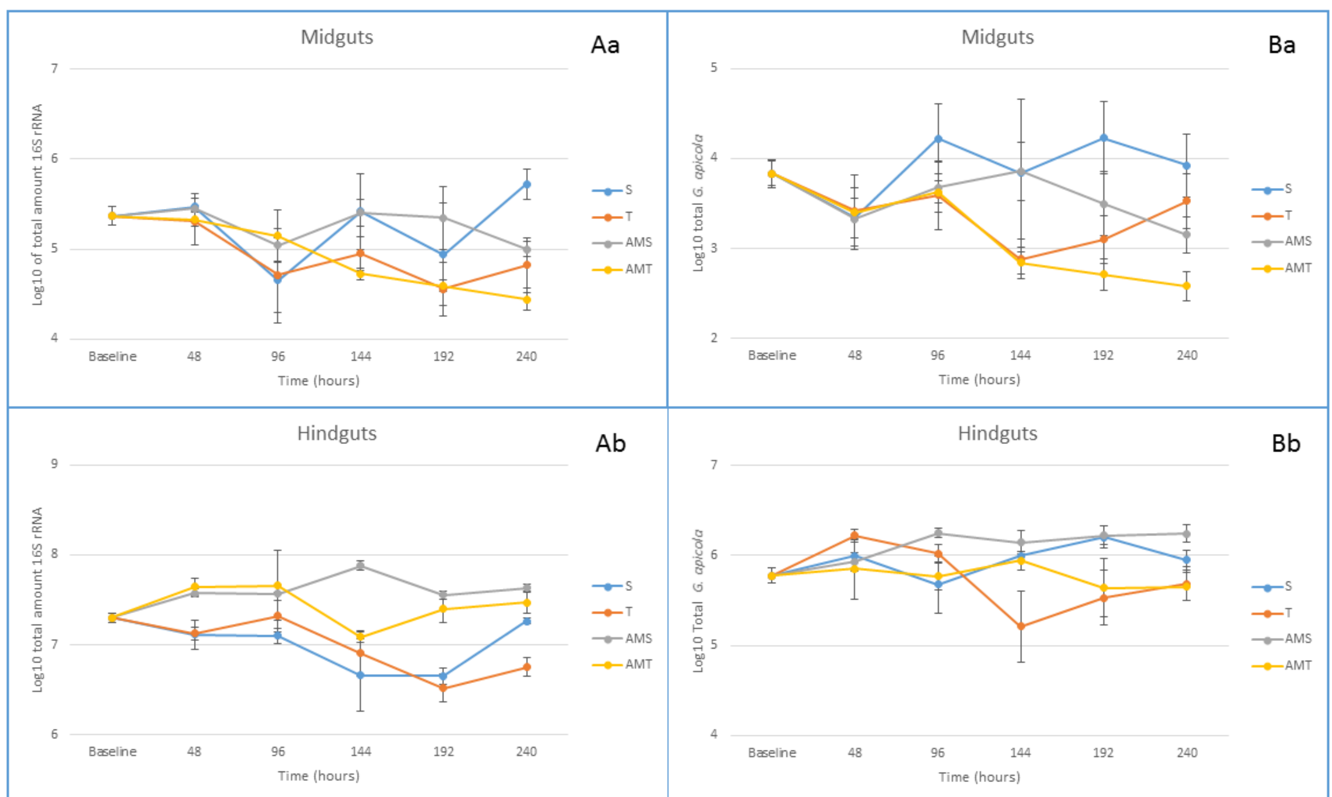


Figure 3.1: Total amount of log10 copy number of 16S rRNA (A) and *G. apicola* (B) in midguts (a) and hindguts (b) per 2 µl sample (Mean ±SEM). S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

MANOVA analysis gave significant effect of treatment with tetracycline (p-value= 0.034) and treatment with *G. apicola* (p-value = 0.046) in total number of 16S rRNA in midgut samples. For hindgut samples significant effects of treatment with *G. apicola* was observed on total number of 16S rRNA (p-value = 0.0009), in addition to effect of tetracycline on total number of *G. apicola* (p-value= 0.033).

3.1.2 Presence of *tetB*

Large individual variations were seen in presence of *tetB* (appendix G). In midguts, copy numbers ranged between 10^1 and 10^6 (mean=1122, median= 524). In average 28 % of all midgut samples were positive for *tetB*, independent of treatment. In hindguts, copy numbers where higher, and ranged between 10^2 and 10^7 (mean= 6918, median= 1659). In average 44 % of all hindgut samples were positive, but between treatment groups, variations were detected with the lowest percentages of positives in the two sugar groups (S and AMS) (33 % and 42 %, respectively). The two groups treated with tetracycline (T and AMT), had higher amounts with positives, with respectively 51% and 49 % positive samples. Detection limit was set to 100 copy numbers, and graphs, based on the mean score values are given in figure 3.2, where (A) and (B) shows presence of *tetB* in midgut and hindgut samples, respectively. A baseline measure for comparison was obtained from samples from time points 0h and 24h (n= 18), which had not received antibiotic treatment.

MANOVA analysis showed significant effects of number of *tetB* positive bees in both midguts and hindguts treated with American *G. apicola*, with p-values of 0.008 and 0.012 respectively. No significant effect of treatment with tetracycline was detected.

The graph show an increase of number of bees positive for *tetB* over time in hindguts treated with *G. apicola.*

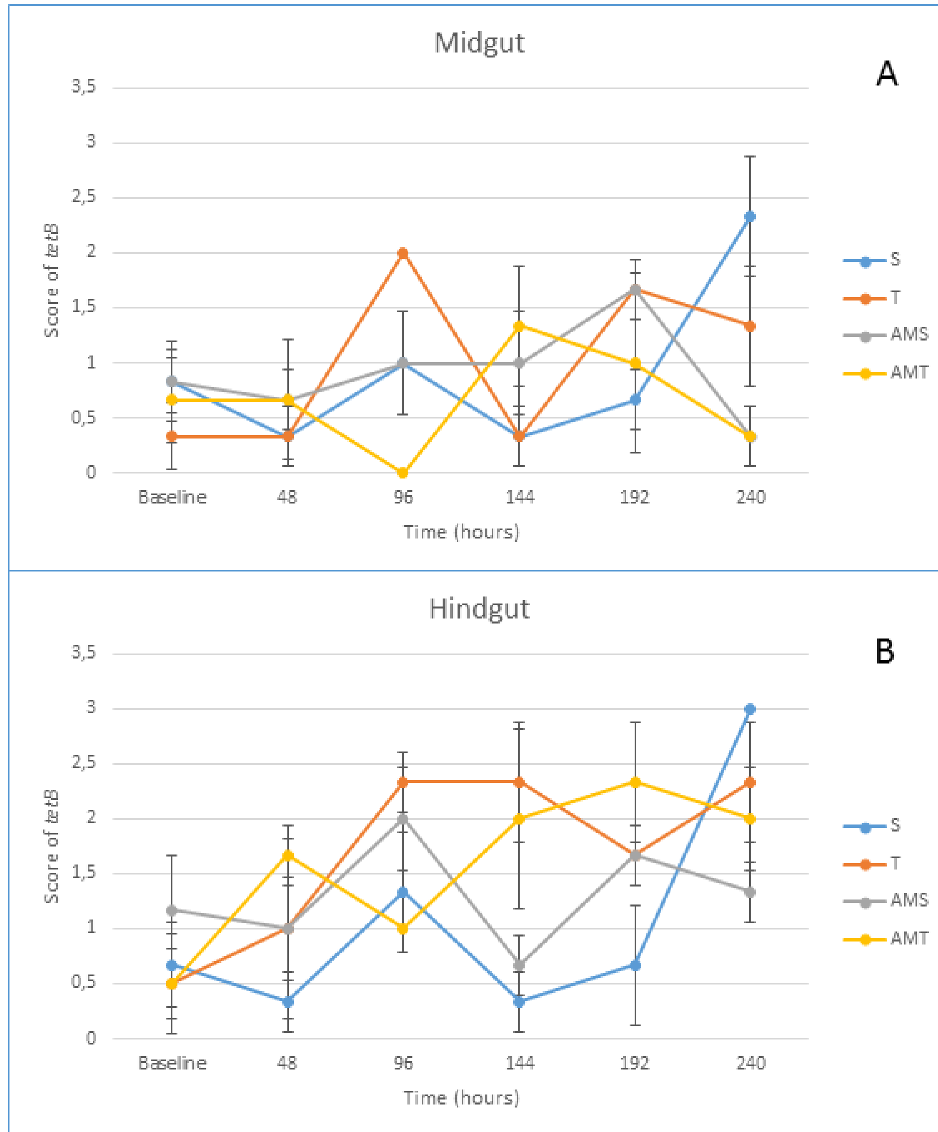


Figure 3.2: Presence of *tetB* (mean ± SEM) at each sampling time point in midgut (A) and hindgut (B) samples. Abundance is given by a scoring measure, where positive and negative samples for *tetB* are scored by 1 and 0, respectively, for each sampled gut part from each cage (three bees sampled from three cages with same treatment), thus leading to a maximum possible mean score of 3. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

3.1.3 Detection of *G. apicola* strains given in sugar solution

The Norwegian strain was detected frequently in all treatments, with copy numbers ranging between 60 and 10^5, where positives for the strain was found in 94 % of the bees, independent of treatment (data not shown).

Calculated copy numbers of the American strain of *G. apicola* is given in appendix H.

Detection limit was set to 15 copy numbers. The groups who had received bacterial culture of American *G. apicola,* copy numbers ranged between 15 and 3811 (mean=241, median=15) in the midguts, and 15 and 443 (mean=41, median=15) in the hindguts. Respectively, 22% and 32 % of all midgut, and hindgut samples, from bees who had been treated with the bacterial culture, were positive for the strain. In contrast, respectively 4% and 2% of the midgut and hindgut samples, from bees who had not been treated with the bacterial culture, were positive for the strain. Results are shown figure 3.3, were a clear trend is seen in both the midgut (3.3.A) and hindgut (3.3.B) samples. For comparison, a baseline measure was obtained from samples collected at time point 0h (n= 9), which had not received any treatments. A peak is seen in in the two groups who received the bacterial culture (AMS and AMT) at time point 24h in the midguts, and time point 48h in the hindguts.

MANOVA analysis showed significant effect of treatment with American strain *G. apicola* in both midgut and hindguts, with p-values of 0.0008 and 0.0001, respectively.

Further testing by ANOVA, gave significant effect of time in the AMS group in both midguts (p-value= 0.0037) and hindguts (p-value=0.0029), in addition to the AMT group in midguts (p-value <0.0001). Post-hoc testing with Bonferroni tests, gave significant differences in midguts treated with AMS between 0h and24h (p-value= 0.0193), hindguts treated with AMS between 0h and 48h (p-value= 0.0023) and midguts treated with AMT between 0h and 24 hours (p-value= <0.0001).

In addition significant differences were seen between time points 24 and 240 h in the midguts treated with AMS, and AMT (p-values respectively 0,0039 and <0.0001), and between time points 48 and 240 h in the hindguts treated with AMT (p-value= 0.0082).

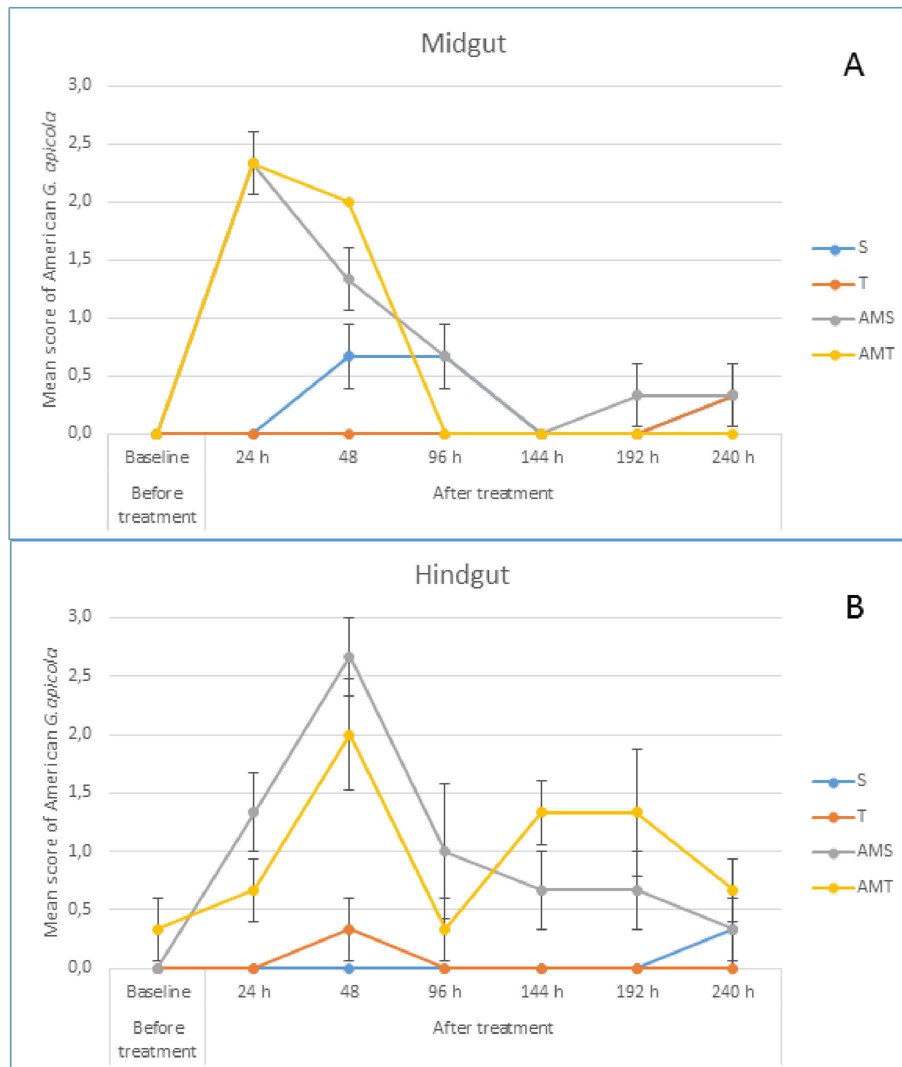


Figure 3.3: Presence of American strain of *G. apicola* (mean ± SEM) in midgut (A) and hindgut (B) samples. The presence is given by a scoring procedure, were positive and negative samples are given a score of 1 and 0, respectively. A maximum mean score of 3, applies to all samples being positive for the strain for each treatment at given time point. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

3.2 Metagenome analyses of the 16S rRNA gene

All midgut and hindgut samples from the colony, the two groups receiving sugar (S and AMS), and the two groups receiving tetracycline (T and AMT) were sequenced, giving a total of 534 samples.

In total 8 381 830 sequences passed the filtering in QIIME. Further editing was done by removing samples with less than 1000 sequences in total. In total, 230 OTUs were generated.

3.2.1 Dominating OTUs in gut samples

To compare samples treated and not treated with tetracycline, all samples from time points 0h and 240h, was chosen for further analysis, in addition to samples from the colony.

Sequences that constituted ≤ 1% of sequences in any sample were removed (≤ 10 sequences in any sample). In addition, sequences present between 1 and 1.5 % in less than 3 samples were removed. This resulted in 71 remaining OTUs of the 230 in total. From these sequences, 44 OTUs were dominating.

Due to lack of 16S rRNA sequences of bacteria isolated from the honey bee in the Greengenes database, resulting in classification for only the family level for some of the OTUs, the dominating OTUs were searched for homologue sequences in the BLAST search algorithm blastn (http://blast.ncbi.nlm.nih.gov/). The search in BLAST had a higher success rate of classification, leading to classification on the genus, and also species level for some of the dominating OTUs.

The 44 dominating OTUs are shown in a phylogenetic three in figure 3.4. Accession numbers from GenBank are included.

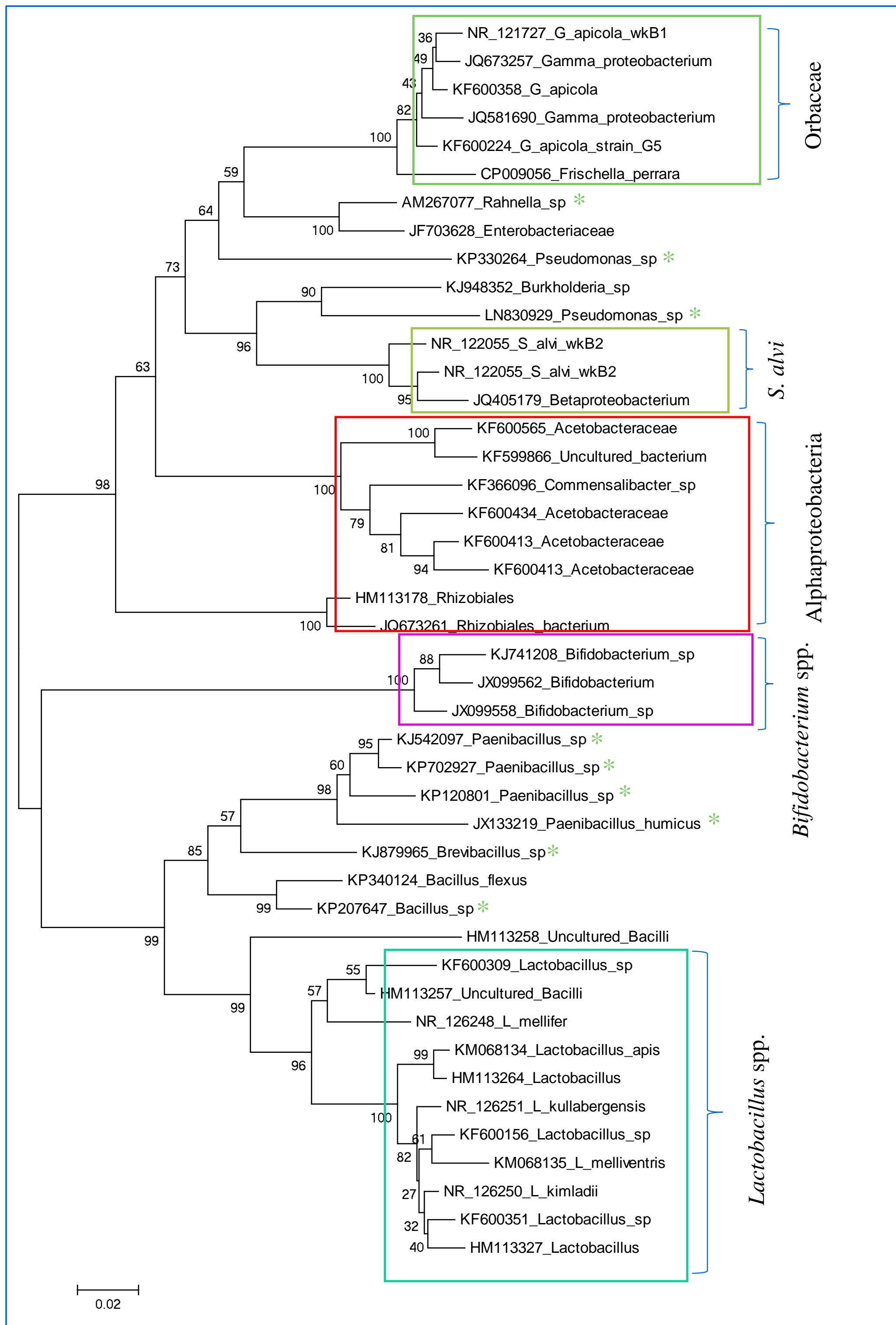


Figure 3.4. Distance based Neighbor-Joining phylogeny based on 16 S rRNA gene multiple sequence alignment. The tree includes 44 sequences representing the dominating OTUs in all samples from time points 0h and 240h. Multiple alignment was generated with ClustalW and manually edited in BioEdit (Hall 1999), and the tree was generated in MEGA6 (Tamura et al. 2013). Bootstrap values are indicated for 1000 replicates. Species or genera marked with a star, indicates that these were only present in colony samples. Accession numbers from BLAST hits are given for each OTU. The frames indicate the most dominating bacterial groups found in the samples.

Six OTUs belonged to the family Orbaceae (five *G. apicola* and one *F. perrara)*, while three belonged to *S. alvi.* In addition three Actinobacteria were observed (all belonging to the genus *Bifidobacterium*), eleven OTUs was identified as *Lactobacillus,* and eight OTUs belonged to the class Alphaproteobacteria, with *Acetobacteraceae* as the main family.

3.2.2 Comparison of relative abundance of bacteria in the different groups

Relative abundance of the dominating bacteria in the different groups are shown in percentage of total amount of bacteria in bar charts (figure 3.5). Both midgut and hindgut samples are presented, in respectively (A) and (B). A baseline measure of bacterial composition was obtained, including all samples taken from time point 0h (n= 36), to compare to different treatments.

Midgut samples from the hive showed high abundance of bacteria belonging to *Pseudomonas*, *Paenibacillus* and *Bacillus* (~10 %, ~5 %, and ~5 %, of total amount of bacteria, respectively), which was absent in caged bees at both time points 0h and 240h.

Somewhat different composition are also observed between different treatments, such as the two different treatments with tetracycline (T and AMT), where the midguts in the AMT group harbour a larger amount of *Burkholderia* spp., and also a larger composition of *Lactobacillus* spp., and fewer *G. apicola*. Largest amount of Alphaproteobacteria is seen in the colony groups. In hindgut samples, also the AMT group show a higher abundance of *Lactobacillus.*

In hindguts, fewer groups of bacteria are present, and all bacterial groups are found in all treatments.

In midguts, abundance of *G. apicola* varies between groups, but the amount in all groups constitutes percentages between 20% and 50% of total amount of bacteria, and is overall the most abundant species. Second most abundant species is bacteria belonging to *Lactobacillus,* making up 13% to 50 % of total amount of bacteria. *S. alvi* is abundant in percentages between 7% and 26 %, while species belonging to Alphaproteobacteria represents 5% to 22 % of total amount.

In hindguts, *Lactobacillus* spp. are most abundant, and represents relative abundances between 30- 60 % of total microbiota. *G. apicola* is the second most present bacterial group and is present in relative abundances between 20- 30 %. Bacteria belonging to Alphaproteobacteria presents the third most abundant bacterial group.

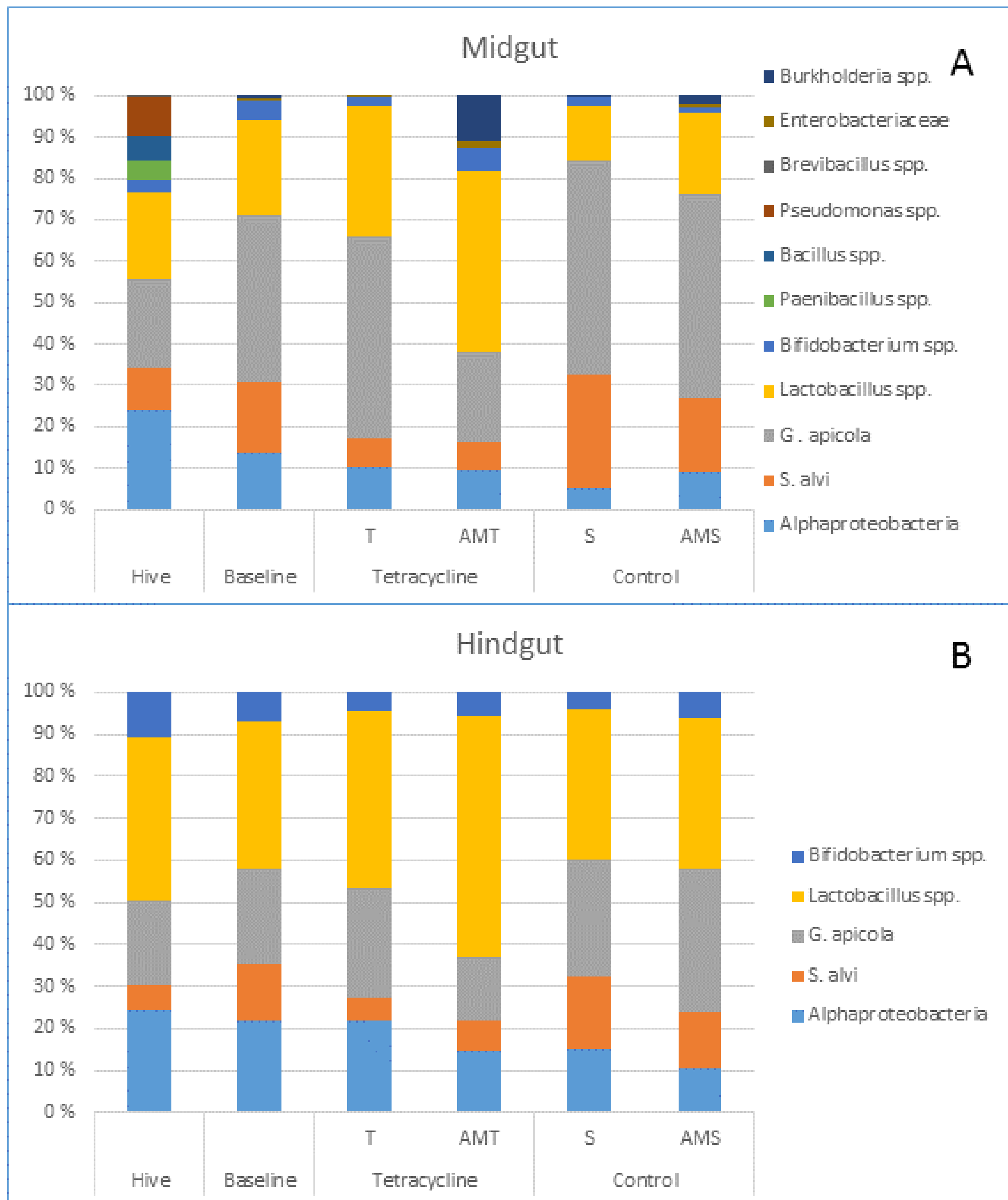


Figure 3.5. Bar graphs showing relative abundance of bacteria in midguts (A) and hindguts (B) in different treatment groups obtained by 16S rRNA sequencing. The OTUs were searched for homologues in BLAST (99% identity). Data presented shows the abundance of bacteria compromising >96 % 1000 sequences in the midguts, and >98 % of 1000 sequences in hindguts. Baseline presents composition of microbiota in samples from time point 0h (n=36), while the tetracycline and control group samples are sampled at time point 240h. S=treatment with sugar (n=9), T=treatment with tetracycline (n=9), AMS=treatment with American *G. apicola* and sugar (n=9), AMT= treatment with American *G. apicola* and tetracycline (n=9).

3.2.3 Effect of treatment determined by MANOVA

MANOVA analysis revealed significant effects of tetracycline (p-value= 0.0001), treatment with *American G. apicola* (p-value=0.0001), and time (p-value=0.004).

3.2.4 Comparative analyses of the main bacterial groups

The four most dominating bacteria belonging to *G. apicola, S. alvi, Lactobacillus* spp., and Alphaproteobacteria were chosen to investigate significant effects of tetracycline. Relative abundance of bacterial groups, according to treatments are presented in figure 3.5- 3.8. The abundance is shown in both midgut and hindgut samples, where (A) shows samples treated with tetracycline, and (B) shows controls, where tetracycline was not administered, in each figure. A baseline measure was included as a basis for comparison in each graph, presenting the average abundance of each of the bacterial species in all samples at time point 0 (n= 36).

G. apicola (figure 3.6) shows an overall higher abundance in midguts, compromising around 40 % of total amount of bacteria, compared to around 20 % in hindgut samples. Significant differences in the relative amount of *G. apicola* was seen in the midguts treated with AMT (p-value: 0.027). In addition there was a significant difference in abundance in midguts between the two different groups T and AMT (p-value=0.04). No significant differences were seen in hindguts treated with tetracycline. In controls, a significant difference was observed in one of the groups (p-value= 0.016).

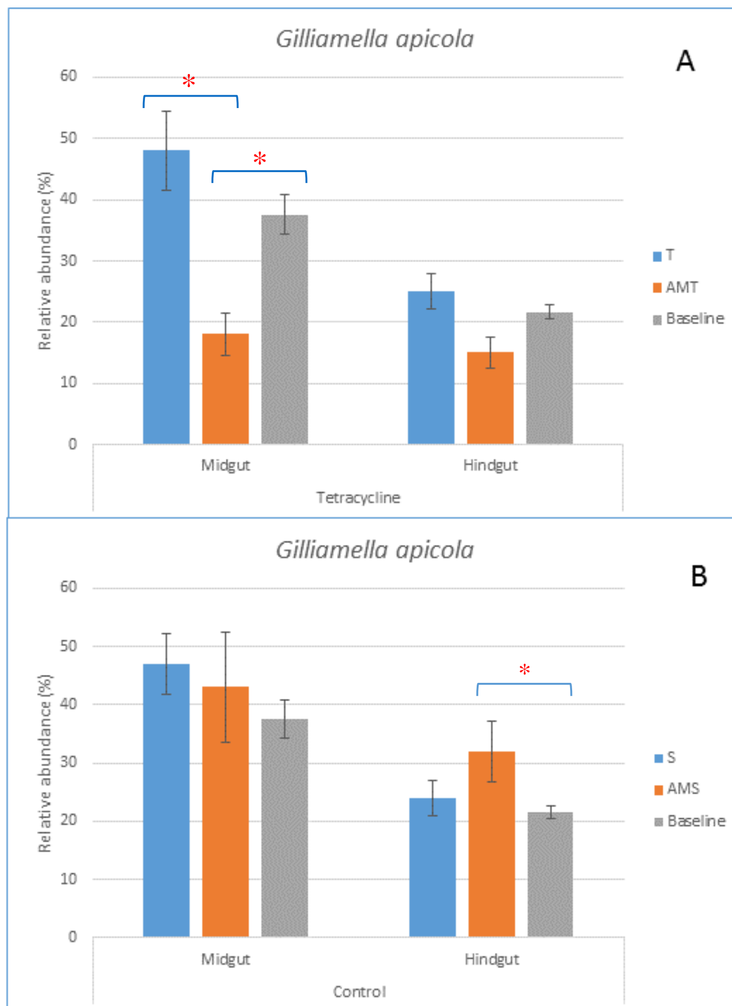


Fig 3.6: Relative abundance of *G. apicola* (mean ± SEM) in samples. Samples from bees treated with tetracycline are shown in (A), while the controls are shown in (B). *p-values < 0.05, determined by t-test. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

S. alvi showed a lower abundance in the tetracycline treated groups in both midguts and hindguts (figure 3.7), where significant differences were seen in the hindgut samples treated with tetracycline (p-values 0.022 for T, and 0.054 for AMT). No significant difference was seen in midguts, nor in any controls.

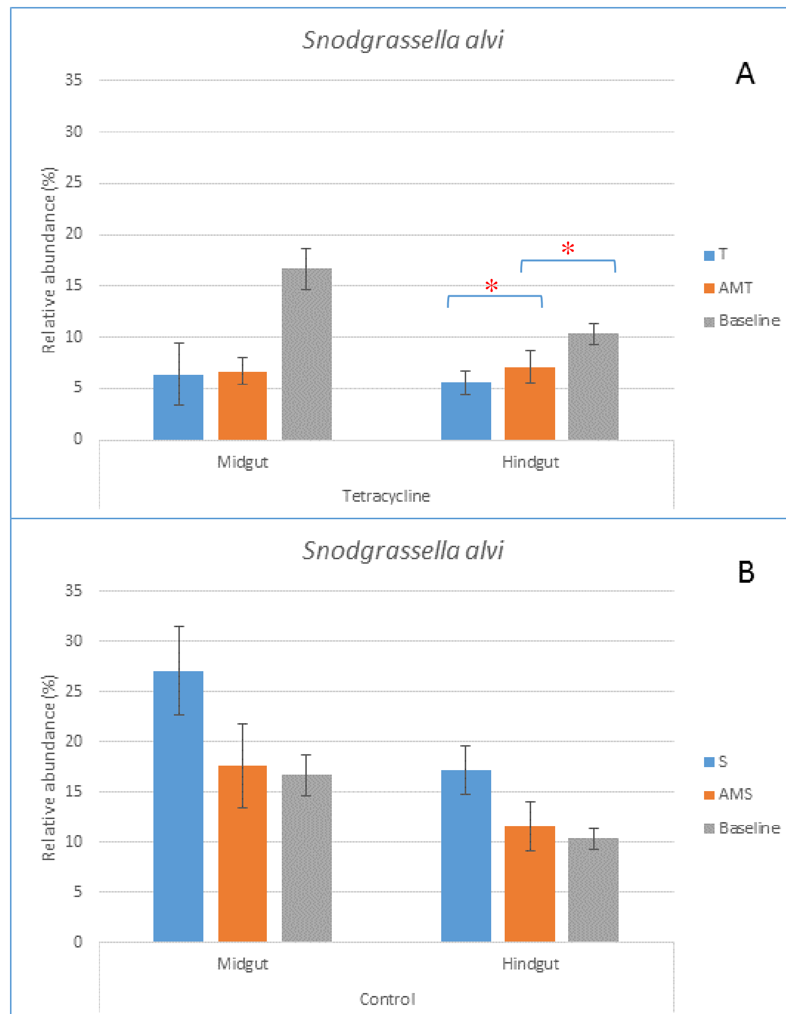


Fig 3.7: Relative abundance of *S. alvi* in samples (mean ± SEM). Samples for bees treated with tetracycline are shown in A, while controls are shown in B. *p-values < 0.05, determined by t-test. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

For *Lactobacillus* spp., an increase was seen in samples treated with tetracycline (figure 3.8). Significant effects were seen in both midgut and hindguts treated with AMT (p-values respectively 0.001 and >0.0001), and hindguts treated with T (p-value=0.029). In the midguts, the AMT group showed an increased abundance of 45 %, compared to the baseline group.

In addition there was a significant difference between hindguts treated with T and AMT (p-value= 0.012. For the controls, significant effect was seen in midguts in one of the groups (S) (p-value= 0.027).

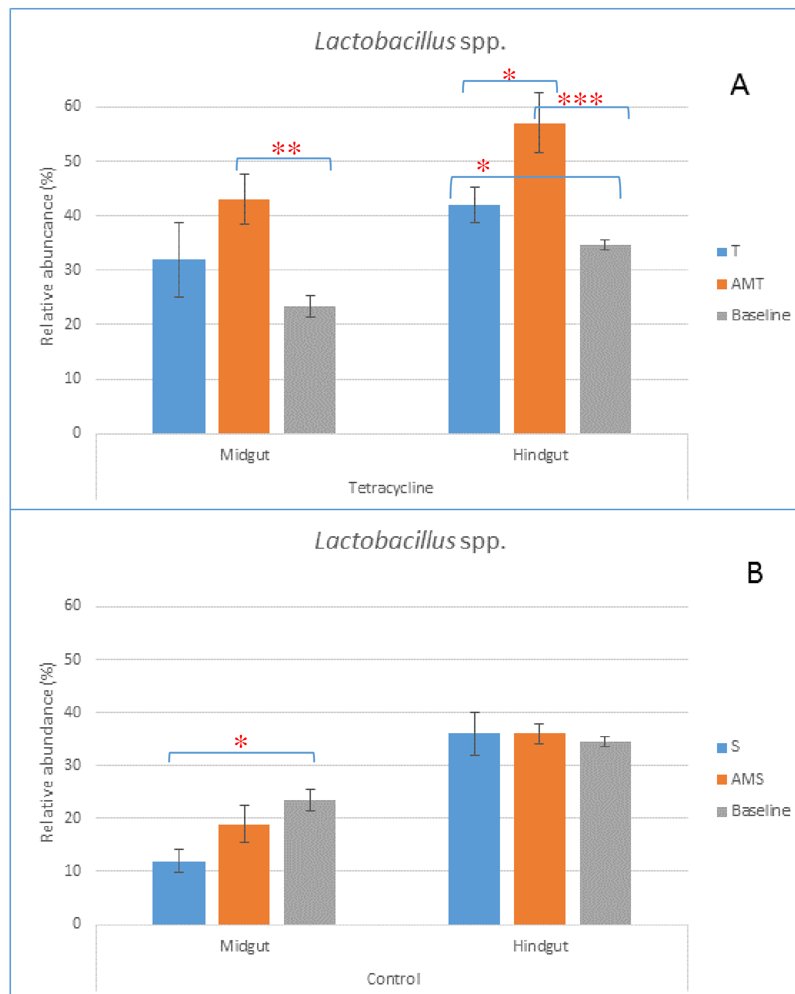


Fig 3.8: Relative abundance of *Lactobacillus* spp. in samples (mean ± SEM). Samples, according to gut compartments treated with and without tetracycline are shown in (A) and (B), respectively. *p-values< 0.05, **p-values<0.01, ***p-values<0.001. P-values were determined by t-test. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

Relative abundance of bacteria belonging to the class Alphaproteobacteria (figure 3.9) was shown to be higher in the hindgut samples, where the bacterial class constituted between 15% and 25% of total amount bacteria. Midgut samples had a 50 % lower abundance, compared to hindguts. No significant differences in abundance of the bacterial class were seen in midguts or hindguts from bees treated with tetracycline. However, a significant decrease was detected in the hindguts in one of the control groups (AMS) (p-value= 0.016).

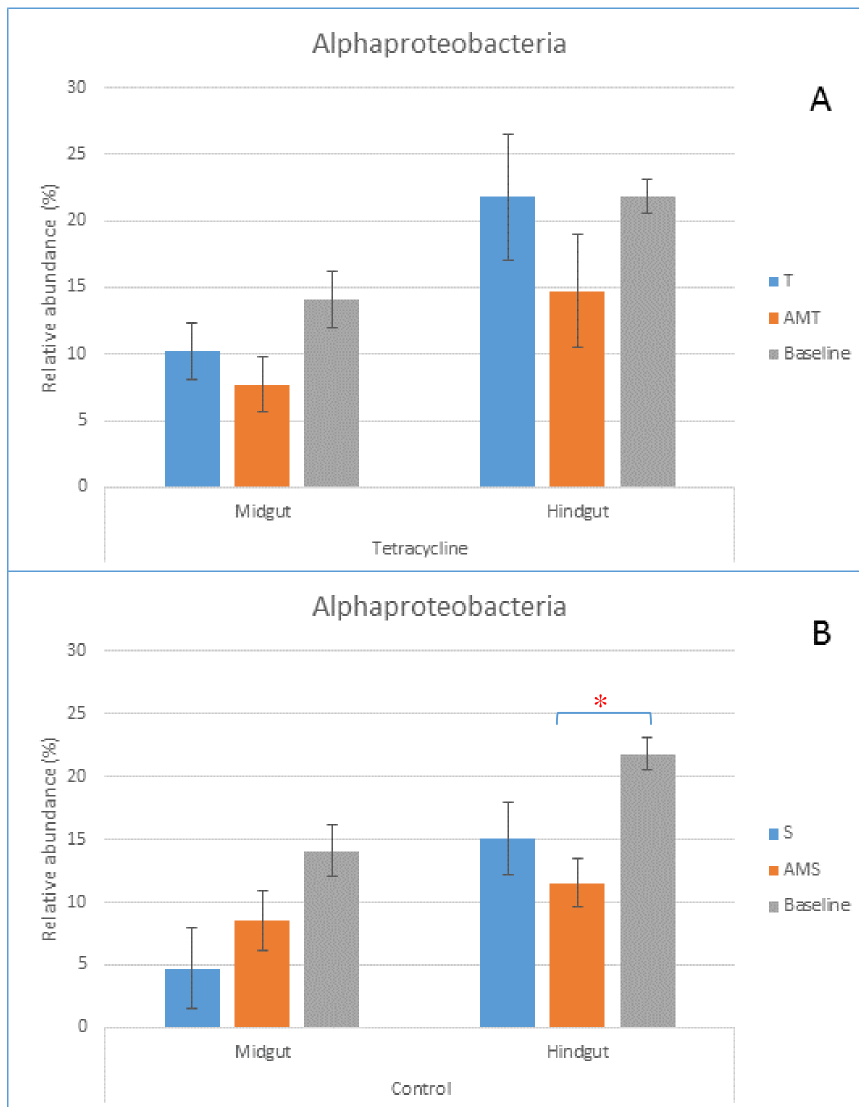


Figure 3.9: Relative abundance of Alphaproteobacteria in samples (mean ± SEM). Samples treated with and without tetracycline are shown in (A) and (B), respectively. *p-value<0.05, determined by t-test. S=treatment with sugar, T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

3.2.5 α-diversity

Alfa- diversity measurements of the diversity within the samples according to different gut parts and treatment with or without tetracycline are presented in rarefaction curves (figure 3.10). Data was obtained from QIIME. Midgut samples showed higher diversity than hindgut samples. In addition, midgut samples from bees treated with tetracycline had higher diversity than midgut samples from bees not treated with tetracycline, although this difference was not. In contrast, hindgut samples showed somewhat higher diversity in samples not treated with tetracycline.

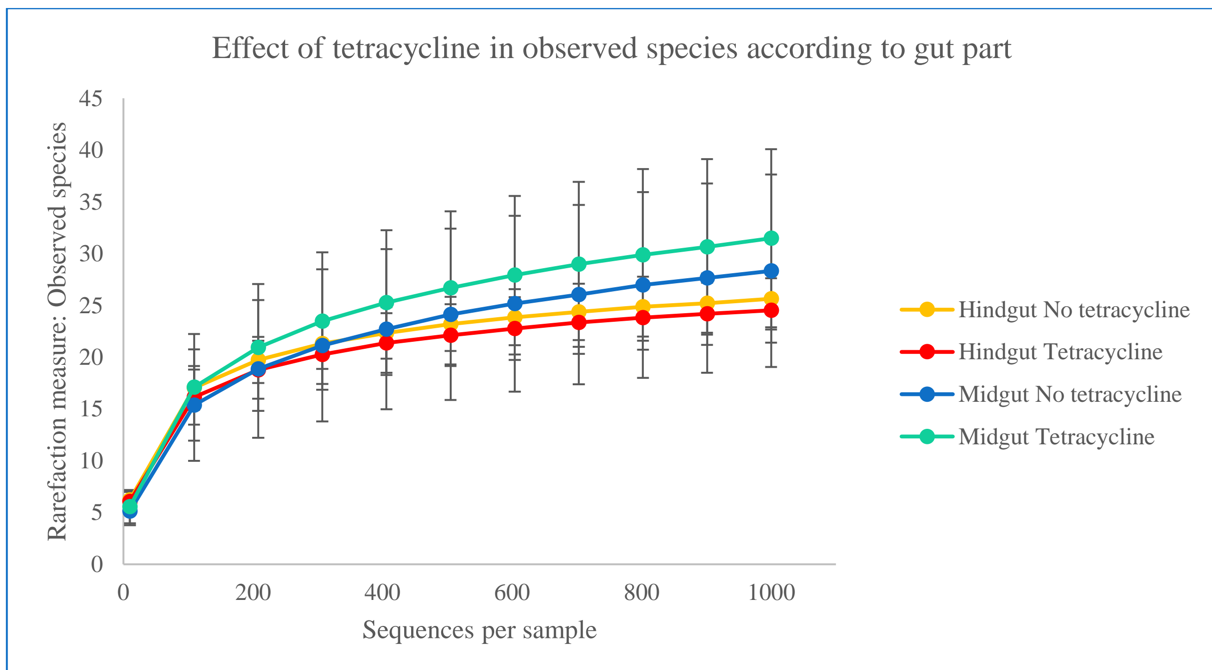


Fig 3.10: Rarefaction curves of observed species in the different gut compartments treated with or without tetracycline.

3.3 Sanger sequencing of the *tetB* gene

To detect possible origin of *tetB*, sequencing of a fragment of the gene (659bp) was performed. A sequence alignment of the gene fragment had been performed in advance, and an unique mutation was observed in *tetB* belonging to the American strain used in this study.

In total, 48 samples were chosen for sequencing of *tetB*. Only samples with the highest amount of the gene were selected i.e. samples containing >10 000 copies of the gene. Both samples from the midgut and hindgut were included, from the treatments S, T, AMS and AMT.

Sequencing results showed one sample positive for the American *tetB*. A full length alignment of the consensus (consensus 1-34) and the American strain is given in appendix D.

The positive sample originated from a bee who had received the bacterial culture, which also was the sample where highest copy numbers of the strain was detected.

3.4 Primer optimization

Searches for candidates of *G. apicola* strains to use in strain specific qPCR assays (one Norwegian, and one American strain), were done by Professor Knut Rudi, and primers were designed by PhD student Jane Ludvigsen. In addition, the potential unique sequence for detection of the American strain were tested for specificity by doing sequence similarity searches in a local database with whole genomes of 76 isolates containing *tetB* (Jane Ludvigsen, unpublished work). The isolates included whole genomes of both American and Norwegian *G. apicola and S. alvi* Optimum annealing temperature of chosen primer pairs were chosen by evaluation of bands from gel electrophoresis of the PCR products from gradient PCR. Specificity of primer pairs were tested by doing PCR using the same 76 isolates as earlier described. In addition qPCR was performed on the same isolates with the primers designed for the Norwegian strain. Results of the positives isolates for each of the strains are presented in the table 3.2. All positives belonged to *G. apicola*: Image of agarose gels of positive strains from PCR, is given in appendix E.

Table 3.2: *G. apicola* isolates containing *tetB* positive for the unique strains of *G. apicola*

Isolate ID	Country of origin	Cq value	Gel-band by doing dPCR (+/-)	Whole genome search (+/-)	Positive for
N-9-4[6]	Norway	17.51	+	n.d.	Norwegian strain
N-12-12	Norway	21.98	+	n.d.	Norwegian strain
N-15-12	Norway	21.87	+	n.d.	Norwegian strain
A-3-12	U.S	n.d.	+	-	American strain
Aw-15	U.S	n.d.	+	-	American strain
A-12-24	U.S	n.d.	+	-	American strain
A-6-24	U.S	n.d.	+	-	American strain
A-8-24	U.S	n.d.	+	-	American strain
A-9-12	U.S	n.d.	+	+	American strain
A-1-24[7]	U.S	n.d.	+	+	American strain
A-2-24	U.S	n.d.	+	+	American strain
A-7-12	U.S	n.d.	+	+	American strain

Cq = Quantification cycle

n.d. = No data available

[6] Norwegian strain given in bacterial culture in this thesis
[7] American strain given in bacterial culture in this thesis

4. Discussion

4.1 Presence and origins of the *tetB* gene

A significant association with number of bees positive for *tetB* and treatment with *G. apicola* was found, while no significant effect of antibiotic treatment were observed. The baseline level of *tetB* was quite high, with the gene being detected in 40-50 % of all bees, independent of treatment. Furthermore, we found no evidence for HGT in our data. This, however could be due to the short time-span of our experiment. Findings from Tian et al. (2012), where a selection pressure caused by tetracycline is suggested to be the cause of higher prevalence of tetracycline resistance genes seen in American honeybees compared to others, is probably a result of many years of exposure to the antibiotic.

Furthermore, since Norwegian honeybees not have been treated with tetracycline, the occurrence of resistance genes towards the antibiotic is probably of natural origin, and it is suggested that these are a part of their normal microbiota. Although antibiotic treatment has been shown to cause accumulation of resistance genes, the presence of such resistance genes can be due to other factors. In nature, resistance genes are widely distributed, which may be explained by the fact that many organisms found in the environment are naturally producers of secondary metabolites with antimicrobial properties (Davies & Davies 2010). A study done in 2008 (Dantas et al. 2008), revealed soil bacteria as reservoirs for resistance traits, where screenings of resistant traits of hundreds of soil bacteria was done by growth experiments on antimicrobial agars, and results showed that also species belonging to *Burkholderia* and *Pseudomonas* grew efficiently on antimicrobials. Both genera are commonly found in the gut of the honeybee, due to their interaction with the environments, and could possibly be the origin of such genes in gut symbionts.

4.2 Detection of *G. apicola* given in sugar solution

The American strain was successfully detected in both midguts and hindguts from bees treated with bacterial culture. However, a few samples from bees not treated was also positive for the strain. This could be due to presence of highly similar strains found in Norwegian bees.

The frequent detection of the Norwegian strain across all treatments could be explained by the fact that this strain, was more similar to other Norwegian strains, than the American strain. This was also supported with the screening of the strain in t*etB* isolates, where the American strain did not match any Norwegians trains of *G. apicola,* whereas the Norwegian one did. High similarity between different strains of same species, as proposed by Engel et al (Engel et al. 2014), probably are dependent on, and also different according to different geographical areas.

Presence of the American strain peaked at 24 hours, and 48 hours, in midguts and hindguts, respectively, but the strain differed in terms of presence at later time points in the different compartments. In contrast to the midgut, where the strain only was detected in one of the nine samples in one of the *G. apicola* treated groups after 240 hours, the hindgut compartments showed presence of the strain in three of the nine samples. A more stable, and nutrient rich environment could be the explanation to this, in addition to the gut wall differences in the two different compartments, where adherence of the bacteria to the gut wall have shown to be minor in the midgut, compared to ileum and rectum. As suggested by Martinson et.al, there are indications for bacterial attachment to the gut wall, and their hypothesis is that the Betaproteobacteria i.e. *S. alvi* serve as a basis for other bacteria to adhere to, such as *G. apicola* (Martinson et al. 2012). The clumping behavior, possibly due to short hair-like structures, as discussed by Kwong and Moran (2013), makes it likely that the bacteria is able to adhere to the gut wall.

4.3 Differences in bacterial compositions in midgut and hindgut

All honeybees harboured the characteristic bacterial phylotypes in their gut. This support findings from earlier studies (references listed in introduction). The five most dominating bacteria in both midguts and hindguts belonged to *Bifidobacterium,* Alphaproteobacteria*, S. alvi, G. apicola and Lactobacillus*. However the different compartments revealed different profiles. In contrast to the microbiota in the hindgut samples, which seemed to remain quite stable, and harbour mainly the mentioned bacteria in both caged and bees from the hive, midgut samples also included other species. Big differences was also observed in midgut samples in

caged versus colony bees, where bees from the hive harboured the three environmental bacteria commonly found e.g. in soil; *Bacillus, Paenibacillus and Pseudomonas.* These three genera together compromised 20% of the total microbiota of the midguts from the hive. This may indicate that the honeybee gut also are colonized opportunistically by bacteria they interact with in the environment, which have also been shown in other insects such as midguts from the gypsy moth larval (Broderick et al. 2004), and caterpillars of the cabbage white butterfly (Robinson et al. 2010). The higher abundance of environmental bacteria in the midgut suggest that this compartment of the GI tract, is more susceptible to bacteria from the environment. However, these bacteria are most likely not belonging to the established microbiota, and are just passing through, entering through nutrition, from the crop, which is also known to harbor such bacteria. In this study, a shift in the midgut microbiota was seen in a relatively short time period (three days), which support these bacteria to be transient, and not members of the core microbiota.

The hindgut, which consist of about 90-97% of the total number of bacteria found in the GI tract of the honeybee (Martinson et al. 2012), probably presents a more stable environment, partly due to a nutrient-rich environment, where nutrients are continuously flowing as e.g. partly digested pollen, modified sugars, and waste products, but also, due to the basis of the gut-wall serving as a good spot for adherence. In this way, the microbiota is more stable, and less affected by external stress.

The lower diversity of species in the midguts of caged honeybees versus midguts from honeybees sampled directly from the hive, indicates that the microbiota in the midgut is strongly affected by the caging conditions. This is not surprising, due to the fact that the bees are taken out of their natural habitat. The honeybees, with their combination of group dynamics and hive physiology has been referred to as a superorganism (Anderson et al. 2013), and they are assumed to be highly dependent on the whole colony, for their health and well-being, which again most likely, is highly dependent on a healthy gut microbiota. The observed differences detected in the midgut microbiota in this study, should be considered in future studies on midgut microbiota, due to the effect caging has on the bacterial community on this gut compartment.

Total amount of bacteria was shown to be 100-10 000 times lower in the midguts, than in the hindguts, which is consistent with earlier done studies (Anderson et al. 2013; Martinson et al. 2012). The lower abundance of bacteria in the midguts, compared to hindgut, is probably due to the presence of digestive enzymes, differences in pH, and also, as earlier mentioned, bacterial adherence to the gut wall.

However, compared to other studies, somewhat higher numbers of bacteria in the midgut were found in this study, compared to other studies (Martinson et al. 2011). An explanation to this could be presence of parts of the pylorus in the samples, which have been shown, by doing FISH microscopy, to harbor most midgut bacteria are located posterior, near the pylorus. For further work, and repeated experiments, precautions should be made in advance to avoid incorrect sampling, and preferably the gut should be cut by using e.g. a stereomicroscope.

4.4 Effects of tetracycline on gut microbiota

Treatment with tetracycline gave significant differences in composition of the microbiota. According to measurements of total bacterial abundance over time, treatment with tetracycline tend to decrease the total amount in the midgut, whereas hindgut compartments remain more stable, where the total amount of bacteria did not seem to be affected by antibiotic treatment. Diversity measurements by rarefaction curve plots, showed higher diversity within samples in the midguts, according to antibiotic treatment, whereas the hindgut samples did not show a big difference in diversity, which also support a more stable microbial environment in the hindgut.

For the main bacterial groups, differences were observed in abundance relative to tetracycline treatment. Strangely, abundance of *G. apicola* significantly decreased in the midguts group treated with the American strain and tetracycline, where this group showed a 50 % lower abundance than baseline, while the other group, only treated with tetracycline had a significant increase of the bacteria. Unfortunately, a possible explanation for this is lacking.

The same was also observed in control groups given only sugar. The observed differences, is not easily explained, but one explanation could be that a few bees, with high numbers of a specific genus or species could impact the average results, giving somewhat incorrect average community profile.

For the dominating bacterial groups, species belonging to *Lactobacillus,* were the species where biggest differences were observed, where a significant increase of abundance was detected in both midgut and hindgut samples, when bees had been treated with tetracycline. Resistance traits of the genera towards tetracycline have been shown in other studies, e.g. in five strains of *Lactobacillus* normally found in the honeybee GI tract, including *L. kunkeii (Vásquez et al.*

2012). Tian et al. also observed the recovered *Lactobacilli* when the bacterium was plated on medium with tetracycline (Tian et al. 2012). The genus have shown to be important for honeybee health, by e.g. exploiting a vast array of antimicrobial traits, protecting the honey bee towards pathogens such as *Paenibacillus* and *Pseudomonas,* which bees are continuously exposed to through the environment (Forsgren et al. 2010; Olofsson et al. 2014).

In addition, it has appeared that honeybees harbor the largest collection of novel species from the genera *Lactobacillus*, ever discovered in insects (Vásquez et al. 2012). Due to their own antimicrobial traits, a reason for its good survival in the GI tract post antibiotic treatment, could be that they harbor resistance mechanisms, therefore that they do not get affected by their own antibacterial traits, thus also are not altered by antibiotic treatment. Lactic acid bacteria relative to human gut are also known to harbor conjugative transposons to confer resistance to antibiotics, including tetracycline (Broaders et al. 2013).

4.5 Methodological considerations

Designing primers that specifically target species or strains of interest, despite the presence of closely related bacteria, is a challenge in qPCR development for this purpose. The primers designed to target the Norwegian strain, had a rather low annealing temperature, which could lead to increased sensitivity, but also decreased specificity (Evans et al. 2013).

Efficiency and accuracy of the qPCR also depend on DNA quality. The main obstacles for obtaining good quality DNA from bacterial communities in the environment, are co-extraction of PCR-inhibitory substances and bad recovery of the total genomic DNA (Rinttilä et al. 2004; Zoetendal et al. 2001). In addition, copy number calculations obtained from high Cq give higher uncertainty. However, HMR analysis are assumed to be rather accurate, and these were checked continually in this study, especially when Cq values were low. Also, replicates could advantageously been included, but the large number of samples in this study made this an uneasy task to fulfil.

Another aspect of the qPCR method, relates to the use of the standard curve, where the prepared standards, and the sample are quite different, due to the fact that the standards are made from pure PCR-products of nucleic acid fragment of interest, while the sample contains a vast array of multiple species, genera, and in this study, both prokaryotic and eukaryotic DNA. The

assumption of a constant E for both standards and sample, is necessary not a fact in real life (Brankatschk et al. 2012).

The feeding and sampling procedures also give challenges, due to the fact that caged bees with unlimited access to the feed, are hard to control for feeding regimes on individual basis, and sampling of gut contents cannot be done repeatedly from the same individuals. In studies were supplements are given in feed, and time is of interest to monitor, isolation experiments of individuals could be an alternative, where the bees are given the feed in a highly controllable manner. However, such studies are not ideal, due to the extreme unnatural conditions for the bees, leading to a short lifespan, thus only short lasting experiments.

4.6 Future work

The gut microbiota of the honeybee presents an interesting microbial community, and deserves its increased attention. Moreover, the microbiota is highly relevant for future studies related to resistance development, not only due to their high interaction with the environment, but also due to their unique, and relatively simple microbiota. In addition, they are fairly simple to maintain in the lab.

For studies involving commensal bacteria, and resistance development, several of the core-species could be of interest to explore in more detail. In addition to *S. alvi* and *G. apicola*, also species belonging to *Lactobacillus* would be suitable candidates for such studies.

Furthermore, studies directed towards finding more about the mobile element type associated with the gene needs more attention, since the mechanisms is not known to date, but e.g. *tetB* is assumed to be associated to a transposon on a conjugative plasmid. Study of transfer of antibiotic resistance genes in natural settings is not well established, and research in this field is lacking.

Conclusion

Treatment with tetracycline was not found significant for number of bees positive for *tetB*, but a significant association with number of positive for the gene was found in bees treated with the bacterial culture. Furthermore we found no evidence for HGT. Comparisons of the different gut compartment showed a more stable microbiota in the hindguts, whereas the midguts showed to be more unstable and was highly affected by caging conditions. In addition, midguts samples showed higher diversity and harbored more species than the hindgut samples. We suggest that the observed caging effects seen on the midgut microbiota, should be taken in consideration when using caged bees in studies done on midgut microbiota.

In addition, treatment with tetracycline gave significant effects in the microbiota of both midguts and hindguts, where species belonging to *Lactobacillus* increased in both compartments post treatment with the antibiotic. Strangely, abundance of *G. apicola* was strongly affected by treatment with bacterial culture and tetracycline, but unfortunately, we do not have any good explanation for this.

For further work, it is suggested to perform isolation experiments, to investigate possible gene transfer, and conditions triggering such events, in a more controlled manner.

References

Allen, H. K., Cloud-Hansen, K. A., Wolinski, J. M., Guan, C., Greene, S., Lu, S., Boeyink, M., Broderick, N. A., Raffa, K. F. & Handelsman, J. (2009). Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. *DNA and cell biology*, 28 (3): 109-117.

Aminov, R., Chee-Sanford, J., Garrigues, N., Teferedegne, B., Krapac, I., White, B. & Mackie, R. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Applied and Environmental Microbiology*, 68 (4): 1786-1793.

Anderson, K. E., Sheehan, T. H., Mott, B. M., Maes, P., Snyder, L., Schwan, M. R., Walton, A., Jones, B. M. & Corby-Harris, V. (2013). Microbial Ecology of the Hive and Pollination Landscape: Bacterial Associates from Floral Nectar, the Alimentary Tract and Stored Food of Honey Bees (Apis mellifera). *PLoS ONE*, 8 (12): e83125.

Beekman, M. & Ratnieks, F. L. W. (2000). Long-range foraging by the honey-bee, Apis mellifera L. *Functional Ecology*, 14 (4): 490-496.

Benjamin, A. & McCallum, B. (2009). *A World Without Bees*: Guardian Books.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nat Rev Micro*, 13 (1): 42-51.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R. & Abebe, E. (2005). Defining operational taxonomic units using DNA barcode data. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360 (1462): 1935-1943.

Blázquez, J., Couce, A., Rodríguez-Beltrán, J. & Rodríguez-Rojas, A. (2012). Antimicrobials as promoters of genetic variation. *Current opinion in microbiology*, 15 (5): 561-569.

Brankatschk, R., Bodenhausen, N., Zeyer, J. & Bürgmann, H. (2012). Simple Absolute Quantification Method Correcting for Quantitative PCR Efficiency Variations for Microbial Community Samples. *Applied and Environmental Microbiology*, 78 (12): 4481-4489.

Broaders, E., Gahan, C. G. M. & Marchesi, J. R. (2013). Mobile genetic elements of the human gastrointestinal tract. *Gut Microbes*, 4 (4): 271-280.

Broderick, N. A., Raffa, K. F., Goodman, R. M. & Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environmental Microbiology*, 70 (1): 293-300.

Buermans, H. P. J. & den Dunnen, J. T. (2014). Next generation sequencing technology: Advances and applications. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1842 (10): 1932-1941.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K. & Gordon, J. I. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, 7 (5): 335-336.

Casadevall, A. & Pirofski, L.-a. (2000). Host-Pathogen Interactions: Basic Concepts of Microbial Commensalism, Colonization, Infection, and Disease. *Infection and Immunity*, 68 (12): 6511-6518.

Channaiah, L. H., Subramanyam, B., McKinney, L. J. & Zurek, L. (2010). Stored-product insects carry antibiotic-resistant and potentially virulent enterococci. *FEMS microbiology ecology*, 74 (2): 464-471.

Chopra, I. & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews*, 65 (2): 232-260.

Cox-Foster, D. L., Conlan, S., Holmes, E. C., Palacios, G., Evans, J. D., Moran, N. A., Quan, P.-L., Briese, T., Hornig, M. & Geiser, D. M. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, 318 (5848): 283-287.

Dade, H. A. (2009). *Anatomy and dissection of the honeybee (Revised edition)*. Cardiff, UK: Bee Research Association.

Dantas, G., Sommer, M. O., Oluwasegun, R. D. & Church, G. M. (2008). Bacteria subsisting on antibiotics. *Science*, 320 (5872): 100-103.

Davies, J. & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews : MMBR*, 74 (3): 417-433.

Davis, C. F. (2004). *The honey bee inside out*: Bee Craft Ltd.

Dillon, J. R. & Dillon, V. M. (2004). The Gut Bacteria of Insects: Nonpathogenic Interactions. *Annual Review of Entomology* 49: 71-92.

Dubnau, D., Smith, I., Morell, P. & Marmur, J. (1965). Gene conservation in Bacillus species. I. Conserved genetic and nucleic acid base sequence homologies. *Proceedings of the National Academy of Sciences of the United States of America*, 54 (2): 491.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26 (19): 2460-2461.

Ellegaard, K. M., Tamarit, D., Javelind, E., Olofsson, T. C., Andersson, S. G. & Vásquez, A. (2015). Extensive intra-phylotype diversity in lactobacilli

and bifidobacteria from the honeybee gut. *BMC Genomics*, 16 (284).

Engel, P., Martinson, V. G. & Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences*, 109 (27): 11002-11007.

Engel, P., James, R. R., Koga, R., Kwong, W. K., McFrederick, Q. S. & Moran, N. A. (2013). Standard methods for research on Apis mellifera gut symbionts. *Journal of Apicultural Research*, 52 (4): 1-24.

Engel, P., Kwong, W. K. & Moran, N. A. (2013). Frischella perrara gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honeybee, Apis mellifera. *Int J Syst Evol Microbiol*, 63 (Pt 10): 3646-51.

Engel, P. & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev*, 37 (5): 699-735.

Engel, P., Stepanauskas, R. & Moran, N. A. (2014). Hidden diversity in honey bee gut symbionts detected by single-cell genomics. *PLoS Genet*, 10 (9): e1004596.

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., Yao, J. D. C., Wengenack, N. L., Rosenblatt, J. E., Cockerill, F. R., et al. (2006). Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing. *Clinical Microbiology Reviews*, 19 (1): 165-256.

Evans, J. D., Schwarz, R. S., Chen, Y. P., Budge, G., Cornman, R. S., De la Rua, P., de Miranda, J. R., Foret, S., Foster, L. & Gauthier, L. (2013). Standard methods for molecular research in Apis mellifera. *Journal of apicultural research*, 52 (4): 1-54.

Fan, W., Hamilton, T., Webster-Sesay, S., Nikolich, M. P. & Lindler, L. E. (2007). Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. *Molecular and cellular probes*, 21 (4): 245-256.

Forsgren, E., Olofsson, T. C., Vásquez, A. & Fries, I. (2010). Novel lactic acid bacteria inhibiting Paenibacillus larvae in honey bee larvae*. *Apidologie*, 41 (1): 99-108.

Fox, G. E., Magrum, L. J., Balch, W. E., Wolfe, R. S. & Woese, C. R. (1977). Classification of methanogenic bacteria by 16S ribosomal RNA characterization. *Proceedings of the National Academy of Sciences*, 74 (10): 4537-4541.

Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology*, 3 (9): 722-732.

Gallai, N., Salles, J.-M., Settele, J. & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, 68 (3): 810-821.

Gilliam, M. (1971). Microbial sterility of the intestinal content of the immature honey bee, Apis mellifera. *Annals of the Entomological Society of America*, 64 (1): 315-316.

Guo, J., Wu, J., Chen, Y., Evans, J. D., Dai, R., Luo, W. & Li, J. (2015). Characterization of gut bacteria at different developmental stages of Asian honey bees, Apis cerana. *Journal of Invertebrate Pathology*, 127 (0): 110-114.

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Nucleic acids symposium series. 95-98 pp.

Huddleston, J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infection and Drug Resistance*, 7: 167-176.

Jeyaprakash, A., Hoy, M. A. & Allsopp, M. H. (2003). Bacterial diversity in worker adults of Apis mellifera capensis and Apis mellifera scutellata (Insecta: Hymenoptera) assessed using 16S rRNA sequences. *Journal of invertebrate pathology*, 84 (2): 96-103.

Kačániová, M., Chlebo, R., Kopernický, M. & Trakovická, A. (2004). Microflora of the honeybee gastrointestinal tract. *Folia Microbiologica*, 49 (2): 169-171.

Kadavy, D. R., Hornby, J. M., Haverkost, T. & Nickerson, K. W. (2000). Natural antibiotic resistance of bacteria isolated from larvae of the oil fly, Helaeomyia petrolei. *Applied and environmental microbiology*, 66 (11): 4615-4619.

Keeling, P. J. & Palmer, J. D. (2008). Horizontal gene transfer in eukaryotic evolution. *Nat Rev Genet*, 9 (8): 605-618.

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C. & Tscharntke, T. (2007). *Importance of pollinators in changing landscapes for world crops*, vol. 274. 303-313 pp.

Koch, H. & Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences*, 108 (48): 19288-19292.

Koch, H., Abrol, D. P., Li, J. & Schmid-Hempel, P. (2013). Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. *Molecular ecology*, 22 (7): 2028-2044.

Kuzina, L. V., Peloquin, J. J., Vacek, D. C. & Miller, T. A. (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, Anastrepha ludens (Diptera: Tephritidae). *Current microbiology*, 42 (4): 290-294.

Kwong, W. K. & Moran, N. A. (2013). Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of Snodgrassella alvi gen. nov., sp. nov., a member of the family Neisseriaceae of the Betaproteobacteria, and Gilliamella apicola gen. nov., sp. nov., a member of Orbaceae fam. nov., Orbales ord. nov., a sister taxon to the order 'Enterobacteriales' of the Gammaproteobacteria. *Int J Syst Evol Microbiol*, 63 (Pt 6): 2008-18.

Li, J., Qin, H., Wu, J., Sadd, B. M., Wang, X., Evans, J. D., Peng, W. & Chen, Y. (2012). The Prevalence of Parasites and Pathogens in Asian Honeybees <italic>Apis cerana</italic> in China. *PLoS ONE*, 7 (11): e47955.

Liu, L., Chen, X., Skogerbø, G., Zhang, P., Chen, R., He, S. & Huang, D.-W. (2012). The human microbiome: A hot spot of microbial horizontal gene transfer. *Genomics*, 100 (5): 265-270.

Lowe, C. F. & Romney, M. G. (2011). Bedbugs as vectors for drug-resistant bacteria. *Emerging infectious diseases*, 17 (6): 1132.

Marshall, B. M., Ochieng, D. J. & Levy, S. B. (2009). Commensals: underappreciated reservoir of antibiotic resistance. *Microbe*, 4 (5): 231-238.

Martinson, V. G., Danforth, B. N., Minckley, R. L., Rueppell, O., Tingek, S. & Moran, N. A. (2011). A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*, 20 (3): 619-628.

Martinson, V. G., Moy, J. & Moran, N. A. (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and environmental microbiology*, 78 (8): 2830-2840.

McClintock, B. (1950). The Origin and Behavior of Mutable Loci in Maize. *Proceedings of the National Academy of Sciences of the United States of America*, 36 (6): 344-355.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., Andersen, G. L., Knight, R. & Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*, 6 (3): 610-618.

McGregor, S. E., Agriculture, U. S. D. o., Service, U. S. A. R., Science, U. S. & Administration, E. (1976). *Insect Pollination of Cultivated Crop Plants*: U.S. Department of Agriculture, Agricultural Research Service.

Milinovich, G. J., Burrell, P. C., Pollitt, C. C., Klieve, A. V., Blackall, L. L., Ouwerkerk, D., Woodland, E. & Trott, D. J. (2008). Microbial ecology of the equine hindgut during oligofructose-induced laminitis. *The ISME journal*, 2 (11): 1089-1100.

Mohr, K. I. & Tebbe, C. C. (2006). Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. *Environmental Microbiology*, 8 (2): 258-272.

Moran, N. A., Hansen, A. K., Powell, J. E. & Sabree, Z. L. (2012). Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees. *PLoS ONE*, 7 (4): e36393.

Nadkarni, M. A., Martin, F. E., Jacques, N. A. & Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*, 148 (1): 257-266.

Nikolich, M. P., Hong, G., Shoemaker, N. B. & Salyers, A. A. (1994). Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Applied and Environmental Microbiology*, 60 (9): 3255-3260.

Norman, A., Hansen, L. H. & Sørensen, S. J. (2009). Conjugative plasmids: vessels of the communal gene pool. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364 (1527): 2275-2289.

Olofsson, T. C., Butler, È., Markowicz, P., Lindholm, C., Larsson, L. & Vásquez, A. (2014). Lactic acid bacterial symbionts in honeybees–an unknown key to honey's antimicrobial and therapeutic activities. *International wound journal*.

Powell, J. E., Martinson, V. G., Urban-Mead, K. & Moran, N. A. (2014). Routes of acquisition of the gut microbiota of Apis mellifera. *Applied and Environmental Microbiology*.

Rankin, D. J., Rocha, E. P. C. & Brown, S. P. (2011). What traits are carried on mobile genetic elements, and why[quest]. *Heredity*, 106 (1): 1-10.

Reybroeck, W., Daeseleire, E., De Brabander, H. F. & Herman, L. (2012). Antimicrobials in beekeeping. *Veterinary microbiology*, 158 (1): 1-11.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L. & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology*, 97 (6): 1166-1177.

Robinson, C. J., Schloss, P., Ramos, Y., Raffa, K. & Handelsman, J. (2010). Robustness of the Bacterial Community in the Cabbage White Butterfly Larval Midgut. *Microbial Ecology*, 59 (2): 199-211.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230 (4732): 1350-1354.

Salyers, A. A., Gupta, A. & Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol*, 12 (9): 412-6.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74 (12): 5463-5467.

Santos, C. G. & Serrão, J. E. (2006). Histology of the ileum in bees(Hymenoptera, Apoidea). *Braz. J. morphol. Sci.*, 23 (3-4): 405-413.

Schacker, M. (2008). *A Spring without Bees: How Colony Collapse Disorder Hs Endangered Our Food Supply*: Lyons Press.

Shendure, J. & Ji, H. (2008). Next-generation DNA sequencing. *Nat Biotech*, 26 (10): 1135-1145.

Shoemaker, N. B., Vlamakis, H., Hayes, K. & Salyers, A. A. (2001). Evidence for Extensive Resistance Gene Transfer amongBacteroides spp. and among Bacteroides and Other Genera in the Human Colon. *Applied and Environmental Microbiology*, 67 (2): 561-568.

Snodgrass, R. E. (1910). *The Anatomy of the honey bee*: Kessinger Publishing, LLC.

Steinbakk, M., Sunde, M., Urdal, A. M., Barkbu, K. N., Sørum, H., Lunestad, B.-T., Bonhorst, J. Ø., Nielsen, K. M., Lindbæk, M. & Bjørnholt, J. V. (2014). Antibiotikaresistens - kunnskapshull, utfordringer og aktuelle tiltak. Oslo.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, 30 (12): 2725-2729.

Tetteh-Quarcoo, P. B., Donkor, E. S., Attah, S. K., Duedu, K. O., Afutu, E., Boamah, I., Olu-Taiwo, M., Anim-Baidoo, I. & Ayeh-Kumi, P. F. (2013). Microbial carriage of cockroaches at a tertiary care hospital in Ghana. *Environmental health insights*, 7: 59.

Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K. & Moran, N. A. (2012). Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. *MBio*, 3 (6): e00377-12.

Totland, Ø., Hovstad, K. A., Ødegaard, F. & Åström, J. (2013). Kunnskapsstatus for insektpollinering i Norge - betydningen av det komplekse samspillet mellom planter og insekter. Norge.

Varley, A. J., Sule, J. & Absalom, A. R. (2009). Principles of antibiotic therapy. *Continuing Education in Anaesthesia, Critical Care & Pain*, 9 (6): 184-188.

Vásquez, A. & Olofsson, T. C. (2009). The lactic acid bacteria involved in the production of bee pollen and bee bread. *Journal of apicultural research*, 48 (3): 189-195.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L. & Olofsson, T. C. (2012). Symbionts as Major Modulators of Insect Health: Lactic Acid Bacteria and Honeybees. *PLoS ONE*, 7 (3): e33188.

Wallberg, A., Han, F., Wellhagen, G., Dahle, B., Kawata, M., Haddad, N., Simoes, Z. L. P., Allsopp, M. H., Kandemir, I., De la Rua, P., et al. (2014). A worldwide survey of genome sequence variation provides insight into the evolutionary history of the honeybee Apis mellifera. *Nat Genet*, 46 (10): 1081-1088.

Wannigama, D. L., Dwivedi, R. & Zahraei-Ramazani, A. (2013). Prevalence and antibiotic resistance of Gram-negative pathogenic bacteria species isolated from Periplaneta americana and Blattella germanica in Varanasi, India. *Journal of Arthropod-Borne Diseases*, 8 (1): 10-20.

Williams, G. R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D. P. & Medrzycki, P. (2013). Standard methods for maintaining adult Apis mellifera in cages under in vitro laboratory conditions. *Journal of Apicultural Research*, 52 (1): 1-36.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences*, 87 (12): 4576-4579.

Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Tse, H. & Yuen, K. Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, 14 (10): 908-934.

Yu, Y., Lee, C., Kim, J. & Hwang, S. (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnology and Bioengineering*, 89 (6): 670-679.

Zoetendal, E. G., Ben-Amor, K., Akkermans, A. D. L., Abee, T. & de Vos, W. M. (2001). DNA Isolation Protocols Affect the Detection Limit of PCRApproaches of Bacteria in Samples from the HumanGastrointestinal Tract. *Systematic and Applied Microbiology*, 24 (3): 405-410.

Zurek, L. & Ghosh, A. (2014). Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. *Applied and environmental microbiology*, 80 (12): 3562-3567.

Appendix

APPENDIX A: Feed compositions in the different experiments

Table A.1: Feed compositions of the different treatments in October pilot study.

Cage/ Treatment	**1**	**2**	**3**	**4**
Feed composition	Sugar solution w/o supplements	Sugar solution w/ 10^7 CFU/ml N. *G. apicola*	Sugar solution w/ 10^7 CFU/ml N. *G. apicola* *and* 6 µl/ml tetracycline	Sugar solution w/ 10^7 CFU/ml N. *G. apicola* *and* 100 µl/ml tetracycline

Table A.2: Feed compositions of the different treatments in September pilot study.

Cage/ Treatment	**1**	**2**	**3**	**4**
Feed composition	Sugar solution w/o supplements	Sugar solution w/ 0,25 µl/ml tetracycline	Sugar solution w/ 12 µl/ml tetracycline	Sugar solution w/ 100 µl/ml tetracycline

Table A.3: Feed compositions of the different treatments in main experiment.

Cage/ Treatment	**1**	**2**	**3**	**4**	**5**	**6**
Feed composition	Sugar solution w/o supplements	Sugar solution w/ 100 µl/ml tetracycline	Sugar solution w/ 10^7 CFU/ml N. *G. apicola*	Sugar solution w/ 10^7 CFU/ml N. *G. apicola* *and* 100 µl/ml tetracycline	Sugar solution w/ 10^7 CFU/ml A. *G. apicola*	Sugar solution w/ 10^7 CFU/ml *A. G. apicola* *and* 100 µl/ml tetracycline

N. =Norwegian strain
A. = American strain

APPENDIX B: Preparations of sugar solutions

Preparation of sugar solution with tetracycline

A stock solution of 10 mg/ml was prepared by dissolving 0.1 gram Tetracycline (Sigma Aldrich, Germany) in 10 ml 70% ethanol. The solution was filter sterilized by a 0.45 µm filter (Sarstedt, Germany) before a final dilution of 1mg/ml was prepared (1:10 in milliQ water).

The stock solution was further diluted to a final concentration of 100 µg/ml directly in the sugar solution (wt/v), in 15 ml plastic tubes, and stored at -20 °C, until introduction into honeybees.

Preparation of sugar solution with viable *G. apicola*

The cultivated *G. apicola* cells were harvested with a sterile inoculated loop and resuspended in 40 % sugar solution to obtain a cell density of 10^7 CFU/ml. The cell density was measured by a McFarland spectrophotometer (bioSan, Latvia). The freshly prepared bacterial solution was immediately provided to the bees.

APPENDIX C: PRK Illumina primer sequences for index PCR

PRKi forward (5'-3'):

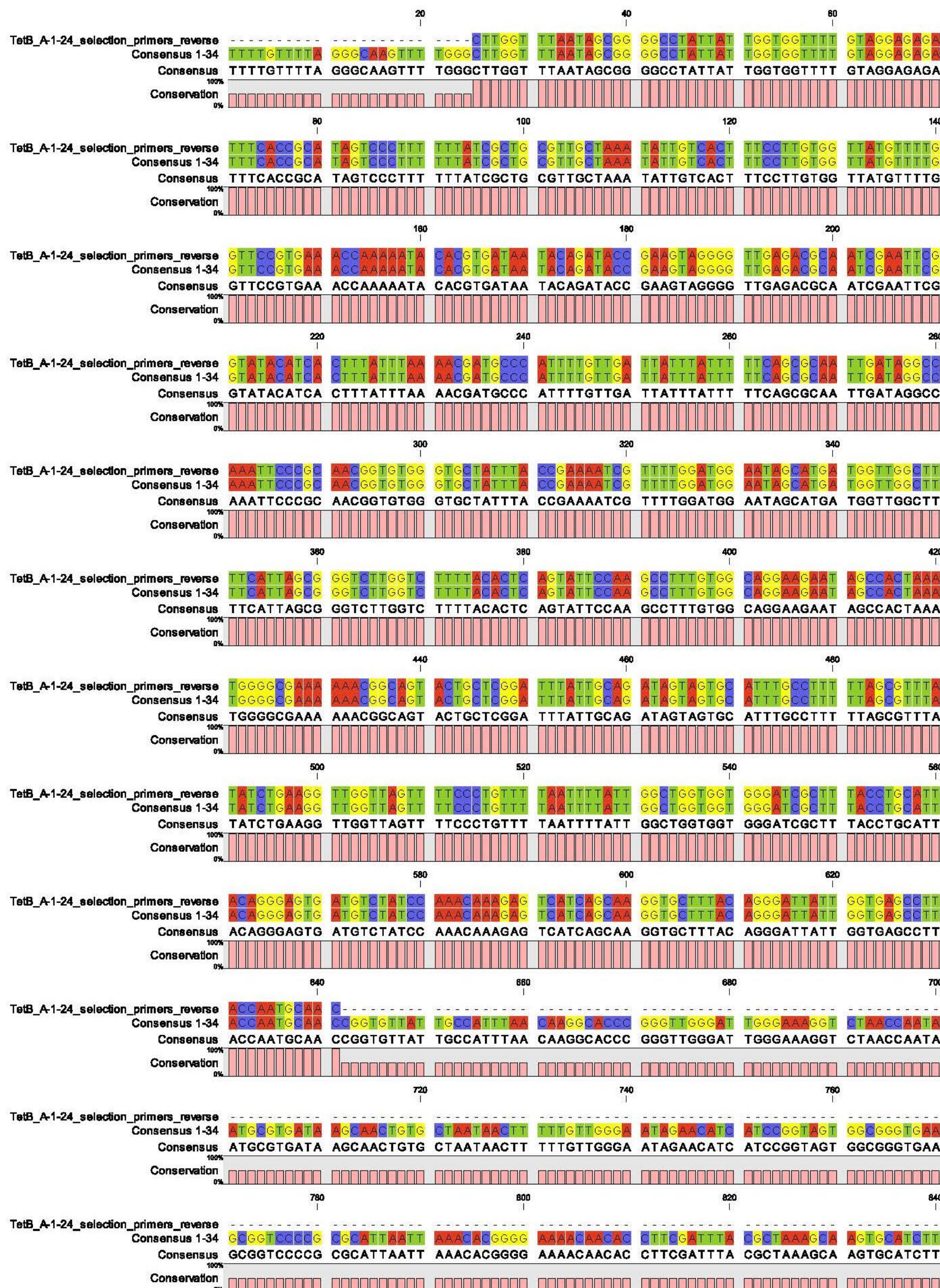
1. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagtcaaCCTACGGGRBGCASCAG
2. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagttccCCTACGGGRBGCASCAG
3. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgtcaCCTACGGGRBGCASCAG
4. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctccgtccCCTACGGGRBGCASCAG
5. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtagagCCTACGGGRBGCASCAG
6. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtccgcCCTACGGGRBGCASCAG
7. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtgaaaCCTACGGGRBGCASCAG
8. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtggccCCTACGGGRBGCASCAG
9. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtttcgCCTACGGGRBGCASCAG
10. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcgtacgCCTACGGGRBGCASCAG
11. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgagtggCCTACGGGRBGCASCAG
12. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctggtagcCCTACGGGRBGCASCAG
13. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctactgatCCTACGGGRBGCASCAG
14. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgagcCCTACGGGRBGCASCAG
15. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctattcctCCTACGGGRBGCASCAG
16. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctcaaaagCCTACGGGRBGCASCAG

PRKi Reverse (5'-3'):

1. caagcagaagacggcatacgagatCGTGATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
2. caagcagaagacggcatacgagatACATCGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
3. caagcagaagacggcatacgagatGCCTAAgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
4. caagcagaagacggcatacgagatTGGTCAgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
5. caagcagaagacggcatacgagatCACTCTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
6. caagcagaagacggcatacgagatATTGGCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
7. caagcagaagacggcatacgagatGATCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
8. caagcagaagacggcatacgagatTCAAGTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
9. caagcagaagacggcatacgagatCTGATCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
10. caagcagaagacggcatacgagatAAGCTAgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
11. caagcagaagacggcatacgagatGTAGCCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
12. caagcagaagacggcatacgagatTACAAGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
13. caagcagaagacggcatacgagatTTGACTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT

14. caagcagaagacggcatacgagatGGAACTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
15. caagcagaagacggcatacgagatTGACATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
16. caagcagaagacggcatacgagatGGACGGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
17. caagcagaagacggcatacgagatCTCTACgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
18. caagcagaagacggcatacgagatGCGGACgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
19. caagcagaagacggcatacgagatTTTCACgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
20. caagcagaagacggcatacgagatGGCCACgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
21. caagcagaagacggcatacgagatCGAAACgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
22. caagcagaagacggcatacgagatCGTACGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
23. caagcagaagacggcatacgagatCCACTCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
24. caagcagaagacggcatacgagatGCTACCgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
25. caagcagaagacggcatacgagatATCAGTgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
26. caagcagaagacggcatacgagatGCTCATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
27. caagcagaagacggcatacgagatAGGAATgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
28. caagcagaagacggcatacgagatCTTTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
29. caagcagaagacggcatacgagatTAGTTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
30. caagcagaagacggcatacgagatCCGGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
31. caagcagaagacggcatacgagatATCGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
32. caagcagaagacggcatacgagatTGAGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
33. caagcagaagacggcatacgagatCGCCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
34. caagcagaagacggcatacgagatGCCATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
35. caagcagaagacggcatacgagatAAAATGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT
36. caagcagaagacggcatacgagatTGTTGGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAA

APPENDIX D: Sequence alignment of *tetB*



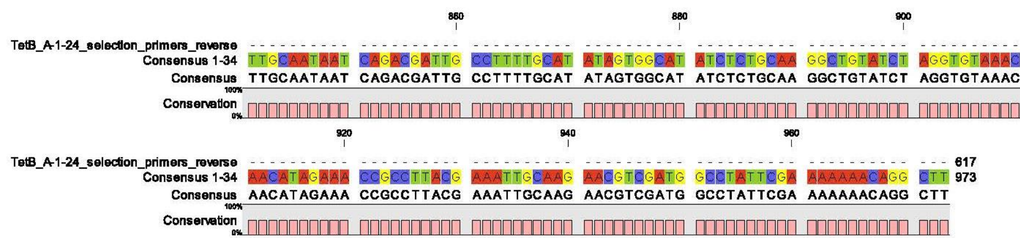


Figure D.1: Full alignment of the matching sequence (Consensus 1-34) and *tetB* from American strain.

APPENDIX E: Gel electrophoresis of positive strains with strain specific primers

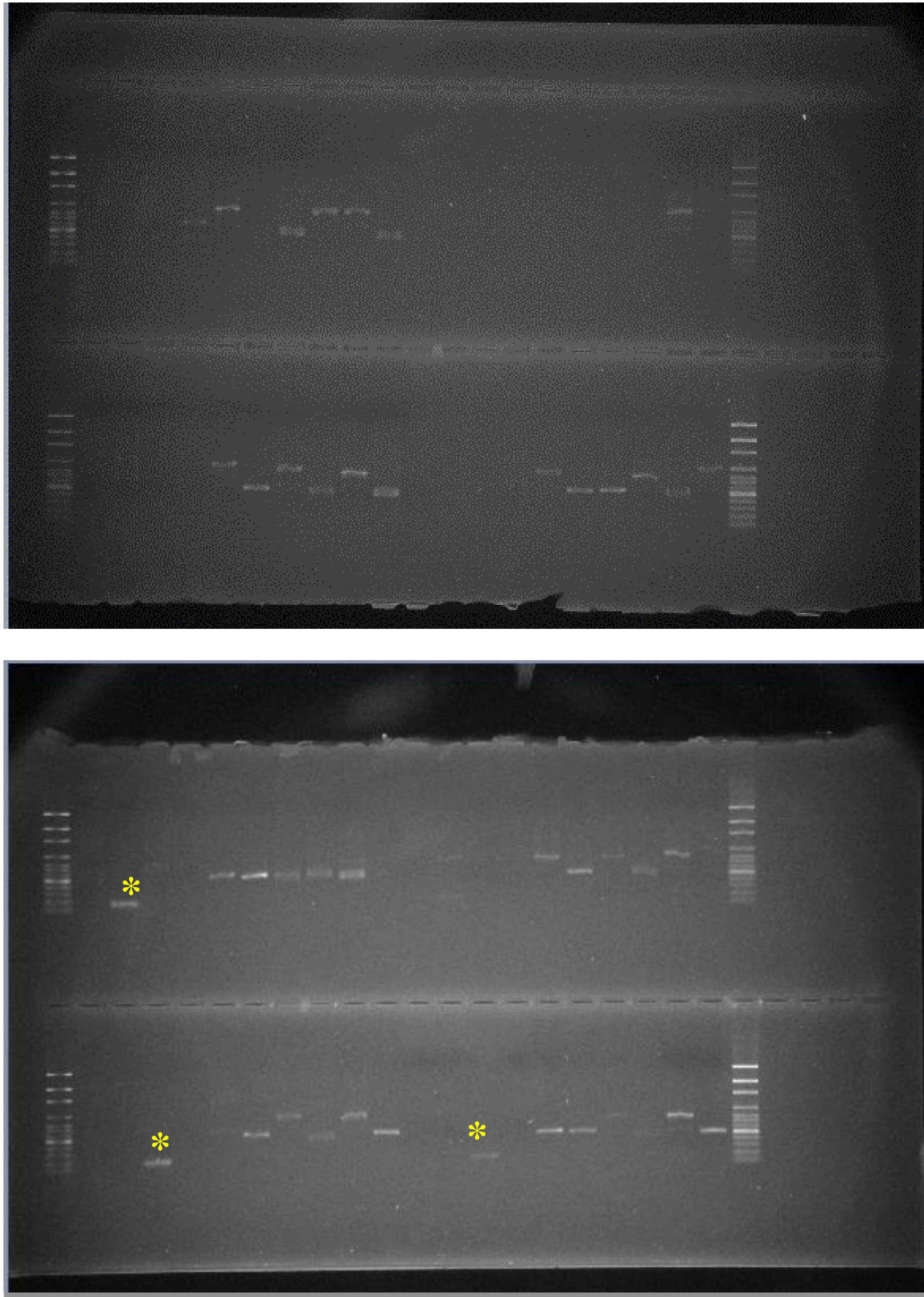


Figure E.1: Picture of gel from the gel electrophoresis of PCR products obtained by using primers designed for detection of Norwegian *G. apicola* strain on 76 bacterial isolates containing *tetB*. Three positives were found (marked with yellow asterisk), with correct amplicon size (179bp).

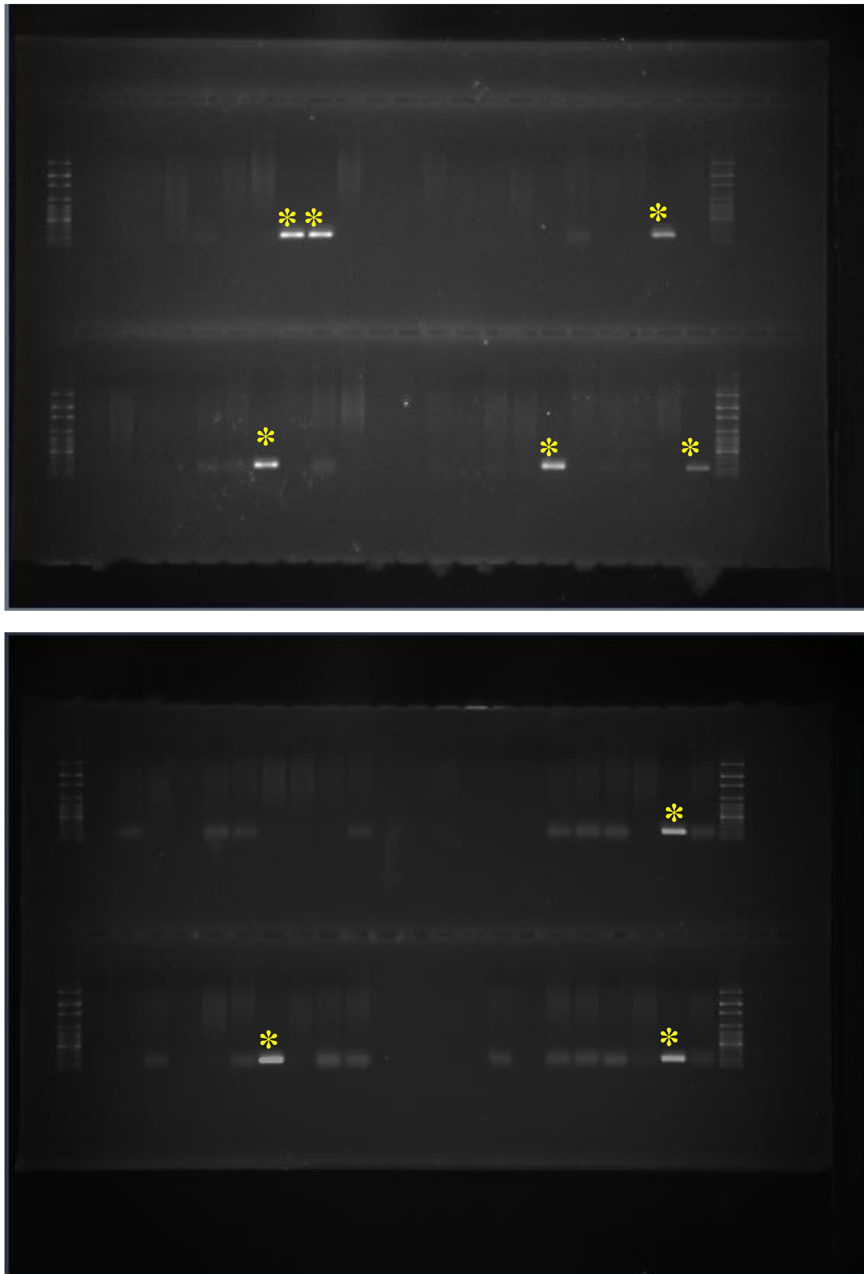


Figure E.2: Picture of gel from the gel electrophoresis of PCR products obtained by using primers designed for detection of American *G. apicola* strain (330 bp). Nine positives were found (marked with yellow asterisk), with correct amplicon size (330bp).

APPENDIX F: Controls and standard curves

DNA quality and controls

Qubit measurements of genomic DNA ranged between 0.57 and 8.22 ng/µl (mean=4.51, median=4.64) for the midgut samples, and 0.39 and 4.65 ng/µl (mean=3.09, median=3.11) for the hindgut samples.

All negative controls used in qPCR assays showed Cq values >34.

Standard curves

The standard curves used for copy number calculations for each of the genes are listed in table B.1. Number of gene targets per sample are all given in amounts per 2 µl sample.

Table F.1: Quantities of amplicons used to construct standard curves for copy number calculations.

Target gene	Slope	C_q- intercept	R^2	Amplification efficiency	Linear range (gene copies per 2 µl DNA)[8]
16S rRNA	-3,85	43,5	0.986	82 %	3,24 x 10^2 – 3,24 x 10^9
TetB	-3,66	40,9	0.998	88 %	4,25 x 10^2 – 4,25 x 10^8
G. apicola	-3,75	41,2	0.998	85 %	2,40 x 10^2- 2,40 x 10^8
American G. apicola	-3,70	39,4	0.987	86 %	1,00 x 10^2- 1,00 x 10^8
Norwegian G. apicola	-3,76	41,7	0.989	84 %	4,00 x 10^2- 4,00 x 10^8

[8] Calculated according to equation 1 in material and methods.

APPENDIX G: Copy numbers of the *tetB* gene in midgut and hindgut

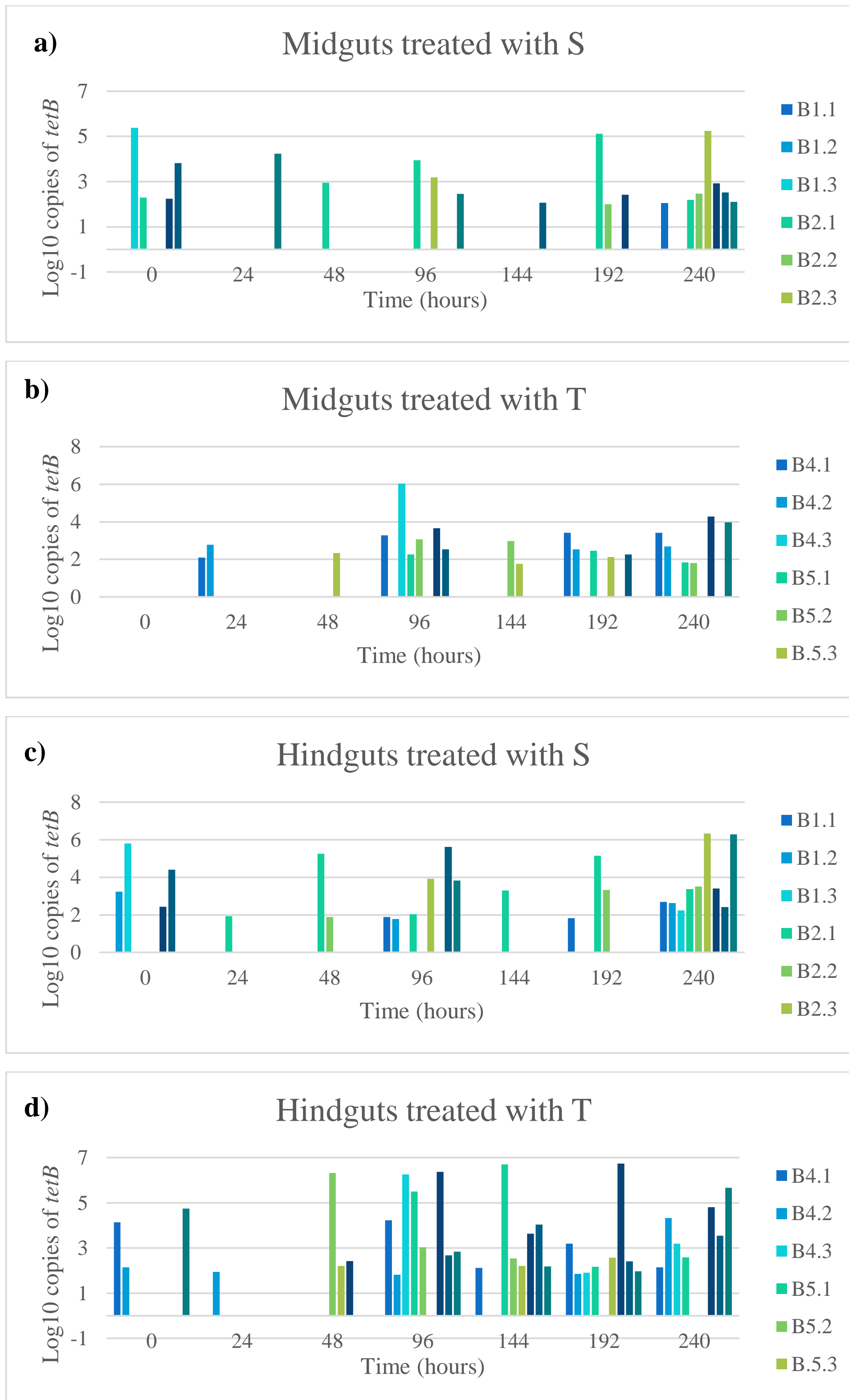


Figure G.1: Log10 copy numbers of *tetB* in samples. T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

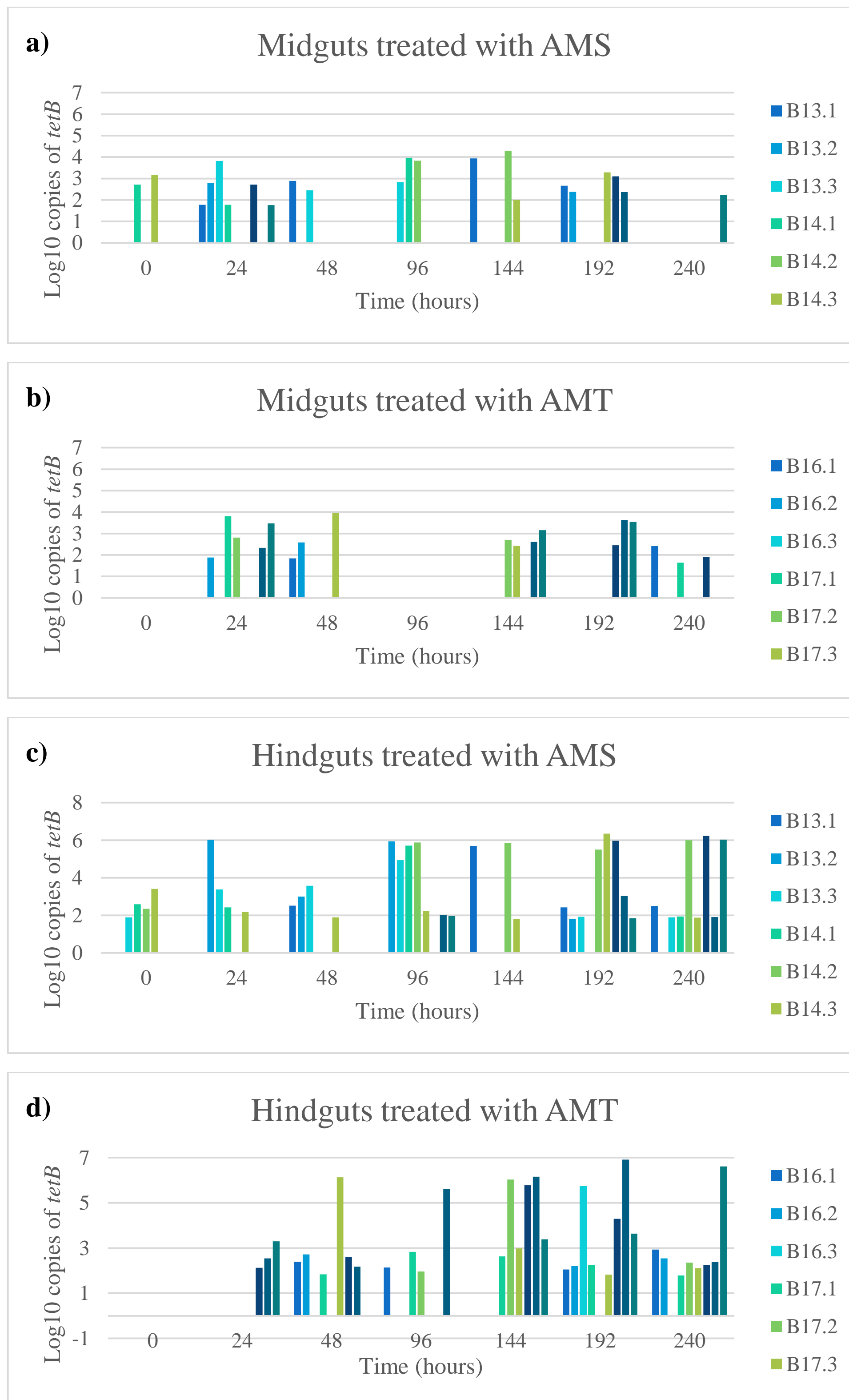


Figure G.2: Log10 copies of *tetB* in samples. T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

APPENDIX H: Copy numbers of American strain of *G. apicola*

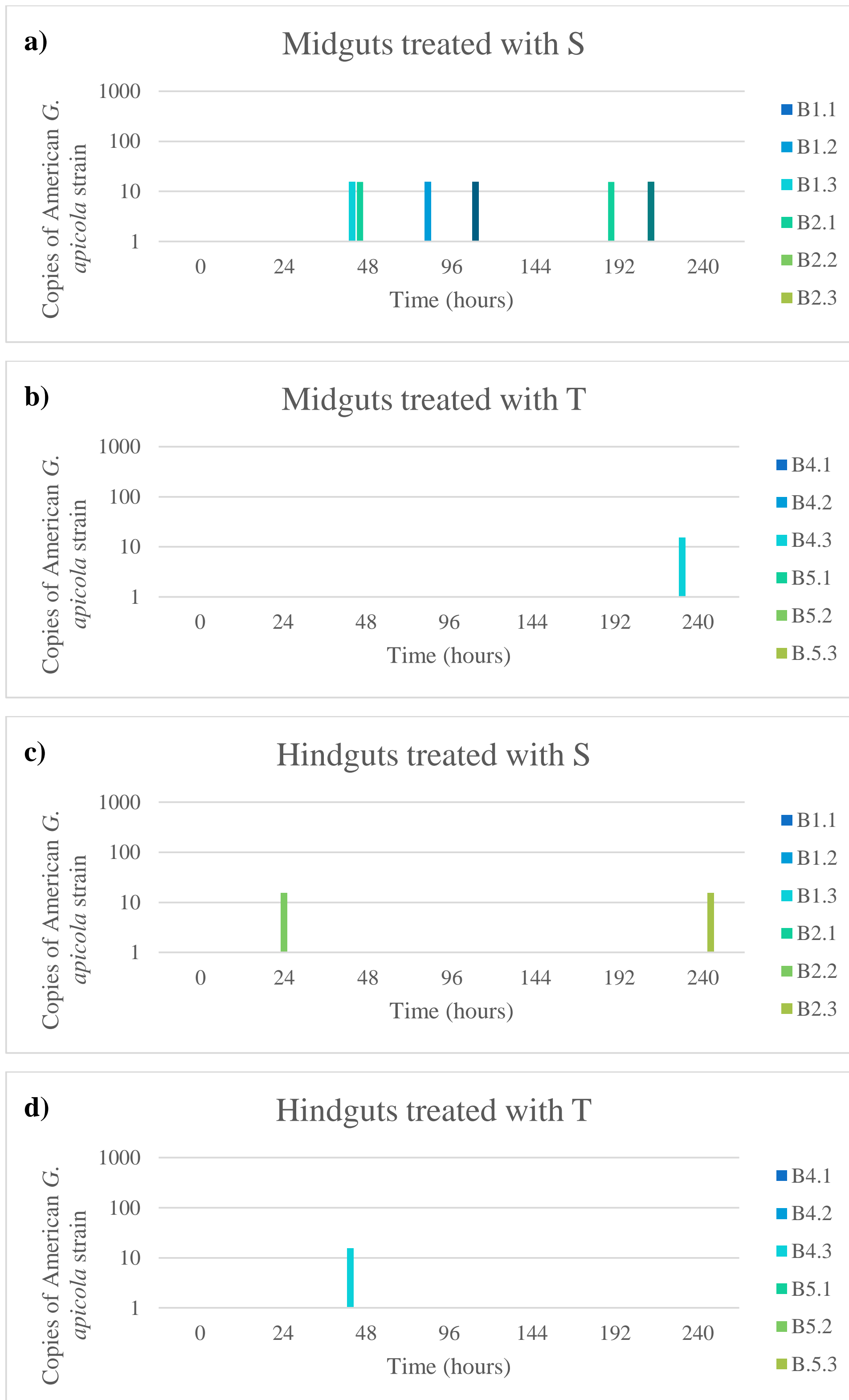


Figure H1: Copy number of American *G. apicola* strain in samples. T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.

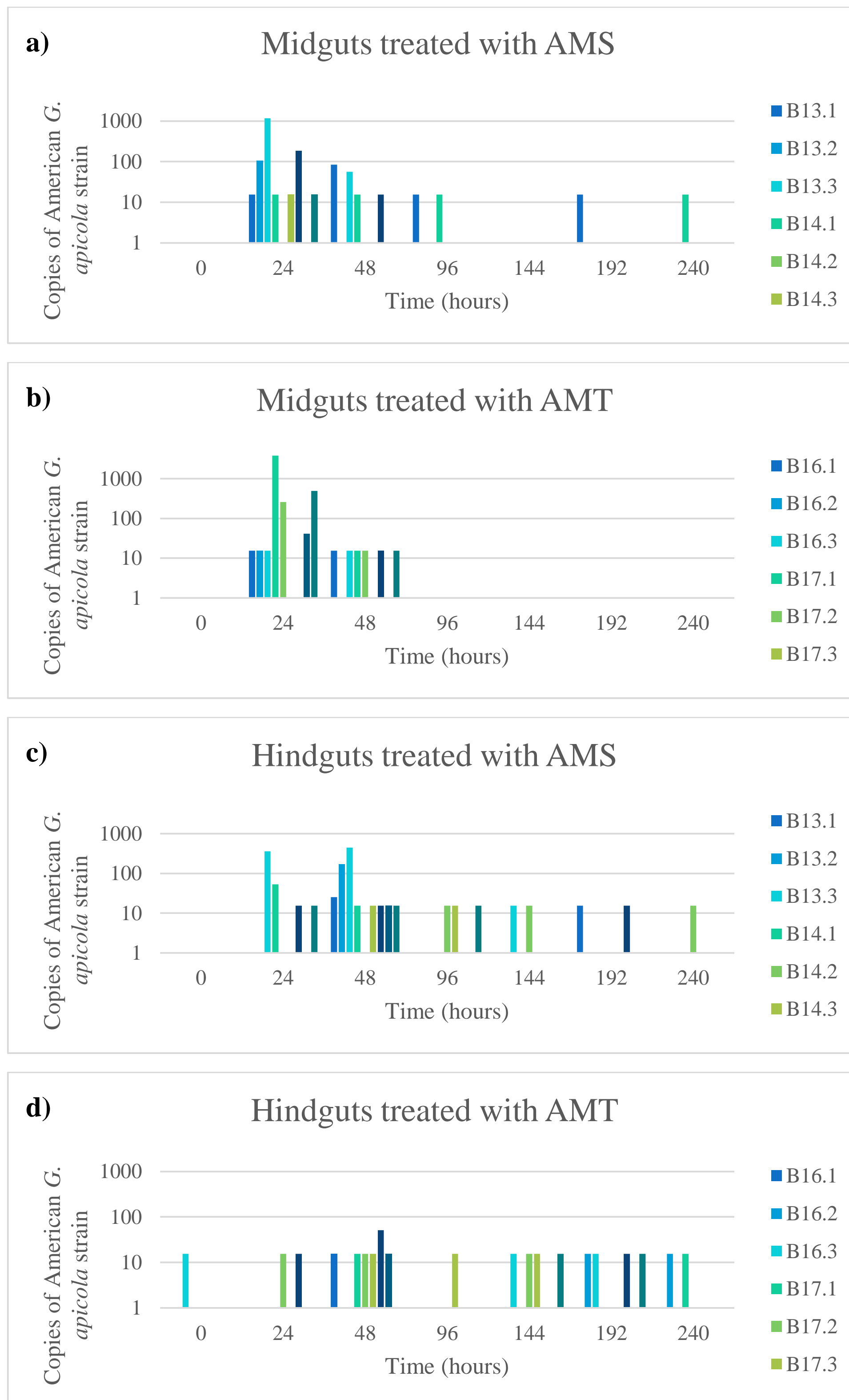
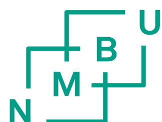


Figure H.2: Copy numbers of American *G. apicola* strain in samples. T=treatment with tetracycline, AMS=treatment with American *G. apicola* and sugar, AMT= treatment with American *G. apicola* and tetracycline.



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no