Microbiota in the honey bee gut and their association with bee health

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

in

Ecology

at

Massey University

School of Agriculture and Environment

Palmerston North, New Zealand

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2020

Preface

In 1998 I stood in the middle of a field in Paradise, Montana, USA. I was dividing hundreds of honey bee colonies to increase the number of hives for Californian almond pollination. The scene was overwhelming; millions of European honey bees swirling overhead, the intense sound of their wings beating in my ears, and hive boxes strewn over the field. At the end of the day I witnessed a phenomenon that changed my life. The chaos around me settled back in to some resemblance of normal life. I was completely captivated by the ability of these individual bees to use visual cues, smell, sound and taste, to find their way back to their hives, reform their communities and function as eusocial insects, cooperatively caring for their brood with division of labour across castes. And who was at the centre of this organisation? A single bee, aptly named the queen. Since this Massey Scholarship experience, my fascination has developed into awe as I endeavour to understand how honey bees respond and adapt to external factors throughout the season, including availability of food resources, human management practices, and pests and disease. We rely on these incredible insects to enrich our world and since the worldwide spread of the parasitic mite *Varroa destructor*, bees now rely on us to aid their survival. Nothing functions in complete isolation and this dependence has led me to search for novel prospects that may ensure honey bees remain healthy. Through discussions with a remarkable colleague around the advancing area of human gut research, my research topic was conceived from the revelation that individual honey bees rely on communities, both external and internal, for survival.

Abstract

European honey bees (*Apis mellifera*) are the most prevalent bee species globally. Honey bees play a key role in human welfare as their pollination services support both the ecological viability of wild and native plants, and the economic viability of numerous nut, fruit, and vegetable crops. A decline in unmanaged pollinators in both natural and managed ecosystems, has resulted in an increased reliance on honey bees.

Despite economic globalisation and increased demand for food over the past several decades contributing to an increase in the total number of honey bee colonies worldwide, annual colony mortality is high and has been attributed to seasonal conditions, poor management practice, outbreaks of pest and disease, pesticide poisoning, and the cost of management. It has been globally hypothesised that the cause of unexplained 'rapid' and 'incremental' colony loss, may result from interactions with bee pathogens (such as *Nosema* spp.), environmental factors and beekeeping management. The social and foraging behaviour of bees ensures that the gut, with its bacterial residents, is the conduit for assimilating of nutrients, antibiotics and oral poison, as well as the ingress and potential reservoir for gut pathogens. Characterisation of the bacterial community within the honey bee gut may provide further insight as to how these factors may affect bee health.

New Zealand honey bees have been largely bred in isolation from the rest of the world, and thus potentially developed their own gut microbiome in response to factors specific to New Zealand. These include sources of native floral nectars (e.g. mānuka and rātā), the prohibition of antibiotics for disease management, and the absence of some global honey bee gut pathogens. My research is the *first* to characterise the bacterial profiles and identify the relative abundance of core and less dominant bacteria in the gut of New Zealand honey bees.

Diet and pathogens known to cause poor honey bee health were examined for their influence on gut bacteria. Bacterial phylotypes in the honey bee gut were identified by sequencing a fragment of the 16S rRNA gene. The problematic assignation of reliable taxonomic information for recently characterised honey bee gut bacteria was overcome by developing a customised 16S rRNA BLAST database that is compatible with QIIME2 sequencing software. This database has now been made available for other users. The five dominant core honey bee gut bacteria identified internationally were present in all apiaries/regions within New Zealand. Eight phylotypes were only identified in colonies deemed 'sick' by beekeepers. Three phylotypes may potentially be used as indicators of poor bee health: the family Rhizobiaceae, and the genera *Serratia* and *Acetobacter*. Although each apiary was broadly similar in bacterial composition, the environmental conditions of each apiary appeared to influence the bacterial composition, in particular the available foraging sources.

In contrast to international reports, the microsporidian gut pathogen *Nosema ceranae* that shifted from the Asian honey bee *Apis ceranae* to the European honeybee in 2004 and was identified in New Zealand around 2007, does not appear to have outcompeted *Nosema apis*. The latter was likely brought to New Zealand with the first bees in the 19th century. In my survey *N. apis* was identified in all sick apiaries whereas *N. ceranae* was only identified in one of the sick apiaries.

Comparison of the gut bacteria in New Zealand bees with those from a pilot trial conducted in Connecticut, USA demonstrates that the dominant core bacteria are internationally widespread, and suggests that they have remained stable within an isolated population for over 60 years. This highlights the importance of the symbiotic relations that these gut bacteria have with honey bees. However, nine phylotypes were present only in the New Zealand samples, suggesting that some phylotypes may have adapted to New Zealand conditions or that dysbiosis may have occurred within New Zealand or elsewhere. This is the first example in the honey bee literature of DNA being analysed using different hypervariable regions. The variation between the number of amplicon sequence variants and their relative abundances highlight the importance of comparing data extracted using similar methodologies.

I observed seasonal variation in the bacterial composition by examining five hives throughout a 12-month period. Gut bacteria in summer bees were the most diverse, autumn and winter bees had lesser diversity, and spring bees had the least diversity. This suggests that the increased bee population in spring may result in a cleansing of less prevalent bacteria for the year ahead. The relative abundance of *G. apicola* and *S. alvi* did alter within individual bees throughout the year suggesting that these species may alter their abundance in response to occurrences within the gut and this may ultimately influence bee health and metabolism. The relative abundance of Rhizobiaceae peaked in winter when the bees live longer and often have elevated pathogen infections. The relative abundance of Rhizobiaceae exceeded that of all dominant core phylotypes, except *Lactobacillus* spp. This supports my hypothesis that Rhizobiaceae may be a useful early indicator of poor bee health.

Sucrose-rich diets, often fed to bees during periods of scarce food supply, were shown to increase the relative abundances of three less dominant core bacteria; Rhizobiaceae, Acetobacteraceae, and *Lactobacillus kunkeei*, and decreased the relative abundance of the core species *Frischella perrara*. In combination, these diets significantly altered the bacterial composition. Acetogenic bacteria from the Rhizobiaceae and Acetobacteraceae families increased two- to five-fold when bees were fed sucrose, suggesting that sucrose fuels the proliferation of specific low-abundance primary sucrose-feeders.

The gut pathogen *N. apis* did not appear to disrupt the development of *Gilliamella apicola*, which normally forms the outer layer of the biofilm in the luminal surface of the honey bee ileum. A gut slurry inoculation from older worker bees increased abundance

of bacterial phylotypes in newly emerged workers (NEWs), thus supporting the limited literature that NEWs acquire gut bacteria from worker bees. This study also confirms that NEWs are not axenic when they emerge from their cells as their guts contain low levels of *G. apicola*, *S. alvi*, *L. apis*, *L. mellis*, *Lactobacillus* spp., *Bifidobacterium* spp., *Serratia* spp., *Acetobacter* spp., Rhizobiaceae, and Cyanobacteria. Finally, this research also identified a correlation between the lack of abundant bacteria in the honey bee gut with an increase in the opportunistic colonising bacteria Rhizobiaceae and *Serratia*. This is further evidence in support of my suggestion that the family Rhizobiaceae contains opportunistic bacteria and that the relative abundance of this family in honey bee guts may be a useful indicator of poor bee health.

This work is thus the first study to examine gut bacteria in New Zealand honey bees and I have demonstrated that environmental factors and diet influence gut bacterial composition which may influence honey bee health and metabolism.

Acknowledgements

I am so grateful to Plant and Food Research for investing in me. You not only gave me time to conduct my PhD, supplied all my bee, office and lab requirements, but you have also funded my salary which enabled me to maintain a work-life balance that motivates me each day. After 19 years I still enjoy coming to work and not only am I inspired by the new found knowledge that my PhD has established, but the prospects that this has created for our team.

To all of you that funded my PhD expenses, thank you for believing in me and providing me with the flexibility to follow where the research led and to attend conferences. I could not have done this without you: Margaret Hogg-Stec Memorial Scholarship, New Zealand Apples and Pears Inc., the branches of the New Zealand Beekeepers Association (Auckland, Bay of Plenty, Waikato, Southern North Island, Nelson, Canterbury, Otago), Honey NZ (Young Mee Yoon), Beeline Apiaries (Fiona and Jeremy O'Brien), Galatea Apiaries (Cameron and Rose Martin), NZ Beeswax Ltd, The Honey Industry Charitable Trust, Hawkes Bay Research Trust, and Arataki Honey – Hawkes Bay.

My supervisory team have made this PhD experience so enjoyable. You have been such a pleasure to research with and your skills are inspiring. Thank you for sticking with me through this long process of a part-time PhD. It has been delightful getting to know you and my only regret is that our meetings were not in person with coffee and cake.

The acumen of my primary supervisor, Associate Professor Alastair Robertson brought clarity and perspective to my PhD in an especially kind manner. Alastair, you made the planning of my PhD exciting and I appreciate the way you helped me draw a line between commercial research and academia so that I could determine what was within scope and what was not. Without this, the trajectory of my PhD completion was heading towards 2025. Thank you for pushing for the important points and for ensuring my family was a primary focus. I have enjoyed getting to know you and look forward to future collaborations.

My interest in gut bacteria was inspired by a discussion about human gut bacteria with my esteemed colleague, Dr Shanthi Parkar. Little did she know that this discussion would land her a supervisory role for a PhD regarding the bacteria in the honey bee gut. Little did I know that her enthusiasm for gut bacteria was so justified, that her attention to detail would inspire me to explore paths previously unknown, and that her well-timed guidance in QIIME2 analysis would encourage me to find connections within honey bee microbiota and ultimately recognise their importance for bee health. Thank you, Shanthi, you are an incredible role model and friend.

My supervisor Associate Professor Patrick Biggs blew my mind by introducing me to the world of bioinformatics. This enabled me to explore the interactions within the honey bee gut and I remain in awe of how you unfold the story of gut microbiota from a tiny piece of DNA using code. Thank you, Patrick, for your patience as I have learnt this new 'language'. I am no longer terrified of trying new code. This was a huge divergence from my normal bee world but one that I grateful for as this powerful tool provided an important balance for my future research and this PhD. For this and the interest that you showed in my bee world, I will always be grateful. Thank you.

I would also like to thank my supervisor, Professor Ravi Ravindran, who provided thought-provoking discussion at the start of this PhD which helped shape the structure and enabled me to understand nutrient flow through the honey bee gut.

After working at PFR for so long I have so many wonderful colleagues to thank for their help and support throughout this PhD. Firstly, thank you to my amazing team for your continued interest in this project. David, your leadership is appreciated and inspiring. Heather, you have supported me throughout my shifting labs 6 times and assisted my set up for the mānuka and *Nosema* trials, along with Milena, Theo and Max. Without you all, these trials would not have been accomplished in the required time frames. Sarah and Grant, thank you for making hives available for my trials. Melissa, thanks for the guidance on the R plots. James, thanks for discussions regarding the DNA extraction for the *Nosema* trial and for supporting the completion of my PhD as my new manager. Crystal, Tama, Mateusz and Ashley, thank you for the support over lunch with the rest of the team. Your conversations have kept me interested in my PhD and have been a welcome distraction when needed.

I am most indebted to Kate Richards, my R guru. Not only have you patiently taught me the ways of R and the power of google R, you have given me a lifelong gift that enables me to unfold the bioinformatics story of bacteria. Although I will stick to bees and you will stick to stats and spatial modelling, I hope that we can regularly find projects to work on together, or at least have lunch.

A huge thank you to my other colleagues for their help along the way and your tea-room support throughout the five years. Specifically, Dan Jones (establishing an initial PFR QIIME2 workflow), Carel Jobsis (PCR information and provision of supplies), Paul Blatchford (discussions re sequencing bioinformatics), Duncan Hedderley (statistical reasoning of sampling 80 bees and Genstat discussions re bacteria in emerging bee gut trials, soon to be published), Janet Yu and Deirdre Cornish (lab use and DNA extraction techniques), Jodene Gonano (admin assistance), Lynn Mae De Leon (ensuring my funding remained mine so that I could attend conferences and publish), Donna Gibson (making the quality of my figures and photos suitable for publication), Carol Dolman and Jill Bradburn (editorial tips for Word), and Laura Ward (teaching me DNA extractions and quality analysis).

Dr Mark Goodwin, thank you for the 18 years we worked together. Your insight, focus and ability to synthesise honey bee health and pollination continues to inspire me to ensure bee health is at the heart of all beekeeper management. Your interest in the industry constantly reminds me that people are the reason we conduct science and why we need to ask “Why?” Thank you, my friend, for opening many doors that set me on a path that I love. I miss you.

I would like to thank Peter Molan for helping me in to the beekeeping industry when I arrived back in New Zealand after studying honey bees in Montana. He trusted me with his emails, his lab and then gifted me with his extensive Endnote library re all things mānuka before passing away. I will always appreciate the robust discussions and I trust the completion of this PhD would have made him proud.

Honey bees now only exist because good beekeepers manage their colonies. I am grateful to each of you who collected bee and pollen samples, and helped fund my PhD. Without you the first two chapters of this PhD would have been less robust. Northland: Harlan Cox (NL1), Bobby Leef (NL2), and Syd Greeves (NL3). Waikato: Cameron Martin (W1), and Fiona and Jeremy O’Brien (W2). Whanganui: Neil Farrer (WN1), Allan Richards (WN2), and Chris Valentine (WN3). Hawkes Bay: John Berry (HB1), James and Mary-Anne Ward (HB2), and Tom Taylor (HB3). Nelson: Murray Elwood (N1), Rae Butler (N2), and Hector Urquhart (N3). Canterbury: Brian Lancaster (C1), Roger and Linda Bray (C2), and Geoff Bongard (C3). Otago: Frans Laas (O1), Peter Scott (O2), and John Stevenson (O3). Tauranga: although these samples were not included, thank you, Ross Carroll, Tony Roper, and Neil Mossop. As you reliably followed the instructions, I look forward to enlisting you for future trials. Thank you also, Hiku Honey (Allan and Barbara Pimm) for supplying the analysed mānuka honey, and Natural Sugars for the invert sugar.

Thank you, my lovely friends who have been there to celebrate my wins, encouraged me to dig deep when things were overwhelming, and covered me with prayer. I love you dearly.

Throughout my PhD my beautiful family has blessed and supported me with love, prayers and encouragement. I cannot describe how blessed I am to have such incredible people in my life. Byron, my amazing husband, thank you for ensuring our family has stayed close. You have biked to my work with the girls for ice cream breaks, met me for dinner in random spots at random times, planned fun activities, learnt to cook, and taken every opportunity to laugh with me and provide perspective. These have been huge boosts, especially when the trials ran late in to the nights, analysis took longer than expected, and deadlines loomed. Thank you. To my delightful daughters Aimee-Grace and Emily, you are now five years older and you make me realise how precious time is. The journey of this PhD has been far more important than the completion and I thank you for being part of this journey. Your kind words, thoughtful questions and your desire to bring people in to our home to keep me connected has

inspired me to finish my PhD. It has also given me some encouragement that this process has not completely stuffed you up, but actually inspired you both to pursue your own precious dreams and never give up. You are my treasures and I am proud of who you are.

Statement of contribution

Although this PhD was conceived and written by me, my supervisors have played a crucial role in directing my lines of enquiry and editing my chapters. In particular, Shanthi Parkar did a large portion of editing the reviewer comments for the published paper in Chapter 6 when the turnaround was tight and the others were unavailable.

I produced a bioinformatics pipeline using a Jupyter notebook to analyse the sequences of the 16S rRNA gene fragments. Information and written code was combined from several sources: Patrick Biggs, Dan Jones, Shanthi Parkar, Paul Blatchford, Marcus Davy, and the websiteQIIME2docs (https://docs.qiime2.org/2019.10/). Patrick Biggs played a major role, specifically writing new code or fixing broken code to aid the flow of the pipeline. He also helped me enable other researchers to identify recently characterised species currently unavailable in the four taxonomic databases by creating Perl code, now publically available, to produce customised 16S rRNA BLAST reference datasets that would be compatible with QIIME 2.

Sequencing of the 16S rRNA gene from the V3V4 hypervariable regions was conducted by the Massey Genome Services, Palmerston North, New Zealand.

Kate Richards played an important role in identifying the R code needed to conduct the statistical analysis of the bioinformatics information as well as fixing my broken R code.

Chapters 3 and 4 required DNA from bees sampled throughout New Zealand within a two-week period. Seventeen beekeepers collected these bee samples for me as per detailed instructions.

The carbohydrate trial described in Chapter 6 required the assistance of a research associate to replace the sucrose feeders and count bee mortality in the middle of the trial.

At the conclusion of the bacteria versus pathogen trial described in Chapter 7, I required the assistance of technicians to label and fill microcentrifuge tubes with DNA/RNA shield so that I could extract the guts and store them immediately. These tubes were unable to be set up earlier as the shipment of the DNA/RNA shield had been delayed for weeks which delayed the trial. The trial immediately commenced on arrival of the shield to ensure the results were not confounded by the use of ‘winter’ bees that are morphologically different to ‘summer’ bees.

Contents

List of tables

List of figures

List of abbreviations and acronyms

Abbreviation	Meaning
16S rRNA	16S ribosomal RNA (ribonucleic acid)
AFB	American foulbrood disease
ASV	Amplicon sequence variant
ATCC	American Type Culture Collection
AZ	Arizona, USA
BHI	Brain Heart Infusion agar
bp	Base pair
Cq	qPCR quantification cycle
CT	Connecticut, USA
DNMT3	Deoxyribonucleic acid-methyltransferase
DW	Distilled water
DWV	Deformed wing virus
EFB	European foulbrood
h	Hour(s)
H_2	Hydrogen
H_2O_2	Hydrogen peroxide
HG	Hypopharyngeal glands
ID_{50}	The infective dose for 50% of a population
ID_{100}	The infective dose for 100% of a population
KG	Knowledge gaps
MA	Massachusetts, USA
MALDI-TOF	Matrix-assisted laser desorption/ionization using a time-of-flight analyzer
MD	Maryland, USA
MGO	Methylglyoxal
MGS	The Massey Genome Service
MIC	Minimum inhibitory concentration
Min	Minute(s)
MMRA	Maximum mean relative abundance
MRM	Microbial Resource Management

NEW	Newly emerging worker bee
NMDS	Non-metric multidimensional scaling
NZ	New Zealand
O_2	Oxygen
PERMANOVA	Permutational multivariate analysis of variance
PFR	The New Zealand Institute for Plant and Food Research
qPCR	Real-time polymerase chain reaction
RH	Relative humidity
s	Second(s)
SBV	Sacbrood virus
Spp.	Species
V	A hypervariable region (V1 to V9) of the 16S rRNA gene
WST-1	Cell Proliferation Reagent

Glossary

Anoxiated	An absence of oxygen reaching the tissues. Used to make the bees sleep temporarily.
Axenic	Bacteria free
Bacterial community	In the context of this PhD it describes all bacteria within the digestive tract of the honey bee from crop to rectum, unless specified otherwise.
Bacteriome	Specialized organ that hosts endosymbiotic bacteria whilst protecting the host.
Bee bread	Pollen pellets collected by honey bees that are stored in the wax cells with some honey and glandular secretion. This is later eaten by newly emerging workers.
Brood	Honey bee larvae (uncapped) and pupae (capped) growing inside the cells of the wax comb.
Brood box	One or two of the boxes at the base of the stack that contains nine or ten brood frames.
Brood nest	The central area of comb in the hive that is used to rear brood. It is therefore tended to by nurse bees. Normally it is located within the lower brood boxes.
Colony	A group of honey bees that are led by a single queen and live in a single hive.
Comb	The hexagonal wax cells that contain bee brood and honey.
Cell	Bacterial cell.
Drone	A male honey bee that is produced from an unfertilized egg.
Dysbiosis	An unhealthy shift in the composition of a bacterial community.
Eclose	When the pupa emerges as an adult honey bee from the wax cell; the bee chews through the wax capping and climbs out of the cell.
Eusocial	A social group of organisms with cohabitation of overlapping generations that cooperatively care for their brood and display division of labour across their reproductive castes.
Frame	A section of bee brood or honey that is edged by a wooden or plastic frame.

Greengenes	A 16S rRNA gene database.
Hive	The material components used to house a colony of honey bees. These normally consist of wooden boxes, each with nine wax frames, a floorboard and a lid.
Hypopharyngeal gland	Glands that are located in the head of the worker bee behind the eyes. These are fully developed when protein is consumed, normally > 5 days, and produce glandular secretion which the workers feed larvae.
In vitro	In the context of this PhD it describes a trial conducted using the honey bee gut isolated from the abdomen.
In vivo	In the context of this PhD it describes a trial conducted on living honey bees in a cage or colony.
MALDI-TOF MS Biotyper	MALDI Biotyper systems provide high-speed, high-confidence identification and taxonomical classification based on proteomic fingerprinting using high-throughput MALDI-TOF mass spectrometry.
Microbiome	In the context of this PhD, it is the genetic material of all the microbes such as bacteria, microsporidia, and viruses that live in the honey bee gut.
NCBI	National Center for Biotechnology Information. A 16S rRNA gene database.
Nurse bee	A young worker bee, approximately 13 days old, which feeds brood royal or worker jelly produced by their hypopharyngeal glands.
Operational Taxonomic Unit	Definition used to classify a group of closely related individuals.
Overwintering	Colonies during winter reduce egg laying and foraging. The bees require sufficient honey and pollen, and they cluster for warmth.
pH	A measure of hydrogen ion concentration to measure the acidity or alkalinity of a solution.
Propolis	The waxy resin collected by honey bees from newly formed leaves. The bees use this in their colonies to reduce gaps between the hive components.
Queen	The dominant female honey bee produced from a fertilized egg and fed royal jelly. She produces pheromones to control the colony.
Queen excluder	A selective barrier inside the beehive that allows worker bees but not the queen or drones to traverse the barrier.

Queen right	The honey bee colony contains a laying queen.
RDP	Ribosomal Database Project. A 16S rRNA gene database.
Royal jelly	The glandular secretion from the hypopharyngeal glands in the head of the nurse bee. This is fed to queens, workers and drones by nurse bees.
SILVA	A 16S rRNA gene database.
Split	To make additional colonies from a parent colony, some sealed brood frames covered with bees and honey are removed and combined with a newly mated queen or a queen cell.
Sterna	Ventral abdominal plates on the honey bee.
Super	A box on the hive used to store honey. Normally positioned above the brood boxes and contains nine or ten frames of honey.
Terga	Dorsal abdominal plates on the honey bee.
Wax cell	The wax cell within which honey bees raise their brood or store hive products such as honey and pollen.
Worker	A female honey bee that is produced from a fertilized egg.

Chapter 1

Introduction: honey bees, their health and the bacteria associated with their digestive tracts

1.1 Global importance of *Apis mellifera*

Globally there are likely to be more than 20,000 bee species (Michener 2007) but only eleven of these belong to the genus *Apis*, and only *Apis* are considered true honey bees (Michener 2007). Of these eleven, the eusocial European honey bee (Insecta, Hymenoptera, Apidae, *Apis mellifera* Linnaeus) is the most prevalent globally (Engel 1999) and it plays a key role in human welfare. Not only are they one of the first insects that we learn to identify as children, but they are the global producers of honey and providers of pollination services. Their pollination services support both the ecological viability of some wild and native plants (Morse and Calderone 2000; Gallai et al. 2009; Potts et al. 2010a) and ensure the economic viability of numerous nut, fruit, and vegetable crops (McGregor 1976; Southwick and Southwick 1992; Free 1993; Morse and Calderone 2000; Gallai et al. 2009). Large-scale monoculture farming which reduces the natural habitat of pollinating insects (Klein et al. 2003; Klein et al. 2007) has contributed to the recent reduction of wild pollinators (Butchart et al. 2010; Rucker et al. 2012; Ollerton 2017), such as native bees and wasps (Ollerton et al. 2014), flies (Biesmeijer et al. 2006), and moths (Fox 2013). In turn, our reliance on honey bees as pollinators for managed ecosystems has increased. The role of honey bees in pollinating natural ecosystems is not well elucidated. The term 'managed ecosystem' includes 'direct' crop pollination and 'indirect' pollination. The latter is where crops, such as carrots, can be grown from seed harvested from pollinated inflorescences.

Of the 115 leading global food crops used directly for human consumption, 76% require pollination and more significantly, 35% of global food production relies on pollinators (Klein et al. 2007). Without insect pollinators, the yields of some fruit, seed, and nut crops decrease by more than 90% (Southwick and Southwick 1992). In 2005 the economic value of insect pollination was estimated to be worth €100 billion (Gallai et al. 2009). Currently there is no global valuation for the economic impact of using honey bees as pollinators. However, in 2012 the agricultural industry in the United States of America (USA) estimated the value of direct crop pollination by honey bees to be $11.68 billion, and indirect pollination of crops was estimated to be $5.39 billion for (Calderone 2012).

1.2 Global beekeeping trend

Since 1961, the number of domesticated honey bee colonies have increased globally by 45% according to the 2008 Food and Agriculture Organization of the United Nations database (Aizen and Harder 2009). This increase is thought to be driven by economic globalisation and demand rather than by biological factors that improve colony health (Aizen and Harder 2009). This global increase has occurred despite the well-publicised colony losses that have resulted from seasonal conditions, management practices that cause malnutrition (Smart 2015; Frias et al. 2016), outbreaks of pest and disease (Dahle

2010; Evans and Schwarz 2011; McMenamin and Genersch 2015), and pesticide poisoning (Babendreier et al. 2007; Dively et al. 2015). The upward global trend highlights how adept the beekeeping industry is at replacing these losses by propagating colonies annually through a process called colony 'splitting', where one colony is divided into two or more colonies. The global trend also conceals regional colony losses that occur because it is uneconomic to replace them, as well as unexplained rapid colony losses historically observed in parts of Europe and America (Biesmeijer et al. 2006; Cox-Foster et al. 2007; Currie et al. 2010). The effect of rapid loss may be experienced for several years. In contrast, the effect of incremental regional loss may be more permanent; during 1985 and 2005 the number of colonies reduced by 25% in central Europe, despite populations in Scandinavia and the Mediterranean increasing (Potts et al. 2010b). USA also observed a 59% reduction of colonies. This occurred incrementally between 1949 and 2007 (vanEngelsdorp et al. 2008). However, during this time, parasitic mites, in particular *Varroa destructor* (hereafter *Varroa*) contributed to this decline (Anderson and Trueman 2000). It is likely that the changes in beekeeping required to offset the increased costs to control *Varroa*, such as colony replacement and the chemical treatments, have played a part in the reduction of commercial beekeepers and therefore colonies in the USA.

Unexplained rapid colony loss has also occurred in North America. During 2006–2007, 29% of beekeepers reported colony losses of up to 85% (Stokstad 2007; vanEngelsdorp et al. 2008). The phenomenon was termed Colony Collapse Disorder (CCD) (Stokstad 2007), but the cause is still ambiguous. There have been multiple hypotheses as to the cause of CCD such as pathogens, e.g. Israeli acute paralysis virus (IAPV) (Chen and Evans 2007), parasites, pesticides and immune system disorder (Stokstad 2007). It has also been suggested that the cause of widespread colony loss may be more complicated than a single factor, and may result from interactions between bee pathogens, environmental factors and beekeeping management (Neumann and Carreck 2010). It is therefore necessary to understand the components of these key factors and how they affect the health of honey bees with the intention of globally curtailing localised colony decline and rapid colony loss.

1.3 Beekeeping in New Zealand

1.3.1 Industry dynamics

The upward trend in the New Zealand (NZ) beekeeping industry is comparable to the global trend of increased honey bee colonies. However, the high value of mānuka (*Leptospermum scoparium*) honey, driven by international demand, has resulted in unprecedented industry growth, and illustrates well the economic globalisation discussed by Aizen and Harder (2009). Over the past five years the number of beehives and beekeepers in NZ has almost doubled to 881,185 and 8,552 respectively (Figure 1.1 and 1.2) (New Zealand Government 2018), and for the years 2012–2017, the compound annual growth rate of export revenue for NZ honey was 22.19%. A further advantage of the NZ beekeeping industry is the pollination service that they provide for numerous domestic and export crops totalling approximately NZ$3B pa (New Zealand Government 2017).

Until this year, the rapid growth of NZ's beekeeping industry and the high honey prices have mitigated the annual colony mortality that is primarily caused by pests and pathogens, poor management, pesticide poisoning, or poor economics. In 2017 the annual colony loss recorded in NZ was 9.8% (86,356 colonies) (Brown and Robertson 2018). The losses were variously attributed by beekeepers to problems with the queen (43.3%), *Varroa* (14.0%), starvation (10.1%), and predation by wasps (*Vespulid* spp.) (7.3%). However, to determine the cause of colony death post-mortem, sound beekeeping knowledge is required. As half of NZ beekeepers are new to the industry in the past five years, the accuracy of the percentage of colony losses assigned to each factor may be questionable. High colony mortality is regarded as a sign of poor colony management. Therefore, although the total number of dead colonies recorded may be reliable, the percentages may be skewed so that beekeepers can save face; beekeepers my favour factors that they have limited control over, such as queen problems and wasp predation. Beekeepers who participated in the survey represented 30.9% of all NZ beekeepers. This survey represented 30.1% of the colonies managed by commercial beekeepers, and 33.8% of commercial beekeepers with > 400 colonies. Of these, a few commercial beekeepers from all North Island regions reported colony mortality of 30–40%. (Brown and Robertson 2018).

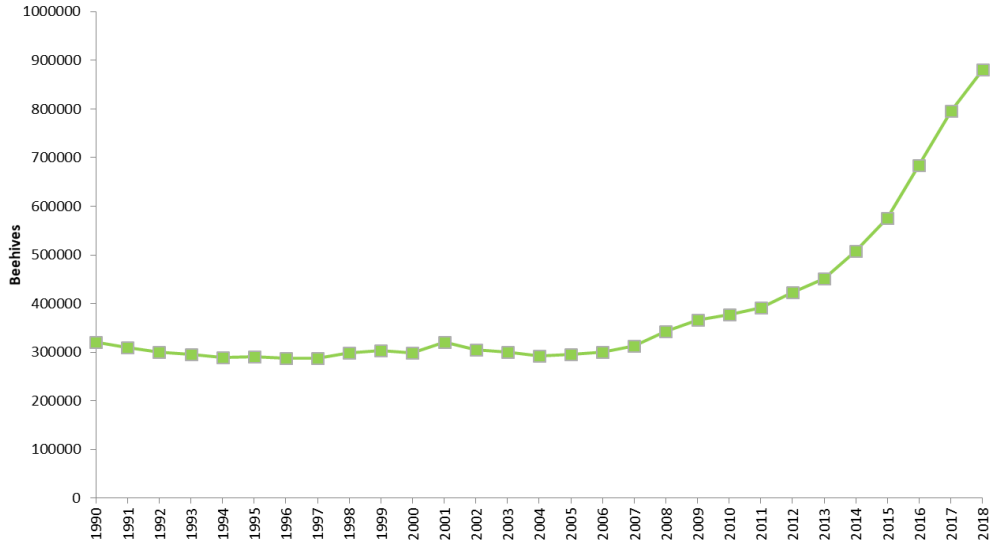

Figure 1.1 | Number of beehives present in New Zealand.

Data were collected by AsureQuality Limited (New Zealand Government 2018).

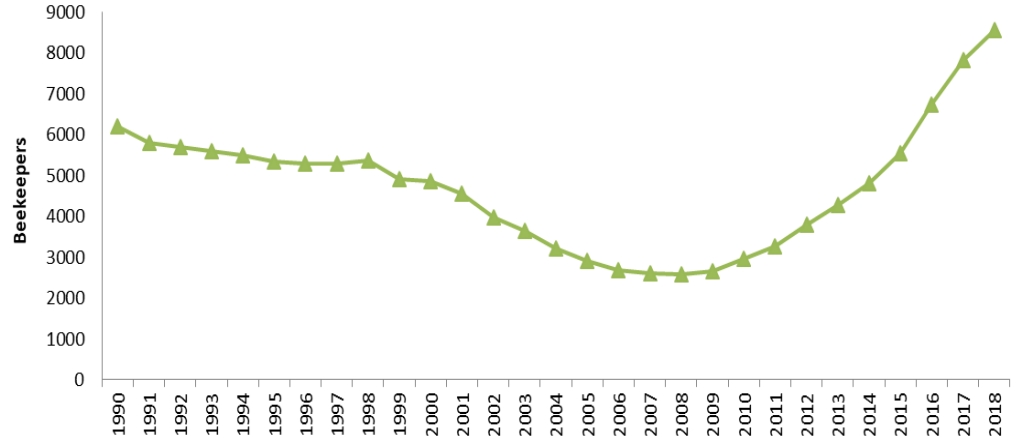

Figure 1.2| Number of beekeepers present in New Zealand.

Data were collected by AsureQuality Limited (New Zealand Government 2018).

To date, one of the biggest regional losses that the NZ beekeeping industry has experienced is the 16% (22,000) colony reduction in the upper North Island between 2000 and 2004. This was caused by the introduction of *Varroa* which was detected in Auckland, April 2000 (Stevenson et al. 2005). The *Varroa* mite is an ectoparasite that feeds and reproduces on honey bees, increasing throughout the season to detrimental levels in autumn (Watanabe 1994; Winfree et al. 2007). However, the decline of the beekeeping industry was short-lived as industry growth was stimulated by the international demand for mānuka honey.

Fortunately, the NZ beekeeping industry as a whole has not experienced the rapid colony loss of > 30% p.a., as observed internationally (vanEngelsdorp et al. 2008; Currie et al. 2010). However, the estimated annual loss of 86,356 colonies from NZ is still a concern. For the individual beekeepers who have experienced large colony mortality, and for those that will, it is critical to understand the factors that influence colony survival and how to manage them. The management of colony health will therefore not only mitigate international colony loss, it will reduce the current NZ annual loss and ensure that the NZ beekeeping industry remains resilient and globally competitive.

1.3.2 Colony management

Honey bee colonies are typically housed in Langstroth hives consisting of one or more rectangular, wooden boxes (Figure 1.3) (Langstroth and Dadant 1922). Each box contains eight to ten wooden or plastic frames (Figure 1.4). Each frame is inserted with a wax foundation that is printed on both sides with a horizontal hexagonal pattern. The worker bees 'draw out' combs of wax cells on this pattern(Figure 1.5) using wax produced from glands on their abdomen (Kurstjens et al. 1985). The boxes are stacked vertically and contain either brood frames (brood box) or frames of honey (a super). The supers are normally positioned above one or two brood boxes.

Figure 1.3 | A standard commercial hive.

Two brood boxes at the base are separated from the honey super on top by a queen excluder.

Figure 1.4 | A single brood box containing nine frames of wax cells.

Varroa control strips (Bayvarol®) are being inserted between two frames in this hive.

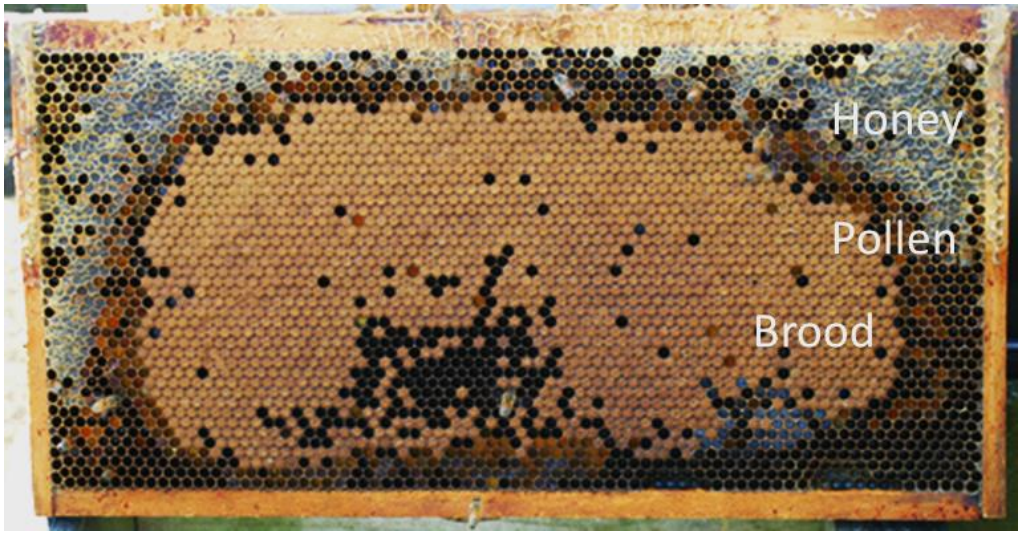

Figure 1.5 | A wooden honey bee brood frame consisting of wax hexagonal cells.

The sealed cells around the top and sides of the frame contain honey. Beneath this is a thin arch of pollen and in the centre of the frame is a large oval of wax-capped brood dispersed with some open larval cells.

1.3.3 Nectar sources and native honeys

Honey bees commonly consume carbohydrates from nectar, honey, sucrose, and invert sugar. In New Zealand, floral nectar is the main source of carbohydrates collected by honey bees. Honeys from native New Zealand plants including mānuka, rātā, and pōhutukawa are recognised internationally. Mānuka honey is produced from the nectar of the plant *Leptospermum scoparium* and is NZ's largest native honey export earner (New Zealand Government 2018) as it is reknowned for its antibacterial activity. Thus, mānuka honey provides a valuable income for many New Zealand beekeepers.

A sucrose solution (≥ 40%) and honey with a 10 – 20% sucrose-equivalent concentration, have comparable antibacterial activity *in vitro* (Molan 1992a; Kwakman et al. 2010). The anti-bacterial action of honey with a sucrose-equivalent concentration ca. of 80% (v/v), is attributed to hydrogen peroxide produced by the enzyme glucose oxidase that the bees add to nectar (Molan 1992a). When hydrogen peroxide is accounted for, the additional antibacterial activity of mānuka honey (Willix et al. 1992; Kwakman et al. 2010) is attributed primarily to the chemical methylglyoxal (MG) with a concentration > 0.15mg/g. The precursor for MGO is Dihydroxyacetone (DHA) (Mavric et al. 2008; Adams et al. 2009; Atrott et al. 2012) and mānuka honey less than one year old has an MG concentration between 0.102 and 0.793 mg/g. After one year, or if the honey has been heated, this can increase to 1.541 mg/g (Majtan et al. 2012). In the presence of mānuka honey, resistant strains of bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faeciumas*, are unable to proliferate (Kwakman et al. 2008). Prior to this thesis, nothing was known about how this unusual honey might affect the microbiome of the honey bee.

1.4 Indicators of colony health

Healthy honey bee colonies look and behave differently to sick colonies. Beekeepers discern these key differences throughout the year and alter their management practices to reduce colony loss and maximise colony productivity. In particular, beekeepers observe colony development throughout each season, bee behaviour, the appearance of both the brood frames and adult bees, annual honey production, and pollen collection. The following section outlines the expected appearance and behaviour of healthy colonies. Deviations from these patterns indicate that the colony may be sick.

1.4.1 Honey bee castes

A healthy colony consists of three castes that vary in both phenotype and function: the queen (Figure 1.6), female workers (Figure 1.7), and male drones (Figure 1.8) (Graham 1992). A young laying queen is central to the colonies long-term survival. The workers collect all of the food and water for the colony and tend to the brood. The drones ensure the survival of their species and their major role is to fertilise the queen's eggs. Further detail regarding each of these castes is provided in Box 1.1: Honey bee biology.

Figure 1.6 | Honey bee queen.

A blue age tag is glued to her thorax. Approximate length is 20 mm.

Figure 1.7 | Worker honey bee.

Approximate length is 18 mm.

Figure 1.8 | Drone honey bee.

Approximate length is 23 mm.

Box 1.1 | Honey bee biology

Queen honey bee

The queen honey bee is a fertilised female that grows from egg to adult in 16 days. She can live up to eight years, but on average will survive for four to six years. In commercial beekeeping operations the queens are replaced annually. The queen mates in flight with seven to 44 individual drones (Taber III 1954; Koeniger et al. 1979; Schlüns et al. 2005) during one to four mating flights that normally occur within the first week after emerging from her queen cell (weather dependant) (Roberts 1944). Occasionally two mated queens exist within a single colony, but normally there is just one. The queen lays between 175,000 and 200,000 eggs annually, each within a single wax cell (Figure 1.5). All fertilised eggs produce females (worker bees or a queen) and unfertilised eggs produce male drones (Haydak 1970; Graham 1992).

Worker honey bees

Worker honey bees make up the majority of a colony with typically more than 60,000 bees in summer. They eclose from the egg as larvae at day 3, the cell is capped with wax at day 8.5, then they develop into pupae. On day 21 they chew through the wax cap and emerge from the cell as adult bees (Graham 1992). The emerging workers conduct age-related tasks: comb building, cell cleaning, tending brood, capping brood, attending the queen, receiving nectar, packing pollen, cleaning debris, corpse removal, ventilating the hive, guarding the entrance, and foraging (Winston 1991; Graham 1992). Peak foraging activity occurs at 15–32 days post eclosure (Harrison

1986). Throughout summer, worker longevity averages from 15 to 38 days, whereas workers during winter survive >140 days (Fukuda and Sekiguchi 1966; Graham 1992).

The worker bee larvae are supplied with a pool of jelly comprising vitamins, lipids, amino acids, and a family of proteins called Major Royal Jelly Proteins, thought to be crucial for reproductive maturation (Maleszka 2008). The jelly is secreted by the hypopharyngeal glands (HG) of nurse bees (young worker bees between 4 and 12 days old (Johnson 2008)) and is also fed to queens, although for a longer duration and with a slightly different composition. The worker and queen phenotypes result from the diet fed during the larval stage. This was recently shown by silencing the expression of the enzyme DNA-methyltransferase (DNMT3) in the diet of larvae destined to become workers which resulted in the larvae developing into queens (Kucharski et al. 2008; Chittka and Chittka 2010; Mukherjee et al. 2015).

Pollen consumption is highest in nurse bees and this develops their HG for the production of royal jelly. Once the workers commence foraging at two weeks of age, they consume mainly carbohydrates, consuming little or no pollen (Vasquez and Olofsson 2009; Brodschneider and Crailsheim 2010), suggesting they have limited requirements for protein. However, foragers require the amino acid proline that is found in pollen for metabolism during flight (Micheu et al. 2000). It is unknown whether pollen is the only source of proline. A review by Rortais et al. (2005) suggests that during the first three days of development, worker larvae consume ~ 30 mg of food (Nelson et al. 1924), followed by 120 mg over the next two days (Bishop 1961). Most of this food consumption contributes to growth as the larvae weigh on average 150 mg after 5 days (Jay 1963). The review also identifies that sugar concentration of the jelly fed to the workers first 3 days of larval development is thought to be 18% sucrose and fructose, increasing to 45% in the last two days of development. However, these concentrations may need to be revised because Rortais et al. (2005) suggest this information was first published over a century ago by von Planta (1888) and later cited by Haydak (1968).

Drone honey bees

Drones are male bees produced by the queen from unfertilised eggs. There are normally between 300–3000 drones present per colony that are are produced in spring and autumn for the sole purpose of mating with queens. Drones grow from egg to adult in 24 days and are mature for mating at day 42. The average longevity of a drone is 21 to 32 days during summer. As the colony prepares for winter all drones are discarded from the colony by worker bees in autumn (Fukuda and Ohtani 1977).

1.4.2 Population size

In temperate climates, such as NZ, a typical spring honey bee colony has a few thousand worker bees. This expands to > 60,000 bees over summer with the population size peaking in early autumn. The population then reduces throughout autumn and winter (Haydak 1970; Graham 1992), despite suitable winter conditions enabling workers to forage on available resources (Rinderer 1988).

An atypical population at any time during the year is a key indicator that the colony is inhibited by one or more of the colony loss factors discussed previously. Population decline in spring is associated with excessive *Varroa*, *Nosema* infection (Mattila and Otis 2006), and viruses, such as deformed wing virus (DWV) (Highfield et al. 2009). Further information regarding these pests/diseases is available in Box 1.2: Major pests and diseases present in NZ honey bees. In Canada *Varroa* has been identified as the primary cause for population decline and mortality of overwintered colonies (> 85%) in spring and early summer (Guzmán-Novoa et al. 2010). This is likely because viral outbreaks are progressed by *Varroa* vectoring viruses that persist at low levels within bees (Tentcheva et al. 2004). This association has been demonstrated in NZ with autumn collapse occurring in colonies where both Kashmir Bee virus (KBV) and *Varroa* occur (Todd et al. 2007). Controlling *Varroa* is therefore imperative for colony production and survival. However, *Varroa* treatments applied in late autumn may fail to prevent colony loss during winter because adult bees infested as pupae, do not fully develop physiological features of long-lived wintering bees (Amdam et al. 2004). The absence of the queen that produces all of the brood obviously will cause population decline and colony mortality.

NZ honey bees harbour just a subset of the pests and diseases that are present worldwide. This is presumably because NZ is geographically isolated and its border security has prohibited the importation of honey bees, hive ware and bee products since the 1950s (Stevenson et al. 2005). The four diseases/pests that negatively affect colony size are the parasitic mite *Varroa*, *Paenibacillus larvae* subspecies *larvae*, *Nosema apis*, and *Nosema ceranae* (for details see Box 1.2).

Box 1.2 | Major pests and diseases present in New Zealand honey bees

Varroa destructor are parasitic mites that breed in the sealed wax cell of the honey bee pupae and parasitize the adult bees. *Varroa* infest brood to reproduce, feed on fat body tissue in the adult (Ramsey et al. 2019), and vector bee viruses, such as Deformed wing virus (DWV) and Sacbrood virus (SBV). Colonies must be treated in spring and autumn using synthetic treatments or the colonies die (Downey and Winston 2001; Goodwin and Taylor 2008). Resistance to these chemicals is developing (Goodwin et al. 2005). Damaged fat body cells and two morphologically distinct bacteria have been detected below the feeding site (Ramsey et al. 2019). The bacteria are currently uncharacterised so their association with *Varroa* or the honey bee is unknown.

Paenibacillus larvae **subspecies** ***larvae*** (class Bacilli) are the causative agent of American foulbrood disease (AFB) in honey bee larvae/pupae (Alippi and Reynaldi 2006). In NZ, colonies with a single clinical symptom of AFB must be destroyed by fire within seven days of identification, along with the hive ware (New Zealand Government 1998). Bee larvae are infected by consuming food contaminated with AFB spores. These germinate in the larval gut, penetrate the gut wall, and consume the pre-pupal or early pupal tissue (Bamrick 1967).

Nosema **spp**. (two species) infect the epithelial cells of the honey bee midgut and reduce brood rearing, adult longevity, colony size, and honey production (Higes et al. 2007; Botías et al. 2013; Huang and Solter 2013; Eiri et al. 2015). They are obligate intracellular single-cell spore-forming parasitic microsporidians that belong to the fungal phylum Microsporidia. *Nosema apis* was first introduced to NZ at least 100 years ago (Zander 1909). *Nosema ceranae* was likely introduced to NZ between 2005 and 2007 (Klee et al. 2007). *Nosema ceranae* causes increased food consumption (Martín-Hernández et al. 2011), then immune suppression (Antúnez et al. 2009), degeneration of the epithelial cells in the gut and then reduced lifespan. Ultimately, this results in decreased population size and reduced honey production (Higes et al. 2007).

Sacbrood Virus (SBV) is an infectious disease caused by the *Morator aetatulas* virus that affects honey bee larvae widespread virus that prevents larvae from pupating so that the unshed cuticle fills with fluid, forming a sac (Bailey et al. 1964). SBV does not cause symptoms in adult bees (Bailey 1969), but infected workers fly earlier and collect limited pollen (Bailey and Fernando 1972).

Ascosphaera apis (Chalkbrood) is a fungus that mummifies honey bee pupae and appears either black or white. Chalkbrood is not normally lethal to the colony but it can cause a 5–37% reduction in honey yield (Heath 1982; Yacobson et al. 1991;

Zaghloul et al. 2005). The mummified pupae are removed from the cells by adult honey bees. Although Chalkbrood has significant effect on the honey bee immune system (Xu et al. 2019), the effect of this fungi on the gut bacteria in adult honey bees is unknown.

Internationally, additional species cause population decline. These include *Melissococcus plutonius* (European foulbrood disease) (Bailey 1963), *Acarapis woodi* (Tracheal mites) (McMullan and Brown 2005), and *Tropilaelaps clareae* (*Tropilaelaps* mites). These are further described below in Box 1.3: International honey bee pests and diseases. The 'honey bee pathosphere' diagram by Evans and Schwarz (2011) provides a clear overview of the recognised parasites community that affects the health of honey bees.

Box 1.3 | International honey bee pests and diseases

Melissococcus plutonius (European foulbrood disease) is one of the two characterised bacteria that affect honey bee larvae. It is the infective agent of European foulbrood disease (EFB). EFB kills honey bee larvae but not necessarily the entire colony (Bailey 1963). EFB has not been detected, and is therefore likely absent from NZ. The spores of *M. plutonius* are transferred in honey, bees and hive ware. This is one of the reasons why the importation of international honey is illegal in NZ.

Acarapis woodi (Tracheal mites) live in and cause scarring of the trachea of honey bees. Tracheal mites are lethal to colonies when initially introduced to a region. Once control treatments are introduced, colony death is sometimes observed when the colonies have overwintered in cold temperate climates (McMullan and Brown 2005).

Tropilaelaps mites (*Tropilaelaps clareae*) are brood ectoparasites of honey bees native to Asia (Atwal and Goyal 1971). The primary host is *Apis dorsata* but *Tropilaelaps* mites were discovered on *A. mellifera* in the Philippines (Delfinado and Baker 1961). *Tropilaelaps* are particularly pathogenic in *A. mellifera* as they are similar to *Varroa* in that they infect brood, suck haemolymph and are likely to transmit viruses.

1.4.3 Bee behaviour

The movement of bees in and out of the hive entrance is the initial indicator of a colonies health. No bees, bees staggering around or falling out of the hive the entrance, or bees that look wet or are crawling through the grass indicate the bees are diseased or have been poisoned. Additionally, if there is either excessive or limited movement in or out of the colony and the floorboard and entrance is covered with chewed wax, then it is likely that this weaker colony is being or has been 'robbed out' by a stronger honey bee colony, or wasps (*Vespula* spp). When the colony is opened, the low-pitched tone of the worker bees coupled with the bees conducting their jobs in an ordered fashion indicate that the queen is producing sufficient pheromones to control colony behaviour. A higher-pitched tone from the worker bees and erratic worker behaviour indicates the queen is absent or failing (Gary 1992), or that the colony may be experiencing the effects of disease, pests, pesticide or malnutrition.

Individual bees also show signs of disease; *Nosema* infection causes bees to tremble, have dilated abdomens, and produce faecal deposits on the combs (Bailey 1967). Sick or dead bees can also be seen outside the colony, and the colony has decreased brood production, predominantly in spring. Viral infections such as DWV and Acute Bee Paralysis Virus respectively cause wing deformities or paralysis.

1.4.4 Appearance of the wax frames

Spotty brood-patterns on the frame indicate that the colony may be affected by one or more pests, brood diseases or viral infections (Boxes 1.2: Major pests and diseases present in NZ honey bees, and 1.3: International honey bee pests and diseases); The brood cells can appear uncapped or sunken, and may contain mummified larvae, discoloured or c-shaped larvae or pupae, or sac-like pupae. Defecation on the frames may also indicate disease. In NZ, the appearance of the wax frames can be used to identify the following pests and disease. These are listed in order of annually attributed colony losses; the *Varroa* mite (Spivak and Gilliam 1998; Goodwin and Taylor 2008), the pathogenic larval/pupal bacterium *P. larvae* subspecies *larvae*, two microsporidia (*Nosema apis and Nosema ceranae*) that infect the midgut epithelial cells of adult honey bees, the virus Sacbrood (*Morator aetatulas*), and the fungus Chalkbrood (*Ascosphaera apis*).

Honey bee larvae are supplied with a pool of royal jelly secreted by the hypopharyngeal glands (HG) (Figure 1.9) of nurse bees (Terra and Ferreira 1994). The development of the HG requires pollen (protein) that the nurse bees consume (DeGrandi-Hoffman et al. 2010). If the colonies experience malnutrition, the larval diet is compromised, the larvae or pupae are often cannibalised (Webster et al. 1987), and brood rearing is reduced (Keller et al. 2005; Mattila and Otis 2007; DeGrandi-Hoffman et al. 2010). The brood-

pattern therefore appears spotty, as the cells are cleared for new eggs to be laid. Malnutrition also causes bees to forage earlier (Toth et al. 2005). This reduces the length that bees perform other age-related tasks and reduces adult longevity (Rueppell et al. 2007; Woyciechowski and Moroń 2009), ultimately reducing the size of the colony.

Figure 1.9 | Hypopharyngeal gland.

This hypopharyngeal gland was removed from behind the compound eye of a worker honey bee. The white oval acini are each attached to an axial collecting duct.

1.5 Assessing bee health

Measures more specific than those outlined above can be used to assess colony development and bee health, such as worker survival, worker weight, the lifespan of workers, growth and development of fat bodies (Maurizio 1954; Wahl and Ulm 1983; Schmidt et al. 1987), and brood rearing success (Herbert et al. 1970; Wahl and Ulm 1983). The suitability of protein sources for worker bee development can be compared by measuring the size of the acini on the HG in worker bees on day eight (de Groot 1953; Standifer 1967). HG are undeveloped in newly emerging workers (NEWs), absent in drones, and rudimentary in queens. The glands synthesise brood-proteins which nurse bees feed to developing larvae. If the workers have consumed pollen, the HG are fully developed and physiologically functional in nurse bees by the 5^{th} or 6^{th} day post-eclosure (Standifer 1967; Knecht and Kaatz 1990). However, complete development of the HG does not indicate that the secretion produced is nutritious for larvae (Standifer 1967).

1.6 Gut bacteria associated with bee health

With annual NZ colony loss at 9.8% and international colony losses even higher, a rapidly developing field of scientific research proving to be fundamental to animal health is the microbiome in the digestive tract. This includes microsporidia, other micro-eukaryotes and in particular, the bacteria proliferating in the digestive tract (Guarner 2005). Bacterial communities within insect digestive tracts are predominantly composed of commensal bacteria that likely aid host nutrition, digestion, reproduction and/or protection against enteric pathogens and toxins (Dillon and Dillon 2004; Engel and Moran 2013b). The composition of bacterial communities can be influenced by numerous factors. Examples of this occur in the fruit fly, *Drosophila*, as gut bacteria are influenced in part by the composition of bacteria in the environment (Corby-Harris et al. 2007), and in turn these are regulated by *Drosophila* genes (Ryu et al. 2008). The composition and function of gut bacteria are also affected by host diet in crickets (Santo Domingo et al. 1998), termites (Warnecke et al. 2007) and gypsy moths (Broderick et al. 2004). The bacterial communities differ within the gut compartments of termites (Schmitt-Wagner et al. 2003). This also occurs in honey bees, suggesting that pH, digestive enzymes, and/or redox conditions may influence bacterial composition (Babendreier et al. 2007).

When I commenced this PhD in 2014, the literature regarding the honey bee gut microbiome was limited to the recognised pathogens that affect bee health including fungi (*Nosema apis*, *Nosema ceranae*, *Ascosphaera apis,* and *Aspergillus* spp.), protozoans (*Crithidia mellificae*, *Malpighamoeba mellificae*, *and* Gregarines), and bacteria (*Melissococcus plutonius, Paenibacillus larvae, Paenibacillus alvei, Spiroplasma apis, Spiroplasma melliferum*, *Pseudomonas aeruginosa*, *Achromobacter euridice*, *Enterococcus faecalis*, and *Brevibacillus laterosporus*) (Evans and Schwarz 2011). Less well known were the innate bacteria that were considered a small stable community that were either pathogenic or specific to sections within the honey bee gut (Babendreier et al. 2007; Martinson et al. 2011; Moran et al. 2012). The effect of bacteria on other insects, coupled with preliminary research on bumble bees (*Bombus* spp.) that suggested gut bacteria prevent infection of the parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011b), validated the exploration of bacterial communities within the honey bee gut. Over the past five years this knowledge base has significantly increased. The following summarises the relevant key foundations for bacteria in the honey bee gut.

1.6.1 Culture-based identification of bacteria

Historically, identification and classification of bacteria in the digestive tract (gut) of the adult honey bee was conducted using culture-based methodologies. The limitations of culture-based technology meant that bacteria were cultured if they were amenable to the culturing conditions provided or if their culture conditions were known. The information collected from culture-based studies tends to focus on cell structure, cellular metabolism or components within the cell such as DNA, fatty acids, and/or pigments. Culturing initially suggested that the gut was 70% Gram-negative bacteria, including various members of the phylum Bacteroidetes: class Flavobacteria (Tysset and Durand 1968) (translated by Snowdon and Cliver (1996)), and the phylum Proteobacteria: including the class β-proteobacteria, and various genera in the class γ-proteobacteria: *Citrobacter*, *Enterobacter*, *Erwinia, Klebsiella, Proteus, Pseudomonas*, and the species *Escherichia coli.* The remaining bacteria were 29% Gram-positive bacteria from the phyla Firmicutes and Actinobacteria, and 1% yeast-like microbes. The phylum Firmicutes included *Bacillus*, *Lactobacillus* and *Streptococcus* from the class Bacilli, and *Clostridium* from the class Clostridia. The phylum Actinobacteria was represented by species of *Bifidobacterium* and *Corynebacterium* within the class Actinobacteria (Snowdon and Cliver 1996; Gilliam 1997).

Culture-based studies using various agar media incubated in both aerobic and anaerobic conditions suggest that the bacterial community consists predominantly of anaerobic bacteria (10^8–10^9 viable cells per gram of intestine) as opposed to aerobic bacteria (10^4–10^5 cells/g) (Rada et al. 1997).

In contrast, molecular studies identified that the honey bee gut was dominated by facultative aerobic or aero-tolerant bacteria (Mohr and Tebbe 2006; Ahn et al. 2012), and estimated that the average number of bacterial cells in 1-day-old bees increased from 1.1 x 10^5 to a more stabilised level in 16-day-old bees of between 5 x 10^8 and 2.2 x 10^9 bacterial cells (Powell et al. 2014). At this time the discrepancy in the microbial counts between culture-based and molecular studies was because the growing conditions were understood for only some of the core gut bacteria, and without these only a small subset, approximately 0.1–1%, of bacteria from an environment, will grow *in situ* (Staley and Konopka 1985). In the case of the honey bee gut, culture-based techniques were unable to simultaneously simulate all of the growth conditions required by each bacterium, including O_2, H_2, pH, and redox gradients (Brune 1998). Thus, only bacteria that grew in the provided conditions were observed, not necessarily the full complement of bacterial species present in the environment (Gilliam and Valentine 1976; Gilliam et al. 1990; Gilliam 1997). Additionally, culture-based methodology creates ambiguity among and within species as most bacteria lack distinct structures, and many experience lateral gene transfer between unrelated species, thus blurring the boundaries among the phenotypes of taxa (Boucher et al. 2003).

Although culture-based methods are unable to determine species abundance in relation to each other or study interactions at a community level, they are still relevant as they enable species-specific research to be conducted. This includes identification, classification, and inoculation trials using axenic (without bacteria) bee guts, and to improve understanding of individual bacteria present in the honey bee gut (Gilliam 1997; Olofsson and Vásquez 2008; Killer et al. 2009; Koch and Schmid-Hempel 2011b; Engel et al. 2012; Engel et al. 2013b; Kwong and Moran 2013).

1.6.2 Molecular identification of bacteria

The 2006 rapid colony loss experienced in the USA highlighted the dearth of information regarding honey bees and their gut microbiota. This prompted research using culture-independent methods to understand the association of honey bees with both symbiotic and pathogenic microbes (Cox-Foster et al. 2007; Evans et al. 2009; Runckel et al. 2011).

Technological advancement has enabled the use of molecular methods to more accurately identify and place bacterial species into clades. These methods typically utilise a region of DNA present in all bacteria called the 16S ribosomal RNA (16S rRNA) gene. This gene codes for the RNA component of the 30S subunit of the bacterial ribosome (Egert et al. 2003; Jeyaprakash et al. 2003). It is ideal for prokaryotic species identification as it tends to be highly specific to bacterial and archaeal species and tends to remain conserved within species (Olsen et al. 1994).

A variety of molecular techniques have been used to identify and classify bacteria in the honey bee digestive tract based on the 16S rRNA gene including Sanger sequencing (Sanger et al. 1977; Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Babendreier et al. 2007; Cox-Foster et al. 2007; Olofsson and Vásquez 2008; Martinson et al. 2011; Disayathanoowat et al. 2012), terminal restriction fragment length polymorphism (T-RFLP) (Babendreier et al. 2007; Disayathanoowat et al. 2012), single-strand conformation polymorphism (SSCP) (Mohr and Tebbe 2006), gene clone library analysis (Martinson et al. 2011), and more recently, pyrosequencing and other next-generation sequencing (NGS) techniques (Ahn et al. 2012; Moran et al. 2012). These NGS technologies, which began to emerge in 2004 with the first Roche 454 machines and 2005 with the Solexa Genome Analyzer, have been increasingly employed for microbiome research because they generate numerous short sequencing reads from across millions of fragments from the same sample. As advancement in technology continues to reduce the number of reads with errors, molecular identification is now considered more accurate and reliable than culturing (Ross et al. 2013). The identification of numerous unique amplicon sequence variants (ASVs) identified in a single sequencing run, provides a much more detailed picture of the gut microbiome. However, the diversity of NGS approaches used to characterise the gut microbial

assemblage often precludes direct comparisons; the sampling methods vary, DNA extraction methods vary, and any one of the nine hypervariable regions of the 16S rRNA genes may be analysed (Mattila et al. 2012).

International data from the sequencing of 16S rRNA gene amplicons from the guts of individual worker bees suggests that 99% of the bacterial community in the honey bee gut can be classified by eight to ten distinct species/phylotypes. However, this may not be a complete picture worldwide as the bacterial community in New Zealand bees has not been studied.

Molecular nomenclature in the literature uses both ‘species’ and ‘phylotype’ interchangeably to distinguish a group of closely related strains that have ≥ 97% sequence identity or exact ASV in their 16S rRNA gene amplicons (Babendreier et al. 2007; Cox-Foster et al. 2007; Martinson et al. 2011; Moran et al. 2012; Sabree et al. 2012; Engel and Moran 2013a). Hereafter, the term ‘phylotype’ will be used to identify the most specific taxonomic rank that can be identified for each bacterium in the honey bee gut; some phylotypes correspond to known species, others to species complexes where unidentified species cluster within a genus, while others can only be classified to family, order, class or phyla.

1.7 Movement of nutrients through the digestive tract

Before describing the eight to ten core bacterial phylotypes present in the honey bee digestive tract, the bee diet and the process of nutrient consumption need to be summarised. This provides context for the colonisation of the gut compartments within the digestive tract and how these bacteria may interact. From here on, the term 'bacterial community' refers to all bacteria within the digestive tract of the honey bee from crop to rectum, unless specified otherwise.

The adult honey bee diet consists of protein, carbohydrates and water (Graham 1992). Pollen is the natural protein source that bees collect from flowers using the scopae of their hind legs to transfer it to their colony. Nectar is the predominant source of carbohydrate but during periods of dearth, bees feed on their stored honey or alternative carbohydrate sources such as dry sucrose, sucrose solution, invert sugar or high-fructose corn syrup (DeGrandi-Hoffman et al. 2010). For further details see Box 1.4: The honey bee diet and the production of honey.

Box 1.4 | The honey bee diet and the production of honey

Some beekeepers supplement pollen with pollen patties made from ingredients such as crude protein, fat, ash, granulated or liquid sucrose, fondant sugar (sucrose and dry fructose), and tap water (Graham 1992). Natural carbohydrates are collected from flowers in the form of nectar. Nectar predominantly consists of sucrose, its monosaccharide components glucose and fructose, water and pollen grains (Wykes 1952; Baker 1982). Although total sugar concentrations in nectar range from 4 to > 70% (w/w), depending on plant species and environmental conditions (Wykes 1952; Percival 1961; Nicolson 1998), bee-pollinated flowers tend to produce nectar with > 35% sugar (Baker 1982). Within the hive, bees reduce the nectar's moisture content to about 17% (range 13–24%) (White et al. 1962) before they cap the honey-containing cells (Haydak 1970; Nicolson and Human 2008). This results in honey with a concentrated mix of sugar consisting of about 69% monosaccharides (approximately 38% fructose and 31% glucose) (Doner 1977), and < 15% disaccharide (sucrose) (White et al. 1962). Nectar is temporarily stored in the honey bee crop, which is essentially an inflatable bag that mixes nutritional resources collected from the external environment, hive stores, and from other bees. Sources of carbohydrate are utilised by the bee for energy and the surplus is stored in the wax cells and dehydrated to form honey.

From here on the term ‘gut’ will be used to identify the entire digestive tract of the honey bee. The gut consists of six main compartments that are located within the abdomen (Figure 1.10): crop, proventriculus, midgut/ventriculus, a short region called the pylorus, the ileum, and the rectum. The term hindgut refers to both the ileum and the rectum.

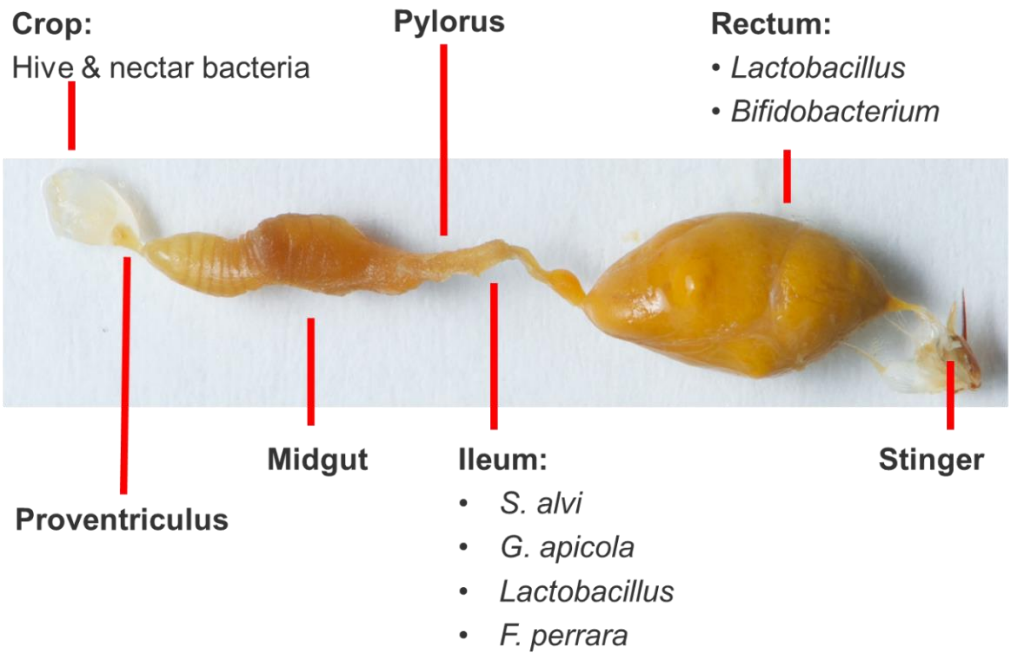

Figure 1.10 | Dissected honey bee digestive tract.

The head and thorax of the honey bee would be attached to the left of the crop. The main bacteria associated with the hindgut are listed.

The type of bacteria within the crop are limited to those usually found in nectar or the surrounding environment. They are rarely found in the rest of the gut and normally grow in aerobic conditions (Wang et al. 2015). It was initially thought that digestion did not occur in the crop, as host proteolytic enzymes were not detected (Graham 1992). However, with the identification of *Bacillus* spp. in the crop, it has been suggested that microbial metabolites may be produced to assist digestion or aid storage (Lee et al. 2015; Saraiva et al. 2015). However, it is more likely that *Bacillus* are present from external sources, such as the nectar, as the pH of the gut (5.5–6.5) (Muszynska and Leznicka 1992) may not support the growth of *Bacillus* spp. which typically require pH conditions > 6 (Gordon et al. 1973).

The nutrient flow from the crop into the midgut (ventriculus) is regulated by the lips and hairs of the proventriculus, a flexible pipe that compresses the pollen grains into a bolus and pushes it through the sphincter muscle of the stomodeal valve (Bailey 1952; Barker and Lehner 1972). In the midgut the bolus is covered by a peritrophic membrane to form a compartment of digestion (Moritz and Crailsheim 1987) that contains immobilised aminopeptidases (Peters and Kalnins 1985). The majority of digestion and absorption occurs here (Peng et al. 1985). Energy from digestion is stored in the form of glycogen

and triglycerides by the fat body (Arrese and Soulages 2010) and within 2 min of consumption, the fat body breaks down glycogen to glucose which is then converted to trehalose (Gmeinbauer and Crailsheim 1993), a non-reducing disaccharide available as an energy source in the haemolymph (Wegener et al. 2003). The midgut substrate, known as the peritrophic membrane, is an unstable chitinous material that sheds continuously so limited bacteria are found in this region (Engel and Moran 2013b). The pylorus is where the boluses accumulate and the Malpighian tubules excrete electrolytes and nitrogenous waste. Bacteria such as *Snodgrassella alvi* (β-proteobacteria), *Gilliamella apicola* (γ-proteobacteria), and *Frischella perrara* (γ-proteobacteria) thrive on this waste (Bradley 1985). From this point on, > 99% of the bacterial cells reside. The boluses pass into the hindgut. The first compartment of the hindgut is the ileum that is dominated by *S. alvi* and *G. apicola* and also contains *Lactobacillus mellis* and *L. mellifer*. The rectum is the final compartment and is dominated by *Lactobacillus* and *Bifidobacterium*. The rectum of worker bees, at least when purging flights are possible, have a pH value below 5.5, whereas toward the end of winter and the boluses accumulate, the pH value ranges from 5.6 to 6.5 (Muszynska and Leznicka 1992). It is from here that the solid wastes are excreted (Winston 1991; Graham 1992). The pollen mass passes through the midgut within 1–3 hours (Bailey 1952; Barker and Lehner 1972) but normally stays in the rectum until the honey bee flies out of the colony and defecates.

Due to the specific functions that occur within the compartments of the digestive tract, it is likely that the bacteria within each are specialised to these conditions. Terminal restriction fragment length polymorphism analysis (T-RFLP) was used to show clear differences between the bacterial communities of the midgut, ileum, and rectum, where 28% of the total variance was explained by differences between the midgut and the hindgut (Babendreier et al. 2007). Deep sequencing of 16S rRNA genes identified that the crop and midgut contain < 5% of the relative abundance of the core bacterial community, whereas the ileum and rectum combined contain > 95% (Martinson et al. 2012); the average number of bacterial gene copies observed in the pylorus was 1.14 x 10^9, the ileum contained ~2.83 x 10^8, and the rectum contained ~3.70 x 10^8 (Engel et al. 2015).

1.8 Core bacteria

The predominant phyla are Proteobacteria (Engel et al. 2013b; Kwong and Moran 2013; Kešnerová et al. 2016), Firmicutes (Babendreier et al. 2007; Martinson et al. 2011) and Actinobacteria (Engel et al. 2012; Martinson et al. 2012; Moran et al. 2012; Sabree et al. 2012; Engel and Moran 2013a). As knowledge and functional understanding of the core bacteria present in the honey bee gut continue to evolve, taxonomic affiliations of these bacteria have been reassigned; taxonomy of strains identified and published prior to 1980 (Tysset and Durand 1968; Gilliam and Valentine 1976) have been updated and the identification and culturing of new strains have led to several re-classifications (Engel et al. 2012; Engel et al. 2013b; Wu et al. 2013). To clarify the currently used classifications, the historical nomenclature of the dominant core phylotypes and the less prevalent/inconsistently present phylotypes within the honey bee gut, are listed in Table 1.1.

Table 1.1 | Core phylotypes in the digestive tract of adult honey bees.

Phylum	Class	Order	Family	Genus & species	Previous nomenclature (Moran 2015)
Proteobacteria	γ–proteobacteria	Orbales	Orbaceae	***Gilliamella apicola*** (Kwong and Moran 2013)	Gamma1, γ–proteobacteria-1, Pasteurellaceae*, Orbaceae*
Proteobacteria	β-proteobacteria	Neisseriales	Neisseriaceae	*__Snodgrassella alvi__ (Kwong and Moran 2013)	Beta, β-proteobacteria, Neisseriales
Actinobacteria		Bifidobacteriales*	Bifidobacteriaceae	*__Bifidobacterium asteroides__ (Jeyaprakash et al. 2003), (Bottacini et al. 2012)	Bifido
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*__Lactobacillus spp.__ (Ahn et al. 2012), (Sabree et al. 2012) __Lactobacillus mellis__ (Olofsson et al. 2014) __Lactobacillus mellifer__ (Olofsson et al. 2014)	Lactobacillus Firm-4
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	***Lactobacillus*** Firm-5: __Lactobacillus apis__ (Killer et al. 2014) __Lactobacillus helsingborgensis__ (Olofsson et al. 2014) __Lactobacillus kimbladii__ (Olofsson et al. 2014) __Lactobacillus melliventris__ (Olofsson et al. 2014)	*Lactobacillus Firm-5, strain 1 F1
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	__Lactobacillus kunkeei__ (Neveling et al. 2012)	Fructophilic lactic acid bacteria
Proteobacteria	γ-proteobacteria	Orbales	Orbaceae	__Frischella perrara__ (Engel et al. 2013b)	Gamma-2, Orbales
Proteobacteria	α-proteobacteria	Rhizobiales	Bartonellaceae	__Bartonella apis__ (Ahn et al. 2012)	Alpha1, Bartonellaceae, Rhizobiales
Proteobacteria	α-proteobacteria	Rhodospirillales	Acetobacteraceae	__Bombella apis__ (Yun et al. 2017)	Acetobacteraceae, Alpha-2.2, *Parasaccharibacter apium* (Corby-Harris et al. 2014b)
Proteobacteria	α-proteobacteria	Rhodospirillales	Acetobacteraceae	__Gluconobacter__–related spp. group (Corby-Harris et al. 2014b)	Alpha 2.1

The currently used nomenclature is in boldface. * indicates the dominant core bacterial phylotypes.

Of the eight to ten core phylotypes, five are considered dominant core bacteria as they are present in most adult honey bee workers, and are rarely found outside of the honey bee gut: *Gilliamella apicola*; *Snodgrassella alvi*; *Lactobacillus* Firm-4; *Lactobacillus apis* (previously known as *Lactobacillus* Firm-5); and *Bifidobacterium asteroides* (Babendreier et al. 2007; Martinson et al. 2011; Bottacini et al. 2012; Kwong and Moran 2013). These five are observed in *Bombus* spp. and other *Apis* spp. (Ahn et al. 2012; Koch et al. 2013; Lim et al. 2015), but microbial transplantation experiments indicate that most strains are specific to their host and are unable to proliferate in other *Apis* spp. (Kwong et al. 2014; Kwong and Moran 2015). An additional four core phylotypes are less prevalent and more variable: *Frischella perrara* (Engel et al. 2013b), *Bartonella apis* (class α-proteobacteria) (Kešnerová et al. 2016), and a *Gluconobacter*-related species group (class α-proteobacteria) that contains Alpha 2.1, which in the following chapters of this thesis is referred to as Acetobacteraceae, and a species within the Alpha 2.2 group, *Parasaccharibacter apium* (Corby-Harris et al. 2014a). Although *P. apium* has recently been published as *Bombella apis* (Yun et al 2017), I continue to use *P. apium* throughout this thesis to prevent confusion between *Bartonella apis* and *Bombella apis*. These core bacteria are reviewed in detail in section 1.7: Core Bacteria. Additional bacteria associated with the crop, specifically *Lactobacillus kunkeei* (class Bacilli) is reviewed in lesser detail (Corby-Harris et al. 2014a).

Studies of the relative abundance of the core gut phylotypes and the sequencing methods utilised to obtain these data have been reviewed by both Sabree et al. (2012) and Corby-Harris et al. (2014a). The information was compiled from research of *Apis mellifera* populations in South Africa (Jeyaprakash et al. 2003), Germany (Mohr and Tebbe 2006), Switzerland (Babendreier et al. 2007), Australia (Cox-Foster et al. 2007), Sweden (Olofsson and Vásquez 2008), United Sates of America (Cox-Foster et al. 2007; Vasquez and Olofsson 2009; Martinson et al. 2011; Mattila et al. 2012; Moran et al. 2012), and Thailand (Disayathanoowat et al. 2012). Two additional phylotypes: Gamma-3 and Gamma-4, were initially identified as part of the dominant gut bacteria (Moran et al. 2012) but as they are rare, infrequently observed and have not been further classified, they will be discussed as additional bacteria.

None of the initial studies mentioned above identified all nine core phylotypes in a single colony or from a single location. Two publications identified all of the dominant phylotypes but these samples were collected from more than one location: Cox-Foster (2007) analysed bees from Australia, USA and Hawaii, and Moran et al (2012) analysed bees from Arizona (AZ) and Maryland (MD), USA. *G. apicola* and *Lactobacillus* Firm-5 phylotypes were present in all of the above-listed studies. The South African populations did not identify *F. perrara* or *Lactobacillus* Firm-4 (Jeyaprakash et al. 2003). *Bartonella apis*, *F. perrara* and *Lactobacillus* Firm-4 were not discussed in the German study (Mohr and Tebbe 2006). *B. apis*, Alpha-2 and *S. alvi* were not discussed in the Swedish study (Olofsson and Vásquez 2008) or in the study from Arizona, USA where *F. perrara* was also not identified (Vásquez et al. 2009). *B. apis* and Alpha-2 were not identified in the

reanalysis of data from Massachusetts, USA, by Mattila et al. (2012). *B*. *asteroides* was absent from the Swiss study and *B. apis*, Alpha-2, *F. perrara* and *Lactobacillus* Firm-4 were absent in the Thai study. *G. apicola* and *F. perrara* were the most frequent and abundant bacteria detected in Switzerland (Babendreier et al. 2007).

It is likely that the differences between the phylotypes present and the relative abundance observed in these studies are associated with both methodology; age of bees sampled, DNA extraction methods, DNA sequence methods, and sequence analysis, as well as which phylotypes were reported.

Table 1.2 summarises the methodology used in several studies that helped structure this PhD as they provided context for expected presence and potential relative abundance of dominant core bacteria for the analysis in Chapters 3–5.

Table 1.2 | Summary of key methods used to conduct research on the bacterial phylotypes in the digestive tract of honey bees (*Apis mellifera*).

	(Martinson et al. 2011)	(Ahn et al. 2012)	(Martinson et al. 2012)	(Moran et al. 2012)	(Anderson et al. 2013)	(Corby-Harris et al. 2014a)	(Engel et al. 2015)	(Kapheim et al. 2015)	(Jones et al. 2018b)
Location	AZ, USA	Suwon, Korea	AZ, USA	AZ & MD, USA	AZ, USA	AZ, USA	CT USA & Switzerland	IL, USA	Sussex, UK
Bee type/age (days)	W	W	9–30	~16	Honey, nectar, bee bread, W	PF	5–29	F, N	F
Gut Section	E	M, H	C, M, I, RM	M–RM	E	C	M–H	H	M–H
Sample type (number of bees)	P (80)	Ind (30)	Ind (18)	Ind (40)		P (10)	Ind (21–140)	Ind (3) & P (4)	P (3)
DNA extraction method	LI, DNeasy kit (QI), & P/C	FastDNA SPIN Kit for Soil (MPB)	LI, DNeasy kit (QI), or PC	B, PCI	FG Genomic DNA Purification Kit	B, LI, FG Genomic DNA Purification Kit	CTAB/phenol extraction	MB PowerSoil DNA isolation kit	ZR DNA MiniPrep kit
Phylotype identity method	UBP, qPCR: 27F & 1391R	P454	Custom primer pairs (100–250 base pairs), qPCR	P454	DNA Analyzer	P454	qPCR	P454	Illumina MiSeq
Region of DNA analysed		V1-9F/V3–541R		V6–V8	V1V2	V1V2		V1–V3	V4
Gene sequence database	Greengenes	BLAST	NA	Greengenes	RDP	Silva		BLAST	Greengenes & Silva
Total reads	NA	M: 3626 H: 5135	NA	329,550	1723	1,616,883		4,402,282	11,636,723
Gilliamella apicola	Abundant	M: 48.9 H: 5.1	M: dominant (47)	11.9	31.5	S 5.8, A 12.1 C: 6		4th abundant	~ 22–65
Snodgrassella alvi	Abundant	30.5	I: dominant (42)	9.1	8.2	S 13.8, A 14.5 C: 5		2nd abundant	~10–35
***Lactobacillus* spp. Firm-4**	Abundant	M: 17 H: 84.2	NS	23.2	17.5	S 5.5 A 2.5		3rd abundant	~2–12
Lactobacillus spp. Firm-5	Dominant	17–84.2	C: 69 R: 81	45.4	34	S 69.6 A 55.4		Dominant F: 30.4, N: 49.4	~5–35

	(Martinson et al. 2011)	(Ahn et al. 2012)	(Martinson et al. 2012)	(Moran et al. 2012)	(Anderson et al. 2013)	(Corby-Harris et al. 2014a)	(Engel et al. 2015)	(Kapheim et al. 2015)	(Jones et al. 2018b)
						C: 8			
***Bifidobacterium* (*B. asteroides*)**	Abundant	1.6–3.9	NS	5.4	5.7	S 1.2 A 1.2		0	
Frischella perrara	0	M: 30.5 H: 2.9	NS	2.0	2.1	S 0.0006 A 0.0005	24-80% infected with > 25% infection on day 29	Present	~5–65
Bartonella apis	NS	0	NS	1.0	Low abundance	NS		Present	
***Gluconobacter*–related species group (Alpha 2.1)**	NS	NS	NS	NS	1	S 2.0 A 8.5		Present	~2–20
Parasaccharibacter apium	0	3	NS	1.0	Low abundance	C: dominant (42)		NS	~< 1

The relative abundance for each phylotypes is listed as a percentage. Some phylotypes were not applicable (NA), were not detected (0), or were not specified (NS). The samples may have been collected in spring (S) or autumn (A). USA locations: Arizona (AZ), Connecticut (CT), Illinois (IL), Maryland (MD). Bee type/age: queen (Q), larvae (L), nurse bee (N), worker bee (W), forager (F), and pollen forager (PF). Gut section: entire gut (E), crop (C), midgut (M), hindgut (H), ileum (I), rectum (RM), and whole abdomen (A). Sample type: pooled (P) and individual bees (ind). DNA extraction method: Lysozyme incubation (LI), Cetyltrimethylammonium bromide (CTAB), bead beating (B), phenol/chloroform/isoamyl alcohol (PCI), Phenol/chloroform (PC), Gentra PureGene Kit, Qiagen Inc. (QI), MoBio (MB), MP Biomedicals (MPB), Fermentas GeneJet (FG), ZymoResearch (ZR). Phylotype identity method: Universal bacterial primers (UBP), qPCR Light Cycler (qPCR), and Pyrotags 454 (P454). Region of DNA analysed: hypervariable region (V), forward primer (F), and reverse primer (R). Amplicon taxonomy databases: Basic Local Alignment Search Tool (BLAST), Ribosomal Database Project (RDP).

1.8.1 Dominant core bacteria

Despite early studies indicating a wide range in relative abundance for the majority of the dominant core bacterial species, the relative abundance in later studies, where the bacteria in the entire gut is sequenced using molecular methods, suggests less variation (Table 1.2). Additional details for these, including their location in the gut, are summarised below.

1.8.1.1 *Gilliamella apicola*

Gilliamella apicola (class γ-proteobacteria) is one of the most abundant bacterial species in the honey bee gut (Jones et al. 2018b). They are facultative anaerobes with a Gram-negative cell wall structure. They are highly diverse in both *Apis* spp. and *Bombus* spp. (Ludvigsen et al. 2018). Within individual bees there are multiple strains of *G. apicola,* with type strain wkb1 being the first *Gilliamella* species to be isolated and described from honey bees (Moran et al. 2012; Kwong and Moran 2013; Zheng et al. 2016). At the time of writing, the genomes of more than 48 of the 100-plus strains listed on the National Center for Biotechnology Information (NCBI) database, had been sequenced (Ludvigsen et al. 2017).

G. *apicola* forms a thick biofilm-like layer in the luminal ileum along the epithelium, growing over a basal layer of *S*. *alvi* (Martinson et al. 2012); (Kwong et al. 2014). Both *G. apicola* and *S. alvi* appear to partition resources in the ileum so they may cooperate for nutrients (Kwong et al. 2014), whereas *G. apicola* and *F. perrara* may compete for resources as they both colonise the ileum and appear to gain energy from anaerobic fermentation of carbohydrates (Engel et al. 2013b). *G. apicola* produces enzymes that may aid pollen digestion (Engel et al. 2012).

Molecular sequencing of the relative abundance of *G. apicola* within the gut of 16-day-old bees identified a range between 0.6%–30% (Moran et al. 2012). When the DNA was extracted from pooled honey bee guts, this increased to 65% (Jones et al. 2018b).

In vitro, *G. apicola*, grows optimally in microaerophilic conditions forming smooth, white, round colonies, with an approximate diameter of 2.5 mm after 2 days' growth at 37°C and 5% CO_2. They also form filaments > 10 µm long (Kwong and Moran 2013). The susceptibility of *G. apicola* to 12 antibiotics was assessed using disc diffusion assays. *G. apicola* was susceptible to oxytetracycline and tylosin (Kwong and Moran 2013), both of which are used internationally to control *P. larvae* subspecies *larvae*.

1.8.1.2 *Snodgrassella alvi*

Snodgrassella alvi (class β-proteobacteria) are rod-shaped, Gram-negative bacteria that are ~1.0 µm long and 0.4 µm wide (Kwong and Moran 2013). They are present in the gut of both *Apis* and *Bombus* spp. (Martinson et al. 2011; Ahn et al. 2012; Martinson

et al. 2012; Moran et al. 2012; Sabree et al. 2012; Koch et al. 2013; Ludvigsen et al. 2018). Within the honey bee, *S. alvi* is located in the luminal ileum and forms a thick biofilm-like layer along the epithelium, which is then overlaid by *G. apicola* (Martinson et al. 2012). Within individual bees there are multiple strains of *Snodgrassella* (Moran et al. 2012) and the *S. alvi* type strain wkB2^T is present and abundant in every honey bee (Martinson et al 2012).

In vitro, this strain grows well at 37°C in microaerophilic conditions (CO_2-enriched) with pH 6.0–6.5 on blood agar (tryptic soy agar (TSA) infused with 5% sheep blood), TSA, heart infusion agar (HIA), lysogeny broth agar (LBA), and brain heart infusion agar (BHI) (Kwong and Moran 2013). The colonies are smooth, white and round, with an approximate diameter of 1 mm after 2 days. They test positive for urease, nitrate reductase and catalase activity (Kwong and Moran 2013). Susceptibility of *S. alvi* to 12 antibiotics was assessed using disc diffusion assays and was susceptible to the same two as *G. apicola*: oxytetracycline and tylosin (Kwong and Moran 2013).

S. alvi may cooperate with *G. apicola* for nutrients as they both appear to partition resources in the ileum (Kwong et al. 2014). Using molecular sequencing, the relative abundance of *S. alvi* is 6.7% from pooled guts (Sabree et al. 2012), and ranges between 0.6–39% when the DNA were extracted from 16-day-old bees (Moran et al. 2012).

1.8.1.3 Lactic acid bacteria

Lactobacillus spp. are one of the most important lactic acid bacteria (LAB) in humans, insects and other animals with > 175 species (Hammes and Hertel 2006). They are often found in food and fermentation processes where they are generally considered beneficial and produce acid metabolites, such as lactate and acetate, that exclude competing organisms, many of which are pathogens (Jack et al. 1995). In humans and animals they modulate their host's immune response and produce antimicrobial metabolites that protect their host (Servin 2004; Ventura et al. 2009).

LAB are present in the honey bee crop and midgut, and contribute to the dominant fermentative bacteria in the hindgut (Martinson et al. 2012; Anderson et al. 2013). Thirteen species of LAB from *Lactobacillus* and *Bifidobacterium* were identified by culturing the crops of bees from Sweden and the USA (Olofsson and Vásquez 2008). Of these, 11 were also found in pollen and bee bread (eight of the 11 species in pollen and nine in bee bread), which is pollen that has been collected by bees and had glandular secretions added before storing in the wax cells (Vásquez et al. 2009). These 13 LAB are not specific to the crop since a subset of LAB are present in the mid and hindgut (Corby-Harris et al. 2014a).

Lactobacillus spp. (Firm-4), *Lactobacillus apis* (Firm-5) and *Bifidobacterium* dominate the rectum (Martinson et al. 2012; Anderson et al. 2013). This may occur because they are facultative anaerobes that are acid tolerant (Muszynska and Leznicka 1992), and

unlike other bacteria, they may be more metabolically versatile, able to metabolise the pollen walls and nitrogenous wastes that are stored in the rectum before excretion (Winston 1991; Graham 1992).

In vitro, Lactobacillus spp. at 37°C form small, round, opaque, white colonies on BHI agar after one day or on De Man, Rogosa and Sharpe (MRS) agar after 48–72 h. The colonies are both catalase- and nitrate-negative, the pH values for the growth of specific strains are similar, 3.8 ± 0.5, but the concentration of lactic acid generated by each strain is specific (Audisio et al. 2011).

Lactobacillus Firm–4

Lactobacillus Firm–4 (class Bacilli) include *L. mellis* and *L. mellifer*. It is a group of predominantly unclassified *Lactobacillus* species that are acid-tolerant, Gram-positive bacteria that are capable of fermenting sugars. They are present in the lumen but more abundant in the rectum (Martinson et al. 2012; Powell et al. 2014).

Using molecular sequencing, the relative abundance of *Lactobacillus* Firm-4 ranges between 2.0 and 23.2%, the lower abundance when the DNA was extracted from the mid through hindgut of individual forager bees from Sussex, UK (Jones et al. 2018b), and the higher abundance when DNA was extracted from bees outside the brood nest, approximately 16 day old bees, from the USA (Moran et al. 2012).

Lactobacillus Firm–5

Lactobacillus Firm–5 (class Bacilli) includes *L. apis*, *L. melliventris*, *L. kimbladii*, *L. kullabergensis*, and *L. helsinborgensis* (Bonilla-Rosso and Engel 2018). The early composition of the adult honey bee gut is dominated by *L. apis* (Anderson et al. 2016), as are the mouthparts, the HG, and the rectum (Maes et al. 2016). Further characterisation of the species grouped within Firm–5 of the genus *Lactobacillus* is expected. This is because nine unique ASVs have already been identified from the V1 – V3 hypervariable of the 16S rRNA gene (Kapheim et al. 2015) using BLAST (Altschul et al. 1997). The relative abundance of *L. apis* ranges between ~5% when the DNA is extracted from mid through hindguts (Jones et al. 2018b) and 69.6% in pollen forager DNA (Corby-Harris et al. 2014a).

Bifidobacterium spp.

Bifidobacteria (class: Actinobacteria) are Gram-positive, anaerobic, branched or pleomorphic rod-shaped, non-spore-forming bacteria that adhere to the epithelial cell wall in the digestive tracts of humans and other animals (Biavati et al. 2000). Similar to other LAB, *Bifidobacteria* break down carbohydrates to produce both acetate and lactate (Kandler 1983). *Bifidobacteria* also modulate the immune system and strengthen the epithelial barrier in honey bees so they either compete directly with pathogens or they produce bacteriocins (toxins) to kill the pathogens and prevent damage (Vásquez

et al. 2012). Species of *Bifidobacterium* dominate the rectum alongside *Lactobacillus* Firm–4 and Firm–5 (Martinson et al. 2012; Anderson et al. 2013). These include *B. coryneforme*, *B. asteroides*, and *B. indicum* (Biavati and Mattarelli 2006; Bottacini et al. 2012). *B. asteroides* stimulate the production of hormones required for bee development (Kešnerová et al. 2017).

The relative abundance of *Bifidobacterium* spp. ranges between 1.2% in the DNA of pollen foragers (Corby-Harris et al. 2014a) and 5.4% when the DNA was extracted from a sample of pooled honey bee guts (Moran et al. 2012; Sabree et al. 2012).

1.8.2 Less prevalent core bacteria

1.8.2.1 *Frischella perrara*

Frischella perrara is a Gram-negative bacterium that is closely related to *G. apicola.* It specifically colonises the pylorus (~7.29 x 10^7 cells/g) and the ileum (~7.67 x 10^6 cells/g), and its presence is negatively correlated with bee survival and development (Engel et al. 2015; Maes et al. 2016). *F. perrara* uses energy from anaerobic fermentation of carbohydrates (Engel et al. 2013b), suggesting it may compete directly with *G. apicola* for resources (Martinson et al. 2012; Powell et al. 2014). Scab formation on the luminal surface of the epithelium in the bee pylorus, 0.5 mm behind the attachment of the Malpighian tubules at the start of the ileum, occurs after exposure to *F. perrara* (Engel et al. 2015). The scabs were first described in 1946 and appear as an incomplete yellow/brown/black band on the gut perimeter (Engel et al. 2015). These are similar to melanisation that occurs as part of the insect immune system (Cerenius et al. 2008). Melanisation in insects is observed in wound healing, encapsulation of parasites and causes oxidative stress (Nappi and Christensen 2005).

A study of bees from the USA and Switzerland observed bees with scabs ranged between 24% and 82% and correlated with abundance of *F. perrara* in foraging honey bees (Engel et al. 2015). Engel et al. (2015) found scabs were not obvious in NEWs but were present in 20% of 5-day-old bees and 70% of 7-day-old bees. The percentage of bees with scabs remained constant thereafter until day 29. The same study showed *F. perrara* was the dominant bacterium in the pylorus (> 50%) in 2- to 7-day-old bees, which then declined over time to < 25% and thereafter *G. apicola* and *S. alvi* dominated the bacterial community in the pylorus (Engel et al. 2015). *F. perrara* is widely distributed throughout Europe, North America and Asia (Cox-Foster et al. 2007; Ahn et al. 2012; Moran et al. 2012) but is less prevalent than *G. apicola* or *S. alvi* (Moran et al. 2012; Sabree et al. 2012; Corby-Harris et al. 2014a) and is sometimes absent, as shown when DNA was extracted from both pooled guts and entire 16-day-old honey bees from Arizona, USA (Martinson et al. 2011). Up to 65% relative abundance was observed when the DNA was extracted from individual honey bees (Martinson et al. 2012; Jones

et al. 2018b). However, the presence of this bacterium is variable and < 25% is more common (Martinson et al. 2012; Moran et al. 2012; Engel et al. 2015).

1.8.2.2 *Bartonella apis*

Bartonella apis are occasionally observed in the honey bee gut. They are rod-shaped bacteria approximately 1.2–1.8 µm. *Bartonella* adapt to limited carbohydrates by deriving carbon and energy from the catabolism of amino acids rather than glucose (Chenoweth et al. 2004).

In vitro, bacterial colonies form under microaerophilic conditions at 35–37°C on solid media with defibrinated sheep blood, although the growth of some strains is inhibited by aerobic conditions. The strain PEB0122T is positive for catalase, cytochrome-c oxidase, urease and nitrate reductase (Kešnerová et al. 2016).

The relative abundance of *B. apis* ranges between 0% when the DNA was extracted from a sample of pooled honey bee guts (Sabree et al. 2012), and 1% in the DNA extracted from 16-day-old bees (Moran et al. 2012).

1.8.2.3 *Gluconobacter*-related species group (Alpha 2)

The family Acetobacteraceae contains the Alpha 2 group which comprises numerous strains (Corby-Harris et al. 2014b). Alpha 2.1 are gut specialists whereas members of Alpha 2.2 grow in external environments such as nectar, bee bread, honey and the larval gut (Anderson et al. 2013). Nine groups of Acetobacteraceae Alpha 2.2 bacteria were isolated from first-instar honey bee larvae (Corby-Harris et al. 2014b). From these, 44 bacterial isolates were successfully cultured and all grew well over a 48 h period in slightly acidic SDA medium and 5% CO_2 (Corby-Harris et al. 2014b). *Parasaccharibacter apium* has recently been classified within this Alpha 2.2 group. It is a generalist bacterium which is acid resistant and osmotolerant (Corby-Harris et al. 2014b). It is ubiquitously present and abundant in the queen gut, the HG of worker bees which produce royal jelly, the royal jelly itself, developing larvae, and occasionally in the worker crop and in stored pollen (Corby-Harris et al. 2014b). *P. apium* are negligible in the worker mid and hindguts (Corby-Harris et al. 2014b), and in low numbers among castes sampled in spring and late summer (Babendreier et al. 2007), bees from colonies at different locations (Mattila et al. 2012; Moran et al. 2012), and among bee populations in different countries (Jeyaprakash et al. 2003; Babendreier et al. 2007; Olofsson and Vásquez 2008). This was confirmed using both culture and pyrosequencing techniques (Corby-Harris et al. 2014b).

1.8.3 Additional bacteria

Lactobacillus kunkeei

Lactobacillus kunkeei are rarely found in the bee gut but are considered mutualistic symbionts of honey bees (Rangberg et al. 2012). They are present in honey, bee-collected pollen, royal jelly and this species is the dominant LAB in bee bread (Anderson et al. 2013; Asama et al. 2015). They are acid-resistant, fructophilic bacteria that produce lactate, acetate and ethanol (Neveling et al. 2012). These traits enable them to grow in the crop in the presence of nectar and honey, both of which have an average pH level of 3.9 (ranges from 3.4 to 6.1) (Snowdon and Cliver 1996).

1.9 Honey bee acquisition of gut bacteria

The social behaviour of honey bees is important for the acquisition of gut bacteria as NEWs are thought to contain a limited bacterial community in their gut when they eclose (Martinson et al. 2012). Transmission, propagation and longevity of gut bacteria are facilitated by the social behaviour of bees and the management practices of beekeepers who swap wax frames with bees between colonies and move colonies to other regions for pollination (Gilliam 1971; Powell et al. 2014).

The bacterial community is thought to be acquired through exposure to hive surfaces, nurse bees, and hindgut material transferred via proctodaeal (anus to mouth) trophallaxis; trophallaxis is the social transfer of food or faeces from one adult bee to another which generates a common stomach that enables all bees within a colony to obtain knowledge of the nutritional status of sister workers (Crailsheim 1991), and to acquire gut bacteria. Without these exposure routes, bees lack a substantial gut community even after eight days (Powell et al. 2014). Both honey bee and bumble bee workers raised in isolation after pupal emergence have been shown to lack specific gut microbiota (Gilliam 1971; Koch and Schmid-Hempel 2011a; Martinson et al. 2012), with potentially detrimental consequences such as higher parasite susceptibility (Koch and Schmid-Hempel 2011a; Koch and Schmid-Hempel 2012). The dominant Gram-negative species *S. alvi*, *G. apicola*, and *F. perrara* require the presence of nurse bees or exposure to hindgut material through proctodaeal trophallaxis, whereas the transfer of some Gram-positive species is aided by exposure to hive surfaces (Powell et al. 2014). However, the bees in the Powell et al. (2014) study were removed from the cells before they chewed through the wax cell-cap, which they must do in a colony. It is therefore unclear whether the initial colonisation of the gut in a colony is determined by the gut composition of the nurse bees and the hive environment, or whether chewing through the wax cell-cap provides the initial seed-bacteria required to develop the bacterial composition.

Within the first 2 days post-eclosure, the ileum colonises before the rectum with non-core and core γ-proteobacteria and fungi (Gilliam 1971; Powell et al. 2014). Within 4 to 6 days post-eclosure, full species colonisation occurs rapidly with total bacterial counts of $1.1x10^8$ 16S rRNA gene copies. These are dominated by the core bacteria, *S. alvi*, *G. apicola*, and *F. perrara* (Powell et al. 2014). The gut of adult honey bee workers, 9 to 30 days post-eclosure, contains a large bacterial community consisting of β-proteobacteria, Firmicutes and γ-proteobacteria. This community is known as BFG and specifically includes *S. alvi*, *L. apis*, and *G. apicola*, respectively (Martinson et al. 2012). Powell et al. (2014) and Martinson et al. (2012) characterised BFG throughout the life-cycle of the honey bee, and also among compartments within the digestive tract. BFG were almost non-existent in the crop and midgut, with almost 95% of the BFG being located in the hindgut (Martinson et al. 2012). NEWs in contact with only material from hive frames and NEWs that had access to only hindgut material from nurse bees both had significantly more bacteria after eight days than NEWs that had simultaneous contact with entire nurse bees and frames, or those that only contacted the heads of nurse bees, allowing stomodeal (oral) trophallaxis (Powell et al. 2014). This supports the theory that stomodeal trophallaxis may cause suppression of environmental bacteria (Vásquez et al. 2012). It is unknown what part of stomodeal trophallaxis inhibits the bacteria. It may be enzymes or bacteria within the crop that affect the bacteria or it may be the propolis, the antibacterial waxy resin that bees collect from leaf buds that they mould with their mouthparts to use as a glue for their hive (Grange and Davey 1990).

The digestive tracts of honey bee larvae, NEWs (Disayathanoowat et al. 2012), and their major protein source (bee bread), all contain minimal bacterial phylotypes (Martinson et al. 2012). Only 7% of the gut bacteria in adult Africanised honey bees (a hybrid of *A. mellifera* and the African honey bee, *Apis mellifera* subspecies *scutellata*) overlap with bacteria found in bee bread (Saraiva et al. 2015). This suggests that protein in the diet is not a common source of bacteria for NEWs but there is no understanding as to whether carbohydrate sources have any effect on the bacterial composition. However, the mouthparts and HG may act as reservoirs for both hive and gut-associated bacteria, and this may also harbour strain variability (Maes et al. 2016).

Engel and Moran (2013a) hypothesised that the initial colonisation of the honey bee gut may prime the immune system to protect bees from enteric pathogens. They also hypothesised that bacterial functions related to nutrient absorption may influence the social behaviour of bees. The reasoning was because bacterial metabolism of the diet may increase nutrient availability, therefore increasing insulin signalling which is involved in regulating division of labour in bees (Ament et al. 2008). It has since been shown that initial gut colonisation affects immune gene expression (Kwong et al. 2017).

1.10 Importance of bacteria in the honey bee gut

The importance of the honey bee gut is becoming more apparent as we obtain understanding of how bacterial phylotypes within the honey bee gut function, their influence on the bee, how these phylotypes interact, and what they are influenced by. In contrast with the human gut, the simplicity of the honey bee bacterial community (Martinson et al. 2011) provides opportunities to understand the processes that regulate the specialised gut communities and how these communtiies effect host biology (Zheng et al. 2018). Specifically, the bee gut is a powerful model system for the human gut as bees can be raised with limited bacteria (Powell et al. 2014), enabling the effect of specific bacteria on the host to be studied in isolation. Additionally, the bacterial communities are similar in that there is a high concentration of microbiota in the distal part of the gut, some bacteria are host-specific, and the bacteria is socially transmitted among individuals

Experimental studies using individual phylotypes or entire bacterial communities are best designed by assimilating prior knowledge and identifying the research gaps. Sections 1.10 – 1.12 provide a brief overview of what is currently known about the bacterial community in the honey bee gut and highlights some knowledge gaps (KG) that I used to form the principal objectives for this thesis.

1.10.1 Bacterial variation among *Apis* species

Apis species have different gut bacterial compositions. In contrast to the significant variation observed among the bacterial community in pooled mid- and pooled hindguts of 30 adult *A. mellifera*, the bacterial community in the pooled midgut of 40 *Apis ceranae* bees, the closely related Asian (Eastern) honey bee, was similar to the pooled hindgut samples (Ahn et al. 2012). This study also showed that the bacterial community in *A. cerana* was less diverse than that in *A. mellifera* (Ahn et al. 2012). Unfortunately, this Korean study only analysed bees from one colony of each species. However, they two colonies were located in the same apiary, therefore mitigating the effect of environmental factors. In contrast to the Ahn et al. (2012) study, Disayathanoowat et al. (2012) found that the midgut bacterial communities of *A. cerana* differed significantly between three apiaries in Northern Thailand, but those of *A. mellifera* did not. However, because the midgut of *A. mellifera* contains < 5% of the entire bacterial community, this lack of variation is unlikely to accurately represent what occurs in the bacterial community of the hindgut, let alone the entire bee gut (Ahn et al. 2012; Disayathanoowat et al. 2012). The Korean study also showed that the proportion of Proteobacteria in 20 larval bee guts (77.4–97.1%) was be larger than that in adults (13.3–81.1%). These studies show that the developmental stage of the bee, gut compartment, and bee species are all factors that alter the relative abundance of bacterial groups in the mid and hindguts of *A. mellifera* (Ahn et al. 2012).

Prior to publishing Chapter 6 of this thesis (Taylor et al. 2019), the bacterial composition in the gut of New Zealand honey bees was unidentified. As there are no longitudinal studies comparing gut bacteria over time, the physical isolation of honey bee colonies in New Zealand since the 1950s (Stevenson et al. 2005) may be the only way to determine whether bacterial selection may have occured over time. This lack of knowledge therefore precludes the statements that the guts of honey bees worldwide contain only eight to ten phylotypes.

Knowledge gap 1: No NZ data

It is unclear whether both community composition and relative abundance observed in these studies were the expected variation between different populations (NZ included), or whether they were a response to external biotic and / or abiotic factors associated with the apiary.

Cox-Foster et al. (2007) conducted a metagenomic study on bees from USA, Hawaii and Australia, which identified all genetic material in the samples. The graphs indicate that Lactobacillus spp. was absent, and G. apicola was more abundant in colonies with Colony Collapse Disorder (CCD) compared with healthy colonies. Recently, Lactobacillus spp. and Leuconostoc spp. (Lactobacillales, Firmicutes) have been associated with healthier colonies, whereas bacteria from the genus Arsenophonus (γ-proteobacteria) are associated with poor health (Budge et al. 2016). It is therefore possible that some bacteria may be useful indicators of colony health. This change in bacterial abundance suggests that gut dysbiosis (an unhealthy microbial imbalance) may have occurred, so that normal dominating bacteria are replaced with underrepresented species (Joossens et al. 2011).

Knowledge gap 2: The affect of external factors

Although bacterial species are associated with poor health, it is unknown whether bacterial composition in the gut alters bee health or whether the composition of gut microbiota alters in response to external factors, which in turn may enable pathogenic bacteria to proliferate.

Colonies headed by queens inseminated with semen from multiple drones are on average less impacted by the larval gut bacterium *P. larvae* subspecies larvae and have larger bee populations than those headed by queens inseminated with semen from a single drone (Seeley and Tarpy 2007). A study of colonies from Massachusetts, USA, headed by queens inseminated with semen from either a single drone or 15 drones, identified a novel bacterium within the *Oenococcus*, *Leuconostoc* and *Weissella* clade

(Lactobacillales, Firmicutes) and showed strain variation within core species of bacteria (Mattila et al. 2012). It was proposed that healthy colonies with diverse gut bacterial communities were correlated with the genetic sources carried by the queen bee (Mattila et al. 2012). However, reanalysis of these sequence data indicated that the novel bacteria and the proposed correlation were unsubstantiated (Sabree et al. 2012).

Knowledge gap 3: Variation in subdominant species

Although strain variation within core gut species has been observed, it has not been identified for sub dominant core species. It is also unknown whether genetic variation in the queen is associated with bacterial colonisation in worker bees.

1.10.2 Variation in relative abundance

The studies listed in Table 1.2 provide insight into the variation between the presence/absence of phylotypes as well as variation in relative abundance of individual phylotypes. These data cannot be easily compared as different DNA extraction methods and PCR primers have been used. A comparison of five methods used to extract bacterial DNA from human faecal samples showed that DNA extraction techniques can favour either Gram-positive or Gram-negative organisms (McOrist et al. 2002). They recommend that a DNA extraction technique should be carefully selected based on the specimen type. Additionally, method choice affects alpha-diversity and consistency of community composition (Hermans et al. 2018). Therefore, the relative abundance of phylotypes identified by Moran et al. (2012) using the bead-beating method that effectively releases the DNA from Gram-positive organisms (those within the *Lactobacillus* Firm–4 and Firm–5 clusters. In 2012 the species within these clusters were not individually identified), should not be compared with those from the RNA analysed by Cox-Foster et al. (2007) (*G. apicola* was the most abundant phylotype). As the foundation of bacterial phylotypes in the honey bee gut is reasonably established and the technology to analyse sequences is more reliable, moving forward the consistency between trial designs and analysis should be sought as this would enable more accurate comparison of the relative abundance within the body of literature.

Additional studies provide some evidence that subtle differences in community composition may be related to season (Ludvigsen et al. 2015), diet (Maes et al. 2016); (Jones et al. 2018b), host age (Martinson et al. 2012; Tarpy et al. 2015), caste (Kapheim et al. 2015), geography (Ludvigsen et al. 2017), pathogens (Sabaté et al. 2009; Audisio and Benítez-Ahrendts 2011; Killer et al. 2014), and the health status of the bee (Cox-Foster et al. 2007). Why these studies may have observed these variations is discussed in further detail in the following chapters of this thesis. For now, relative abundance has

been observed to vary between bees and colonies (Moran et al. 2012). From 40 individual honey bees – five bees each from four colonies in both AZ and MD, USA – 11 unique ASVs accounted for 98.5% of the bacterial reads. There was no significant difference in ASV richness or evenness between the apiaries, but on an individual level, every bee contained seven of the eight dominant phylotypes present, and the relative abundance of each phylotype varied between bees and colonies (Moran et al. 2012). At a colony level, the composition of bacterial communities was significantly different, especially at the AZ apiary. *G. apicola* and *S. alvi* were present in every bee, but their abundance ranged from 0.6–30% and 0.6–39%, respectively. Indicator-species analysis identified *G. apicola* and *L. apis* to be prominent in the MD samples, whereas two Enterobacteriaceae, Gamma-3 and Gamma-4, were prominent in the AZ samples. The phylotypes Alpha 2, *Bifidobacterium* and *F. perrara* were found in low abundance in most bees but the presence of Gamma-3, Gamma-4, and *B. apis* were variable across individuals.

Knowledge gap 4: Comparative analysis between NZ and international bees

Composition and relative abundance of some bacterial species in the honey bee gut vary within bacterial communities. The cause of this variation is unclear. Varying methodology precludes comparison among some studies. Comparative analysis between NZ bees and international bees would provide context within the world domain.

1.11 Functions of the dominant honey bee gut bacteria

1.11.1 Potential functions identified through metagenomic sequencing and other methods

An initial picture of the potential functions of the dominant honey bee gut bacteria was first ascertained through sequencing the honey bee metagenome. This identified all DNA within the samples, including that of the bacteria. The honey bee metagenome was used to reconstruct metabolic pathways to predict their possible functions based on other species (Koch and Schmid-Hempel 2011a; Engel et al. 2012). Initially, they were compared to the genes and their associated functions from the microbiota of five mammals and four other insects. The bacterial species were grouped by common genes or functions in these other animals (Clusters of Orthologous Groups (COG)) and the majority of functions were related to assisting nutrient utilisation by the host, assisting host immunity, and pathogen defence (Markowitz and Kyrpides 2007). Additional research suggests that the dominant gut bacteria may also play key roles in worker and

larval health, hive hygiene, and food storage (Anderson et al. 2013; Corby-Harris et al. 2014b; Engel et al. 2016).

Functions related to biofilm formation and host interaction were predicted to correspond with both γ-proteobacteria: *G. apicola* and *F. perrara*, and β-proteobacterium: *S. alvi* (Engel and Moran 2013a). Both *Gilliamella* and *Snodgrassella* spp. are significantly enriched in the functions of 'intracellular trafficking, secretion, vesicular transport and cell motility' (Engel et al. 2012). *Snodgrassella* also has enriched genes that encode RTX (repeats-in-toxin) exoproteins which assist in host cell interaction and can act as bacteriocins that inhibit competing bacteria. As both *G. apicola* and *S. alvi* are microaerophilic, they may play a role in modulating the gut environment by maintaining anaerobic conditions in the gut lumen. They do this by consuming the available oxygen, as has been observed of bacteria in the termite gut (Brune et al. 1995). Both bacteria also form biofilm-like aggregations on the lining of the ileum (Martinson et al. 2012), and this, coupled with the observation that strains of these two species dominate the ileum of *Bombus* spp. (Martinson et al. 2012) and protect the bees from the intestinal trypanosomatid pathogen *Crithidia bombi* (Koch and Schmid-Hempel 2011a; Cariveau et al. 2014), suggests the *Gilliamella*-*Snodgrassella* biofilm may function as a protective layer against protozoa in honey bees.

To support the above predictions made from analysis of the gut metagenome, Lee et al. (2015) pioneered the study of the honey bee gut metatranscriptome: the total RNA extracted from multiple honey bee guts within a sample was transcribed to its cDNA and subjected to Illumina sequencing to identify active microbial members and infer community metabolic functions (Lee et al. 2015). The dominant focus identified from the transcriptome was carbohydrate metabolism (Lee et al. 2015). Three major bacterial classes were found to be active in the gut: γ-proteobacteria; Actinobacteria; and Bacilli. γ-proteobacteria and Actinobacteria were predicted to synthesise all essential and non-essential amino acids, whereas Bacilli were predicted to uptake amino acids derived from diet or synthesised by other microbiota (Lee et al. 2015). Lee et al. (2015) also predict that these classes assist in the degradation of complex molecules such as polysaccharides (glucose) and polypeptides, and the resulting fermentation that produces metabolites, such as short-chain fatty acids and alcohol. In support of this, bacterial isolates from the *Gilliamella* genus have been shown to have the potential to digest pectin, a polysaccharide found in pollen tubes (Engel et al. 2012).

1.11.2 Functions identified through *in vitro* studies and other methods

1.11.2.1 Pollen digestion

Pollen digestion may require the supply of enzymes to break down the pollen walls. For example, cellulase and hemicellulase, which are normally associated with degrading plant cell walls, or pectinase.Some *Gilliamella* strains contain genes that are involved in pectin degradation (Engel and Moran 2013b) and *in vitro* culturing of these bacteria confirm this activity (Engel et al. 2012). *Bacillus* spp. are not members of the dominant gut bacteria as they are likely to originate in nectar but some *Bacillus* produce the enzyme amylase, which assists honey bees to digest carbohydrates (Welker and Campbell 1967). *In vitro*, increasing the number of *Bacillus* spp. in nectar, after isolating it from nectar and the crop, resulted in increased amylase values (Wang et al. 2015).

1.11.2.2 Carbohydrate utilisation

The phylogenetic groups γ-proteobacteria, Firmicutes and *Bifidobacterium* are associated with several carbohydrate-related functions, the most abundant category being 'carbohydrate metabolism and transport' (Engel et al. 2012). These functions include several components of the phosphotransferase systems (PTS) which are responsible for the import of sugars from the environment (Engel et al. 2012), and the 'arabinose efflux permease' function (Engel et al. 2012) which is a sugar-transport system that assists with the import/export of antimicrobial, amino acid and sugar compounds, including the mannose family of phosphotransferase (Law et al. 2008). Mannose is toxic to NEWs and, like several other monosaccharides found in nectar including xylose, arabinose, and rhamnose, it reduces adult honey bee longevity (Barker and Lehner 1974). However, *in vitro*, some strains of *G. apicola* utilise mannose, xylose, arabinose, and rhamnose as their sole energy source (Zheng et al. 2016). Mannose utilisation requires the gene that encodes for mannose-6-phosphate isomerise. This observation suggests some *G. apicola* strains may enable the detoxification of some nectar components or enable the digestion of complex carbohydrates in pollen such as pectin. Pectin, which is normally indigestible by bees, is in the cell wall of pollen and pollen is the sole source of protein for honey bees (Engel et al. 2012; Zheng et al. 2016). Although the breakdown required for nutrient absorption is not fully understood, the phylogenetic cluster of *G. apicola* correlated with genes coding for pectin-degrading enzymes (Engel and Moran 2013a). As with mannose, not all *G. apicola* isolates are able to degrade pectin, suggesting isolates have functional differences (Engel et al. 2012; Engel et al. 2013b).

1.11.2.3 Immune response

The immune system of insects consists of cellular (cell to cell), and humoral (extracellular) immune responses that are regulated by immunity-related genes (Hoffmann 2003). The humoral response occurs when antibodies produced by β-cells destroy extracellular microorganisms and prevent the spread of intracellular infection. Immune mechanisms have been linked with changes in the gut microbiota of Drosophila melanogaster (Zaidman-Rémy et al. 2006). They also occur in honey bees that are infected with the bacterium Escherichia coli, as native, beneficial gut bacteria induce immune responses that increase bee longevity (Kwong et al. 2017). The honey bee immune system is also activated by the scabbing that forms in the pylorus by F. perrara (Engel et al. 2015). In vitro, four honey bee genes that encode the antimicrobial peptides abaecin, apidaecin, hymenoptaecin, and defensin, show inhibitory activity against bacteria (Casteels-Josson et al. 1994; Evans and Lopez 2004). Two of these antimicrobial peptides also up-regulate after natural exposure of honey bees to *P. larvae* subspecies larvae (Evans and Lopez 2004).

Reviews of insect microbiome to manage insect-related problems (Hamdi et al. 2011; Crotti et al. 2012) reveal that *Bifidobacteria* and *Lactobacillus* modulate the immune system and strengthen the epithelial barrier by secreting antimicrobial compounds, such as bacteriocins, that either inhibit pathogen contact, and/or directly compete with microorganisms such as pathogenic bacteria and fungi (Mitsuoka 1992; Gilliam 1993; Reynaldi et al. 2003; Reynaldi et al. 2004; Biavati and Mattarelli 2006). They show that species of *Lactobacillus* that adhere to the cell wall of the intestine are implicated in vitamin production, regulation of healthy gut microflora, and the enhancement of the immune system. This supports the potential for gut bacteria in honey bees to be biological control agents. Preliminary studies of *Bacillus* suggest that bacteriocin-like compounds in *B. cereus* and *B. licheniformis*, and enzymatic activity in *B. laterosporus* and *B. megaterium* may inhibit the pathogenic bacterium *P. larvae* subspecies larvae (Alippi and Reynaldi 2006).

1.11.2.4 Protection against pathogens

The theory that native gut bacteria have mutualistic interactions with their honey bee host is supported by the early establishment of *S. alvi* that potentially excludes *P. apium* and *F. perrara* from the ileum (Maes et al. 2016), especially since *F. perrara* is directly associated with scabbing in the honey bee gut (Engel et al. 2015). The consumption of pollen diets stored in the frames for more than 14 days by caged bees showed that *S. alvi* were positively correlated with bee survival and development, whereas *F. perrara* and *P. apium* were negatively correlated (Maes et al. 2016).

More specifically, the consumption of aged diet was associated with an increase of *Bifidobacterium* and *Lactobacillus* Firm-4 in the rectum, an increase of *P. apium* in the mouthparts, and an increase of *Bifidobacterium*, *F. perrara*, and *P. apium* in the HG

(Maes et al. 2016). *P. apium* is normally associated with larval feeding and present in larval jelly (Corby-Harris et al. 2014b), so the increased prevalence of *P. apium* in the HG, ileum and rectum of adult honey bees fed aged diet suggests that it acts opportunistically in unhealthy adults. This replacement of normal dominating bacteria with underrepresented species, previously referred to as dysbiosis, has been linked to specific diseases in vertebrates, such as Crohn's disease in humans (Joossens et al. 2011), and invertebrates (Mukherjee et al. 2015; Anderson and Ricigliano 2017). Dysbiosis in adult honey bees is further likely as the study by Maes et al. (2016) also showed that the presence of certain *Bifidobacterium* and *Lactobacillus* strains in the rectum were associated with the reduced infection level of the honey bee pathogen *Nosema* (species not identified)**,** despite no change in brood production, food stores or foraging rates (Corby-Harris et al. 2016).

Mutualistic interactions between bacteria and the honey bee are also supported by inhibition of *P. larvae* subspecies larvae by native gut bacteria, as shown by several *in vitro* studies. Audisio et al. (2011) showed that the acidity produced by *Lactobacillus johnsonii* inhibits the growth of *P. l. larvae*, and Killer et al. (2014) showed that two strains of *Lactobacillus apis* inhibited both *P. larvae* subspecies larvae and *Melissococcus plutonius* (Firmicutes), the infective bacteria that cause European foulbrood (EFB) disease in honey bees (Killer et al. 2014). Three *B. subtilis* strains, isolated from both honey and gut samples, produce natural surfactin, which is a biosurfactant and an antibiotic with antitumoral and antiviral action that inhibits the vegetative cells of *P. larvae* subspecies larvae (Sabaté et al. 2009). *P. larvae* subspecies larvae grown on agar plates has also been shown to be inhibited by *L. kunkeei*, three phylotypes related to *B. asteroides*, one related to *B. coryneforme* and six phylotypes related to *Lactobacillus*. These phylotypes individually displayed different inhibition levels and when a combination of all LAB were fed to honey bee larvae in the laboratory that were previously infected with *P. larvae* subspecies larvae, total inhibition was observed (Forsgren et al. 2010).

Knowledge gap 5: The affect of diet on gut bacteria

These studies suggest that diet may influence the health of the honey bee colony by altering the presence of pathogen-inhibiting bacteria, and also that bacteria associated with the honey bee gut has the potential to prophylactically control honey bee pathogens.

1.11.2.5 Foraging behaviour

Bacteria in floral nectar are occasionally identified in the honey bee crop, such as *Asaia astilbis* (α-proteobacteria), *Erwinia tasmaniensis* (γ-proteobacteria), and *L. kunkeei* (Good et al. 2014). When given the choice, honey bees avoid consuming synthetic nectar inoculated with 200 cells per µl of these three bacteria. Bees apparently avoided the nectar because of the chemical changes that the three bacterial species generated in the nectar. However, it is difficult to determine whether the bees would naturally choose to avoid nectar containing these bacteria as the natural concentration in floral nectar is less than that tested (30 bacterial colony forming units per µl) (Good et al. 2014). For each day of experimentation, the pH level reduced in the nectars containing each of the three bacteria but not the control nectar. The glucose and fructose concentrations increased in the *A. astilbis* treatment, suggesting that they metabolised the sucrose to glucose and fructose. However, as the metabolites were not measured, it is difficult to speculate whether acidity or spoilage caused this avoidance.

Knowledge gap 6: Affect of nectar chemistry on foraging behaviour

Bees avoid nectar inoculated with bacteria that are not native to the honey bee gut. Thus bacteria in nectar may alter nectar chemistry that ultimately influences bee foraging behaviour and may possibly affect the efficacy of *A. mellifera* as pollinators. How foraging behaviour may be influenced is unsubstantiated but I suggest that this may also occur with any other source of carbohydrate.

1.11.2.6 Honey bee physiology

The metabolism of honey bee gut microbiota was found to affect honey bee growth, hormonal signalling, behaviour and chemical conditions within the honey bee gut (Zheng et al. 2017); in comparison to germ-free bees, the gut microbiota in bees fed hindgut homogenates from nurse bees increased the weight of both the gut and the entire bee, and potential mechanisms include modulation of vitellogenin, insulin signalling and the gustatory response within the bee. Zheng et al. (2017) also identified an oxygen gradient within the intestine and found this to be formed by the bacteria adjacent to the gut wall. The metabolism of the bacteria in this study was found to produce short chain fatty acids (SCFA), which reduced gut pH and redox potential, therefore altering the environment within each gut compartment. Wu et al. (2017) suggest that these microbial metabolic pathways assist with the degradation of ingested polysaccharides in pollen, and the metabolites then contribute to host nutrition. Specifically, *B. asteroides* has been found to stimulate the production of host hormones such as Juvenile hormone III (Kešnerová et al. 2017) which regulate the pace of the developmental maturation from young nurse bees to older forager bees (Robinson et al. 1989).

1.12 Factors that affect gut bacteria

1.12.1 Chemicals

Chemicals are used within the hive for the purpose of controlling pests. In NZ, fluvalinate, flumethrin, amitraz, thymol, oxalic acid and formic acid are used to control *V. destructor*. Overseas, oxytetracycline is used to control *P. l. larvae*. Chemical properties are also introduced to the hive environment when the bees return with contaminated nectar, pollen, or water (Taylor et al. 2007), or propolis that contains natural antibacterial properties. Bees exposed to antibiotics have perturbed gut bacteria and increased mortality (Raymann et al. (2018a). It is unknown whether gut bacteria is affected by the antibacterial properties in mānuka nectar.

A review of several older studies show that bees fed pollen containing the endotoxin (Cry1Ab) from the soil bacterium *Bacillus thuringiensis* (Bt) had no negative effects on bees (Malone and Pham-Delègue 2001). The diversity of adult honey bee gut bacteria was also unaffected in both NEWs and free-flying bees when fed either maize pollen containing Cry1Ab from Bt or non Bt-maize pollen, suggesting that the effect of Bt-pollen on honey bee gut bacteria is limited (Babendreier et al. 2007). These endotoxins are powerful biopesticides that effect the digestion process of numerous flies and moth larvae as Bt-toxin forms pores in intestinal epithelial cells that disrupt intestinal function.

1.12.2 Pests and diseases

In vitro, both *P. larvae* subspecies larvae and *N. ceranae* are inhibited by honey bee gut bacteria. Previously discussed in section 1.11.2.4: Protection against pathogens, *P. larvae* subspecies larvae growth is inhibited by *L. johnsonii* (Audisio and Benítez-Ahrendts 2011), and two strains of *Lactobacillus apis* (Killer et al. 2014). Reduced levels of *N. ceranae* occur when bee food is supplemented with species of *Lactobacillus* and *Bifidobacterium* (Baffoni et al. 2016). This suggests that bacteria can be utilised or harnessed to enhance honey bee health, also referred to as Microbial Resource Management (MRM) (Verstraete 2007; Hamdi et al. 2011; Crotti et al. 2012). It is anticipated that MRM could be used to both indicate colony health (Budge et al. 2016) and help to reduce the effects of honey bee related pests and diseases that currently do not have long-term solutions. Thus, ultimately mitigating colony loss worldwide. Specifically, NZ honey bee diseases that do not have long-term solutions include *Varroa*, *N. apis*, and *N. ceranae.*

1.12.3 Effect of diet on gut bacteria

Ludvigsen et al. (2015) showed that the bacterial composition in the midgut/pyloric section was seasonally altered by diet and the surrounding environment. As previously explained, the crop and midgut regions represent < 5% of the entire bacterial community (Martinson et al. 2012). Therefore studies on the entire gut are required. Aged pollen diets have been shown to cause gut dysbiosis and this dysbiosis is correlated with increased *Nosema* disease (Maes et al. 2016).

1.12.3.1 Carbohydrate and pollen requirements

Honey bee larvae and NEWs feed on food stores within the hive (Beetsma 1985), and adult bees adapt their brood-care and foraging behaviour in response to the carbohydrate and protein needs of the hive (Schmickl and Crailsheim 2004). Larvae are fed HG secretions that are essential for growth and development (de Groot 1953; Standifer 1967; Haydak 1970; Brodschneider and Crailsheim 2010), optimising worker longevity (DeGrandi-Hoffman et al. 2010), pathogen resistance (Ritz and Gardner 2006; Rowley and Powell 2007), and ultimately the productivity of the colony (Kleinschmidt and Kondos 1976). The surrounding environment of the colony therefore impacts on colony welfare since the quality of the HG secretions depends on quality pollen sources in the landscape. In the prairie region of North Dakota, the annual proportion of colony survival is positively correlated with both the area of uncultivated land surrounding the apiaries and the mean amount of pollen collected per day over summer (Smart 2015). The proportion and quantity of protein, amino acids, vitamins, minerals, trace elements, enzymes, fatty acids, and carbohydrates in pollen varies between plant species. Pollen composition is also altered by environmental factors, collection methods, and storage conditions (Crailsheim 1990; Day et al. 1990; Roulston and Cane 2000; Human and Nicolson 2006). Some pollens contain distinctive fatty acid profiles dominated by one or more fatty acids (Manning 2001). This suggests that not all pollen is nutritionally equal (Crailsheim 1990), not all pollen is able to be digested by bees, and not all sugars in the pollen, as well as nectar, can be utilised equally (Haydak 1970; Wheeler and Robinson 2014).

The potential physiological functions of dominant bacteria within the honey bee gut and the fermentative processes identified by metatranscriptome sequencing (Lee et al. 2015) highlight the need for a deeper understanding of the functions of specific bacteria. Practically, this may enable the pollination industries that utilise honey bees for pollination to better manage the pollinators. Currently, some hive, orchard and crop management techniques employed to increase crop production alter the natural foraging behaviour of honey bee colonies for up to six weeks. Some of the management techniques currently employed that reduce food sources are caging crops, cutting the sward beneath the crop, and the establishment of large monoculture crops. Six weeks

are equivalent to two complete brood cycles (42 days), meaning that new larvae and NEWs that are produced during this time may consume only a single pollen source for their entire life. In the case of kiwifruit pollination, the pollen is nutritionally poor (Jay and Jay 1993), and may therefore affect honey bee health and subsequently hive production throughout the remainder of the season.

1.12.3.2 Carbohydrate types

The compostion of carbohydrate sources consumed by bees vary. For example, mānuka honey contains 80% monosaccharides and only about 10% disaccharides, which includes sucrose (Weston and Brocklebank 1999). Sucrose and honey are both carbohydrate sources that kill bacteria *in vitro* when in sufficient concentration (Molan 1992b, a). Both are also used effectively to heal mammalian wounds and ulcers (Willix et al. 1992; Molan 2006; Emsen 2007; Visavadia et al. 2008). However, not all carbohydrates are utilised by bees and/or their gut microbial residents (Haydak 1970; Wheeler and Robinson 2014). Although, the effect of sucrose or honey on honey bee gut bacteria is unknown, bees fed honey, have hundreds of active genes compared with those fed either high fructose corn syrup (HFCS) or sucrose (Wheeler and Robinson 2014). It is possible that this gene activity may regulate gut bacteria, as observed in *Drosophila* where their genes, in part, regulate gut bacteria (Ryu et al. 2008). This suggests that the structure of the carbohydrates in the treatments may differentially effect bee health. In comparison with sucrose, honey with a carbohydrate concentration ca. 80% (w/v), is recognised to have additional antibacterial activity due to hydrogen peroxide (H_2O_2) (Molan 1992a). Mānuka honey displays even greater antibacterial activity (Willix et al. 1992; Kwakman et al. 2010) due to the active ingredient methylglyoxal (MGO) (Mavric et al. 2008).

Synergistic effects between pathogens have also been observed in bees fed different diets. DWV increases when bees inoculated with *N. ceranae* receive a restricted pollen diet. This synergistic effect was both dosage- and nutrition-dependent (Zheng et al. 2015). Numbers of DWV in caged workers also increased with bee age, and were highest in those fed sugar syrup and lowest in bees fed pollen (DeGrandi-Hoffman et al. 2010).

Knowledge gap 7: The affect of carbohydrate type on gut bacteria

It is unknown whether the composition of carbohydrate consumed by bees influences the gut bacteria. Further research on the interaction between gut bacteria, diet and bee health is required to determine whether supplementary food sources should be modified so that both the bees and their gut bacteria receive suitable nutrients. Specifically, identifying whether the differences in the types of food supplied, such as

mono- or disaccharide carbohydrates, or the effect of different floral protein sources, alters the bacterial composition.

It us unknown whether the unique floral resources in NZ, including mānuka with its antibacterial properties, and the regular pollination of monoculture crops alters the gut bacterial composition, or affects nutrient utilisation by gut bacteria. Both which may affect honey bee foraging behaviour and bee health.

1.13 Thesis objectives

The studies discussed above show that bacteria in the honey bee gut play numerous roles in digestion, protection against pathogens, foraging behaviour, and host physiology. Since this PhD commenced five years ago, the extent of knowledge regarding these roles, the interactions between bacterial species, and the influence of external factors on the dominant bacteria have increased considerably. As we seek to understand whether honey bee gut bacteria can be manipulated to enhance honey bee health using MRM, the assimilation of species-specific knowledge, particularly regarding the less dominant bacteria, will be integral.

The seven knowledge gaps (KG) identified in this chapter helped determine the principal objectives of this PhD thesis. Prior to the publication of Chapter 6, the bacteria in the gut of NZ honey bees remained uncharacterised. This PhD focuses on characterising the bacteria in the gut of NZ honey bees and identifies how some external factors influence the relative abundance of core honey bee gut bacteria. The response of less dominant gut bacteria were also a focus, specifically in regard to their potential as health-indicators for honey bee colonies.

The international isolation of NZ honey bees provides an opportunity to compare the composition of gut bacteria in bees relating to those that were introduced decades ago, with the bacterial profiles that are currently observed internationally. To provide international context for the bacterial profiles identified in the NZ honey bee gut, the data were compared with data from a small study conducted in Connecticut, United States of America (USA). The influence of NZ-specific, seasonal changes to honey bee gut bacteria was characterised throughout the year and the relative abundance of both dominant and sub donminat phylotypes was identified. After characterising the bacteria, two further lines of enquiry were established. The first determined the effect of carbohydrate consumption by the bee on their gut bacteria. The second identified the interaction between honey bee gut pathogens and the core bacteria *G. apicola*. Measurable criteria such as bee longevity, adult weight, and HG development (see section 1.4.2: Assessing bee health), in combination with the relative abundance of bacteria present in the honey bee gut, were used to assess the two lines of enquiry and correlate them with the concept of ‘bee health’ (Engel et al. 2016).

The principal objectives of this PhD thesis were:

- Establish a temperate-climate dataset that identified the bacterial community within the gut of NZ honey bees and variation associated with factors external to the bee (KG 1, 2, 4, 7).
- Determine whether colonies deemed ‘sick’ by beekeepers display gut microbial dysbiosis that could be used as an indicator of colony health, or whether this ‘sick’ descriptor is associated with the gut pathogens *N. apis* or *N. ceranae* (KG 1, 2, 4, 5, 6, 7).
- Compare the bacterial composition of NZ honey bees with that from a honey bee population in Connecticut, USA (KG 1, 2, 4, 7).
- Establish a small temperate-climate dataset to determine the stability of gut bacteria throughout a 12 month period (KG 1, 4, 5).
- Determine whether natural and processed carbohydrate sources fed to bees, including the antibacterial properties of mānuka honey, alter the relative abundance of core gut bacteria (KG 5, 6, 7).
- Determine whether the gut pathogen *N. apis*, influences the development of *G. apicola*, the bacterium associated with the top layer of the biofilm in the honey bee ileum (KG 2, 4, 5).

The ultimate intention for this PhD was to substantiate the usefulness of gut bacterial studies for researching the health of honey bees, and other pollinators, within NZ. If these studies suggest that gut bacteria can be used to indicate the status of bee health or appear to contribute to poor bee health, further studies will be established to understand whether gut bacteria can be manipulated to enhance the health of honey bee colonies.

Chapter 2

General methods

Methodology is required to explain how the honey bee colonies were maintained for use in the studies and some methods were used in several chapters. Although these methods are interesting, they add bulk to the research narrative of each chapter. Hence, to facilitate the flow of each chapter, the colony management details and the details of the repeatedly used methods are presented in this General Methods chapter and only the salient points are included in each research chapter.

2.1 Colony management

All honey bees within New Zealand (NZ), and all honey bees studied in this PhD thesis are European honey bees, *Apis mellifera ligustica* (Malone et al. 1995). The colonies used in Chapters 1 to 7 were managed in response to the weather conditions; colonies were fed supplementary carbohydrates (50% sucrose solution) in spring and autumn when required, and additional honey supers were added to each hive once the populations had expanded sufficiently in spring. This reduced the likelihood of the colonies swarming as it provided more space, and it also created room for honey collection.

Varroa destructor was controlled in all hives using the synthetic pyrethroid Bayvarol® (Bayer New Zealand Ltd, Auckland, NZ) during spring and autumn each year. Four strips were placed in each brood box (Chapter 1: Figure 1.4) for eight weeks and each strip contained 3.6 mg of the active ingredient flumethrin.

2.2 Worker bee preparation for cage trials

In order to test the effects of carbohydrate diets and gut pathogens on the honey bee microbiome, standard methods for maintaining adult honey bees in cages under *in vitro* conditions (Williams et al. 2013) were modified. These modifications enabled the isolation of newly emerged worker bees as they eclosed (emerged out of their wax cell), so that they could be maintained *in vitro*, separate from the colony where they would otherwise become inoculated with gut microbes (Powell et al. 2014). On other occasions, the NEWs were required to be hive-inoculated and their age was important for the trial. The modifications enabled the NEWs to be marked before being released back in to the parent colony.

2.2.1 Identifying newly emerged workers and inoculating them with gut bacteria from their parent colony

Capped frames of ready-to-emerge honey bee worker brood (18–20 days old, black-eyed pupae) were selected from a colony at Plant and Food Research (PFR) located in Hamilton, NZ. As the average temperature of the brood nest in summer is 35.5°C (Fahrenholz et al. 1989), the frames were incubated at 33–35°C and 65% relative humidity (RH). Within 24 hours (h) of emerging through the wax capping of their cells, the NEWs were marked on their thorax with coloured nail polish (MIKI, Platinum Pharmacy, NZ), caged with approximately 150–300 marked bees and slow-released back in to the parent colony. This enabled the NEWs to fly, defecate and develop a full complement of bacteria in their digestive tracts (Powell et al. 2014). This was required

for Chapter 6: The effect of carbohydrate sources, and Chapter 7: The effect of *Nosema apis* spores on *Gilliamella apicola*. The cages (L100 x H105 x W40 mm) were constructed from metal mesh on one side and removable glass on the other that was held in place by a single-use rubber band (Figure 2.1). The bees in each cage were fed and/or treated using an inverted glass bottle feeder that had two small holes in the lid (Figure 2.1). Whilst the bees were confined to cages during the treatment application in Chapter 7, the base of each cage was layered with absorbent, leak-proof liners (babyU™ disposable change mats, Nice Pak Products Pty Ltd., Victoria, Australia) and these were removed as required.

Figure 2.1 | Metal cage with an inverted feeder and absorbent liner.

The slow release of the bees back to the parent colony occurred throughout a 24–48 h period by plugging the hole used for the inverted feeder with grass and pressing the side of the cage (glass removed) in to a frame of honey. The bees both inside and outside the cage then chewed the wax away from the cage and removed the grass which enabled the NEWs to escape (Figure 2.2). The slow release was necessary as NEWs that were not slow-released were found crawling on the grass out the front of the colony, unable to access food and due to their requirement for food for growth, these bees were unlikely to survive.

Figure 2.2 | Newly emerged worker bees marked with nail polish on their thorax on a wax frame containing honey brood and pollen.

The bees in Chapter 6: The effect of carbohydrate sources were assessed in transparent plastic queen cages (75 x 30 x 15 mm) (Figure 2.3). Inverted glass bottles with two small holes in the lid were used to feed the bees. These were secured on top with a single-use rubber band.

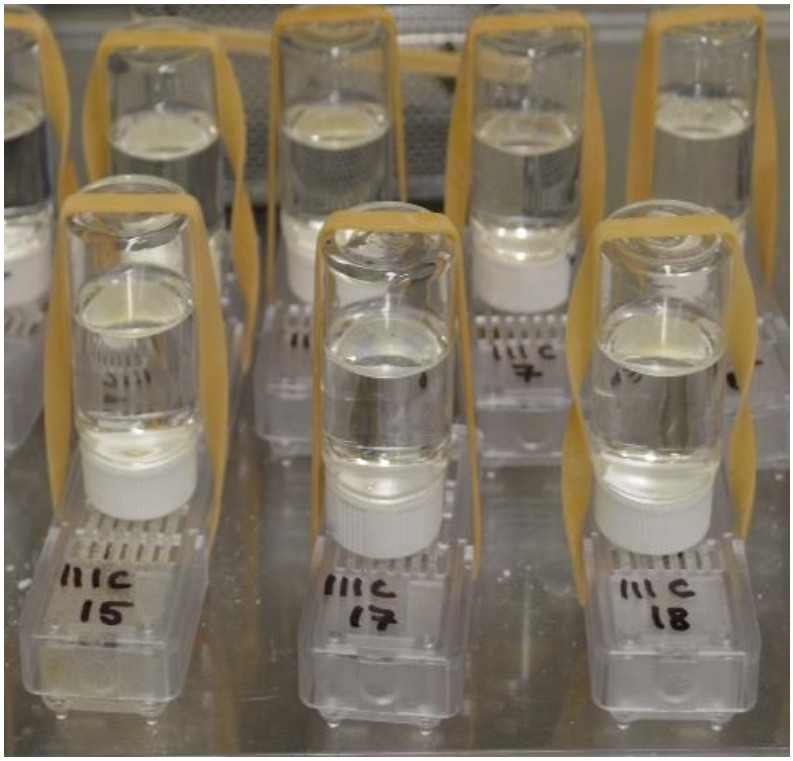

Figure 2.3 | Worker honey bees in plastic cages fed with sucrose solution using a gravity feeder.

2.3 Pollen irradiation

All pollen fed to bees for Chapter 6: The effect of carbohydrate sources, and Chapter 7: The effect of *Nosema apis* spores on *Gilliamella apicola* was collected in 2014 by honey bees located in an apple orchard in Napier, NZ, using pollen traps fitted to the front of the hives. The pollen was frozen at -20°C until gamma-irradiated (27kGy) by Schering-Plough Animal Health Limited (Wellington, NZ). It was then stored at -70°C in 100 g bottles until required. Although pollen frozen for one year does not affect the development of the HG or ovaries in worker honey bees (Pernal and Currie 2000), it is unknown how long pollen can be stored.

2.4 Dissecting the digestive tract from the honey bee abdomen

The collection and handling of bee samples prior to dissection was specific to each trial, therefore this section covers the basic dissection details and specific details are outlined within each chapter. The bacteriome of the entire digestive tract, crop to rectum, of individual and pooled honey bees was studied by aseptically dissecting each honey bee abdomen. This was aided by a dissecting microscope (6.5x to 50x magnification) (Stemi 2000-C, Zeiss) and a cold light source (KL1500 LCD Zeiss) (Figure 2.4a–h). Each bee preserved in 95% ethanol at -70°C was placed in a clean petri dish for 2–5 minutes (min) to thaw the bee and evaporate the alcohol (Figure 2.4a). Two forceps rinsed for > 5 s, wiped with clean wipes and and further dowsed with alcohol were used to separate the exoskeleton of the honey bee abdomen along both sides (Figure 2.4b–e). When the correct tension was used to grasp the stinger end of the abdominal terga (dorsal plates) and sterna (ventral plates), the sides of the exoskeleton separated like domes on a shirt (Figure 2.4d). To extract the digestive tract, the top of the crop was grasped by the forceps and slowly separated from the thorax. Still attached to the rest of the gut, the entire gut was lifted out of the abdomen cavity, and the end of the rectum was then cut from the stinger (Figure 2.4f–g). If the sample consisted of a single honey bee gut (as used in Chapter 7) or five pooled bee guts (as used in Chapter 6), the guts were placed directly into a ZR BashingBead™ Lysis Tube containing 750 µl lysis solution (Catalogue D6010, Zymo Research Corporation (ZR), California, USA). However, if the samples consisted of twenty pooled bee guts (as used in Chapters 3–5) then all of the digestive tracts were placed into a single DNase-free 1.5 ml microcentrifuge tube on ice (Figure 2.4h). This was then macerated and a subsample was removed, placed into the ZR lysis solution, and immediately frozen at -70°C until the DNA was extracted (Section 2.5). To mitigate DNA contaminants, the dissection dish for each sample was new or cleaned using fresh alcohol rubs. Additionally, on removal of each gut from the abdominal cavity, the gut was transferred directly to the Lysis tube, without touching any external surface.

Figure 2.4a | A thawing honey bee covered in ethanol in a petri dish in the light of a dissecting microscope.

Figure 2.4b | Forceps attached to the terga of a honey bee abdomen.

Figure 2.4c | The sterna being separated from the terga of a honey bee abdomen.

Figure 2.4d | The lateral membranes between the terga and sterna have been separated and the severed connection appears dome-like.

Figure 2.4e | The sterna have been folded back to reveal the complete and compact honey bee digestive tract.

Figure 2.4f | The complete honey bee digestive tract is stretched with the crop tucked in to the first terga and sterna on the left.

Figure 2.4g | A stretched honey bee digestive tract with the crop on the left and the stinger on the right.

Figure 2.4h | A DNase-free 1.5 ml microcentrifuge tube containing numerous extracted honey bee digestive tracts (crop to rectum).

2.5 Extracting bacterial DNA from the honey bee digestive tract

The 16S rRNA amplicon sequencing is conducted on either the entire honey bee gut (Martinson et al. 2011; Moran et al. 2012) or on specific gut sections (Corby-Harris et al. 2014a; Engel et al. 2015; Jones et al. 2018a). Throughout this thesis, all sequencing of 16S rRNA gene fragments was conducted on the entire gut for three reasons; the first was the prohibitive cost of sequencing the three main gut sections individually per bee. The second and more important reason is that the core bacteria are linked to specific gut sections (Martinson et al. 2012; Powell et al. 2014), meaning that sequencing of the entire gut would create a more complete picture, as the bacteria could be linked to the gut sections post analysis. The third was that cutting the sections may have resulted in contamination of the sections or loss of bacteria from the sections as they were processed.

The DNA was extracted from each gut sample using the Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kits (Catalogue D6010, Zymo Research Corporation (ZR), California, USA). For each individual gut sample, the entire gut was placed in one ZR BashingBead™ Lysis Tube containing 750 µl of lysis solution. Based on a sample of 11 individually extracted NZ bee guts, the weight of a gut ranged from 20–37 mg and the mean was 26.3 mg. For each pooled sample, the guts were macerated by pipetting the material up and down, and then grinding the material with a pipette tip for 30 seconds (s). A 150 mg gut subsample (28% of the mean total weight of a pooled sample of 20 bee guts (526 mg)) was transferred to a ZR BashingBead™ Lysis Tube containing 750 µl lysis solution. Bead-beating methodology was used as it effectively disrupts the cell walls to release the cell contents, including the DNA from Gram-positive organisms such as *Lactobacillus* and *Bifidobacterium*. For consistency it was also the method used in two of the early Next Generation Sequencing (NGS)-based surveys of honey bee gut microbiome (Mattila et al. 2012; Moran et al. 2012).

The samples were homogenised with a high-throughput cell disrupter, FastPrep®-24 (MP Biomedicals, Seven Hills, Australia), at 6 m/s for 40 s. The FastPrep®-24 was used for Chapters 3–7. 6m/sec for 40 s was tentatively selected as other groups within PFR Research used this speed to extract bacterial DNA. As the DNA was suitably extracted from the honey bee gut using this speed, as determined by the preliminary analysis of the DNA using PCR (Section 2.6.1), 6 m/s for 40 s was used for all FastPrep® homogenisation. When the FastPrep®-24 (MP Biomedicals, Seven Hills, Australia) was undergoing maintenance, a Biospec Minibead Beater-96 (BioSpec Products, Oklahoma, USA), which oscillated at 2400 strokes/min, was used for the “sick” samples from Chapter 3. The oscillation meant that the samples were processed at a lower velocity than those in the FastPrep®-24, but this was accounted for by homogenising

the samples at 3640 rcf for 4 min. The remainder of the recommended ZR protocol was followed:

1. 500 µl of beta-mercaptoethanol was added to 100 ml of *Fecal DNA Binding Buffer.*
2. The base of the *Zymo-Spin™ IV-HRC Spin Filter*, was snapped off, the filter was inserted in to a collection tube (CT), and centrifuged for 3 min at exactly 8,000 x *g*. This was set aside for step 11.
3. The *ZR BashingBead™ Lysis Tube* containing the gut sample(s) was centrifuged for 1 min at ≥ 10,000 x *g*.
4. 400 µl of supernatant was transferred to a *Zymo-Spin™ IV Spin Filter*, in a CT, and centrifuged for 1 min at ~ 7,000 x *g*.
5. 1,200 µl of *Fecal DNA Binding Buffer* was added to the filtrate in the CT.
6. Transfer 800 µl from the CT, to a *Zymo-Spin™ IIC Column* in a second CT. Centrifuge for 1 min at 10,000 x *g*.
7. The flow through from the second CT was discarded and step 4 was repeated.
8. 200 µl of *DNA Pre-Wash Buffer* was added to the *Zymo-Spin™ IIC Column* in a new CT, and centrifuged for 1 min at 10,000 x *g*.
9. 500 µl of *DNA Wash Buffer* was added to the *Zymo-Spin™ IIC Column* in a new CT, and centrifuged for 1 min at 10,000 x *g*.
10. The *Zymo-Spin™ IIC Column* was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of *DNA Elution Buffer* was added directly to the column. This was centrifuged at 10,000 x *g* for 30 s to elute the DNA.
11. The eluted DNA in step 11 was transferred to the prepared *Zymo-Spin™ IV-HRC Spin Filter* in a clean 1.5 ml microcentrifuge tube. This was centrifuged for 1 min at exactly 8,000 x *g*.

The eluted DNA was transferred to 0.2 ml Sapphire qPCR 8-tube strips with optical flat caps (Reference 608281, Lot 16372) (Greiner bio-one, Germany), and stored as 1 x 50 µl and 1 x < 50 µl aliquots at -70°C. These were used for downstream applications, such as DNA analysis using a NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific, NZ), Qubit™ 2.0 Fluorometer with a dsDNA HS Assay Kit (ThermoFisher Scientific, NZ), and Next Generation sequencing through the Massey Genome Service (MGS; Massey University, Palmerston North, NZ).

2.6 Assessing DNA quality and quantity

The purity and quality of the DNA extracted from the bee gut samples were assessed using electrophoresis gels, and a NanoDrop™ full-spectrum UV-Vis spectrophotometer (ThermoFisher Scientific, Auckland, NZ). The quantity of DNA was assessed using a Qubit™ 2.0 Fluorometer (ThermoFisher Scientific, NZ)

2.6.1 Confirming that bacterial DNA was extracted from the honey bee gut

Gel electrophoresis was used in the first instance to determine the success of the DNA extraction from the honey bee gut samples using the Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kits. It was also used to confirm the quality of the DNA of these initial samples prior to extracting the DNA from for the remaining trials, and to assess the effect of thawing on the DNA.

A 1% (w/v) agarose gel was prepared by mixing 0.5 g of Le Agarose powder (A4718 Merck, Darmstadt, Germany) plus 50 ml of 1 x Tris-acetate (TAE) buffer l(40 mM Tris base, 20 mM acetic acid, and 1 mM sodium salt of ethylenediaminetetraacetic acid (EDTA), pH 8.3). This mix was microwaved until dissolved (approximately 60 s), then cooled until the bottle was able to be touched. This was mixed with 2.5 µl of Redsafe™ Nucleic Acid Staining Solution (20,000 x), (#21141, Intron Biotechnology, Gyeonggi-do, Korea). The gel was then cast, cooled until opaque (10 min) and once set, the cast was placed in a tank and covered with 1 x TAE buffer. The DNA was loaded into the wells, which were positioned near the anode (black wire) to allow the negatively charged DNA to run toward the cathode (red wire).

A 5 µl DNA sample was pipetted onto Parafilm™ (Bemis Company, Inc., WI, United States of America) and mixed with 5 µl of 5 x loading dye (2 g of Bromophenol Blue was mixed with 2 ml Xylene Cyanol, 25 ml Ficoll®–400, and water added up to 100 ml. This was diluted to 5 x using dw). Each gel well was loaded with 5 µl of the DNA-dye sample. New tips were used for each sample. A well loaded with 5 µl of a 1 kb DNA ladder was included to confirm the size of DNA fragments. The gel was run at 89 V for 30 min and removed for visualisation with FireReader V10 (UVITEC, Cambridge, England), a high-end, Geldoc system.

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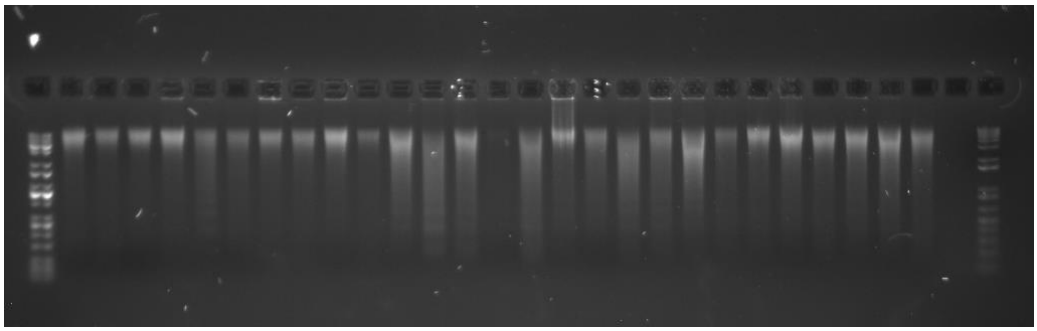

Figure 2.5 | Visualisation of the quality of bacterial DNA that was extracted from honey bee digestive tracts.

Electrophoresis in an agarose gel, as above, were used to determine the quality of extracted DNA. For example, the DNA bands were bright and clean and were closer to the loading wells around the 10 kb band of the DNA ladder. This suggests that the DNA was intact without obvious signs of degradation or shearing. The empty wells were blank controls. The smearing in the wells in the middle and to the right were attributed to DNA samples that thawed during transit. To mitigate this degradation, the freeze thawing of all samples was limited or at least kept the same for every trial. This picture indicates that the methodology used to extract and store the DNA from the honey bee gut was suitable for qPCR amplification and sequencing a fragment of the 16S rRNA gene.

2.6.2 Analysis of DNA quality

A NanoDrop™ full-spectrum UV-Vis 2000c spectrophotometer (ThermoFisher Scientific, NZ) was used to assess the purity and quality of the DNA extracted from the bee gut samples prior to sequencing a fragment of the 16S rRNA gene by Massey Genome Services (MGS) (Palmerston North, NZ).

A 1 µl sample of extracted DNA from the second aliquot for each gut sample was pipetted on to a measurement pedestal and the nucleic acid concentration and the A260/A280 ratio were used to determine whether the DNA samples were suitable for further DNA quantification or whether they required re-extraction. Elution buffer from the Zymo kit (CAT No: D3004–4–10, Lot No: ZRC179 191) and PCR grade water were the controls used to 'blank' the Nanodrop™ below 0.3 ng/µl. To certify that the NanoDrop™ was reading in the correct range, a control solution containing 1.58 x 10^{11} copies/µl of glyceraldehyde 3–phosphate dehydrogenase (GAPDH), which is a stably expressed kiwifruit reference gene (Genebank sequence FG499278) synthesized by Life Technologies (Auckland, NZ), was used as it consistently read between 11.9–13.9 ng/µl.

2.6.3 Analysis of DNA quantity

A dsDNA HS Assay Kit and a Qubit™ 2.0 Fluorometer (ThermoFisher Scientific, NZ) were used to determine the quantity of DNA present in each aliquot prior to the first samples from Chapter 3 being sent for sequencing of a fragment of the 16S rRNA gene. Once the process was determined all further samples were analysed using Qubit™ by MGS.

The working solution was prepared using the following methodology where n = the number of samples plus two DNA standards (S1 and S2), plus one extra to account for pipetting errors (e.g. Testing 4 samples means n = 7). n x 1 µl of dye plus n x 199 µl of the kit buffer were mixed thoroughly using a vortex mixer. Standards 1 and 2 were prepared by mixing 190 µl of the working solution and 10 µl of the either of the supplied DNA standards. DNA samples were prepared by mixing 198 µl of working solution with 2 µl of DNA. The standards were measured in the Qubit™ Fluorometer followed by the samples. The raw reads were then converted to account for the DNA being diluted.

2.7 DNA Identification using quantitative polymerase chain reaction

The bacteria in the DNA samples were identified generically or specifically using real-time quantitative polymerase chain reaction (qPCR) (Rotor Gene 6000, The Bosch Institute, Sydney, Australia). This was conducted using primers that identified specific bacteria or pathogens, such as *Gilliamella apicola* and *N. apis*, or universal primers that identified the amount of all bacteria.

DNA samples extracted in Chapter 7 were analysed using universal primers, as were the samples in the pilot trial used to determine whether bees should be inoculated by individually feeding each bee or by group feeding with *Nosema* spp. or bacteria (see section 7.8.1). The DNA samples from the trials in Chapters 3 and 7 were analysed specifically for *N. apis* and *N. ceranae* using hydrolysis probe assays (TaqMan®) (dnature, Gisborne, NZ).

2.7.1 Primers

Universal bacterial primers were purchased (Life Technologies New Zealand Limited, NZ) in a freeze dried form; Forward primer TCCTACGGGAGGCAGCAGT (89.1 nmol), reverse primer GGACTACCAGGGTATCTAATCCTGTT (47.1 nmol), as were *G. apicola* primers ((Ludvigsen et al. 2015) (Life Technologies New Zealand Limited, NZ) in a freeze dried form; forward primer GTATCTAATAGGTGCATCAATT (95.8 nmol), reverse primer TCCTCTACAATACTCTAGTT (94.7 nmol). The primer stocks were

reconstituted to 100 µM. Each tube was centrifuged for 30 s at 15000 *g* to collect the primer in the base of the tube. Depending on the number of moles in the tube, a specific volume of 10 mMol Tris buffer was added to generate 100 µM stocks; the forward primer for the Universal eukaryote (89.1 nmol) required 891 µl of 10 mMol Tris buffer and the reverse primer (47.1 nmol) required 471 µl of 10 mMol Tris buffer. The forward primer for *G. apicola* (95.8 nmol) required 958 µl of 10 mMol Tris buffer and the reverse primer (94.7 nmol) required 947 µl of 10 mMol Tris buffer. These were rehydrated for 2 min and vortexed for 15 s. Working primer solutions (10 µM) were prepared from 180 µl PCR grade water and 20 µl of 100 µM primer, then stored at -70°C.

2.7.2 Master Mix

Each PCR tube contained 5 µl of a DNA double-strand-specific dye called LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Auckland, NZ), 3.6 µl PCR grade water, 0.2 µl forward primer, 0.2 µl reverse primer, and 1 µl of DNA sample previously diluted 10-fold.

2.7.3 qPCR cycling conditions

To amplify the DNA to identify the bacteria, the following qPCR cycling conditions for the Rotor Gene 6000 real-time qPCR machine were used (Table 2.1); 95°C for 5 min, 40 cycles of two-step PCR at 95°C for 3 s, and 60°C for 20 s. All runs were conducted using the automatically assigned Rotor Gene gain settings of 7.33, 8 or 8.33. The threshold was generated automatically using the Rotor Gene 6000 series software.

Table 2.1 | Cycling conditions utilised in each qPCR run to analyse the number of copies of bacteria present in each sample.

	Temp (°C)	Time (h:mm:ss)	Acquisition
Pre-incubation	95	0:5:00	Off
Amplification (40 cycles)	95 60 72	0:0:30 0:0:20 0:1:00	Off Off On
Melt-curve (1°C per step)	High 95°C Low 60°C		

Three technical replicates of each DNA sample, the no template controls (NTC) ie just buffer, and the *E. coli* standards (1.56 x 10^7 copies/µl) were included in each qPCR run. To identify the samples within each a spreadsheet (Supplementary material 2.10.1) was established and used to populate a qPCR run sheet for each qPCR run (Supplementary material 2.10.2), except the first run that established the standard curve for each bacteria.

2.7.4 Standardisation of the qPCR runs

A standard curve was used to standardise the number of gene copies in each qPCR run. A known concentration for each bacterium discussed below was determined by growing 50 µl of bacteria overnight in 5 ml Tryptic Soy Broth (TSB). The bacterial cells were dyed blue to assist with identification by mixing 10 µl of the bacteria/TSB suspension with 10 µl of Trypan blue (CAS no: 72-57-1, Sigma-Aldrich, NZ). A 1/10 dilution was made (20 µL bacterial suspension plus 180 µL distilled water (DW)) and this was vortexed thoroughly to mix and suspend the cells evenly. If the cells were too numerous to count, further serial dilutions were conducted to give a concentration of 10^{-7} cells.

The bacterial cells were counted using a haemocytometer (1/400 mm 2 x 1/10 mm deep, Improved Neubauer, USA). Ten µl was pipetted under the coverslip and five of the 4 x 4 squares were counted (Figure 2.6) to calculate the mean. The concentration of the original culture was determined using the following calculation; Concentration (cells/ml) = mean number of cells per square x dilution factor x 25 x 10,000. The dilution factor is 10 for 1/10 dilution or 20 if Trypan blue was used. The 25 accounts for the 25 squares in the middle square identified by the green circle. 10,000 is the correction factor specific to the haemocytometer volume within the middle square (depth under coverslip is 1 mm). Thus, if approximately 200 cells are counted, then the count in the tube was 20 x 10^7 cells/ml.

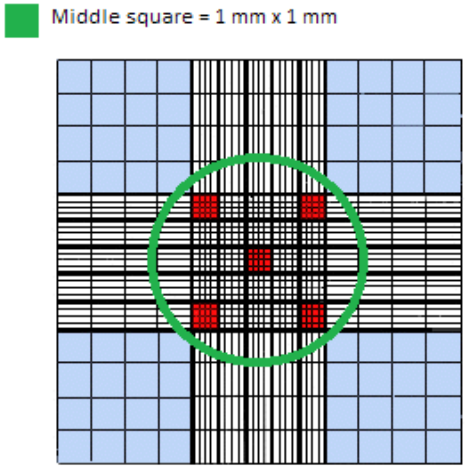

Figure 2.6 | The bacteria in the red 4 x 4 squares on the haemocytometer grid were counted.

The pellet was centrifuged at 1310 rcf for 5 min. The supernatant was then removed and 100 µl of DW or lysis solution was added to make the pellet 1 x 10^9 cells/ml. The DNA was extracted from 50 µl using the Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit, as described in section 2.5.

The NanoDrop™ was used to determine the concentration of DNA/µl. The 260/280 ratio was used to identify the proportion of protein absorption to the DNA where > 1.6 was acceptable.

A 9–point standard curve (1 x 10^9 DNA copies/µl to 1.53 x 10^4 DNA copies/µl) was established from the known DNA concentration using a 4-fold dilution series (10 µl of DNA plus 30 µl of PCR water) (Table 2.4). The DNA was expected to double every cycle and if so then a concentration from the standard curve was then selected to standardise all subsequent qPCR runs.

Table 2.4 | Dilution series used to establish a standard curve for each bacterium.

Dilution Step	Counts (copies/µl)
1	1.00 x 10^9
2	2.50 x 108
3	6.25 x 107
4	1.56 x 107
5	3.91 x 106
6	9.77 x 105
7	2.44 x 105
8	6.10 x 104
9	1.53 x 104

Each concentration was 10 µl of DNA from the dilution step above plus 30 µl of PCR water.

2.7.4.1 Standard curve for universal bacteria primers

DNA extracted from *Escherichia coli* Nissle (PFR, Palmerston North) was used as the control to confirm the universal bacteria primers were positively identifying bacteria. The *E.coli* DNA was extracted using a ZR Fecal DNA Miniprep™ kit on 8 April 2016 from *E. coli* cells, and the concentration was determined using a haemocytometer (Cantwell 1970). A dilution series was established and based on the quantitation analysis and the standard curve, the *E.coli* dilution-point 1.56 x 10^7 copies/µl was included to check between run variations (Figure 2.7). All DNA samples were diluted 10-fold for the qPCR run and accounted for in analysis.

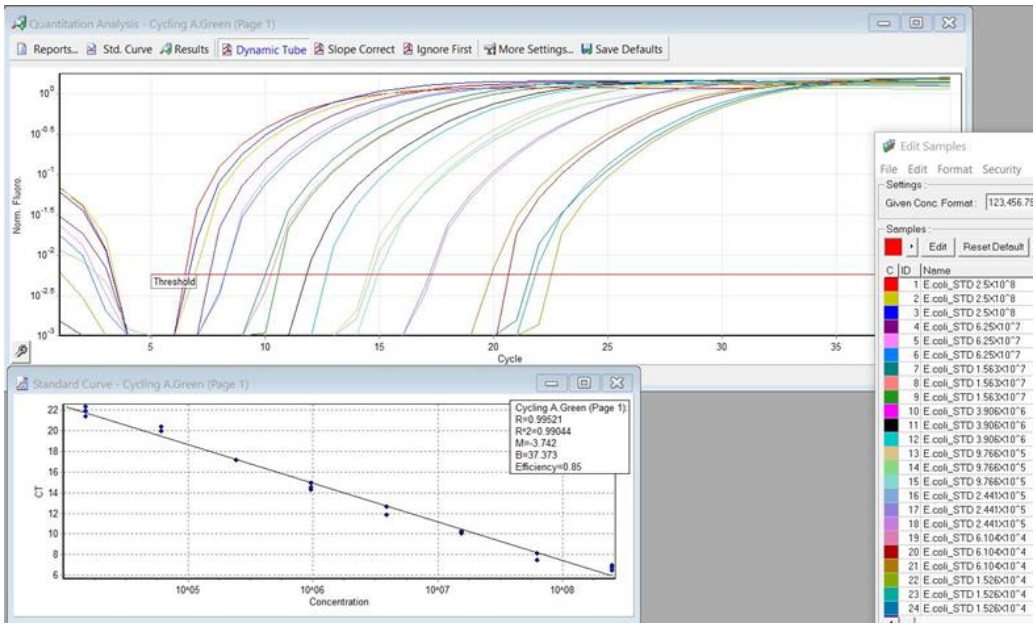

Figure 2.7 | Quantitation analysis and standard curve for the bacterium *Escherichia coli* produced using an R6000 qPCR.

2.7.4.2 Standard curves for *Gilliamella apicola*

To confirm visual identify of *Gilliamella apicola* isolated from NZ honey bees, an international strain of freeze dried *G. apicola* (ATCC® BAA-2448™) was purchased for comparison from the American Type Culture Collection (ATCC) (In Vitro Technologies NZ Pty. Ltd, Auckland, NZ). Both cultures were grown on Tryptic Soy Agar (TSA) plates with 5% sheep blood (Fort Richard Laboratories, Auckland, NZ), in a Level 2 Physical Containment laboratory at PFR, Hamilton, NZ. The growth and DNA extraction methods are discussed in further detail in Chapter 7.

Gilliamella apicola supplied by ATCC

The melt curve for the ATCC *G. apicola* showed that the majority of the DNA product fluoresced at the temperature peak of 85.5°C. A slight shoulder was also observed at 79–80°C (Figure 2.8).

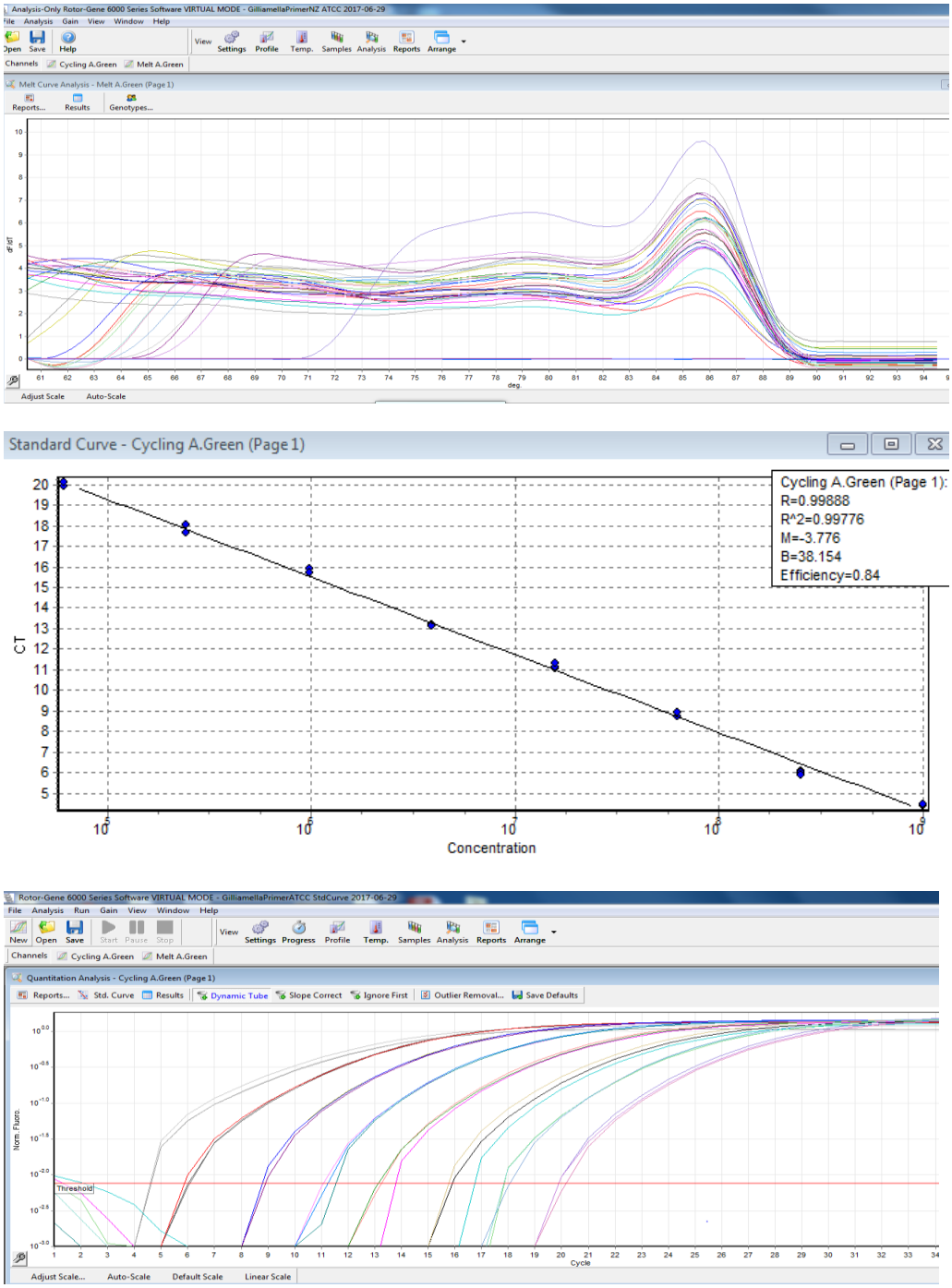

Figure 2.8 | Quantitation analysis and standard curve for the bacterium *Gilliamella apicola* produced using an R6000 qPCR.

Gilliamella apicola from New Zealand

The melt curve for the NZ *G. apicola* showed that there was much less DNA in the samples but that it was still present. The majority of the DNA product fluoresced at the same temperature peak of 85.5°C as the ATCC strain and that the slight shoulder was also present at 79–80°C (Figure 2.9). The NZ *G. apicola* DNA will be sequenced further at the conclusion of this PhD research to confirm its identity, i.e. sequencing all nine hypervariable regions (V1–V9) of the 16S rRNA gene, or sequencing the whole genome. Its likely identity is confirmed in Chapter 7 as Next Generation Sequencing (NGS) of the 16S rRNA gene fragments in the honey bee gut supports the qPCR melt curves.

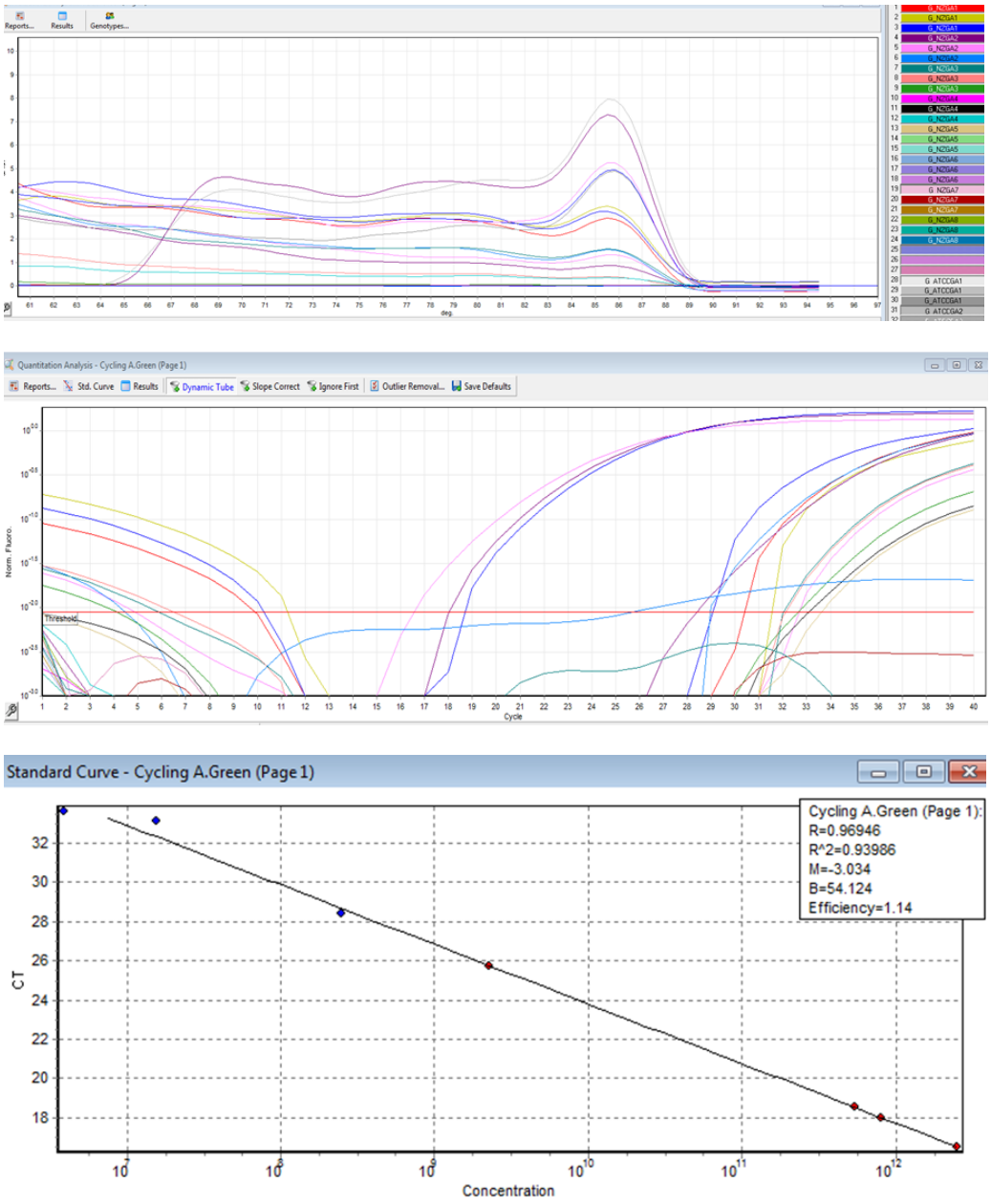

Figure 2.9 | Quantitation analysis and standard curve for the bacterium *Gilliamella apicola* produced using an R6000 qPCR.

2.7.4.3 Standard curves for *Nosema apis* and *Nosema ceranae*

To confirm visual identity of *Nosema apis* and *Nosema ceranae* isolated from NZ honey bees, as well as provide controls for the hydrolysis probe assays used to identify *Nosema* spp. ((TaqMan®, dnature, Gisborne, NZ) section 2.7.5), standard curves were produced for both. The forward primer sequence (5' - 3' direction) used to identify *N. apis* was CCATTGCCGGATAAGAGAGT and the reverse sequence was CACGCATTGCTGCATCATTGAC (Chen et al. 2008). The forward primer sequence (5' - 3' direction) used to identify *N. ceranae* was CGGATAAAAGAGTCCGTTACC and the reverse sequence was TGAGCAGGGTTCTAGGGAT (Chen et al. 2008).

The standard curves for *N. apis* (Figure 2.10) and *N. ceranae* (Figure 2.11) both had R^2 values of 0.98.

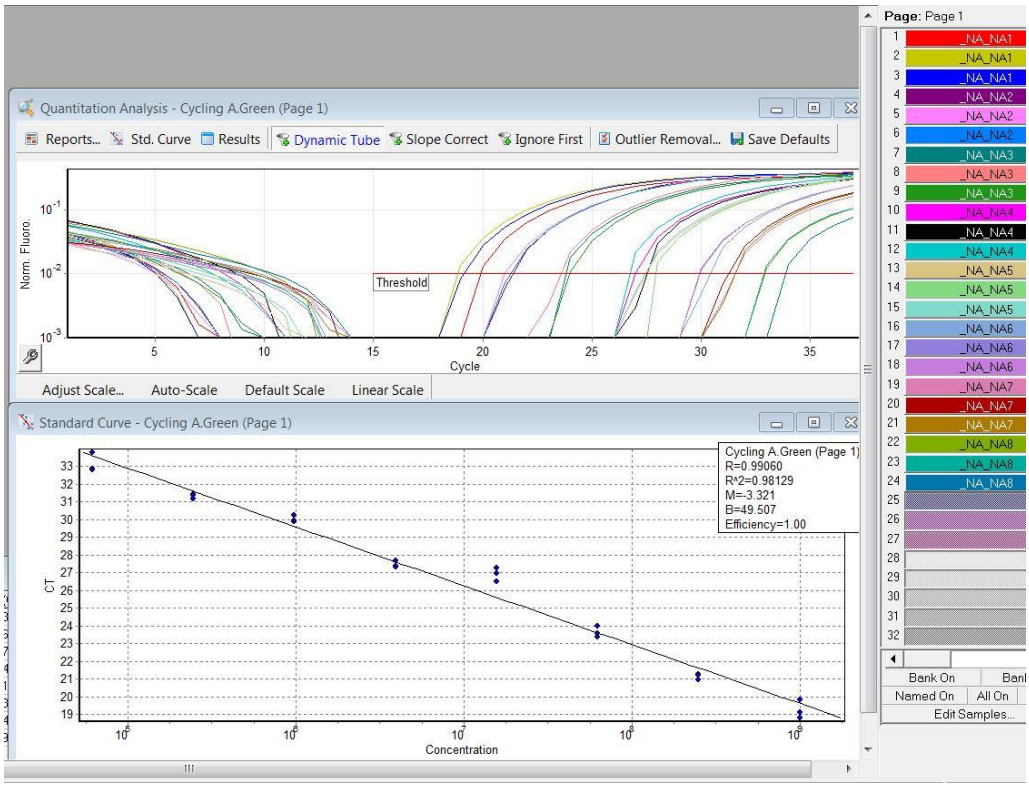

Figure 2.10 | *Nosema apis* standard curve produced using an R6000 qPCR.

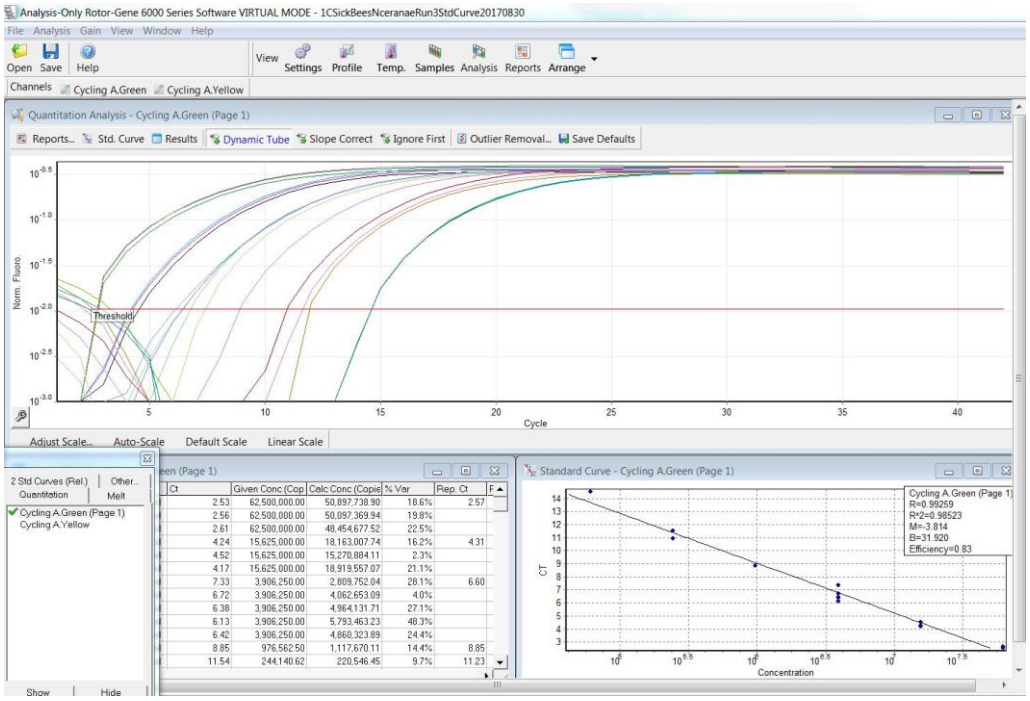

Figure 2.11 | *Nosema ceranae* standard curve produced using an R6000 qPCR.

2.7.5 Hydrolysis probe assays

Nosema apis and *Nosema ceranae* were detected using hydrolysis probe assays (TaqMan®) (dnature, Gisborne, NZ) by amplifying the products of *N. apis* and *N. ceranae* specifically. TaqMan® assays do not require a melting curve. No cross-reaction with other *Nosema* species has previously been reported. A reaction cocktail was prepared by briefly vortexing and pulse centrifugation of the 2 x Mastermix provided by dnature, then combining 5 µl of the 2 x Mastermix, 2.5 µl water, and 0.5 µl 20 x primer probe mix. 8 µl was added to the base of each well and 2 µl of DNA template was added to the middle left hand-side of the well, to separate it from the reaction cocktail. The tubes were sealed and the qPCR was run using a Rotor Gene 6000 (The Bosch Institute, Sydney, Australia). The protocol recommended by dnature (Gisborne, NZ) for a RotorGene using LightCycler 480 was followed; pre- incubation at 95°C for 3 min, 45 cycles of amplification at 95°C for 10 s and 60°C for 25 s. A melt curve was not required. An assay cut-off (C_q) < 37 was deemed positive by dnature for both *Nosema* spp.

2.8 DNA sequencing

DNA sequencing determines the order of nucleotides within a DNA molecule, and specifies the order of the four bases – adenine, guanine, cytosine, and thymine – in a strand of DNA. The development of rapid DNA sequencing methods include Next Generation Sequencing (NGS) technology. NGS is where fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) are incorporated into strands of DNA template during sequential cycles of DNA synthesis. At this point, the nucleotides are individually identified by fluorophore excitation. This reaction is catalysed by DNA polymerase and, unlike older technology that sequence a single DNA fragment, this process is conducted in parallel across millions of DNA fragments (Illumina 2017). To investigate the bacterial identity of the DNA in the digestive tract of the honey bees, a fragment of the 16S rRNA gene was sequenced.

2.8.1 16S rRNA gene processing

The frozen DNA aliquots were sent on ice by overnight courier to Massey Genome Service (MGS) (Massey University, Palmerston North, NZ) for 16S rRNA gene sequencing of the V3V4 hypervariable region (Kozich et al. 2013) on an Illumina MiSeq platform (Illumina Inc., California, USA). This region was selected for two reasons. The first was that the length of the 16S rRNA sequence can affect the accuracy of the taxonomic designations (Dinsdale et al. 2008). For example, when short read alignments of *Bacteroides* spp. from the honey bee gut were compared with distinct 16S rRNA hypervariable regions (V1V2, V3V4, V5V6, and V7 to V9), the total number of *Bacteroides* spp. reads were 5, 0, 2, and 1 respectively (Lee et al. 2015). Lee et al. (2015) conclude that the relative proportions of bacteria are similar across the V1 to V6 regions but not the V7 to V9 regions. The second reason was that the regions V4, V5, V7 and V8 are considered least useful for genus or species differentiation, and that the V3 region most suitably distinguishes all bacteria to the genus level. The exception is the family Enterobacteriaceae (Chakravorty et al. 2007). Hence the inclusion of the V4 region. It is also possible that bacterial reads from any of the hypervariable regions, including V3V4, may not reflect the actual abundance of less prevalent bacteria. However, their relative proportions, rather than the number of reads, are important. Hence, the V3V4 region has been sequenced for all further DNA studies.

Each DNA sample was submitted as a 20 µl aliquot in 96-well plates with each well corresponding to the specified sample name in the sample submission spreadsheet. To ensure sequencing produced > 80K reads per run, a maximum of 100 samples were included per run. The sick samples in Chapter 3, the healthy samples in Chapter 3, and the samples from Chapter 7 were submitted separately. To enable the sequencing of as many samples as possible within the budget, samples from Chapters 4 and 5 were submitted on the same 96-well plate.

To identify the concentration of DNA in each sample, MGS conducted Qubit™ 2.0 Fluorometer analysis using a dsDNA HS Assay Kit for 12 samples per plate. A PCR reaction was then performed using the primers 16Sf_V3 (5' - 3' direction) – CCTACGGGAGGCAGCAG; and 16Sf_V4 (5' - 3' direction) – GTGCCAGCMGCCGCGGTAA with adaptors (Kozich et al. 2013). The PCR products (c. 420-440 base pairs) were purified to generate a library and their concentrations were analysed using Qubit™. The products were pooled in equimolar concentrations and the concentration and size were confirmed with both Qubit™ and LabChip (PerkinElmer, Waltham, MA, USA) analysis, respectively. The latter was conducted using the HT DNA High Sensitivity Reagent Kit (PECLS760672) and the HT DNA 1K/12K/HI Sensitivity Assay LabChip (PE760517). The PCR products were sequenced with a 250-base paired end run on an Illumina MiSeq™ platform (Illumina Inc.) with version 2 chemistry. Illumina PhiX Control v3 (FC-110-3001) was included as the sequencing control. The sequences from Chapter 6 are available in the National Center for Biotechnology Information's (NCBI's) Sequence Read Archive under the project code PRJNA531038: Honey bee gut metagenome.

2.8.2 Gene sequence processing and characterisation of microbial communities

The Illumina de-multiplexed fastq sequence data were processed and trimmed using ea-utils to a 0.01 probability of error, an equivalent Phred score of Q20 (Aronesty 2011), then further processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) analysis suite, version 2018.2 (Caporaso et al. 2010) (https://github.com/PlantandFoodResearch/bioinf_Apis_metabarcoding). The quality of all available sequences for all samples was checked using both FastQC and MultiQC. The reads were run through DADA2 methodology in QIIME2 (Callahan et al. 2016) to filter and trim the paired-end sequences, dereplicate them, and filter chimeras to produce exact amplicon sequence variants (ASVs). A stringency level at the taxonomic level of species was set. The statistics for each dataset where the fragment of the 16S rRNA gene was sequenced are listed in Supplementary material 2.10.3 and the 16S rRNA sequence counts for each honey bee sample associated with Chapters 3–5 and chapter 6–7 are in Supplementary material 2.10.4 and 2.10.5 respectively.

The honey bee microbiome is a relatively new area of research, with new bacterial strains being identified and reclassified frequently. Initial sequencing analysis for the NZ survey data (Chapter 3) in 2015 indicated that some sequences were incorrectly assigned to old nomenclature, or absent in both the Greengenes and SILVA taxonomy reference databases. Differences between taxonomies occur between the four major reference taxonomies (SILVA, RDP, Greengenes and NCBI) because the size and structure of each varies (Balvočiūtė and Huson 2017).

To ensure taxonomic classification of honey bee gut bacteria was current and consistent within this PhD, all analyses were conducted using the 16S RefSeq rRNA BLAST (Basic Local Alignment Search Tool) database, which was downloaded in August 2018 from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). This was customised using Perl script written by Associate Professor Patrick Biggs to make a QIIME 2 compatible reference dataset without intervening taxonomic classifications, such as suborders and tribes (https://github.com/pjbiggs/16SrRNA_taxonomy).

2.8.3 Customised 16S rRNA BLAST database

The NCBI taxonomy classifies some species with additional groups, suborders and tribes. This offsets all subsequent taxonomic assignments by one. For example *Streptomyces klenkii*, is a member of the Actinobacteria phylum, but the full NCBI taxonomy also places it in the Terrabacteria taxon, which is located between the levels kingdom and phylum. This rearrangement prevents the analysis of some species within the six traditional taxonomic levels; phyla through species. To realign the taxonomy, additional groups, suborders and tribes were removed using a customised 16S rRNA BLAST database. This was established by downloading the 16S rRNA RefSeq database from the NCBI server in August 2018 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) and converting it back to a FASTA format file using the BLAST+ tool blastdbcmd (the 2016 version was used so as to retain the GI identifier in the header by the use of the "%f" flag). The header in the FASTA sequence file ("16SMicrobial.fasta") was linked to the GenBank GI accession IDs in the taxonomic file ("16S_id_to_tax.map") using the shell script "id_to_tax_mapmaker.sh" (https://github.com/mtruglio/QIIME_utilities).

The subsequent output mapping file contained two columns, the first being the GI accession ID, and the second being the taxonomy. The data structure of the full taxonomy (including the intermediate taxonomic classifications groups, suborders and tribes) was a character string, delimited by semicolons, and it was this string that was parsed using a customised Perl script (https://github.com/pjbiggs/16SrRNA_taxonomy) and stored in a MySQL database (version 5.7.18). The groups, suborders and tribes were removed using a repetitive three step process whereby the first step was Simple taxonomy, where names were correctly classified between phyla through subspecies (L1-L9). The second step, Complex taxonomy, removed specific taxonomic names. The third step removed 'Group' names. At the end of the three steps, the process was repeated with the initial step resetting the group names for levels L3–L9, where L3 specifies the third taxonomic level of Class, and L7–L9 retained the taxonomic level of species when suborders and tribes had been present in the levels above. This three step process was conducted one at a time as some 'groups' changed their location as they were moved back through the taxonomy; each taxonomic string was split into an array with a variable number of elements. This was loaded into a table within MySQL

and the taxonomy was analysed as a group at the L2 level. Archaea were analysed first, and then bacteria. Whilst these subsets were manually curated to work out the most parsimonious taxonomic situation, the building of a new taxonomic table was done through a MySQL script that processed the taxonomy if required and moved the curated data into a new table. The resulting curated table was used to generate a new taxonomy file that could still be used with the FASTA file as the entries in it were the same.

From the customised dataset a biological observation matrix (BIOM) was created that contained the ASVs identified from the sequencing of each sample associated with the assigned taxonomy. Any ASVs that were unable to be identified taxonomically to species level in the customised dataset were assigned to the closest identifiable taxonomic level.

2.9 Statistical analysis

2.9.1 Bacterial diversity

For each set of sequenced data, phylogenetic diversity was measured within a sample (alpha (α)-diversity), and between samples (beta (β)-diversity) using the web-based tool MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017). To reduce estimation errors that result from the different number of sequences per sample, the data were rarefied to the number of sequences in the smallest sample, such as 52880 in the carbohydrate trial (Chapter 6).

The α-diversity and β-diversity in each sample were based on unfiltered data counts. To reduce estimation errors that result from the different number of sequences per sample, each dataset was rarefied to the number of sequences in the smallest sample. The data were relativised using total sum but were not transformed.

Two measures of α-diversity were calculated at the feature level using Kruskal-Wallis pairwise comparisons. The first was a measure of richness, where the measures Observed ASVs and Chao1 analysed the number of unique species in the community by treating the abundant and rare species equally. The second measure of α-diversity was evenness. The Simpson and Shannon measures both accounted for richness and abundance, although the Simpson measure gives more weight to more abundant species in a sample, and the Shannon method gives more weight to rare species.

Beta-diversity was calculated by generating a distance matrix for every pair of samples with two distance/dissimilarity methods: Bray-Curtis dissimilarity, and Jaccard Index. These compared the number of phylotypes between the two communities without accounting for phylogenetic diversity. For each experimental factor, these distance methods were displayed as 3-D Principal Coordinate Analysis (PCoA) plots. A

permutational MANOVA (PERMANOVA) was used to compare the variation between the samples.

The α-diversity and β-diversity measures are specific to each dataset and these details are presented in each section.

2.9.2 R analysis

Data analysis was conducted in R (version 3.5.1) (R Core Team 2018). Sequences with a minimum total read composition of <0.01% prevalence were filtered from the dataset. This low threshold ensured the inclusion of the majority of less abundant bacteria in the analysis. As each analysis required its own methods to normalise the data and graphically display the results the methods are described in each chapter.

2.10 Supplementary material

2.10.1 | Spreadsheet used to populate each qPCR run

Sample	Treatment	Trial	Treatment	Primer	qPCR well	Rotor Gene descriptor
1	A25	Axenic	Hive +8	Universal	1	Universal_A25
2	A26	Axenic	Hive +8	Universal	2	Universal_A25
3	A27	Axenic	Hive +8	Universal	3	Universal_A25
4	A28	Axenic	Hive +8	Universal	4	Universal_A26
5	A29	Axenic	OnComb0days	Universal	5	Universal_A26
6	A30	Axenic	OnComb0days	Universal	6	Universal_A26
7	A31	Axenic	Pollen	Universal	7	Universal_A27
8	A32	Axenic	WaxCapping	Universal	8	Universal_A27
9	A33	Axenic	Sucrose	Universal	9	Universal_A27
10	A34	Axenic	<2hrs on frame+8	Universal	10	Universal_A28
11	A35	Axenic	<2hrs on frame+8	Universal	11	Universal_A28
12	A36	Axenic	<2hrs on frame+8	Universal	12	Universal_A28
13	A37	Axenic	<2hrs on frame+8	Universal	13	Universal_A29
14	A38	Axenic	<2hrs on frame+8	Universal	14	Universal_A29
15	A39	Axenic	<2hrs on frame+8	Universal	15	Universal_A29
16	A40	Axenic	<2hrs on frame+8	Universal	16	Universal_A30
17	A41	Axenic	<2hrs on frame+8	Universal	17	Universal_A30
18	A42	Axenic	OnComb0days	Universal	18	Universal_A30
19	A43	Axenic	Pre + 9	Universal	19	Universal_A31
20	A44	Axenic	Pre + 9	Universal	20	Universal_A31
21	A45	Axenic	Pre + 9	Universal	21	Universal_A31
22	A46	Axenic	CapEaten8days	Universal	22	Universal_A32
23	STD 1.56X10^7	Axenic	STD	Universal	23	Universal_A32
24	NTC	Axenic	NTC	Universal	24	Universal_A32
					25	Universal_A33
					26	Universal_A33
					27	Universal_A33
					28	Universal_A34
					29	Universal_A34
					30	Universal_A34
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					54	Universal_A42
					55	Universal_A43
					56	Universal_A43
					57	Universal_A43
					58	Universal_A44
					59	Universal_A44
					60	Universal_A44
					61	Universal_A45
					62	Universal_A45
					63	Universal_A45
					64	Universal_A46
					65	Universal_A46
					66	None
					67	Universal_STD 1.56X10^7
					68	Universal_STD 1.56X10^7
					69	Universal_STD 1.56X10^7
					70	Universal_NTC
					71	Universal_NTC
					72	Universal_NTC

2.10.2 | qPCR run sheet established for each run to identify the sample within each well

1	Universal_A25	9	Universal_A27	17	Universal_A30	25	Universal_A33	33	Universal_A35	41	Universal_A38	49	Universal_A41	57	Universal_A43	65	Universal_A46
2	Universal_A25	10	Universal_A28	18	Universal_A30	26	Universal_A33	34	Universal_A36	42	Universal_A38	50	Universal_A41	58	Universal_A44	66	None
3	Universal_A25	11	Universal_A28	19	Universal_A31	27	Universal_A33	35	Universal_A36	43	Universal_A39	51	Universal_A41	59	Universal_A44	67	Universal_STD 1.56X10^7
4	Universal_A26	12	Universal_A28	20	Universal_A31	28	Universal_A34	36	Universal_A36	44	Universal_A39	52	Universal_A42	60	Universal_A44	68	Universal_STD 1.56X10^7
5	Universal_A26	13	Universal_A29	21	Universal_A31	29	Universal_A34	37	Universal_A37	45	Universal_A39	53	Universal_A42	61	Universal_A45	69	Universal_STD 1.56X10^7
6	Universal_A26	14	Universal_A29	22	Universal_A32	30	Universal_A34	38	Universal_A37	46	Universal_A40	54	Universal_A42	62	Universal_A45	70	Universal_NTC
7	Universal_A27	15	Universal_A29	23	Universal_A32	31	Universal_A35	39	Universal_A37	47	Universal_A40	55	Universal_A43	63	Universal_A45	71	Universal_NTC
8	Universal_A27	16	Universal_A30	24	Universal_A32	32	Universal_A35	40	Universal_A38	48	Universal_A40	56	Universal_A43	64	Universal_A46	72	Universal_A46

2.10.3 | Statistics for the 16S rRNA gene amplicons sequenced for each honey bee gut dataset

Chapter	Title	Number of samples	Number of features	Total frequency	Minimum frequency	1st quartile	Median frequency	3rd quartile	Maximum frequency	Mean frequency
3	NZ sick & healthy honey bees	94	5,325	3,121,484	8,239	20,582	25,692	29,146	203,063	33,207
4	V4 NZ & USA	94	1,063	7,977,316	21,586	71,658	89,044	101,621	148,889	84,865
5	NZ Season	20	489	1706354	47,190	80,795	88,785	93,294	108,558	85,318
6	Carbohydrate diet	55	337	5,127,987	58,220	77,373	92,710	108,108	143,085	93,236
7	*G. apicola* vs *N. apis*	149	2,156	6,197,573	12,950	31,611	39,018	50,245	144,277	41,594

2.10.4 | 16S rRNA sequence counts for each honey bee sample associated with Chapters 3–5: identification of bacteria in NZ honey bees

	Chapter 3: NZ sick & healthy		Chapter 4: V4 NZ & USA		Chapter 5: NZ Season	
Sample	Sample name	Sequence count	Sample name	Sequence count	Sample name	Sequence count
1	HB26	116246	N23_S55	148,889	J1	108,558
2	HB36	114931	N22_S54	138,100	S2	102,501
3	NL26	110216	N34_S60	135,635	J4	101,789
4	C36	107044	W34_S24	130,147	D5	94,465
5	WN36	91117	HB22_S30	127,530	J2	93,484
6	O36	91002	HB23_S31	127,374	D4	93,231
7	W16	81898	N32_S58	126,277	J3	93,032
8	N36	71789	C34_S72	114,986	S5	91,169
9	N26	63237	HB34_S36	114,541	D3	90,858
10	HB16	42066	O24_S84	114,415	M1	89,983
11	N23	38366	W23_S19	111,412	D1	87,586
12	N34	37638	N11_S49	110,993	M4	87,408
13	HB23	37018	W33_S23	110,569	J5	86,261
14	N22	35769	HB14_S28	109,659	M2	85,517
15	HB22	34307	C23_S67	107,902	S4	82,996
16	W34	33973	HB11_S25	105,873	D2	74,192
17	W23	33361	N24_S56	105,580	M5	73,389
18	N32	33045	C22_S66	105,181	S3	63,682
19	C22	32574	W21_S17	104,412	S1	59,063
20	O24	31237	N21R_S53	103,827	M3	47,190
21	HB34	29636	N33_S59	103,598		
22	O21	29249	O21_S77	102,795		
23	C34	29157	NL34_S12	102,624		
24	HB14	29111	HB32_S34	102,392		
25	W33	28868	C11_S61	99,306		
26	C23	28469	W11R_S13	99,099		
27	N24	28125	W12_S14	98,641		
28	W12	28096	N13R_S51	97,793		
29	N11	27966	W14_S16	97,663		
30	HB11	27908	NL33R_S11	97,564		
31	C21	27889	NL12B_S1	97,338		
32	NL34	27874	NL22_S6	96,689		
33	W21	27803	C21_S65	96,499		
34	HB32	27555	W22_S18	96,159		
35	C24	27525	HB13R_S27	94,823		
36	N33	27241	HB33R_S35	94,779		
37	WN21	27195	W32_S22	94,429		

	Chapter 3: NZ sick & healthy		Chapter 4: V4 NZ & USA		Chapter 5: NZ Season	
Sample	Sample name	Sequence count	Sample name	Sequence count	Sample name	Sequence count
38	WN32	27052	W24_S20	94,047		
39	O22	27022	C24_S68	93,657		
40	O23	26941	O23_S79	91,957		
41	N13R	26616	C33_S71	91,542		
42	WN23	26462	C32_S70	91,263		
43	NL12B	26287	C14_S64	91,109		
44	N21R	26058	HB12_S26	90,972		
45	W14	26044	WN32_S46	90,746		
46	O34	25869	C13_S63	89,869		
47	W24	25515	NL24_S8	89,680		
48	NL22	25508	NL21B_S5	88,407		
49	NL33R	25135	O22_S78	87,653		
50	W11R	25043	N12_S50	87,283		
51	C32	24892	WN21_S41	87,192		
52	C13	24712	HB21R_S29	85,236		
53	HB13R	24641	O11_S73	84,164		
54	NL24	24576	O34_S83	84,046		
55	WN11R	24331	WN22_S42	83,949		
56	C14	24178	NL14B_S3	83,380		
57	W22	24027	HB24_S32	82,618		
58	HB12	23712	N14_S52	82,337		
59	WN22	23688	WN23_S43	81,034		
60	C11	23500	WN11R_S37	80,882		
61	W32	23473	W13R_S15	80,230		
62	NL21B	23449	NL23_S7	78,158		
63	N12	22734	O33_S82	77,529		
64	C12	22415	C12_S62	77,036		
65	C33	21995	W31_S21	76,278		
66	HB33R	21733	NL32_S10	75,492		
67	O11	21599	NL1580_S4	74,408		
68	O31	21108	O13_S75	72,926		
69	W31	20949	WN24_S44	72,574		
70	N31	20459	O31_S80	71,679		
71	HB24	20349	N31_S57	71,651		
72	HB21R	20140	O12R_S74	69,860		
73	N14	20053	NL13B_S2	67,838		
74	O33	19744	HB31R_S33	66,381		
75	NL1580	19461	WN34_S48	61,771		
76	NL14B	19254	WN33_S47	60,572		
77	NL23	19006	NL31_S9	60,133		
78	NL32	18871	WN14_S40	54,555		
79	HB31R	18733	C31_S69	53,946		
80	O12R	18461	WN13_S39	50,773		
81	NL31	18243	1.60E+04	49,317		

	Chapter 3: NZ sick & healthy		Chapter 4: V4 NZ & USA		Chapter 5: NZ Season	
Sample	Sample name	Sequence count	Sample name	Sequence count	Sample name	Sequence count
82	WN33	18201	O32_S81	49,100		
83	O13	18035	WN12_S38	48,572		
84	NL13B	17846	16P3	48,006		
85	WN24	17837	1.60E+06	47,012		
86	W13R	16901	WN31_S45	45,547		
87	WN34	16057	16P1	43,001		
88	WN14	15799	16P2	39,723		
89	WN12	15605	16P5	37,776		
90	O32	15098	1.60E+05	34,806		
91	C31	14212	16P4	34,390		
92	WN31	14052	1.60E+03	33,738		
93	WN13	8239	O14_S76	32,436		
94	O14		1.60E+02	21,586		

2.10.5 | 16S rRNA sequence counts associated with Chapters 6 and 7: effects of external factors on gut bacteria

	Chapter 6: Carbohydrate sources		**Chapter 7: *G. apicola* vs *N. apis***	
Sample	Sample name	Sequence count	Sample name	Sequence count
1	H5	143,085	GN2-4	144,277
2	IS3	132,700	GN2-5	90,753
3	SI9	131,287	GN3-1	88,023
4	MGO1	128,433	G3-4	86,718
5	MH15-1	123,080	GN2-3	72,745
6	MH17-8	117,134	GN4-1	69,958
7	H1	114,742	GN7-2	68,631
8	MH15-6	114,430	G3-3	67,438
9	SI4	113,983	GN7-3	67,188
10	MH15-2	110,972	N2-4	66,152
11	MH17-4	109,872	G1-2	65,956
12	MH15-3	109,858	B4-3	64,633
13	DHA6	109,684	GN1-4	64,567
14	SI6	108,725	N5-1	62,918
15	SI1	107,491	GN5-1	61,789
16	IS2	104,037	GN7-1	61,474
17	DHA3	103,263	G3-5	60,335
18	DHA5	102,117	B4-5	60,100
19	H2	99,618	B5-2	58,041
20	IS9	98,652	N4-4	56,397
21	SI3	98,650	G2-5	56,149
22	MGO9	98,421	G7-2	56,086
23	DHA7	97,043	GN3-4	54,818
24	H4	95,403	G5-2	54,588
25	MGO2	94,923	B1-1	54,438
26	MGO6	94,764	C7-4	54,330
27	MH15-7	93,838	GN3-5	54,188
28	SI2	92,710	N5-3	53,865
29	SI7	91,295	GN4-2	53,655
30	DHA4	90,075	G2-4	53,534
31	IS4	89,800	GN5-5	52,524
32	MH17-6	88,708	B6-3	51,548
33	MGO8	88,225	N5-2	51,495
34	MGO3	86,967	GN3-2	51,253
35	SI8	86,641	B1-3	50,681
36	SI5	86,221	G1-1	50,566
37	DHA9	86,008	GN5-2	50,363
38	DHA2	84,815	C7-2	50,245
39	MH15-8	79,232	N1-4	49,499
40	IS1	78,133	GN2-2	48,991

	Chapter 6: Carbohydrate sources		Chapter 7: *G. apicola* vs *N. apis*	
Sample	Sample name	Sequence count	Sample name	Sequence count
41	MH17-2	77,878	GN4-4	47,931
42	IS7	76,868	B5-1	47,494
43	DHA1	76,460	G1-3	47,263
44	MGO7	76,260	B6-2	46,987
45	MGO4	76,254	G5-3	46,467
46	DHA8	73,862	GN1-2	46,157
47	IS6	72,506	B2-1	46,005
48	MH17-9	67,233	GN5-3	45,779
49	IS8	66,459	G1-5	45,430
50	MH17-3	66,010	C7-1	45,286
51	MH15-4	65,861	N4-5	44,373
52	MH17-7	64,059	Comp1	44,005
53	H3	63,977	B6-5	43,893
54	MH17-1	61,045	B2-3	43,838
55	MH15-9	58,220	GN4-3	43,698
56			B6-1	43,314
57			G1-4	42,989
58			N6-1	42,665
59			G2-2	42,134
60			N4-1	42,109
61			B5-4	42,084
62			GN1-1	42,026
63			B3-5	41,773
64			G7-5	41,635
65			G5-4	40,636
66			GN3-3	40,396
67			B3-3	40,336
68			N5-4	40,148
69			B2-5	39,902
70			G2-1	39,741
71			N2-1	39,626
72			B5-5	39,610
73			GN5-4	39,412
74			GN7-5	39,388
75			B1-2	39,018
76			N6-5	38,908
77			N5-5	38,759
78			B1-5	38,626
79			B1-4	38,395
80			N1-3	38,352
81			B4-4	38,153
82			G5-5	38,055
83			C6-5	38,051
84			G3-2	38,042
85			B6-4	38,036

	Chapter 6: Carbohydrate sources		Chapter 7: *G. apicola* vs *N. apis*	
Sample	Sample name	Sequence count	Sample name	Sequence count
86			N6-4	37,438
87			B5-3	37,268
88			G7-1	36,833
89			C4-1	36,248
90			GN2-1	36,011
91			C7-5	35,992
92			N2-3	35,847
93			B2-6	35,717
94			N4-3	35,532
95			G5-1	35,352
96			C4-4	35,319
97			GN1-3	35,172
98			N3-1	35,138
99			Comp2	35,062
100			N6-2	34,776
101			GN4-5	34,555
102			B2-2	33,630
103			N6-3	33,223
104			B3-2	32,721
105			C5-4	32,600
106			B4-1	32,402
107			N1-2	31,978
108			C5-1	31,890
109			B3-4	31,785
110			C7-3	31,633
111			C1-1	31,628
112			G2-3	31,611
113			C4-3	31,456
114			N1-1	31,287
115			B3-1	30,993
116			Beegut	29,881
117			C5-5	29,874
118			C6-4	29,242
119			N3-3	28,952
120			C2-3	28,426
121			GN1-5	28,309
122			N2-5	27,848
123			N4-2	27,800
124			B4-2	27,565
125			N3-5	26,762
126			C2-2	26,207
127			N1-5	25,859
128			C6-1	25,339
129			C1-2	25,152
130			C5-2	24,481

	Chapter 6: Carbohydrate sources		Chapter 7: *G. apicola* vs *N. apis*	
Sample	Sample name	Sequence count	Sample name	Sequence count
131			G7-3	24,422
132			C2-5	24,353
133			C6-2	23,817
134			C4-2	23,109
135			N3-4	22,991
136			G7-4	22,825
137			C2-4	21,853
138			G3-1	21,036
139			C5-3	20,711
140			C1-3	20,687
141			G20	20,428
142			C2-1	19,835
143			C1-5	19,180
144			GN7-4	19,165
145			N2-2	18,106
146			N3-2	17,684
147			C4-5	16,931
148			C6-3	16,862
149			C1-4	12,950

2.10.6 | Quantitation report for the standard curve production of *Nosema apis and Nosema ceranae*

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Experiment Information

Run Name	*Nosema ceranae* 2016-09-28 (2)
Run Start	28/09/2016 12:29:54 pm
Run Finish	28/09/2016 1:34:56 pm
Operator	James
Run On Software Version	Rotor-Gene 1.7.87
Run Signature	The Run Signature is valid
Gain Green	5.33
Gain Yellow	10.

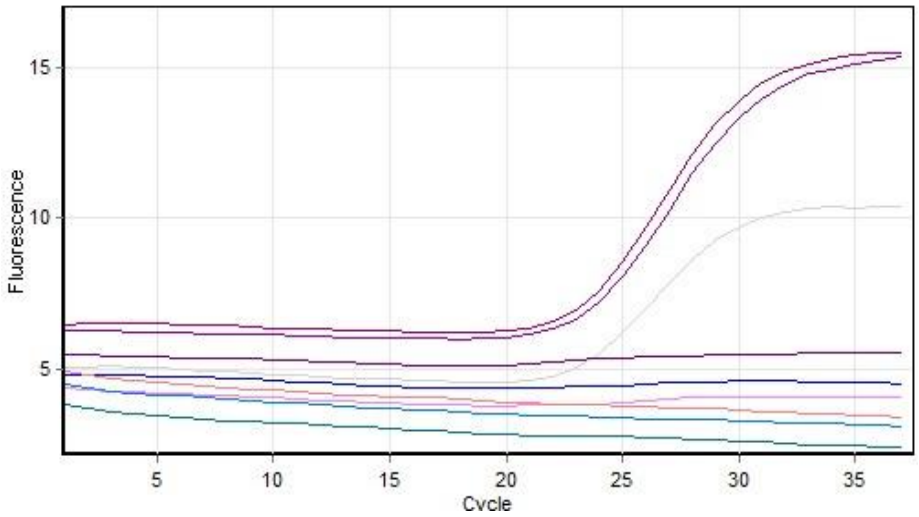
Quantitation Information

Threshold	0.00136
Left Threshold	18.530
Standard Curve Imported	Yes
Standard Curve (1)	conc= 10^(-0.217*CT + 11.419)
Standard Curve (2)	CT = -4.600*log(conc) + 52.526
Reaction efficiency (*)	0.64968 (* = 10^(-1/m) - 1)
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	0%
Reaction Efficiency Threshold	Disabled
Normalisation Method	Standard
Digital Filter	Light

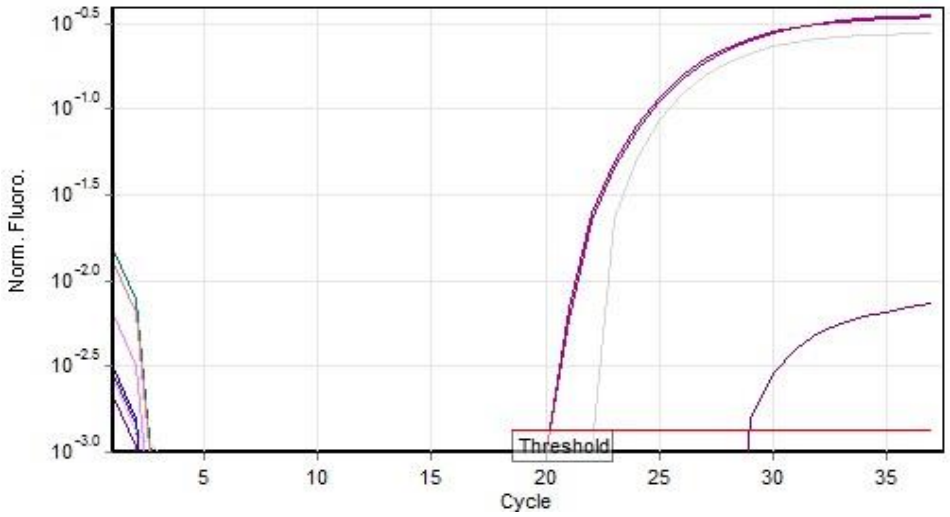
Profile

Cycle	Cycle Point
Hold at 95°C, 3 min	
Cycling (37 repeats)	Step 1 at 95°c, hold 10 s
	Step 2 at 60°C, hold 25 s, acquiring to Cycling A([Green][1][1],[Yellow][2][2])
Melt (72-95°C) , hold 0 s on the 1st step, hold 5 s on next steps, MeltA([Green][1][1],[Yellow][2][2])	

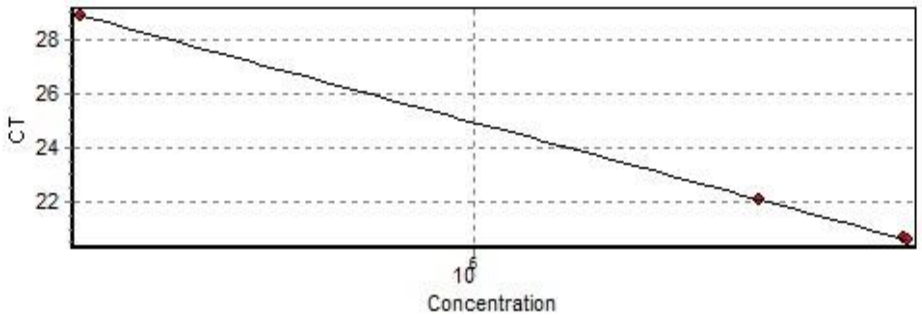
Raw data for Cycling A. Green



Quantitation data for Cycling A. Green



Standard Curve



No.	Colour	Name	Type	Ct	Given Conc (copies/reaction)	Calc Conc (copies/reaction)
58		NC 10-6	Standard	20.63	6.80E+06	8.60E+06
59		NC 10-6	Standard	20.58	6.80E+06	8.80E+06
60		NC 10-6	Standard	22.08	6.80E+06	4.16E+06
67		Michelle - NC	Unknown			
68		Michelle - NC	Unknown	28.87		1.39E+05
69		Michelle - NC	Unknown			
70		NTC	NTC			
71		NTC	NTC			
72		NTC	NTC			

Legend:
NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

Chapter 3

Analysis of honey bee gut microbial profiles across NZ, and a comparison between healthy and sick bee colonies

3.1 Abstract

The bacterial profiles in the guts of New Zealand honey bees were identified from 94 colonies located in 21 apiaries across seven regions. Ten of these colonies were deemed 'sick' by beekeepers. Environmental measures were scored for each apiary including the terrain surrounding the apiaries, classification of the pollen and nectar sources available throughout the year, the length of time the colonies were located at the apiary, and the age of the queen bee. These were assessed collectively with the taxonomic classification of bacterial phylotypes identified using 16S rRNA gene sequencing. The bacterial composition in bees foraging from native plants or mānuka specifically, were found to be distinct from those foraging on exotic floral sources. Twenty-seven bacterial phylotypes were identified including the five dominant core phylotypes present in bees internationally. Rhizobiaceae was present in 100% of the sick colonies but only 27.5% of the healthy colonies from the same apiaries. Eight phylotypes were present only in the sick colonies, two of which may be potential indicators of poor bee health: Serratia and Acetobacter. This study also identified that the gut pathogen *Nosema apis* was associated with all sick colonies but Nosema ceranae, which was recently introduced to New Zealand, was only present in a single colony from the Nelson region, indicating that *N. ceranae* is currently not widespread in New Zealand honey bees.

3.2 Introduction

Individual honey bee (*Apis mellifera* L) colony mortality is often attributed to a single factor including a pest or pathogen (Watanabe 1994; Winfree et al. 2007), pesticide poisoning (Naug 2009), or poor hive management such as poor disease control, or lack of food resource (Smart 2015). In contrast, it is difficult to attribute widespread regional colony loss to a single cause, as observed with the 2007/2008 Colony Collapse Disorder in the United States of America (VanEngelsdorp et al. 2009). It is therefore suggested that widespread colony mortality is more likely to result from multiple factors including bee pathogens, colony management, and environmental factors (Naug 2009; Currie et al. 2010; Neumann and Carreck 2010; Smart 2015). However, the interdependencies between these factors and their effects on colony loss are not well understood. One variable in the honey bee ecosystem that may provide further insight as to how these factors may be linked and how they may effect bee health, is the bacterial community within the honey bee digestive tract (gut). The social and foraging behaviour of bees implies that the gut not only harbours bacteria (Powell et al. 2014) but is the conduit for nutrient sources, antibiotics and poison, and the ingress for gut pathogens like *Nosema* spp., (the spore-forming unicellular fungi from the phylum microsporidia).

The initial body of literature regarding bacteria sequenced from the honey bee gut was primarily from North America and Europe and the data were predominantly collected from a limited number of healthy colonies in one or two apiaries (Mohr and Tebbe 2006; Babendreier et al. 2007; Cox-Foster et al. 2007; Olofsson and Vásquez 2008; Vasquez and Olofsson 2009; Martinson et al. 2011; Mattila et al. 2012). These restricted apiary datasets have helped identify the five dominant core bacterial phylotypes: two Proteobacteria (*Gilliamella apicola* and *Snodgrassella alvi* (Kwong and Moran 2013)), two species clusters of Firmicutes (*Lactobacillus* Firm-4; *Lactobacillus* Firm-5 (Babendreier et al. 2007; Martinson et al. 2011)); and an Actinobacteria species cluster (*Bifidobacterium* (Bottacini et al. 2012)). They have also identified other less consistently present or abundant phylotypes including *Frischella perrara* (Engel et al. 2013b), *Bartonella apis* (Kešnerová et al. 2016), *Parasaccharibacter apium* (Corby-Harris et al. 2014b), and a *Gluconobacter*-related species group designated Alpha2.1 (Martinson et al. 2011). Studies such as the comparison between the gut bacterial community profiles from two apiaries in Arizona and Maryland (total 40 bees) indicate that bacterial communities, although predominantly similar, do differ among apiaries, colonies, and individual bees (Moran et al. 2012). Individual community members are influenced by landscape exposure (Jones et al. 2018b), and are shaped by the interaction with the broader gut community (Kwong et al. 2014). Additionally, antibiotics have been shown to reduce the diversity of the core bacterium *G. apicola* (Raymann et al. 2018a), and sucrose-rich diets have been shown to increase the relative abundances of sub-dominant core bacteria (Rhizobiaceae, Acetobacteraceae, and *Lactobacillus kunkeei*) and decrease the relative abundance of *F. perrara* (Taylor et al. 2019). Similarly, colonies deemed 'sick' (i.e. with a slow population build up in spring) have been observed with less bacterial phylotypes than colonies deemed healthy (Ribière et al. 2019). Therefore, it should not be presumed that the literature typifies the bacterial profile within the digestive tract of honey bees worldwide, and specifically from countries that are geographically isolated, or from colonies that are deemed sick.

New Zealand (NZ) honey bees have been largely bred in isolation, and thus potentially developed their own gut microbiome. Initially, undocumented strains of honey bees were introduced from England (1839 and 1842) and Australia (1842) (Hopkins 1926). Italian honey bees were introduced from California in 1880, and further importations occurred in 1883 from Italy, Switzerland, Syria, Israel, Cyprus, along with the introduction of Carniolan bees (Hopkins 1926). In 1960 the importation of live bees, hive ware, and bee products to NZ were prohibited (Stevenson et al. 2005). Since then the only importation of genetic stock was Carniolan semen during 2005 and 2007. This introduction is a likely source of the gut pathogen *Nosema ceranae* as prior to this *N. ceranae* was not identified in NZ bees (Klee et al. 2007; Huang et al. 2008; Botías et al. 2012).

It is therefore possible that the gut bacteria within bees imported to NZ may have since evolved separately so that the bacterial profiles appear distinct from the current phylotypes described internationally. If this has occurred then the role of dysbiosis within NZ or international bees also needs to be elucidated. Factors specific to NZ that may influence bacterial composition include the floral nectars native to NZ (e.g. mānuka and rātā), the availability and variation among nectar sources throughout the year, the prohibition of antibiotics for disease management (American Foulbrood) (New Zealand Government 1998), and the absence of some honey bee gut pathogens (e.g. European foul brood, and the recently introduced *Nosema ceranae* (Botías et al. 2012). Understanding the profiles of gut bacteria in NZ honeybees is particularly relevant for the Canadian beekeeping industry as for the past few decades they have annually imported between 5,000 and 40,000 one-kilogram packages of NZ honey bees to bolster the Canadian beekeeping industry in spring (New Zealand Government 2018).

Identifying differences in bacterial profiles among studies is often rendered difficult by the disparate methodology of individual studies. Widespread patterns of bacterial profiles within honey bee populations across entire land masses may therefore provide additional information regarding the effect of external factors on gut bacterial profiles and in turn may elucidate the identification of bacteria that could be used as indicators of colony health.

We hypothesise that the honey bee gut bacterial community will vary when the hosts have evolved as an isolated population. Our first objective was to identify the variation in bacterial communities in terms of phylotypes and relative abundances within the guts of honey bees located in seven geographically different regions throughout NZ. The second objective determined whether the surrounding environment of the apiaries, as scored by the associated beekeepers, correlated with differences between bacterial compositions. The third objective was to compare the bacterial composition in the gut of honey bees from colonies deemed by beekeepers as ‘healthy’ and ‘sick’; a colony was defined as ‘sick’ when the bee population had a comparatively slow development in spring, as often attributed to two common honey bee gut microsporidia *Nosema apis* Zander (Zander 1909), and *Nosema ceranae* Fries (Fries et al. 1996; Ribière et al. 2019).

3.3 Materials and methods

3.3.1 Honey bee sampling

Honey bees were collected from five colonies from three apiaries from each of seven geographically and environmentally divergent regions within New Zealand: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Christchurch (C) and Otago (O) (Figure 3.1). All samples were collected between the 6th and 23rd October 2014 (late spring). The 21 apiaries ranged in latitude from 45.854°S to 37.698°S, longitude from 168.930°E to 173.015°E, and altitude from 0m to 3706m. The colonies had almost all been located at these apiaries, and therefore feeding on the local floral sources, for at least one year. The exception was a single apiary in Otago (O1) where the colonies were introduced six weeks prior to bee collection. Each apiary was managed by a different commercial beekeeper according to their own management techniques.

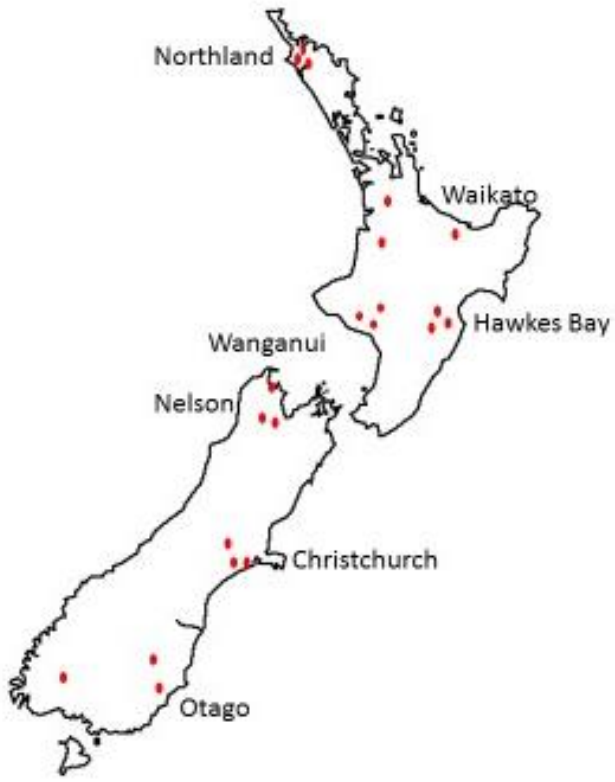


Figure 3.1 | Locations of the 21 apiaries throughout New Zealand from which adult honey bees were sampled from five hives in October 2014.

Bees were sampled from the frames surrounding the brood-nest. Four of the colonies from each apiary were deemed healthy by the beekeepers (total 84 colonies) and one colony from each was deemed sick. However, the details regarding some of the samples were insufficient so only ten sick colony samples were included in the analysis:

one sample from each of the three Hawkes Bay apiaries (HB16, HB26, HB36), one sample from each of the other six regions (NL26, W16, WN36, N26, C36, O36), and a second sample from the Nelson region (N36). The bee samples were immediately stored in 95% ethanol on ice and sent by overnight courier to The New Zealand Institute for Plant and Food Research Limited (PFR), Hamilton, NZ. On arrival, these samples were stored at -70°C.

Metadata were collected regarding the surrounding environment and hive management: apiary location, altitude, the number of spring pollens and nectars, the types of spring pollens and nectars, the type of winter pollen the presence of native bush, the presence of mānuka, the duration that the hives had been at the apiary, and the age of the queen bee.

3.3.2 DNA extraction, amplification, and sequencing of the 16S rRNA gene

DNA was extracted from the honey bee guts and processed as below for analysis of the gut bacterial community. For each of the 94 samples, 20 bees were thawed for 3 min and then the digestive tracts (crop to rectum) were aseptically dissected and pooled in a single DNase- and RNase-free 1.5 ml microcentrifuge tube on ice. Each pooled sample was macerated by pipetting the material up and down, grinding it with a pipette tip for 30 s and then 150 mg (28% of the pooled sample) was transferred to a ZR BashingBead™ Lysis Tube containing 750 µl lysis solution. The samples were homogenised at 6 m/s for 40 s using a FastPrep®–24 (MP Biomedicals, Seven Hills, Australia), and the DNA was extracted using the Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit (Zymo Research Corporation (ZR), California, USA) and the recommended protocol. The eluted DNA samples were stored at –70°C then overnight couriered on ice to Massey Genome Service (Palmerston North, NZ) for 16S rRNA gene sequencing of the V3V4 hypervariable region (Kozich et al. 2013).

A dsDNA HS Assay Kit (12 samples per plate) was used to evaluate the DNA concentration in each sample with Qubit™ 2.0 Fluorometer (ThermoFisher Scientific, NZ) analysis. A PCR reaction was performed using primers with adaptors:

16Sf_V3 (5' - 3' direction) – CCTACGGGAGGCAGCAG and,

16Sf_V4 (5' - 3' direction) – GTGCCAGCMGCCGCGGTAA (Kozich et al. 2013).

A library was generated from the purified PCR products (c. 420-440 base pairs) and their concentrations were analysed using Qubit™. The products were pooled in equimolar concentrations and the concentration and size were confirmed by analysis with Qubit™ and LabChip (PerkinElmer, Waltham, MA, USA). The PCR products were sequenced with a 250-base paired end run on an Illumina MiSeq™ platform (Illumina Inc.) with version 2 chemistry. Illumina PhiX Control v3 (FC-110-3001) was the control. The resulting sequences were used for analysis of bacterial composition and were also deposited in the National Center for Biotechnology Information's (NCBI's) Sequence Read Archive (PRJ TBC).

The healthy samples were dissected, their DNA was extracted, and the samples were sequenced in March 2015 whereas the sick samples were processed and sequenced in September 2017.

3.3.3 Gene sequence processing and characterisation of microbial communities

From here on, the dataset of the healthy NZ honey bee samples is referred to as 'healthy' and the dataset of the sick and healthy NZ honey bee samples is referred to as the 'combined' dataset.

The Illumina de-multiplexed fastq sequence data were processed and trimmed using ea-utils with a 0.01 probability of error and an equivalent Phred score of Q20 (Aronesty 2011). Further processing was conducted with Quantitative Insights Into Microbial Ecology 2 (QIIME 2) analysis suite, version 2018.2 (Caporaso et al. 2010) (https://github.com/PlantandFoodResearch/bioinf_Apis_metabarcoding). To produce exact amplicon sequence variants (ASVs) the paired-end sequences were filtered, trimmed, dereplicated, and the chimeras were filtered using DADA2 methodology (Callahan et al. 2016). The majority of less abundant bacteria were included in the analysis by filtering out sequences with a minimum total read composition < 0.01% prevalence. A total of 3,121,484 paired reads were detected across the 21 apiaries. These were identified as 625 ASVs, with a minimum of 8,239 frequencies and a maximum of 203,063 frequencies.

To ensure current taxonomic classification of honey bee gut bacteria was being used, the customised QIIME 2 compatible reference dataset (https://github.com/pjbiggs/16SrRNA_taxonomy) (Taylor et al. 2019) was used to create a biological observation matrix (BIOM) that contained the ASVs. The nucleotides within the sequence regions from the sick colonies were 100% homologous to those from the healthy colonies except they were truncated by approximately 40 nucleotides (Supplementary material Figure 3.8.1).

The α–diversity for the feature levels health and apiary were calculated using MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017) with the metrics Observed ASVs (simple richness), Chao1, Shannon, and Simpson. β–diversity was determined for the experimental factors health and apiary using the Bray-Curtis dissimilarity method. 3D plots of Principal Coordinates Analysis (PCoA) were used to present β–diversity for the metadata (11 external factors).

3.3.4 Analysis of *Nosema* spp.

The presence of the gut pathogens *N. apis* and *N. ceranae* were identified in the sick colonies using qPCR (Rotor-Gene 6000, QIAGEN, Venlo, The Netherlands) and hydrolysis probe assays (TaqMan®) (dnature, Gisborne, NZ); 5 µl of 2X Mastermix was combined with 2.5 µl water and 0.5 µl 20X primer probe mix. 8 µl of this was added to

each well as was 2 µl of DNA template and the qPCR run was conducted using the recommended protocol (dnature, Gisborne, NZ). An assay cut-off of C_q < 37 cycles was deemed positive. Each qPCR run contained three technical replicates of DNA sample, no-template controls, and *N. apis* DNA standard. The *N. apis* standard was extracted from a *N. apis* spore suspension (3.085 x 10^8 spores/ml) prepared from ten bees from each of three colonies; thirty abdomens were crushed in 30 ml of sterile deionised water then mixed thoroughly with a sterile 18G needle. The suspension was filtered through a 70 µm sterile Falcon cell strainer to remove bee guts, then centrifuged at 5000 *g* for five minutes. The supernatant was removed and the spores were washed in 500 µl sterile water. The wash was repeated twice to produce a *Nosema* suspension which should have had approximately 85% purity according to Fries et al. (2013). The purified spores were re-suspended in 4 ml of sterile water and the spore concentration was determined using a haemocytometer (1/400 mm 2 x 1/10 mm deep, Improved Neubauer, USA) (Cantwell 1970); number of spores / ml = (average total number spores counted / 80) x $4x10^7$ = 506 spores x 500,000 = 253,000,000. DNA was extracted from two 150 µl aliquots of this suspension using a Zymo Research Fecal DNA Miniprep kit (Catalog No D6010, Zymo Research, Irvine, CA, USA). As *N. ceranae* was recently introduced to NZ (Klee et al. 2007), the sick colonies were analysed for *N. ceranae* using a *N. ceranae* – specific TaqMan® (dnature, Gisborne, NZ). The *N. apis* standard also served as a negative control to confirm the absence of cross-reaction between *Nosema* species.

3.3.5 Statistical analyses

Bacterial diversity computed in QIIME2 was measured within a sample (α–diversity), and between samples (β–diversity) using MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017). Data counts were unfiltered but the dataset was rarefied to its minimum library size of 8016. The significance in α–diversity metrics were calculated at the feature levels health and apiary using Mann-Whitney/Kruskal-Wallis pairwise comparisons. A permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) of the Bray-Curtis dissimilarity measures was used to determine the significance in β–diversity of colony health and apiary identity.

Further statistical analysis was conducted in R (version 3.5.1) (R Core Team 2018). Sequences with a minimum total read composition of < 0.01% prevalence were filtered from the dataset. This low threshold ensured the inclusion of the majority of less abundant bacteria in the analysis. To investigate the differences in the number of phylotypes among regions and apiaries, Poisson generalised linear models were used with the number of phylotypes as the response, and region or apiary as a fixed effect. To explore the relationship between phylotypes and apiaries, of which apiary is a factor of region, the data were visually explored using heat maps, where the response was

the sum of the total number of bacterial reads for each phylotype. The interaction of the phylotypes in relation to apiary was further explored using nonmetric multidimensional scale (NMDS) plots where the dissimilarity matrix was calculated using the Bray-Curtis dissimilarity method. The change in relative abundance (proportion of total bacterial abundance) of phylotypes from sick and healthy colonies within apiaries was explored using a simple linear model (lm) in the R package lme4 (Bates et al. 2014). The function 'predictmeans' was used to plot predicted means to enable comparison between apiaries for each phylotype and an ANOVA was used to determine significant differences among apiaries.

3.4 Results

The DNA of the 84 healthy NZ honey bee samples contained 2,070,941 paired reads and the DNA of the 10 sick NZ bee samples contained 1,050,543 paired reads, totalling 3,121,484 paired reads. The reads were filtered to remove < 0.01% prevalence, so the total number of paired reads analysed in all 94 samples was 3,021,057.

3.4.1 Number of amplicon sequence variants and phylotypes

The paired reads from the healthy and sick honey bee samples represented 625 ASVs (Table 3.1). The ASVs within the samples from the healthy colonies were classified as 19 unique phylotypes, excluding the unclassified phylotypes which are referred to simply as Bacteria and depending on the apiary, 15 to 24 phylotypes were present (Table 3.1).

Table 3.1 | The number of unique ASVs and the number of associated bacterial phylotypes within the digestive tract of healthy and sick New Zealand honey bees.

Apiary	ASVs (Healthy)	ASVs (Combined)	Phylotypes (Healthy)	Phylotypes (Combined)
NL1	339	339	18	18
NL2	**359**	**436**	**18**	**24**
NL3	342	342	16	16
W1	**374**	**449**	**14**	**18**
W2	352	352	15	15
W3	405	405	19	19
WN1	340	340	17	17
WN2	326	326	16	16
WN3	**330**	**413**	**16**	**22**
HB1	**368**	**433**	**18**	**24**
HB2	**358**	**446**	**17**	**23**
HB3	**362**	**439**	**15**	**22**
N1	357	357	16	16
N2	**342**	**408**	**14**	**20**
N3	**336**	**413**	**17**	**23**
C1	**320**	**320**	**17**	**17**
C2	363	363	16	16
C3	**363**	**440**	**17**	**22**
O1	**307**	**307**	**14**	**14**
O2	314	314	15	15
O3	306	376	15	21
Total	**625**	**625**	**20**	**28**

Healthy = Healthy dataset. Combined = Sick and healthy dataset. Bee samples were collected from 21 apiaries located in seven regions: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Canterbury (C), and Otago (O). Apiaries that include sick bees are shaded in grey. Variation between the number of ASVs in the healthy and combined datasets are in bold typeface.

Eleven phylotypes within the gut of NZ honey bees were identified as unique species: *Bifidobacterium coryneforme*, *Lactobacillus kunkeei*, *Lactobacillus mellifer, Lactobacillus mellis*, *Ensifer adhaerens*, *Snodgrassella alvi*, *Frischella perrara*, *Gilliamella apicola*, *Escherichia coli*, *Spiroplasma apis*, and *Spiroplasma mirum.* The remaining eight were identified to the taxonomic levels of phylum (Cyanobacteria and Proteobacteria), class (Clostridiales), family (Acetobacteraceae, Rhizobiaceae, and Enterobacteriaceae), or genera (*Lactobacillus* and *Pseudomonas*). These included the internationally identified dominant core species *G. apicola*, *S. alvi*, the genus *Bifidobacterium*, and species from the *Lactobacillus* Firm–4 cluster (*L. mellis*, *L. mellifera*, and *L. kunkeei*). *Bifidobacterium coryneforme* was present but the internationally identified species *Bifidobacterium asteroides* was absent. None of the species from the *Lactobacillus* Firm–5 cluster were found.

Table 3.2 | Number of unique ASVs associated with each bacterial phylotype identified from the healthy and combined datasets from New Zealand honey bees.

Bacterial phylotype	Healthy ASVs	Combined ASVs
Actinobacteria (P)		
Bifidobacterium coryneforme *****	20	24
Bacteroidetes (P)		
Flavobacteriaceae (F)		
Chryseobacterium spp.	–	1
Cyanobacteria (P)	59	66
Firmicutes (P)		
Bacillaceae (F)		
Bacillus pumilus	–	1
Lactobacillaceae (F)		
Lactobacillus spp. *****	126	160
Lactobacillus kunkeei	7	8
Lactobacillus mellifera	8	9
Lactobacillus mellis	12	17
Clostridiales (O)	11	12
Lachnospiraceae (F)		
Lachnoclostridium spp.	–	1
Proteobacteria (P)	13	13
Alpha–proteobacteria (C)		
Acetobacteraceae (F)	26	32
Acetobacter spp.	–	1
Rhizobiales (O)		
Rhizobiaceae (F)	6	7
Ensifer adhaerens	11	12
Beta–proteobacteria (C)		
Neisseriaceae (F)		
Snodgrassella alvi *****	48	63
Gamma–proteobacteria (C)		
Pseudomonas spp.	6	8
Yersiniaceae (F)		
Serratia spp.	–	1
Orbaceae (F)		
Frischella perrara ******	23	29
Gilliamella apicola *****	90	112
Enterobacteriaceae (F)	4	12
Providencia spp.	–	1
Cronobacter sakazakii	–	2
Escherichia coli	4	5
Citrobacter freundii–complex	–	1
Tenericutes (P)		
Spiroplasma apis	10	12
Spiroplasma mirum	6	6
Bacteria (Domain)	9	9
Total (including the domain Bacteria)	**20**	**28**

Healthy = dataset of healthy colonies. Combined = dataset of sick and healthy colonies. Phylum (P), class (C), order (O), family (F), genus (spp.). * indicates core gut bacteria. To aid taxonomic identification higher taxonomic levels without ASVs are shown in bold typeface.

The genus *Lactobacillus* contained the most ASVs (126). The relative abundance of ASVs from *Lactobacillus* species associated with cluster Firm–4 (*L. mellis* (12 ASVs), *L. mellifera* (8 ASVs), and *L. kunkeei* (7 ASVs)) were present in 50% of the healthy colonies from 17 apiaries and ranged from 0.005–10.4% of all reads. *L. apis*, *L. melliventris*, and *L. helsinborgensis* from the *Lactobacillus* cluster Firm–5 were all present but all were below the 0.01% relative abundance threshold, whereas *L. kimbladii*, and *L. kullabergensis*, were not detected. Of the subdominant core bacteria, the species *Frischella perrara* was present (23 ASVs) but *Bartonella apis* was not identified although six ASVs from the same family Rhizobiaceae were identified. The Gluconobacter–related species group, to which *Parasaccharibacter apium* belongs, were also not identified but 26 ASVs from the family Acetobacteraceae were present.

The sick samples contained eight more phylotypes than the healthy samples: three in the family Enterobacteraceae (*Providencia* spp., *Citrobacter freundii*–complex, and *Cronobacter sakazakii*), and one from each of the families Acetobacteraceae (*Acetobacter* spp.), Bacillaceae (*Bacillus pumilus*), Flavobactericeae (*Chryseobacterium*), Lachnospiraceae (*Lachnoclostridium* spp.), and Yersiniaceae (*Serratia* spp.) (Table 3.2). With the exception of *Serratia* spp. (10.4%) and *C. sakazakii* (7.8%), these phylotypes were less prevalent and their relative abundances were much lower (≤ 2.2%) than the core bacteria. The honey bee gut bacterium *Paenibacillus larvae* subspecies *larvae* (Bacteroidetes), the causative agent of American foulbrood disease that is normally identified in larvae or squashed adult bee samples, was absent from all 94 samples.

The Poisson generalised linear model provided no evidence that the number of phylotypes differed among the 84 healthy colonies ($P > 0.8$) but with the addition of the sick colonies a significant difference was observed among the 94 colonies ($P < 0.01$) (Supplementary material Table 3.8.1).

The total number of reads for each phylotype present in the gut of healthy and sick honey colonies shows that the phylotypes *Lactobacillus* spp. and *G. apicola* were the most prevalent but that the relative abundance of both varied among apiaries (Figure 3.2). In contrast the total number of reads for *Spiroplasma mirum*, *S. apis*, *Serratia*, Rhizobiaceae, *Pseudomonas*, *Providencia*, *L. kunkeei*, *Lachnoclostridium*, *Escherichia coli*, Enterobacteriaceae, *Ensifer adhaerens*, *Cronobacter sakazakii*, *Citrobacter freundii*–complex, *Chryseobacterium*, *Bacillus. pumilus*, *Acetobacter*, and the unclassified Bacteria, were below the R-analysis 0.01% inclusion threshold in some apiaries (Figure 3.2).

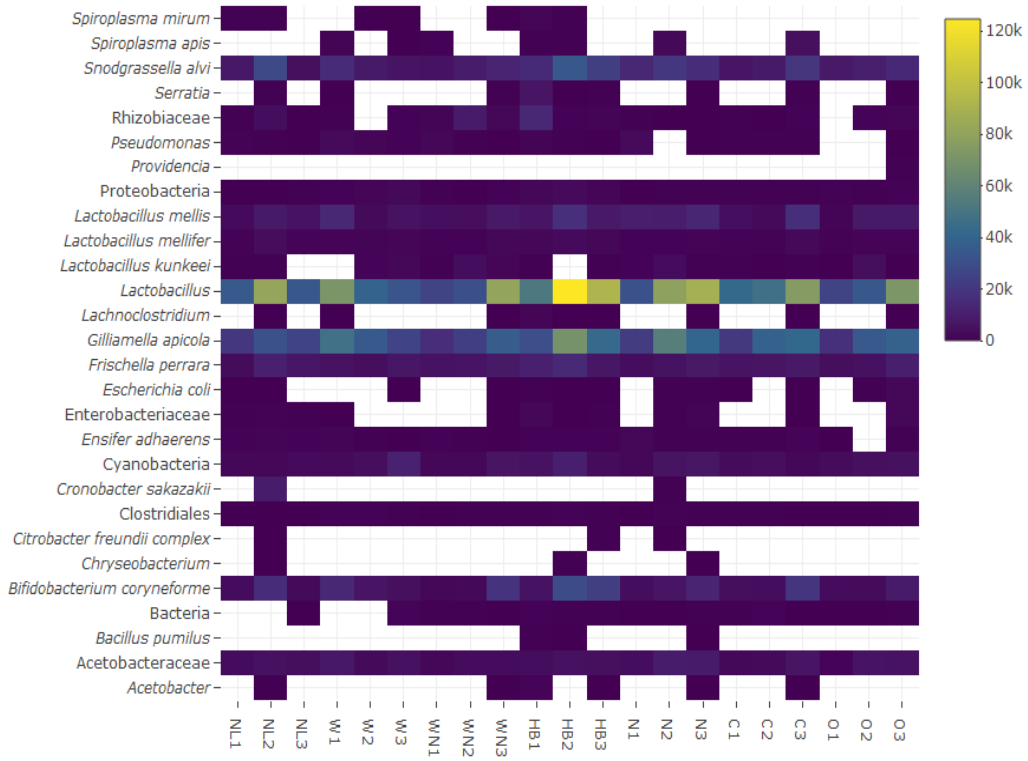


Figure 3.2 | Heatmap of the sum of the total number of reads for each bacterial phylotype in the digestive tract of honey bees from 21 apiaries throughout New Zealand.

The data from the healthy and sick colonies within each apiary were combined. All italicised phylotypes without a species name indicate the genus e.g. *Lactobacillus* indicates members of the genus *Lactobacillus* but which were genetically distinct from the named species in this genus e.g. *L. mellis*. Blank spaces indicate the relative abundance was < 0.01%. Apiary locations: Northland (NL1–3), Waikato (W1–3), Hawkes Bay (HB1–3), Whanganui (WN1–3), Nelson (N1–3), Canterbury (C1–3), Otago (O1–3).

3.4.2 Relative abundance

The mean relative abundance of the top eight abundant phylotypes in the healthy colonies included the dominant core phylotypes; *Lactobacillus* spp. which was the most abundant (31–46.7%), followed by *G. apicola* (17.5–2.5%) and *S. alvi* (4.5–12.6%), and *B. coryneforme* (2.0–7.3%) was sixth most abundant (Table 3.3). A similar pattern of abundance was observed in most apiaries to that of apiary C1 where the relative abundance of *Lactobacillus* spp. was double that of *G. apicola*, 7–fold that of *S. alvi*, and 11–fold that of *Bifidobacterium*. The subdominant core bacteria represented < 10% of the bacterial profiles, where the relative abundance of *Lactobacillus* spp. was 7–fold higher than *F. perrara*, 11–fold higher than *L. mellis*, and 17–fold higher than Acetobacteraceae. The relative abundance of the remaining 13 phylotypes contributed < 5% of the bacterial profile. The exceptions to this pattern were Cyanobacteria in apiary W3 (9.8%) which was 2–fold higher than in apiary WN3 (4.6%), Rhizobiaceae in apiary WN2 (5.6%) which was 4–fold higher than in apiary WN3 (1.4%), and *S. apis* in apiary C3 (9.6%) which was 2–fold higher than in apiary WN1 (4.5%) (Table 3.3).

Table 3.3 | Mean relative percent abundance of phylotypes in the digestive tract of healthy honey bees from 21 New Zealand apiaries.

Phylotype	NL1	NL2	NL3	W1	W2	W3	WN1	WN2	WN3	HB1	HB2	HB3	N1	N2	N3	C1	C2	C3	O1	O2	O3
Lactobacillus spp.	43.1	41.1	39.3	39.2	35.5	31.0	35.4	32.7	42.2	33.1	35.5	41.1	31.0	39.8	43.2	**46.7**	40.4	39.8	40.7	30.7	39.5
Gilliamella apicola	23.9	17.5	27.7	26.6	31.2	24.4	22.9	24.8	24.3	19.6	26.0	24.3	23.4	29.3	22.3	22.5	32.7	22.5	26.1	31.2	25.4
Snodgrassella alvi	7.4	12.6	4.8	7.5	7.1	5.2	8.3	8.8	4.5	9.4	9.5	10.0	**12.9**	9.0	5.2	6.6	6.5	9.2	10.5	8.3	7.8
Frischella perrara	4.0	6.5	6.7	2.9	3.5	5.9	**6.7**	6.0	5.2	6.5	5.4	3.6	3.5	2.6	3.5	6.4	5.2	4.3	6.1	3.9	5.4
Lactobacillus mellis	3.1	2.9	5.4	5.0	2.5	4.5	5.0	3.6	4.0	3.6	3.2	2.5	9.7	3.3	5.5	4.1	2.4	4.1	1.8	6.1	3.3
Bifidobacterium coryneforme	3.8	5.0	3.0	3.1	5.4	3.9	3.2	2.6	4.9	3.3	5.3	**7.3**	2.9	2.0	4.1	4.0	3.0	5.3	3.8	2.8	4.3
Acetobacteraceae	3.3	2.5	4.2	4.5	2.5	4.3	2.4	2.6	1.6	1.8	1.4	1.8	3.1	4.2	3.7	2.7	2.4	2.6	1.5	**5.2**	3.0
Cyanobacteria	1.8	0.9	2.8	1.7	3.3	**9.8**	1.7	2.0	4.6	2.9	4.6	1.5	1.7	2.8	3.6	3.3	3.1	1.2	3.9	3.6	3.2
Lactobacillus mellifer	0.9	1.2	1.0	0.6	1.1	**1.2**	1.1	0.8	0.6	0.9	0.8	0.8	0.7	0.3	0.4	0.6	0.6	0.9	1.1	0.8	0.6
Pseudomonas spp.	**3.9**	0.2	0.0	1.3	6.0	0.3	2.4	0.4	0.1	0.8	0.1	0.0	3.5	<	0.0	0.3	1.8	0.2	<	<	0.0
Ensifer adhaerens	1.2	0.6	0.6	0.7	0.3	0.1	0.9	0.4	0.1	0.4	0.3	0.3	**1.6**	0.2	0.3	0.2	0.3	0.3	0.2	<	0.2
Rhizobiaceae	0.4	1.9[e]	0.1	0.3	<	0.6	1.1	**5.6**	1.4	1.3	0.3	0.5	0.4	0.0	0.1	0.4	0.2	0.6	<	1.4	0.8
Enterobacteriaceae	0.7	0.6	0.5	0.0	<	<	<	<	0.1	0.8	0.0	0.4	<	0.2	1.5	<	<	0.1	<	<	**1.8**
Lactobacillus kunkeei	0.8	0.2	<	<	0.7	1.3	0.3	1.7	0.8	0.8	<	0.1	0.5	1.1	0.4	0.4	0.3	0.2	0.6	**3.1**	0.0
Clostridiales	0.1	0.3[b]	0.1	0.4	0.5	0.1	0.3	0.4	0.1	0.3	**1.2**	0.2	0.0	0.4	0.3	0.2	0.2	0.1	0.3	0.1	0.2
Bacteria	<	<	0.1	<	<	**0.9**	0.9	0.3	0.3	0.7	0.3	0.5	0.2	0.1	0.4	0.6	0.8	0.2	0.2	0.2	0.4
Escherichia coli	0.2	0.1	<	<	<	0.4	<	<	0.0	0.4	0.0	0.0	<	0.4	0.2	0.5	<	0.1	<	0.6	**0.9**
Proteobacteria	0.4	0.3	0.3	0.9	1.2	1.7	1.0	0.6	1.1	1.4	**2.1**	1.4	0.2	0.2	0.5	0.4	0.5	0.4	1.2	0.3	1.2
Spiroplasma apis	<	<	<	0.6	<	0.1	4.5	<	<	0.5	0.8	<	<	3.4	<	<	<	**9.6**	<	<	<
Spiroplasma mirum	0.3	0.4	<	<	0.2	0.3	<	<	0.3	**1.9**	0.8	<	<	<	<	<	<	<	<	<	<

The means were back-transformed and recorded to one decimal place. Relative abundance < 0.01% (<). Apiary locations: Northland (NL1–3), Waikato (W1–3), Hawkes Bay (HB1–3), Whanganui (WN1–3), Nelson (N1–3), Canterbury (C1–3), Otago (O1–3). The maximum relative abundance of each phylotype is in bold typeface.

3.4.3 Phylotypes in the anecdotally sick colonies

The eight phylotypes associated only with the sick colonies all had a high number of reads in at least one of the 10 colonies (Supplementary material Table 3.8.2). The mean relative abundance (MRA) of these eight phylotypes ranged from < 0.01–10.4% (Table 3.4). *Lachnoclostridium* spp. and *Serratia* spp. were identified in nine of the sick colonies (NL26, W16, WN36, HB16, HB26, HB36, N3, C3, and O3. MRA 2.2% and 10.4%, respectively), *Acetobacter* spp. was present in six colonies (MRA 1.8%), *Chryseobacterium* spp., *B. pumilus*, and *C. freundii*-complex were in three colonies (MRA 0.1%, 0.5% and 0.1%, respectively), *C. sakazakii* was present in two sick colonies (NL26 and N26, MRA 7.8%), and *Providencia* spp. was present in one colony (MRA 0.5%) (Table 3.4). In contrast to the other apiaries with a sick colony, HB1 had significantly higher MRA of *Acetobacter* spp. (1.8%), *Lachnoclostridium* spp. (2.2%), *Serratia* spp. (10.4%), and *B. pumilus* (0.5%). The total relative abundance of these phylotypes (~15%) in HB1 is offset by the lowest percentage of *Lactobacillus* spp. (33.1%). The 7.8% *C. sakazakii* in apiary NL2 was offset by a lesser relative abundance of *G. apicola* (17.5%) and Cyanobacteria (0.9%). The relative abundance of *L. kunkeei* in eight of the sick colonies ranged from < 0.01 to 2.85%.

Rhizobiaceae was also identified in all ten of the sick colonies (0.1 to 22.4%) but only 11 (27.5%) of the 40 healthy colonies associated with the same apiaries (0.1 to 23.9%) (Supplementary material Table 3.8.3). The healthy colonies from the 11 apiaries without sick hives were excluded from this count to reduce potential bias resulting from beekeepers that may not have been able to identify a sick colony. The number of Rhizobiaceae reads ranged from 35 to 13,370. The number of reads in healthy colonies never exceeded the number in the sick samples from the same apiary. Thirty-four (40.4%) of the 84 samples from colonies deemed healthy contained Rhizobiaceae. The healthy colony WN2 had the highest relative abundance of Rhizobiaceae (23.9%). The rest of the healthy colonies contained less than that observed in six of the ten sick samples. The relative abundance of Rhizobiaceae was high in four apiaries without sick colonies (NL2, WN3, N3, and O3) and was below the 0.01% inclusion threshold in two apiaries (W2 and O1).

Table 3.4 | Mean relative percent abundance of the phylotypes in sick colonies from ten NZ apiaries.

	NL2	W1	WN3	HB1	HB2	HB3	N2	N3	C3	O3
Lactobacillus spp.	41.1^{a}	39.2^{a}	42.2^{a}	33.1^{a}	35.5^{a}	41.1^{a}	39.8^{a}	43.2^{a}	39.8^{a}	39.5^{a}
Gilliamella apicola	17.5^{a}	26.6^{a}	24.3^{a}	19.6^{a}	26.0^{a}	24.3^{a}	29.3^{a}	22.3^{a}	22.5^{a}	25.4^{a}
Snodgrassella alvi	12.6^{a}	7.5^{a}	4.5^{a}	9.4^{a}	9.5^{a}	10.0^{a}	9.0^{a}	5.2^{a}	9.2^{a}	7.8^{a}
Lactobacillus mellis	2.9^{a}	5.0^{a}	4.0^{a}	3.6^{a}	3.2^{a}	2.5^{a}	3.3^{a}	5.5^{a}	4.1^{a}	3.3^{a}
Frischella perrara	6.5^{a}	2.9^{a}	5.2^{a}	6.5^{a}	5.4^{a}	3.6^{a}	2.6^{a}	3.5^{a}	4.3^{a}	5.4^{a}
Bifidobacterium coryneforme	5.0^{a}	3.1^{a}	4.9^{a}	3.3^{a}	5.3^{a}	7.3^{a}	2.0^{a}	4.1^{a}	5.3^{a}	4.3^{a}
Acetobacteraceae	2.5^{a}	4.5^{a}	1.6^{a}	1.8^{a}	1.4^{a}	1.8^{a}	4.2^{a}	3.7^{a}	2.6^{a}	3.0^{a}
Lactobacillus mellifer	1.2^{a}	0.6^{a}	0.6^{a}	0.9^{a}	0.8^{a}	0.8^{a}	0.3^{a}	0.4^{a}	0.9^{a}	0.6^{a}
Cyanobacteria	0.9^{a}	1.7^{ab}	4.6^{bc}	2.9^{abc}	4.6^{bc}	1.5^{ab}	2.8^{abc}	3.6^{abc}	1.2^{ab}	3.2^{abc}
Rhizobiaceae	1.9^{de}	0.3^{abcde}	1.4^{cde}	1.3^{cde}	0.3^{abcde}	0.5^{abcd}	0.0^{a}	0.1^{ab}	0.6^{abcde}	0.8^{abcde}
Enterobacteriaceae	0.6^{bc}	0.0^{ab}	0.1^{abc}	0.8^{c}	0.0^{a}	0.4^{abc}	0.2^{abc}	1.5^{bc}	0.1^{aabc}	1.8^{c}
Pseudomonas spp.	0.2^{abcde}	1.3^{efg}	0.1^{abcd}	0.8^{defg}	0.1^{abc}	0.0^{ab}	<	0.0^{ab}	0.2^{abcd}	0.0^{a}
Ensifer adhaerens	0.6^{abc}	0.7^{abc}	0.1^{a}	0.4^{abc}	0.3^{abc}	0.3^{ab}	0.2^{abc}	0.3^{abc}	0.3^{ab}	0.2^{ab}
Lactobacillus kunkeei	0.2^{bc}	<	0.8^{bcdef}	0.8^{bcdef}	<	0.1^{b}	1.1^{cdef}	0.4^{bcde}	0.2^{bcd}	0.0^{a}
Clostridiales	0.3^{ab}	0.4^{ab}	0.1^{ab}	0.3^{ab}	1.2^{b}	0.2^{ab}	0.4^{ab}	0.3^{ab}	0.1^{a}	0.2^{ab}
Escherichia coli	0.1^{ab}	<	0.0^{ab}	0.4^{abc}	0.0^{a}	0.0^{ab}	0.4^{abc}	0.2^{abc}	0.1^{abc}	0.9^{c}
Proteobacteria	0.3^{ab}	0.9^{ab}	1.1^{ab}	1.4^{ab}	2.1^{b}	1.4^{ab}	0.2^{a}	0.5^{ab}	0.4^{ab}	1.2^{ab}
Spiroplasma apis	<	0.6^{ab}	<	0.5^{ab}	0.8^{abc}	<	3.4^{bc}	<	9.6^{c}	<
Spiroplasma mirum	0.4^{a}	<	0.3^{a}	1.9^{a}	0.8^{a}	<	<	<	<	<
Acetobacter spp.	0.0^{a}	<	0.0^{a}	1.8^{b}	<	0.0^{a}	<	0.0^{a}	0.1^{ab}	<
Chryseobacterium spp.	0.1^{a}	<	<	<	0.1^{a}	<	<	0.0^{a}	<	<
Lachnoclostridium spp.	0.1^{ab}	0.0^{a}	0.0^{a}	2.2^{b}	0.0^{a}	0.1^{a}	<	0.0^{a}	0.0^{a}	0.0^{a}
Providencia spp.	<	<	<	<	<	<	<	<	<	0.5^{a}
Serratia spp.	0.2^{a}	0.1^{a}	0.2^{a}	10.4^{b}	0.0^{a}	0.2^{a}	<	0.0^{a}	0.2^{a}	0.1^{a}
Bacillus pumilus	<	<	<	0.5^{b}	0.0^{a}	<	<	0.0^{a}	<	<
Citrobacter freundii-complex	0.1^{a}	<	<	<	<	0.1^{a}	0.0^{a}	<	<	<
Cronobacter sakazakii	7.8^{a}	<	<	<	<	<	0.5^{a}	<	<	<
Bacteria	<	<	0.3^{a}	0.7^{a}	0.3^{a}	0.5^{a}	0.1^{a}	0.4^{a}	0.2^{a}	0.4^{a}

The means were back-transformed (1 d.p). < indicates < 0.01% relative abundance. Apiary locations: Northland (NL2–3), Waikato (W1), Hawkes Bay (HB1–3), Whanganui (WN3), Nelson (N2–3), Canterbury (C3), Otago (O3). The eight phylotypes present only in the sick colonies are shaded in light grey. The significant differences among apiaries are identified by superscript letters ($^{a–g}$) and shaded in a darker grey.

A linear model indicated significant differences in the relative abundance of unique phylotypes among apiaries both with and without the sick colonies (P < 0.01) (Supplementary material Tables 3.8.4 and 3.8.5). These differences in mean relative

abundance of phylotypes among apiaries from healthy colonies are further supported by a least significant difference (LSD) > 2.70% (Supplementary material Figure 3.8.2). These differences were observed for all dominant core bacteria: *Lactobacillus* spp., *G. apicola, S. alvi, B. coryneforme* and *L. mellis* (Firm–4). Apiary N1 had significantly more *S. alvi* and *L. mellis* than most other apiaries, whereas apiary C1 had significantly more *Lactobacillus* spp. Significant differences were also observed among the apiaries for the less prevalent bacteria including Acetobacteraceae, Cyanobacteria (high in apiary W3), *F. perrara* (high in apiary WN1), Rhizobiaceae, and *S. apis*. Apiary WN1 had significantly more Rhizobiaceae.

Similar to the healthy colonies, the inclusion of the sick colonies meant that all core bacteria and some of the less prevalent bacteria differed among apiaries (LSD of > 2.98%) (Figure 3.3); Apiary C3 had 1.4–fold more *S. alvi*, and 1.5 to 3–fold more Rhizobiaceae than its healthy counterparts in apiaries C1 and C2. In contrast the sick apiary WN3 had 14–fold less Rhizobiaceae than the healthy apiary WN2.

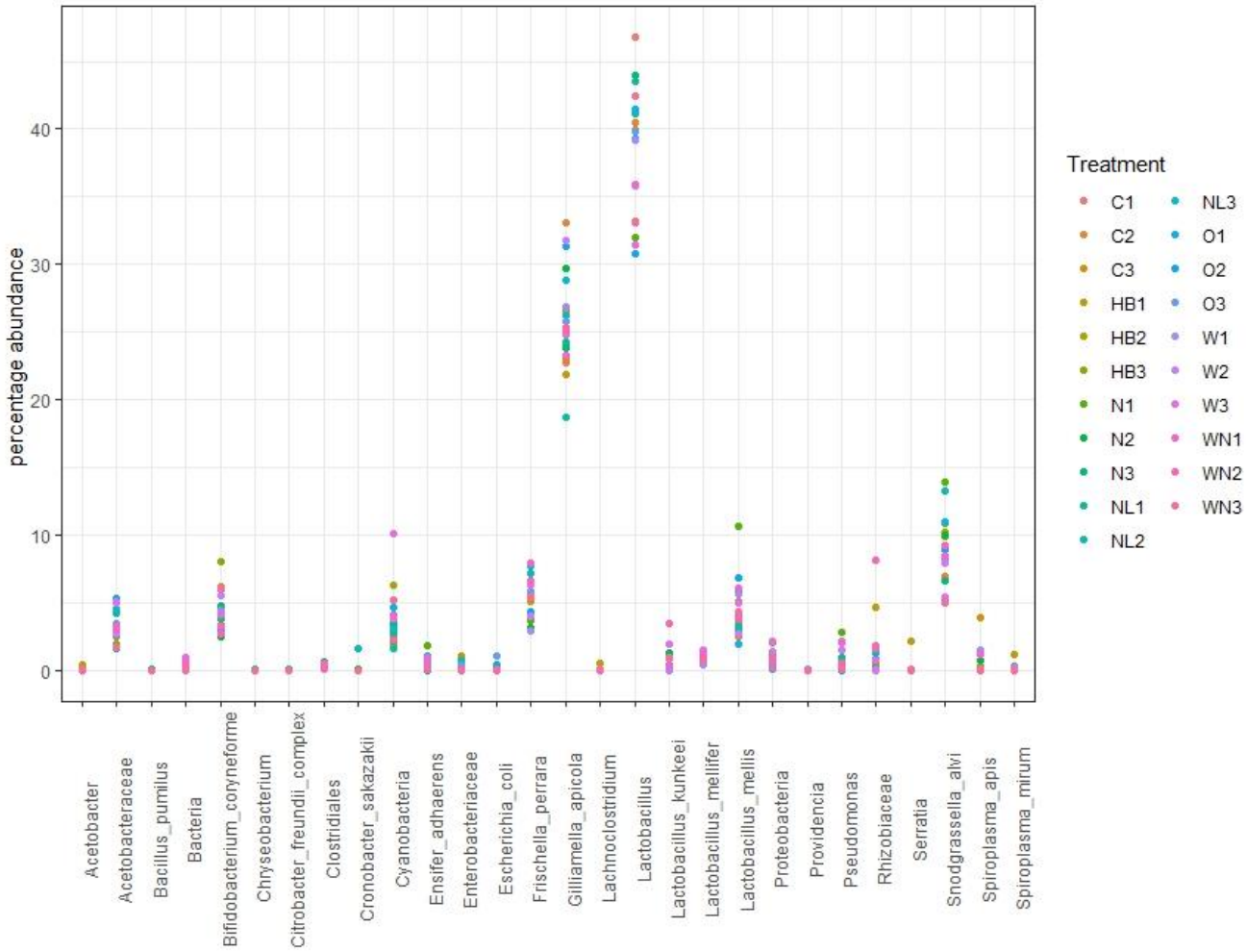


Figure 3.3 | Mean relative abundance of phylotypes in the digestive tract of healthy and sick colonies sampled throughout NZ.
Predicted mean LSD average = 2.70%. Inclusion of data required > 0.01% prevalence.

3.4.4 Diversity analysis

The inclusion of the ten anecdotally sick colonies (NL26, W16, WN36, HB16, HB26, HB36, N26, N36, C36, and O36) significantly ($P < 0.001$) influenced four α-diversity metrics as the bacterial communities clustered based on health; high diversity metrics were observed in the healthy colonies and low diversity metrics were observed in the ten sick colonies (Table 3.5, Figure 3.4). With apiary as the experimental factor, there was no significant difference in α-diversity ($P > 0.1$) (Supplementary material Figure 3.8.3, Table 3.8.6).

Table 3.5 | Alpha -diversity in bacterial communities from healthy and sick NZ honey bee colonies.

Diversity measure	Kruskal-Wallis statistic	*P* – value
Chao1	27.688	< 0.001
Observed ASVs	27.605	< 0.001
Shannon	23.326	< 0.001
Simpson	18.418	< 0.001

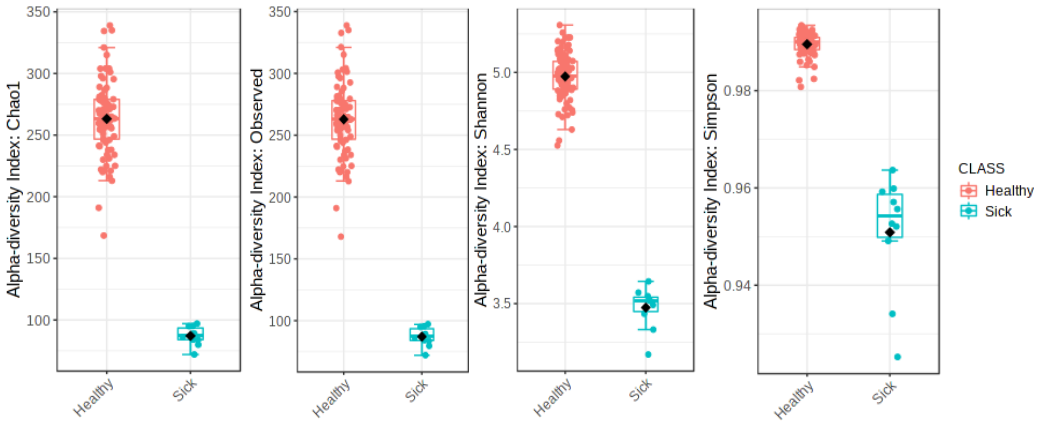


Figure 3.4 | Alpha–diversity for bacterial phylotypes from 21 apiaries throughout NZ.

The inclusion of sick colonies in the β–diversity analysis also had a significant ($P < 0.001$, $R^2 = 0.47$) influence on the metrics as colony health within the apiaries explained 57.4% of the variation (Table 3.6, Figure 3.5).

Table 3.6 | Beta–diversity using health as the experimental factor for the combined dataset.

Diversity measure	*P* – value	*F* – value	R^2	PC% Axis 1	Axis 2	Axis 3
Bray-Curtis dissimilarity	< 0.001	81.222	0.47	47.1	5.5	4.8

A non-parametric multivariate statistical test (PERMANOVA) was used to compare the affect of colony health on bacterial diversity.

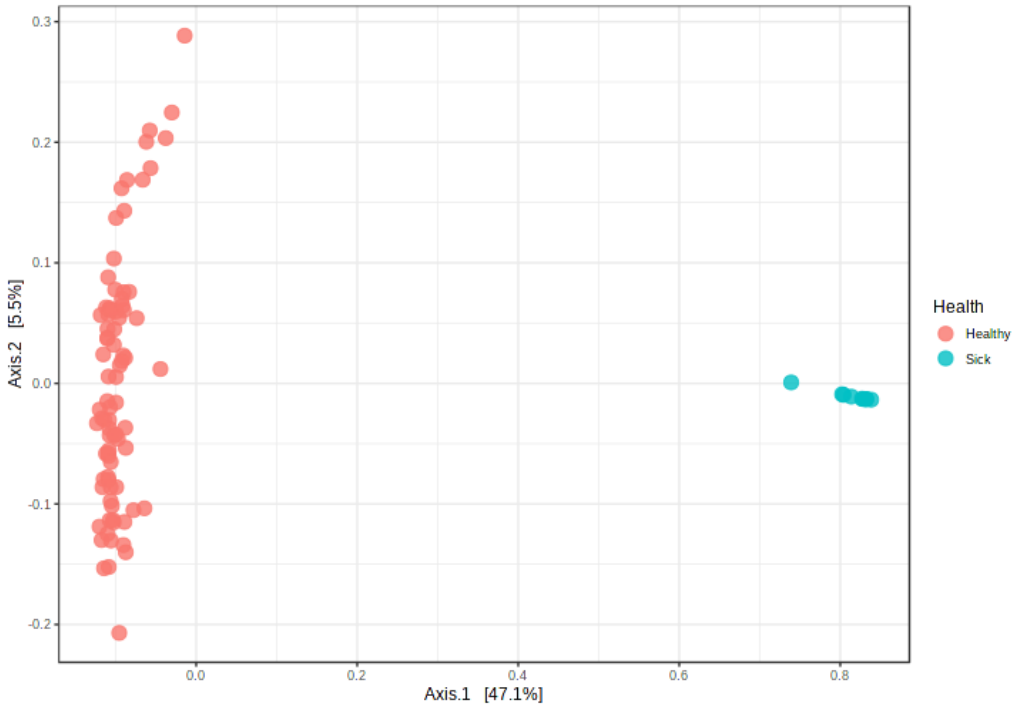


Figure 3.5 | Beta–diversity for honey bee gut bacterial phylotypes from 21 apiaries throughout NZ with colonies deemed healthy or sick.

The apiaries were located in seven NZ regions: Northland, Waikato, Hawkes Bay, Whanganui, Nelson, Canterbury, Otago. Health was the experimental factor and Bray–Curtis dissimilarity was used to conduct the PERMANOVA where $P < 0.001$, $R^2 = 0.29$.

Despite differences in relative abundance among phylotypes in different apiaries, the NMDS analysis suggests only a slight shift in the composition of bacteris was associated with apiary (Figure 3.6). The more obvious shift was between the bacterial composition in apiaries containing only healthy colonies versus those with both healthy and sick colonies; the gut bacterial profile in the healthy apiaries WN1 and O1 were displaced from those in the sick apiaries HB2 and NL2 along axis one of the ordination. These sick colonies converged with a strong representation of *Chryseobacterium* spp. and *C. freundii* complex. The phylotypes Rhizobiaceae, Enterobacteriaceae, *Lachnoclostridium* spp., *B. pumilus*, and *E. adhaerens* strongly diverged from Proteobacteria, and Cyanobacteria that tended towards the opposite direction on axis two, and thus seemed to be less affected by treatment.

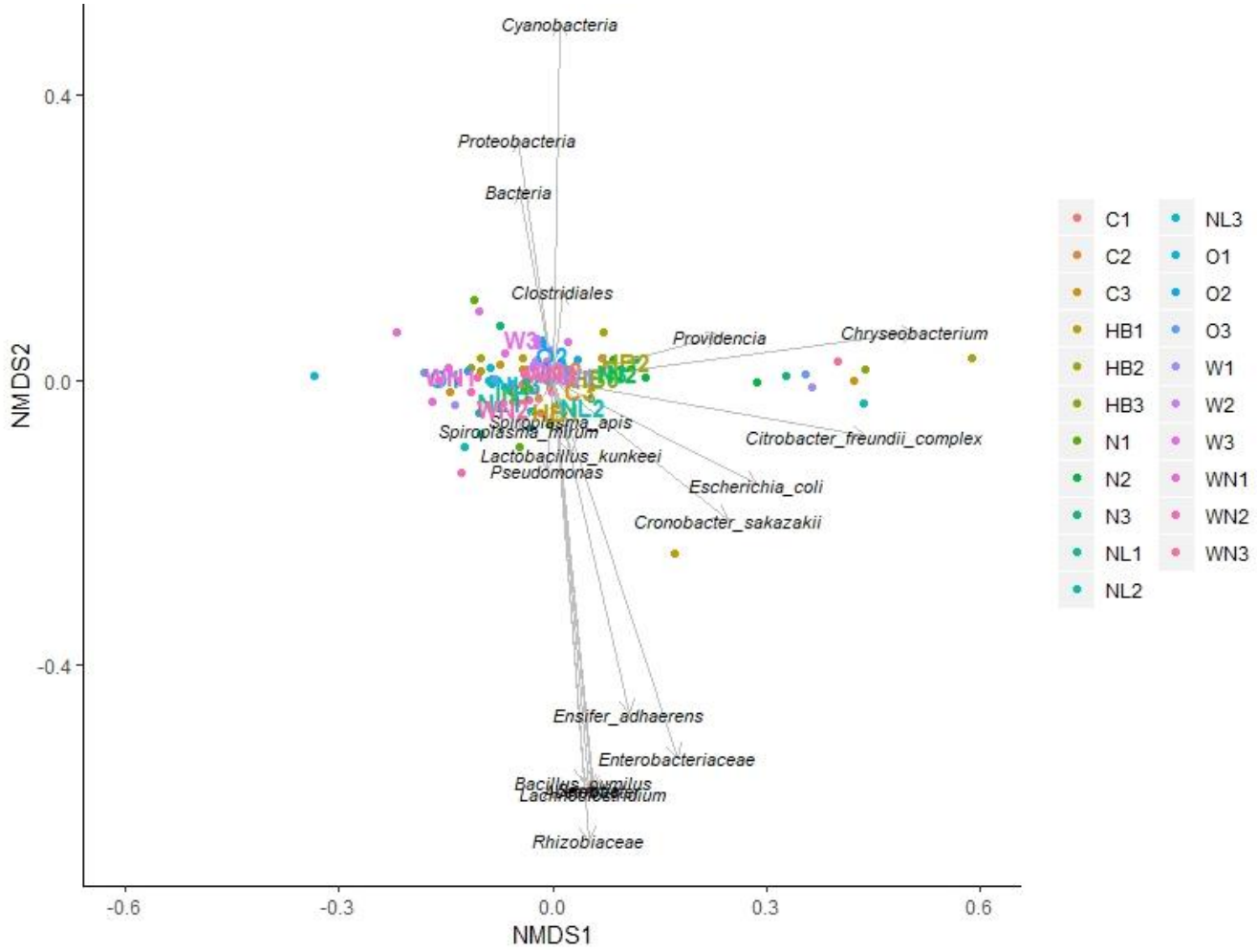


Figure 3.6 | NMDS of relative abundance of phylotypes in the digestive tract of healthy and sick colonies throughout NZ.

The 21 apiaries were located in Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Canterbury (C), and Otago (O).

3.4.5 Survey factors associated with bacterial communities in healthy colonies

The association of the 11 survey factors with the gut microbiome from healthy colonies were all significant (P < 0.01) (Table 3.7). Despite the factors each explaining 29% (Bray-Curtis) of the observed variation among the samples, only apiary (R^2 = 0.43) appeared to have a moderate effect on the bacterial communities as the R–squared values of the remaining factors were low (≤ 0.02) suggesting their effect was only slight (Table 3.7).

Table 3.7 | Beta-diversity using Bray-Curtis Index to calculate differences in bacterial communities for healthy colonies with varying experimental factors.

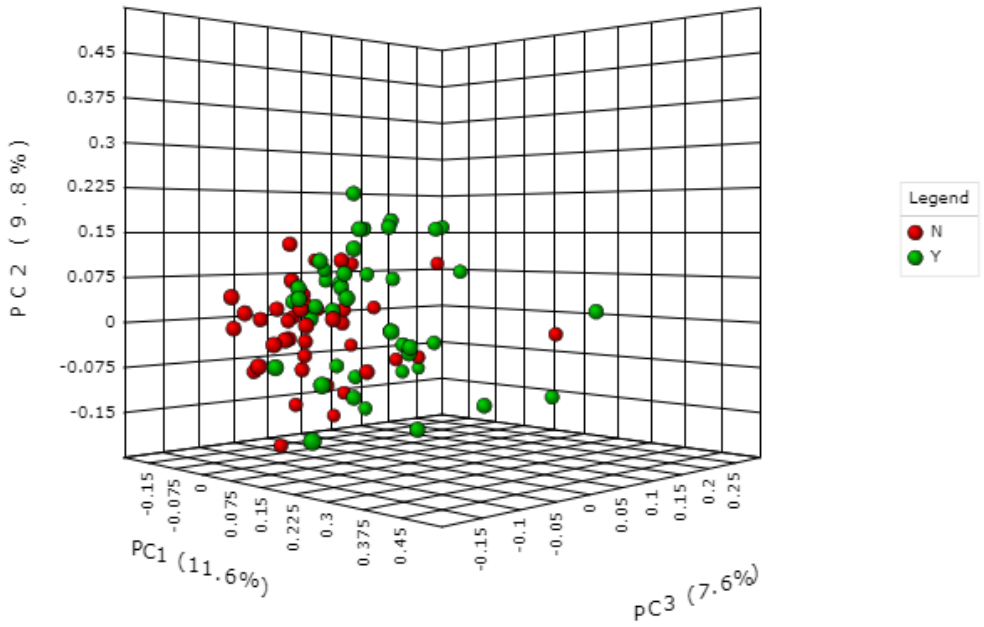
Experimental factor	*P* - value	*F* - value	R^2
Apiary	< 0.001	2.362	0.429
Altitude	< 0.004	1.519	0.054
Queen age	< 0.012	1.800	0.022
Distance from coast	< 0.001	2.593	0.060
Native bush	< 0.001	4.015	0.047
Mānuka	< 0.001	2.755	0.033
Number of spring pollens	< 0.001	1.766	0.062
Number of spring nectars	< 0.001	1.954	0.068
Spring pollen type	< 0.005	1.648	0.058
Spring nectar type	< 0.001	2.542	0.165
Winter pollen	< 0.009	1.511	0.054

PERMANOVA was used to compare groups.

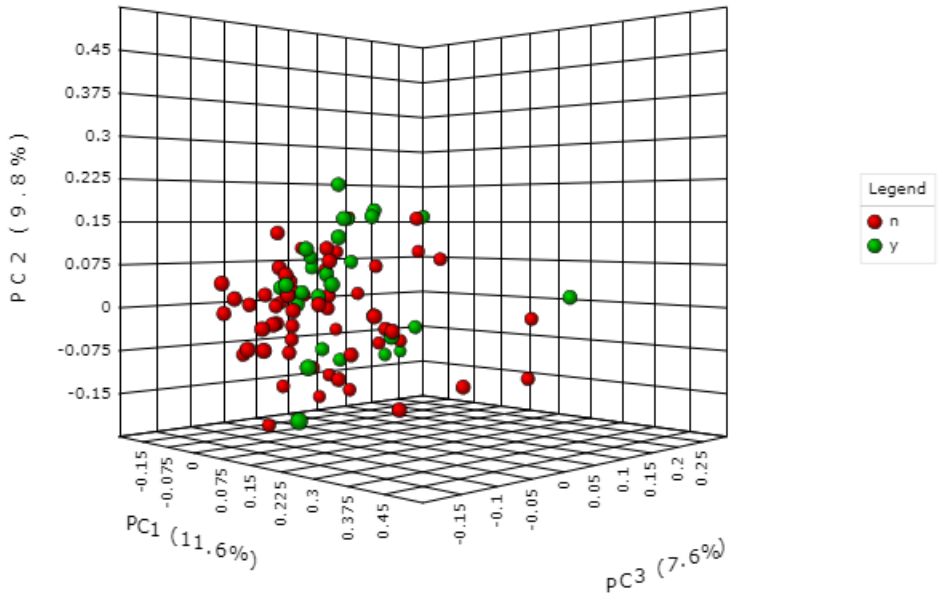
Factors associated with apiary location for the healthy colonies revealed no obvious clustering of bacterial communities from colonies located at different altitudes above sea level (Supplementary material Figure 3.8.4). However, the bacterial communities for apiaries located > 30 km from the coast were clustered slightly separate from those 0 to 10 km from the coast (Supplementary material Figure 3.8.4).

3.4.6 Food resources associated with bacterial communities in healthy colonies

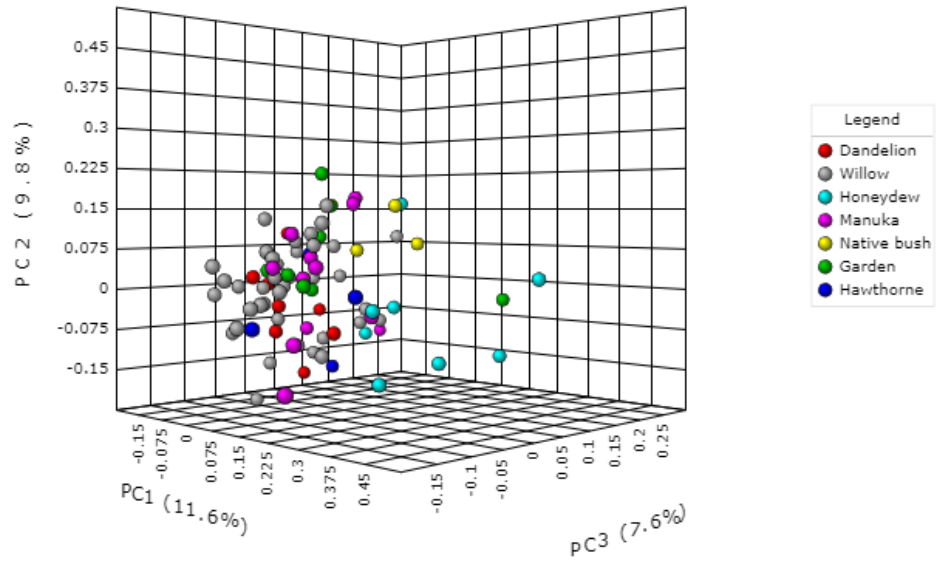
The microbiome in healthy apiaries with access to native bush or mānuka were clustered away from apiaries without these food sources (Figure 3.7A-B). The microbiome in bees feeding on spring willow and dandelion nectar were separated from those feeding on honeydew and native bush (Figure 3.7C). The number of spring nectars and spring pollens in the environment, and the types of both spring and winter pollens did not appear to influence microbiome among apiaries (Supplementary material Figures 3.8.5A-D).



A | Native bush



B | Mānuka



C | Spring nectar type

Figure 3.7 | Beta–diversity associated with the experimental factors for the bacterial phylotypes in healthy NZ honey bees throughout NZ.

Bray-Curtis was the dissimilarity measure and the experimental factors were native bush, mānuka, and the type of spring nectars.

3.4.7 Nosema apis and Nosema ceranae analysis

The ten anecdotally sick samples all contained between 2.46 x 10^7 and 6.29 x 10^{11} gene copies/µl of *N. apis* (Table 3.8), indicating its widespread presence throughout NZ. Only one of the sick colonies (N26) contained copies of *N. ceranae* (17,462 gene copies/µl), suggesting that it is less prevalent in NZ than *N. apis*. Healthy colonies were not assessed for *Nosema* spp. so there is no context for the scale of these results.

Table 3.8 | qPCR copies of *Nosema apis* and *Nosema ceranae* in the DNA from the digestive tract of sick NZ honey bees.

Hive	*Nosema apis* gene (copies/µl)	*Nosema ceranae* (copies/µl)
C36	1.24 x 10^{11}	0
HB16	4.14 x 10^9	0
HB26	6.29 x 10^{11}	0
HB36	2.46 x 10^7	0
N26	4.19 x 10^{11}	1.75 x 10^4
N36	4.22 x 10^{11}	0
NL26	4.22 x 10^{11}	0
O36	2.64 x 10^8	0
W16	7.74 x 10^7	0
WN36	3.32 x 10^7	0

3.5 Discussion

This study is the first comprehensive analysis of the bacterial diversity and relative abundance of individual phylotypes in the digestive tract of adult New Zealand honey bees. Bees from this geographically isolated population were collected from 94 colonies across 21 apiaries from seven regions (Northland, Waikato, Whanganui, Hawkes Bay, Nelson, Christchurch and Otago) and differences were observed between colonies deemed sick and those deemed healthy, and associations with apiary-specific factors were determined. This information is particularly relevant because of NZ's unique flora that sustains these bees as well as the annual exportation of tonnes of live NZ bees to Canada (New Zealand Government 2018). As newly emerged workers acquire their gut communities from both nurse bees and nurse bee faecal material (Powell et al. 2014) it is likely that NZ bees influence the Canadian bee gut microbiome.

Number of amplicon sequence variants and phylotypes

The internationally recognised core bacterial phylotypes (Kwong and Moran 2013), were present within the gut of NZ honey bees, except for phylotypes within the *Lactobacillus* Firm–5 cluster. It is unlikely that they were absent or classified in the genus *Lactobacillus* as Chapter 4 identified *L. apis*, *L. melliventris*, and *L. helsingborgensis* using the same customised 16S rRNA BLAST database that was used in this analysis. It is more likely that the large number of reads analysed may mean that the relative abundance of these species were below the 0.01% inclusion threshold used in this analysis. Indeed, populations of *L. apis* have been shown to vary from 5% to 69.6% (Corby-Harris et al. 2014a; Jones et al. 2018b).

The eight additional phylotypes in the gut of sick NZ honey bees differed between the ten colonies, either because some phylotypes were below the inclusion threshold in some colonies, or the abundance of some phylotypes were much larger. There are three possible explanations for the additional eight phylotypes present in the sick colonies: sampling methodology, the methodology used to classify phylotypes, and actual phylotype differences. The healthy samples were processed two years prior to the sick samples, however, both were processed using the same methodologies during the gut extractions, DNA extractions, and 16S rRNA sequencing. Additionally, prior to gut extraction the sick samples were stored in ethanol at -70°C. As the sick samples had more phylotypes than the healthy samples, DNA degradation does not explain the increase. Thus, methodology is unlikely to explain the difference. Four of the eight additional sick phylotypes were classified further on the taxonomic hierarchy than the healthy samples. For example, *Acetobacter* spp., *Providencia* spp., *C. sakazakii*, and *C. freundii–complex*, were present in the sick samples whereas the healthy samples only had the broader families Acetobacteraceae and Enterobacteriaceae present. As the sequences in the healthy samples had approximately 40 more nucleotides, it was expected that the phylotypes in the healthy samples would be identified more

specifically. This was not the case as they were only classified to the taxonomic level of family. The other four additional phylotypes in the sick colony samples (*Serratia* spp., *Bacillus pumilus*, *Lachnoclostridium* spp., and *Chryseobacterium* spp.), were not classified more broadly in the healthy samples and as all paired-end reads were processed using DADA2 methodology in QIIME 2, exact ASVs were produced. Therefore, if these additional phylotypes had been present in the healthy dataset then they would have been identified, even if only at a broader classification due to sequence trimming. Therefore, the additional phylotypes in the sick dataset are most likely to be additional bacteria in the digestive tract of honey bees from sick colonies.

The relative abundance of the dominant phylotypes and some of the less dominant phylotypes that were either outliers (Cyanobacteria, Acetobacteraceae, Rhizobiaceae, *F. perrara*, and *L. kunkeei)*, or undetected in some apiaries (*Pseudomonas* spp., *E. adhaerens*, Rhizobiaceae, Enterobacteriaceae, *L. kunkeei*, *E. coli*, *S. apis*, and *S. mirum*) differed among the 21 apiaries indicating that bacterial profiles in the honey bee gut vary.

Dominant core bacteria

The decreasing order of the relative abundance of the dominant core bacteria in NZ honey bees is similar to that observed internationally (Martinson et al. 2012; Moran et al. 2012; Jones et al. 2018b) where *Lactobacillus* spp. were the most abundant, followed by *G. apicola*, *S. alvi*, and *Bifidobacterium* spp. As phylotype identification becomes more accurate at the species-level with technology development, the relative abundance of species within *Lactobacillus* Firm–4 and Firm–5 clusters will be further identified, hopefully providing a better understanding of the importance of these species to bee health. The customised 16S rRNA BLAST database was employed rather than the taxonomic classifications SILVA, RDP, Greengenes or NCBI as some of the recently characterised honey bee gut bacteria were absent. This highlights the importance for keeping the databases updated and the need for further species characterisation so that species-specific information can be assimilated to understand their association with the honey bee host as well as their potential effect on bee health. Further classification of genera, in particular the genus *Lactobacillus*, through epidemiological and host experiments is recommended to ascertain their importance for bee health, particularly when the gut is compromised by external factors such as the honey bee diet (Taylor et al. 2019) or gut pathogens (Maes et al. 2016).

Subdominant core bacteria

The relative abundance of the subdominant core phylotypes are often lower than the dominant phylotypes or can be below the inclusion threshold (Corby-Harris et al. 2014a; Kešnerová et al. 2016). Therefore, slight changes in the relative abundance of dominant bacteria significantly affects the relative abundance of subdominant bacteria. Thus, comparative analysis of the relative abundance of subdominant core bacteria among

studies may be less reliable. This extensive study shows that both the dominant and subdominant gut bacteria vary between apiaries and therefore colonies.

The only internationally recognised subdominant core bacteria in the healthy NZ colonies were *F. perrara* and Acetobacteraceae. *F. perrara* abundance peaks in bees aged 2–7 days (> 50%) then declines to < 25% with age (Engel et al. 2015). The observed range of *F. perrara* relative abundance throughout NZ colonies (fifth most abundant with a range of 1.1 to 14.5%) suggests that the bees sampled were consistently older and this gives confidence that the NZ bees contained the full complement of gut bacteria (Powell et al. 2014). In comparison, the relative abundance of *F. perrara* was 4.5–fold more in honey bees from Sussex, UK, where *F. perrara* was the third most abundant phylotype, ranging from c. 2 to 65% in one apiary and 2 to 38% in a second apiary (Jones et al. 2018b). The relative abundance suggests that the age of the Sussex bees may have been younger or more varied. The relative abundance of *F. perrara* may therefore be a useful way to identify the age of honey bees previously undetermined. The presence of *F. perrara* causes scabbing in the epithelial surface of the pylorus of microbiota-free bees (Engel et al. 2015). This scabbing is similar to the innate immune response observed in other insects, where melanisation aids the hardening of damaged tissue (Nappi and Christensen 2005). The association between *F. perrara* and the honey bee immune response suggests that the variation in relative abundance of gut bacteria between colonies in this NZ study could be associated with the bees immune response or external factors that challenge the bacterial community, such as bacteria, gut pathogens, or food sources.

There was no significant difference among the 21 apiaries for the family Acetobacteraceae, which includes *P. apium* and the *Gluconobacter*–related species group that both predominantly inhabit the crop and brood-feeding tissues of worker bees (Corby-Harris et al. 2014b). However, as the samples were derived from the entire gut, the specific gut locations of the bacteria were not confirmed. The relative abundance ranged from fourth highest in apiary N2 to ninth highest in apiary HB2.

The presence of **Rhizobiaceae** in 19 of the 21 apiaries indicates that Rhizobiaceae is widespread. Rhizobiaceae was identified in all ten of the sick colonies but only 27.5% of the healthy colonies from the same apiaries. The cause of variation in relative abundance is unknown. However, as these colonies were deemed ‘sick’ by beekeepers due to delayed spring development, the presence of *N. apis* may explain this delayed start. Rhizobiaceae may therefore be responding to the increase in *N. apis* which may mean it could be utilised as a specific indicator of *N. apis*, or a broader indicator species of poor colony health. Although the number of reads in the healthy samples did not exceed the number observed in the sick samples in the same apiary, 40.4% of the 84 healthy colonies harboured Rhizobiaceae. If Rhizobiaceae does indicate poor health then these results suggest that judgement of sick colonies varies among beekeepers. It

also suggests that these visual symptoms may occur after the gut composition has altered. An increase in Rhizobiaceae may therefore be a response to an impaired gut or the cause of an impaired gut, although the latter is less likely as anecdotal observations suggest Rhizobiaceae is present in colonies that are still considered healthy.

The family Rhizobiaceae has three genera found in honey bees, *Rhizobium*, *Shinella*, and *Ensifer*. Only *Ensifer adhaerens* was identified in NZ honey bees. These genera contain pathogenic, symbiotic and saprophytic species which cannot be identified using physiological or biochemical traits (Ferreira et al. 2011). These potential interactions indicate that an increase in Rhizobiaceae abundance in honey bees may occur in response to beneficial or deleterious plant interactions, or a compromised immune system. Host-challenge trials with Rhizobiaceae and potential pathogens, such as *Nosema*, may elucidate the role of Rhizobiaceae in bee health and determine whether Rhizobiaceae could be used as an indicator species for poor bee health.

Phylotypes present only in the sick colonies

The genus *Serratia* is not part of the core microbiome (Moran et al. 2012). *Serratia marcescens* has been isolated from diseased larvae (El Sanousi et al. 1987), and the gut and haemolymph of honey bee workers (Raymann et al. 2018b). Based on the analysis from four honey bee colonies (one each located in Texas (n = 26 bees), Florida (n = 11), Tennessee (n = 21), and Utah (n = 9)), *S. marcescens* is considered a widespread opportunistic pathogen of adult honey bees (Raymann et al. 2018b). In this USA study *S. marcescens* was present in 22–100% of the bees at a relative abundance of 0.7–9.32%. When this isolate is sprayed on sealed and unsealed brood, it causes larval mortality (El Sanousi et al. 1987), and when administered orally or injected into the haemocoel, it can cause worker bee mortality without elevating the expression of antimicrobial peptides and phenoloxidase genes that encode the melanizing enzyme, phenoloxidase (Raymann et al. 2018b). This suggests *S. marcescens* can evade the honey bee immune system. The presence of *Serratia* spp. in nine of the ten sick NZ colonies (< 0.01–10.4%) from the seven regions, and in none of the 84 healthy samples, provides further support of this widespread opportunistic behaviour, at a relatively low abundance. The lack of identification in the 84 healthy colonies is contrary to most opportunistic pathogens that are usually present in low numbers within their host without causing disease (Sikorowski and Lawrence 1994; Maciel-Vergara et al. 2018). This suggests *Serratia* spp. may be present in the healthy samples of the combined dataset but below the inclusion threshold of < 0.01%, or that they inhabit non-host environments but are taken up by the honey bee host in a process currently undetermined, or that *Serratia* spp. may exploit both the honey bee gut and non-host environments. As *Serratia* spp. are present in both diseased larvae and sick honey bees, it may be hosted in the crop or hypopharyngeal glands, both of which are used by nurse bees to feed developing brood.

Opportunistic pathogens become pathogenic only in susceptible hosts, such as those with weakened immunity or altered microbiome composition (Brown et al. 2012). Exposure to the antibiotic tetracycline severely alters honey bee gut microbiome composition, increases bee mortality in the hive and in the lab, and leads to increased susceptibility to *S. marcescens* (Raymann et al. 2017). Tetracycline is often used internationally to control AFB. This bacterium resides in the larval and pupal gut and causes mortality (White 1906). *Serratia marcescens* shows high resistance to tetracycline, so internationally it is likely to have a selective advantage during a treatment course of antibiotics (Raymann et al. 2017). In NZ, It is illegal to use tetracycline for AFB control (New Zealand Government 1998); so tetracycline regime does not explain the observed increase of *S. marcescens* in the sick colonies. A more likely explanation is the presence of other honey bee gut pathogens that initially weaken the immune system and with whom *S. marcescens* either coexist, or compete. The honey bee gut pathogen *Nosema* spp., is a potential candidate.

The consumption of aged pollen appears to systemically affect honey bees, causing dysbiosis in the ileum, rectum, mouthparts and hypopharyngeal glands. This diet-related dysbiosis is also associated with the establishment of *Nosema* spp., in low abundance (Maes et al. 2016). *Nosema* spp. infect the midgut epithelial cells in honey bees which alter the conditions in the gut and this may aid the proliferation of *S. marcescens*. *Serratia marcescens* efficiently degrades chitin (Tews et al. 1996), a long-chain polymer of N-acetylglucosamine derived from glucose that is a primary component of fungal cell walls, and insect exoskeletons. It is therefore possible that the proteins, including chitinases, that are secreted by *S. marcescens* may potentially degrade increasing populations of other organisms (Tao et al. 2006), such as the microsporidia *N. apis* and *N. ceranae*.

In support of this fungal-bacterial or interkingdom association, all the sick colonies contained *N. apis*, including N26 that did not have *Serratia* spp., and *N. ceranae* was only present in colony N26. This suggests that *S. marcescens* and *N. ceranae* may not coexist or some form of competition may occur. However, as *N. ceranae* was only recently established in NZ (Murray and Lester 2015), the lack of interaction should not be interpreted without further research. Especially since mixed infections of *Nosema* spp. are more virulent than a single species infection (Milbrath et al. 2015), and because *N. ceranae* appears to have replaced *N. apis* worldwide, direct competition does not appear to be responsible (Klee et al. 2007). Host-challenge experiments with the three pathogens would identify whether potential competition is antagonism or synergism. *In vitro* trials that identify the effects of *S. marcescens* metabolites on the proliferation of both *Nosema* species would also help identify the role of these bacteria on honey bee health and whether the excretions from these bacteria could be used to control other bee pathogens, such as *Nosema*. This idea has potential as the supernatant and collected proteins from bacterial cultures of *S. marcescens* isolated from the gut of the

Chinese honey bee *Apis cerana* (Fabricius, 1793), indicate miticidal effects as 100% *Varroa* mortality occurred within five days after treatment (Tu et al. 2010).

The genus ***Lachnoclostridium*** was present at low abundance (≤ 2.2%) in nine of the ten sick colonies. This genus is not common in the current honey bee literature but lead exposure is reported to reduce *Lachnoclostridium* in mice (Zhai et al. 2017). As honey bees forage, they inadvertently collect lead from the environment and this can accumulate in apiary products (Cozmuta et al. 2012). Potential sources of lead in the honey bee environment include water containing lead-based paint, or their hives. Historically, lead-based paint was used to seal the outside of the boxes, this is no longer common practise. However, as the sick colonies increased in *Lachnoclostridium*, it is unlikely that the healthy colonies would all have acquired access to a source of lead that suppressed these bacteria (Cozmuta et al. 2012). The most probable explanation is that these bacteria are low- abundance opportunistic bacteria that have increased in response to a compromised microbiome resulting from infection with another gut pathogen. Further epidemiological research of this bacteria would determine whether *Lachnoclostridium* could be used as an indicator of bee health.

The family **Acetobacteraceae** was present in all ten sick colonies and is known to adapt to sugar-rich environments, although very few of the species are able to completely oxidise ethanol, sugars and polyalcohols (Kersters et al. 2006). The genus *Acetobacter* is frequently associated with honey bees (Lambert et al. 1981; Mohr and Tebbe 2006; Babendreier et al. 2007; Crotti et al. 2010; Martinson et al. 2011). However, in this combined dataset the *Acetobacter* genus was present in only six of the sick colonies (MMRA 1.8%) and below the 0.01% inclusion threshold in the healthy colonies, suggesting that it may behave opportunistically, increasing in relative abundance when the honey bee gut is compromised.

Chryseobacterium spp. was only present in three of the sick colonies. *Chryseobacterium indoltheticum* has been identified in floral nectar from *Amygdalus communis* (almond), and *Citrus paradisi* (grapefruit) (Fridman et al. 2012), both of which are grown in NZ. In contrast, *Chryseobacterium gleum* is present in the Asian honey bee (*Apis cerana*) and the giant honey bee (*Apis dorsata* Fabricius) (Kwong and Moran 2016b), both of which are absent in NZ. Therefore, it is possible that *Chryseobacterium* spp. may have been present in the nectar foraged on by these three sick colonies. Although the significance of why *Chryseobacterium* spp. was not observed in the other colonies is unknown.

Bacillus pumilus sporulates under favourable conditions. It is present in numerous environs, including poultry yards where it efficiently degrades feathers (Reddy et al. 2017), and it has been isolated from corbicula pollen of honey bees, and this was considered non-pathogenic (Gilliam 1997). *B. pumilus* was present in three of the sick

colonies, Foraging behaviour of sick bees differs to that of healthy bees in that they conduct fewer but longer foraging flights (Alaux et al. 2014). Thus the bees may potentially forage further afield than their healthy counterparts. The usefulness of *B. pumilus* as an indicator of bee health would require studies to determine its effect on honey bees, its favourable growth conditions, and its route of transmission.

Three of the phylotypes in the sick colonies are part of the Gram–negative family Enterobacteriaceae. The first, ***Cronobacter sakazakii***, a non-spore forming bacterium was present in two colonies. This species was previously known as *Enterobacter sakazakii* and is considered an opportunistic pathogen that is often food-borne and mainly associated with infections in human infants, but does also occur in children, adults (Lai 2001), fruit flies (Kuzina et al. 2001), and medicinal Syrian spices and plants (Belal et al. 2013). This suggests the reservoir of this species is environmental, primarily associated with plants (Belal et al. 2013). As the abundance of *C. sakazakii* was elevated in the sick NL2 colony, it suggests that the condition in the gut of the honey bees was favourable to this opportunistic pathogen and supports the beekeeper's anecdotal categorisation of sick. What caused this elevated abundance in this colony is unable to be determined from the metadata collected.

The second phylotype within Enterobacteriaceae is ***Citrobacter freundii*-complex**. It was present in three sick colonies at very low abundance and is considered pathogenic to a wide spectrum of animals and humans (Svetlana et al. 2003; Chuang et al. 2006). *Citrobacter* spp. is rarely associated with bees but has been observed in healthy colonies in France (Tysset and Durand 1968), and is associated with bee septicaemia (Lyapunov et al. 2008). *Citrobacter gillenii*, isolated from the honey bee gut, indicates signs of antibiotic resistance (Hleba et al. 2014). This suggests that *Citrobacter* spp. may proliferate in an altered honey bee gut microbiome caused by antibiotics. However, as the use of antibiotics is illegal in NZ honey bee colonies (New Zealand Government 1998), and as the abundance of this species was < 0.01% in this dataset, the relative abundance of *C*. *freundii*-complex is likely to remain low in NZ honey bees, requiring further international studies to understand their role in the gut.

***Providencia* spp.** was the third phylotype within Enterobacteriaceae that was present in only one apiary (MMRA 0.5%). *Providencia* spp. has been identified in the gut of *A. m. jemenitica* from Saudi Arabia (Khan et al. 2017). Both species are rarely mentioned and this may be because until recently the focus has been the dominant core bacteria, as the focus widens the less prevalent phylotypes within the gut of honey bees will start to be researched individually.

The presence of the opportunistic pathogens *Serratia* spp., *Lachnoclostridium* spp., *Acetobacter* spp., and *B. pumilus* in the HB1 apiary may be due to compromised gut conditions. Pesticides are known to cause gut dysbiosis (Kakumanu et al. 2016) and as

apiary HB1 was bordered by agricultural crops, it may be possible that this one sick colony was affected by pesticide use but had not recovered at the time of sample collection. As none of the other colonies were affected, this colony may have been first compromised by another pathogen that caused the colony size to dwindle throughout winter and spring, inhibiting the colony from recovering from pesticide poisoning (Goulson et al. 2015). The higher relative abundance of these less prevalent bacteria appeared to be offset by a reduction in the relative abundance of *G. apicola* and *Lactobacillus* spp. respectively (the abundances of these core bacteria were low in comparison with the other nine apiaries).

In contrast to the increase in the numbers of bacterial phylotypes identified in the sick colonies, a smaller study on the honey bee gut from eight colonies in Ireland observed a reduction in the number of bacterial phylotypes in four colonies deemed 'non-thriving'. Four phylotypes were only associated with the four thriving colonies: The family Orbaceae, the genera *Apibacter* and *Aurantimonas*, and the species *L. kunkeei*. The latter three were present in both gut and whole bee samples (Ribière et al. 2019). Both *Apibacter* and *Aurantimonas* were absent from the NZ colonies.

The beekeepers that supplied bees for analysis, have managed colonies between five to > 45 years. It was therefore expected that their identification of slow colony development in spring was reliable. *L. kunkeei* was present in 50% of the heathy NZ colonies across 17 apiaries, with a widespread abundance (0.005–10.4%), and eight of the ten sick colonies with a lesser spread of abundance (0.004–2.9%). The prevalence of *L. kunkeei* in healthy colonies suggests that *L. kunkeei* may not be a useful health indicator for NZ bees, or in contrast, its presence precedes the visual symptoms that beekeepers use to determine a sick colony, thus making it a useful indicator of poor colony health.

L. kunkeei is a fructophilic acid-resistant bacterium (fructose is the preferred carbon source (Endo et al. 2012)) that produces lactate, acetate and ethanol (Neveling et al. 2012). It is the most abundant bacterium in bee bread, and is present in floral nectar and honey, where the predominant substrates are the monosaccharides glucose and fructose, and the disaccharide sucrose (Wykes 1952; White et al. 1962; Chalcoff et al. 2005), bee-collected pollen, royal jelly, and the crop of adult honey bees (Anderson et al. 2013; Asama et al. 2015). Honey bees challenged with *N. ceranae* and inoculated with *L. kunkeei* have fewer spores of *N. ceranae,* and larvae inoculated with *L. kunkeei* have reduced larval mortality caused by *P. larvae* subspecies *larvae* (Arredondo et al. 2018). It is therefore possible that this bacterium is associated with activities such as food collection and larval provision behaviour, both of which increase in spring with thriving colonies. As there was no apparent correlation with the *Nosema* spp., and *Varroa* had been treated in early spring, *L. kunkeei* may be a better indicator of foraging behaviour rather than pathogen infection.

Effect of pathogens on colony health

The absence of *P. larvae* subspecies *larvae* DNA from all gut samples indicates that *Paenibacilli* was not the reason the colonies were deemed sick.

Nosema infections were seen in all ten sick colonies spread throughout the seven regions, as the pathogen *N. apis* was present. In contrast, *N. ceranae* was only present in one sick colony located in Nelson. As these colonies were deemed 'sick' by beekeepers due to delayed spring development, the presence of the widespread microsporidia *N. apis* in all colonies may explain this delayed start as *N. apis* infection is partially characterised by decreased brood production, predominantly in spring (Mattila and Otis 2006). However, because only the ten sick colonies were analysed for *N. apis*, it may be that *N. apis* was also present in the other 84 colonies. From this small sample size, *N. apis* appears widespread throughout NZ compared with *N. ceranae*. However, further studies are required to detail their distribution throughout NZ. Our study is the first documentation of the recently introduced (2005 – 2007) pathogen *N. ceranae* in Nelson, NZ. The foraging behaviour of bees infected with N. ceranae differs to their healthy counterparts in that the duration of their flights are longer and they spend a larger proportion of each day in the field (Alaux et al. 2014). This suggests that not only may *Nosema*-infected bees forage on resources further afield but that other gut pathogens and or bacteria may also affect bee behaviour. However, this requires further study.

Effect of environmental factors on the bacterial profile

Anecdotal categorisation of healthy and sick honey bee colonies identified differences associated with gut bacteria. However, despite all the categorised hive and environmental factors in this study explaining some variation between the phylotypes, only apiary had a moderate effect on the bacterial communities. Therefore, in this instance anecdotal categorisation of external factors was unable to identify a single factor that primarily influenced the gut bacterial community. It was also unable to determine whether the differences between the healthy and sick colonies was caused by specific environmental conditions associated with the apiaries, external bacteria/pathogens associated with the hive and surrounding environment, or altered conditions within the honey bee gut. The variation in bacterial relative abundance among the 21 widespread NZ apiaries suggests that environmental factors at the apiaries may have had some influence but these factors were not correctly identified. As colonies collect nectar from numerous sources within a season it was anticipated that general categorisation of native bush and garden sourced may have provided sufficient information. However, greater understanding may have been gleaned by further classification of specific nectar sources as the bacterial profile in healthy colonies feeding on mānuka (a subset of native bush) did vary from those feeding on native bush. This suggests that the nectar sources consumed by colonies may alter their bacterial

profiles. This is corroborated by the study where the consumption of mānuka honey, as opposed to sucrose, increased the relative abundance of *F. perrara* and reduced the relative abundance of Rhizobiaceae, Acetobacteraceae and *L. kunkeei* (Taylor et al. 2019). Bacterial profiles have also been observed to vary between environmental landscapes (Jones et al. 2018b) and as the bacterial profiles of apiaries by the coast varied from those further inland, this is further evidence that nectar source may alter gut bacterial profiles of honey bees.

3.6 Conclusion

The presence of the five dominant core phylotypes in all 94 NZ colonies corroborates the current evidence that five dominant core phylotypes consistently reside in the gut of honey bees worldwide. The variation among phylotypes and their associated apiaries appear to be influenced only slightly by the surrounding environment, such as floral resources e.g. native bush versus introduced species. The additional eight phylotypes present in the sick samples, coupled with the variation in relative abundance of the subdominant phylotypes was far more striking and supports the growing body of literature suggesting that external factors associated with bee health may influence the honey bee gut microbiome. Three phylotypes have potential to be used as indicators of poor bee health: the genera *Serratia* and *Acetobacter*, and the family Rhizobiaceae. Rhizobiaceae is a promising indicator of colony health as it was present in 100% of the sick colonies but only 27.5% of the healthy colonies.

This is the first report of the presence of both *N. apis* and *N. ceranae* in NZ honey bee colonies since the introduction of *N. ceranae* and to date the newly introduced *N. ceranae* does not appear to have outcompeted *N. apis* in NZ.

The customised 16S rRNA BLAST database was employed rather than the taxonomic classifications SILVA, RDP, Greengenes or NCBI as some of the recently characterised honey bee gut bacteria were absent. This highlights the importance of keeping the databases updated and the need for further species characterisation so that species-specific information can be assimilated to understand their association with the honey bee host as well as their potential effect on bee health. Further classification of genera, in particular the genus *Lactobacillus*, through epidemiological and host experiments is recommended to ascertain their importance for bee health, particularly when the gut is compromised by external factors such as the honey bee diet (Chapter 6) or gut pathogens (Chapter 7).

3.7 Where to next

To determine whether the bacterial composition in the isolated NZ honey bee population is comparable with the bacterial composition in international honey bee populations, the healthy NZ dataset will be compared with a dataset from Connecticut, USA that was extracted using similar methodology.

3.8 Supplementary material

Using Geneious 10.0.9 (Geneious) the sick ASV ID 2042 with measures of zero had 415 nucleotides, whereas the healthy ASV ID 4399, with measures between 180 and 1342, had 455 nucleotides (Figure 3.8.1). The ASV sequences of *Snodgrassella alvi*, *Frischella perrara*, *L. mellis*, *L. mellifer*, and *L. kunkeei* were also assessed using Geneious 10.0.9 and displayed the same pattern.

A | Sick *Gilliamella apicola* sequence # 2042.

B | Healthy *Gilliamella apicola* sequence # 4399.

Figure 3.8.1 | Comparison of the base pairs between different sequences of several phylotypes using Geneious 10.0.9.

The two sequences of *Gilliamella apicola* are highlighted in purple A | Sick sequences. B | Healthy sequences.

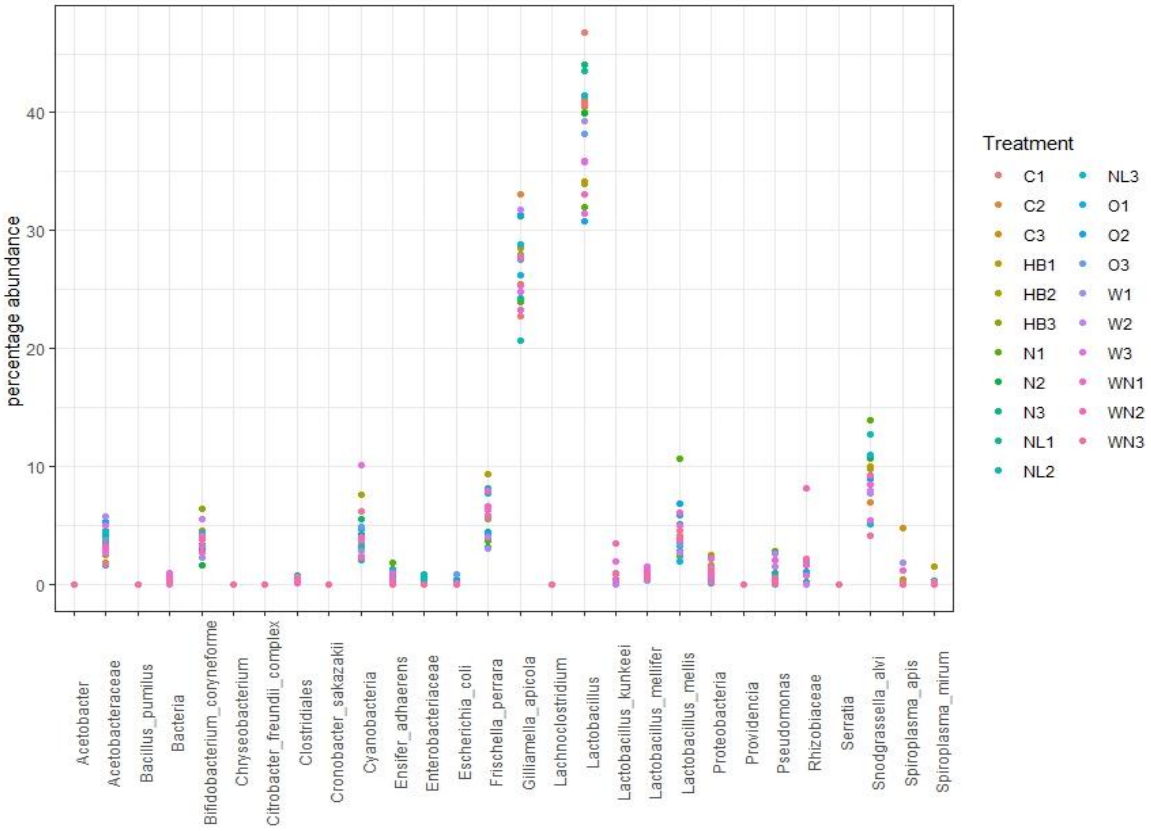


Figure 3.8.2 | Predicted means for relative abundance of phylotypes in the digestive tract of healthy honey bees from 21 apiaries throughout New Zealand.

Inclusion required > 0.01% prevalence. Predicted mean LSD average = 2.70%.The apiaries were located in seven regions: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Canterbury (C), Otago (O). LSD average = 2.98%.

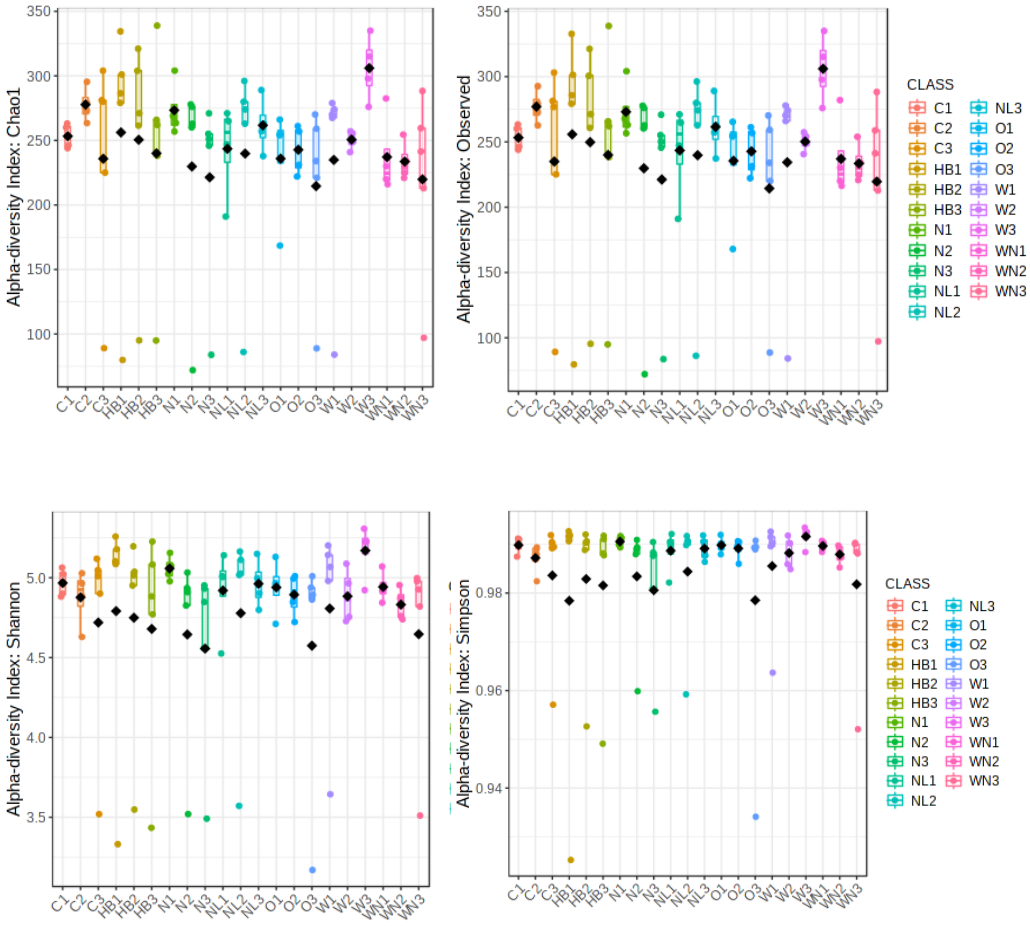
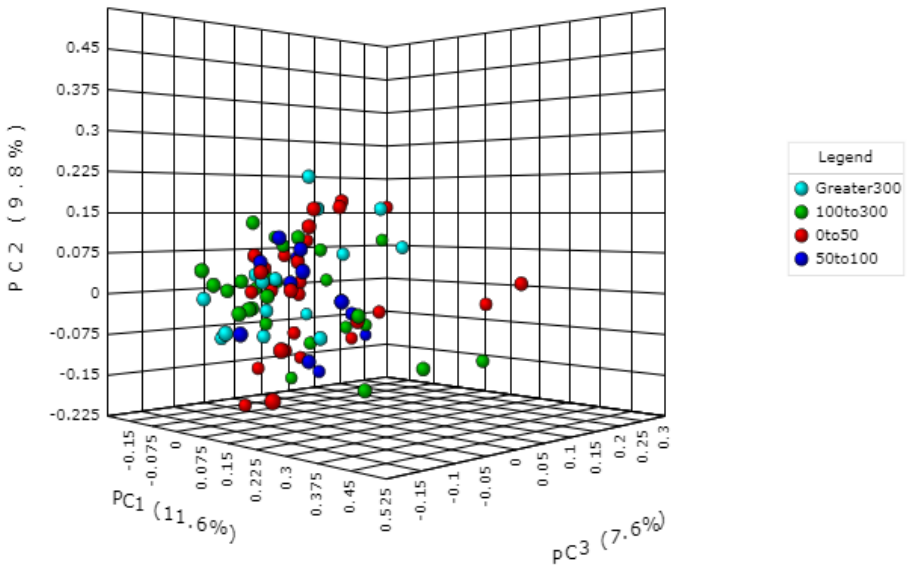
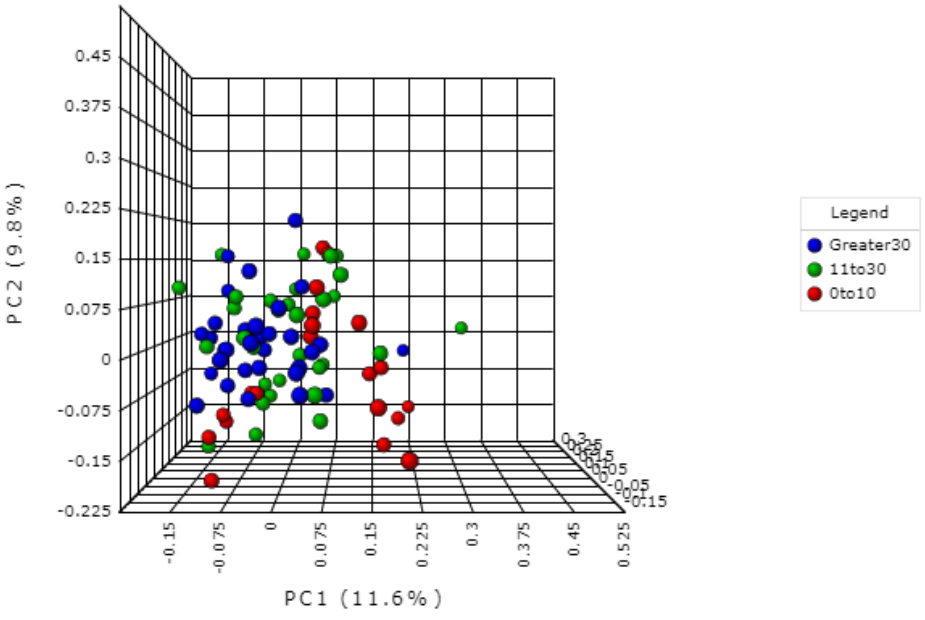


Figure 3.8.3 | Alpha–diversity measures for bacterial phylotypes from healthy and sick conies from 21 apiaries throughout NZ.

Apiary was the experimental factor. The apiaries were located in seven regions: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Canterbury (C), Otago (O).



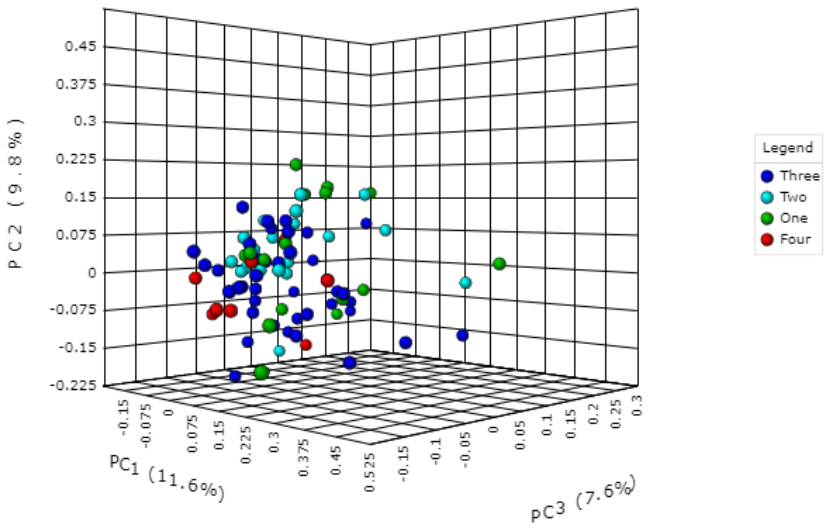
A | Altitude (m)



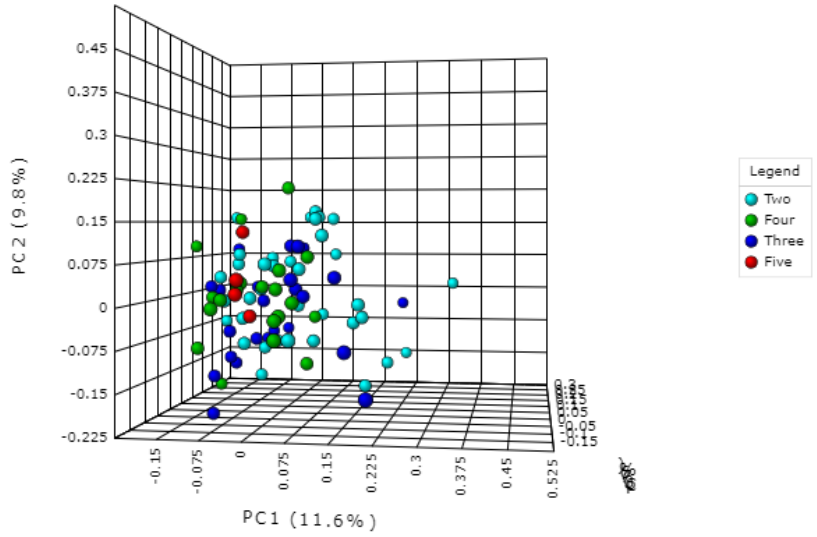
B | Distance from the coast (km)

Figure 3.8.4 | Beta–diversity of the experimental factors associated with hive management for the bacterial phylotypes in healthy honey bees throughout NZ.

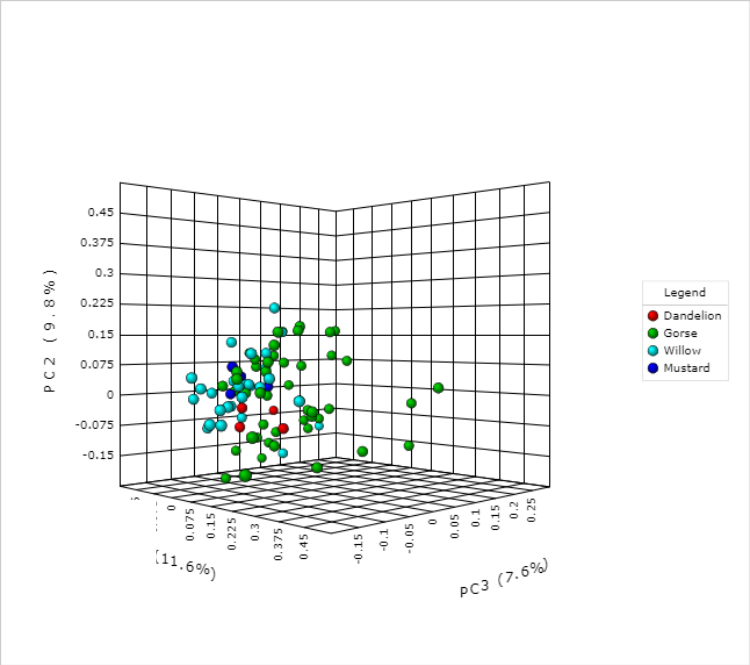
The β–diversity distance measure was Bray-Curtis dissimilarity (BC).



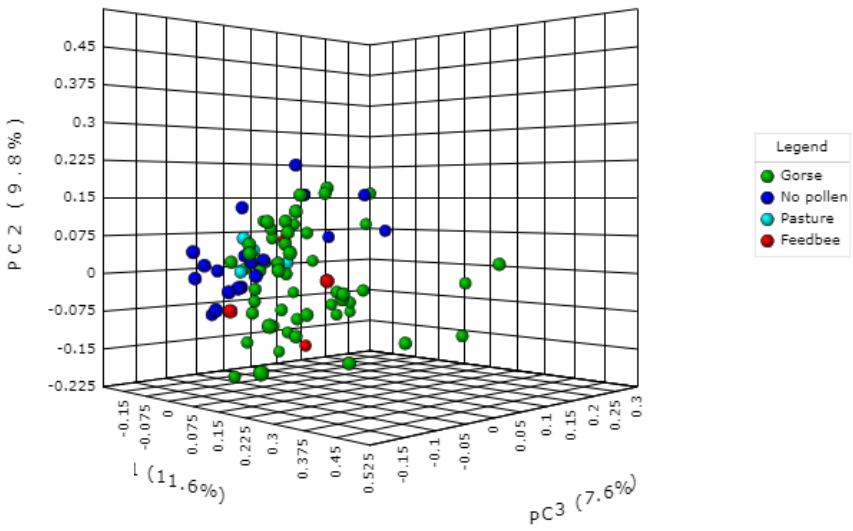
A | Number of spring nectars



B | Number of spring pollens



C | Spring pollen type



D | Winter pollen type

Figure 3.8.5 | Beta–diversity associated with the experimental factors for the bacterial phylotypes in healthy NZ honey bees throughout NZ.

Factors associated with apiary location for the healthy colonies.

Table 3.8.1 | ANOVA table associated with the interaction of the number of phylotypes in the digestive tract of healthy and sick New Zealand honey bees.

Calculated using a Poisson generalised linear model. Significant difference is in bold typeface.

Treatment	Chi squared	DF	*P* – value
Region	3.1736	6	0.787
Apiary	8.377	20	0.989
Colony	16.178	4	**0.003**

Table 3.8.2 | Number of reads for the less prevalent bacteria in the digestive tracts of sick New Zealand honey bees and the associated number of copies identified for *Nosema* spp.

Colony	*Cronobacter sakazakii*	*Acetobacter*	*Bacillus pumilus*	*Chryseobacterium*	*Citrobacter freundii–complex*	*Lachnoclostridium*	*Providencia*	*Serratia*	*Nosema apis*	*Nosema ceranae*
NL26	7604, 1239 (0.5)	46 (0.04)	0	135	133	78	0	220	2.85×10^{10}	0
W16	0	0	0	0	0	44	0	114	7.74×10^{9}	0
WN36	0	46 (0.04)	0	0	0	29	0	158	3.32×10^{7}	0
HB16	0	1061 (1.8)	326 (0.55)	0	0	1307	0	6179	4.14×10^{9}	0
HB26	0	0	15 (0.007)	200	0	16	0	53	6.29×10^{11}	0
HB36	0	50 (0.04)	0	0	154	66	0	262	2.46×10^{7}	0
N26	381 (7.8)	0	0	0	26	0	0	0	4.19×10^{11}	1.75×10^{4}
N36	0	14 (0.02)	11 (0.01)	14	0	4	0	18	4.22×10^{11}	0
C36	0	81 (0.1)	0	0	0	52	0	191	1.24×10^{11}	0
O36	0	0	0	0	0	26	419	86	2.64×10^{8}	0

The mean maximum relative abundance of the phylotype is in parenthesis.

Table 3.8.3 | Number of counts for each Rhizobiaceae read observed in the digestive tracts of New Zealand honey bees sampled from 21 apiaries.

Apiary	H1	H2	H3	H4	S1	% of healthy colonies (H1–H4) containing Rhizobiaceae
NL1	21 (0.1)	0	18, 34 (0.3)	55, 58, 62, 68, 68, 91 (2.3)		
NL2	0	0	72, 79, 89, 97, 104, 123 (3.2)	26, 31, 37, 41, 41, 53 (1.0)	2665 (**2.3**)	50
NL3	0	0	0	21 (0.1)		
W1	0	0	0	0	309 (**0.3**)	0
W2	0	0	0	0		
W3	37, 44, 48 (0.6)	13, 14, 16, 30, 40 (0.5)	41, 52, 55, 61, 77, 83 (1.3)	29, 30, 32 (0.3)		
WN1	124, 127, 138, 139, 145, 174 (3.6)	36, 39, 43, 45, 46, 47 (1.7)	42 (0.3)	18, 22, 22, 25, 26, 27 (0.9)		
WN2	251, 324, 340, 353, 368, 389 (7.5)	0	812, 985, 1013, 1060, 1119, 1198 (23.9)	14, 23, 30, 32, 33, 37 (1.0)		
WN3	0	18, 24, 24, 28, 33, 34 (0.6)	206, 218, 234, 248, 249, 260 (7.9)	0	613 (**0.6**)	50
HB1	26, 35, 38 (0.4)	29, 29 (0.3)	0	0	13370 (**22.4**)	50
HB2	0	0	0	0	687 (**0.3**)	0
HB3	0	0	13, 34, 43 (0.4)	17, 34, 64 (0.4)	996 (**0.9**)	50
N1	0	17, 17, 26 (0.4)	0	11, 36, 46 (0.5)		
N2	0	0	0	0	35 (**0.1**)	25
N3	0	0	44 (0.2)	0	81 (**0.1**)	25
C1	17, 23, 45 (0.4)	0	0	37, 49 (0.4)		
C2	0	34, 43 (0.2)	33 (0.1)	36 (0.1)		
C3	0	0	0	0	634 (**0.6**)	0
O1	0	0	0	0		
O2	24, 34, 35, 38, 41, 50 (0.8)	0	0	112, 115, 131, 147, 151, 165 (2.6)		
O3	0	159, 160, 160, 171, 187, 201 (7.1)	10, 33 (0.2)	0	293 (**0.3**)	50

Healthy colonies (H1-H4). Sick colonies (S1). The apiaries shaded in grey include sick colonies. The percent relative abundance of Rhizobiaceae are in bold typeface and in parenthesis.

Differences among sick and healthy honey bee samples with apiary as the experimental factor

Table 3.8.4 | ANOVA table (Type II tests) for the relative abundance of phylotypes in the digestive tract of healthy and sick New Zealand honey bees.

Response	Sums of squares	DF	F – value	*P* – value
Apiary	0	20	0.0	**1**
Phylotype	184639	27	1479	**$2x10^{-16}$**
Apiary : Phylotype	5123	540	2.05	**$2x10^{-16}$**
Residuals	9447	2044		

Calculated using a linear model. Significant differences are in bold typeface.

Table 3.8.5 | ANOVA table (Type II tests) for the relative abundance of phylotypes in the digestive tract of healthy New Zealand honey bees.

Response	Sums of squares	DF	F – value	*P* – value
Apiary	0	20	2.12	**1**
Phylotype	167571	27	1640.48	**$2x10^{-16}$**
Apiary : Phylotype	4647	540	2.27	**$2x10^{-16}$**
Residuals	6674	1764		

Calculated using a linear model. Significant differences are in bold typeface.

Table 3.8.6 | Alpha–diversity of bacterial profiles in honey bees from healthy colonies using apiary as the experimental factor.

Diversity measure	*P* – value	Mann-Whitney/Kruskal-Wallis statistic
Chao1	0.11699	27.688
Observed ASVs	0.11907	27.605
Shannon	0.27308	23.326
Simpson	0.5599	18.418

Apiary was the experimental factor.

Chapter 4

Comparison between bacteria in the digestive tract of New Zealand honey bees and a population from Connecticut, United States of America

4.1 Abstract

NZ honey bees have been isolated from international bees since 1960, and the last documented importation of honey bees from the United States of America occurred in 1880, thus creating a model system for studying adaptation of bacterial populations within the honey bee as a host. This study compares the bacterial composition of NZ honey bees from seven regions with the bacterial composition in honey bees from Connecticut, USA. The intermingled presence of the dominant core bacteria in all 84 NZ colonies and the two USA colonies not only corroborates the theory that these dominant core bacteria are internationally widespread, but that they have remained stable within an isolated population for over 60 years. The latter highlights the importance of the symbiotic relations that these gut bacteria have with honey bees and provides an opportunity to exploit the bee as a model for human health. Sequence data from the V4 hypervariable region of the 16S rRNA gene from the NZ and USA population were merged in a single dataset and bioinformatic analysis was conducted to enable as direct a comparison as possible of both the presence of phylotypes and their relative abundance between the two countries. Thirty phylotypes were identified, of which 19 were present in both the NZ and USA samples, including the five dominant core phylotypes. The NZ colonies had 28 phylotypes and the USA samples had 21 phylotypes. The genera *Shewanella* and *Halomonas* were only present in the USA samples whereas nine phylotypes were only present in the NZ samples: the order Clostridiales, the family Rhizobiaceae, the genera *Bartonella*, *Escherichia*, *Pseudomonas*, and *Spiroplasma*, and the species *Obesumbacterium proteus*, *Spiroplasma apis,* and *Raoultella planticola.*

4.2 Introduction

The five dominant core phylotypes identified in the gut of honey bees (*Apis mellifera*) include two dominant core species in the phylum Proteobacteria; *Gilliamella apicola* and *Snodgrassella alvi* (Kwong and Moran 2013), two clusters of species from the phylum Firmicutes; *Lactobacillus* Firm–4, and *Lactobacillus* Firm–5 (Babendreier et al. 2007; Martinson et al. 2011), and the species cluster in the phylum Actinobacteria; *Bifidobacterium* (Bottacini et al. 2012; Moran et al. 2012). The subdominant core phylotypes include *Frischella perrara* (Engel et al. 2013b), *Bartonella apis* (Kešnerová et al. 2016), and Acetobacteraceae (*Parasaccharibacter apium*) (Corby-Harris et al. 2014b), and a *Gluconobacter*–related species group designated Alpha 2.1 (Martinson et al. 2011)).

As this field of research is still developing, the detection and abundance of these phylotypes are reported inconsistently within the literature: some studies, such as those conducted in South Africa (Jeyaprakash et al. 2003), Japan (Yoshiyama and Kimura 2009), Korea (Ahn et al. 2012), and Saudi Arabia (Khan et al. 2017), use broad

terminology, such as phyla, to identify some of these five phylotypes. Initially this was because the phylotypes were not well characterised, or culture-methodology was used to characterise specific phylotypes, or more recently a broad structural diversity of different regions, or *A. mellifera* subspecies, were the focus. The five dominant core bacterial phylotypes identified specifically within bees from the USA (Moran 2015; Raymann et al. 2017) and Europe (Jones et al. 2018a), are also present in the gut of NZ honey bees (Chapter 3). However, the presence and relative abundance of the less prevalent phylotypes in the NZ samples appear to differ from those in the literature as *Bartonella apis* and the *Gluconobacter*-related species were not identified.

The consistency of the dominant core bacteria across at least three continents suggests that these bacteria and their symbiotic relationships are important for the function of the bee. This is supported as the metabolism and hormone signalling of dominant core bacteria promote bee weight gain (Zheng et al. 2017), and also because lactobacilli are responsible for the majority of metabolic output (Kešnerová et al. 2017).

The relative abundance of bacterial phylotypes is rarely compared between studies because of the taxonomic biases introduced by differences in methodology; sampling protocols (Hermans et al. 2018), the hypervariable regions sequenced, or the bioinformatics and analysis pipelines that are employed (Pollock et al. 2018). Numerous examples include studies that only sample the crop (Corby-Harris et al. 2014a), or mid to hindgut sections (Jones et al. 2018a), or just the hindgut (Kapheim et al. 2015). Other studies focus on different castes, such as the queen (Tarpy et al. 2015), or larvae (Anderson et al. 2013). In relation to DNA extraction, different methods are biased toward Gram-positive or Gram-negative microorganisms, where the identification of the former is enhanced through a bead-beating step that breaks down the much thicker cell wall, thus enhancing the lysis of diverse Gram-positive microorganisms (de Boer et al. 2010). The use of different chemicals (cetyltrimethylammonium bromide/phenol (Engel et al. 2015) and phenol/chloroform/isoamyl alcohol (Moran et al. 2012)) to extract DNA from the honey bee gut also causes variation. Extraction kits (manufactured by Qiagen or ZymoResearch) may also include a bead-beating step to extract honey bee gut bacteria for 16S rRNA gene sequencing (Moran et al. 2012). Differences may also arise from sequencing different 16S rRNA hypervariable regions such as V1V2 (Sabree et al. 2012), V4 (Jones et al. 2018a), and V6 to V8 (Moran et al. 2012); the relative proportions of bacteria are similar between the V1 to V6 regions, but the V7 to V9 regions favour the identification of Firmicutes sequences but identify fewer Proteobacteria sequences (Lee et al. 2015). The V4, V5, V7 and V8 regions are less useful for identifying bacterial genus or species differentiation in the honey bee, whereas the V3 region suitably identifies all bacteria to the genus level (Lee et al. 2015), and effectively identifies *Lactobacillus* spp. (McFrederick et al. 2013). Therefore, metaanalysis combining data from many studies might have too many unknowns, and thus compromise the reliability of determining potential differences in bacteria among samples. A comparative analysis

between honey bee gut samples from different continents that were collected and processed similarly, was therefore of interest.

The NZ honey bee population has evolved in isolation from international bee communities since 1960 with the implementation of strict border control of honey bees and hiveware (Stevenson et al. 2005). Unknown subspecies of honey bees were first introduced to NZ from England in 1839, with subsequent introductions from England and Australia in 1842 (Hopkins 1926). Italian honey bees (A*pis mellifera ligustica*) were introduced from California in 1880 and Italy in 1883 (Hopkins 1926). Honey bee colonies naturally multiply through a process called splitting; the parent colony raises a second queen but before she emerges the initial queen and a large portion of the worker bees leave the colony in search for a new space to establish new comb and brood. As the bee populations increase, NEWs acquire gut bacteria from their nest mates and from the surfaces of the hive ware (Powell et al. 2014). This suggests the > 850,000 colonies within NZ (New Zealand Government 2018), are somewhat related to those initial introductions, as are the current gut bacteria.

It is therefore possible that the unique environments of Europe, North America, and NZ, may affect the evolution of the bacterial community in the honey bee gut. Specific factors may play a role: 1) floral resources native to each country, for example NZ mānuka has antibacterial properties. 2) In NZ it is illegal to use antibiotics in honey bee colonies (New Zealand 1998), whereas in Europe and the USA it is legal to use antibiotics to control gut pathogens. Antibiotics decrease both bacterial diversity (Raymann et al. 2017) and genetic diversity of the core gut bacteria (Raymann et al. 2018a). Both of these factors may therefore cause an increase in the relative abundance of phylotypes in the gut community that adversely affect bee physiology/health. This is termed dysbiosis (Sartor 2008; Hamdi et al. 2011). Diet-related gut dysbiosis in honey bees also correlates with impaired bee development, increased mortality and *Nosema* disease (Maes et al. 2016).

Open source access to V4 sequences from the gut of USA honey bees, identified by Powell et al. (2014), presents an opportunity to directly compare honey bee gut bacteria from a dataset produced in Connecticut, USA with bacteria in the guts of NZ honey bees. This study is the first attempt at understanding the relatedness of the gut bacterial phylotypes present in the 'closed' NZ honey bee population with a honey bee population from Connecticut, USA. This was achieved by comparing bacterial composition and the relative abundance of core and less prevalent gut bacterial phylotypes using the sequences obtained from the V4 hypervariable region of the 16S rRNA gene. Phylotype differences between these two populations may provide insight into the stability of the core and less prevalent bacterial phylotypes throughout decades.

4.3 Materials and methods

4.3.1 Sample collection and processing, DNA extraction and amplification, and sequencing of 16S rRNA gene amplicons

New Zealand samples

The NZ honey bee samples were the same 'healthy' samples used in Chapter 3. The sampling methodology, DNA extraction and 16S rRNA gene sequencing were all conducted as described in Chapter 3. Briefly, 84 samples of 'healthy' honey bees were collected from the outside frames of each brood nest (bees were 3–11 days old (Moore et al. 1987)) between 6 and 23 October 2014 (late spring). The guts of 20 bees were pooled for each of four colonies located in three apiaries from seven regions across NZ: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Christchurch (C), and Otago (O).

The DNA was extracted from the gut samples using a Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit (Zymo Research Corporation (ZR), California, USA) and then the V3V4 hypervariable regions of the 16S rRNA genes were sequenced by Massey Genome Service (MGS) (Massey University, Palmerston North, NZ). From here on these samples are referred to as NZ samples.

USA samples collected by Powell et al. (2014)

The V4 sequence files produced by Powell et al. (2014) from the Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut, USA, for two colonies were acquired in discussion with the first author and through the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra), BioProject identifier (ID) PRJNA225925.

These USA sequences were obtained by extracting the DNA from five bees aged 16 days old, from two colonies (total of 10 bees) located in West Haven, Connecticut, USA (Powell et al. 2014). The gut was extracted by thawing each bee for 5–10 min and dissecting the ileum and rectum separately using sterile conditions (Powell et al. 2014). Powell et al. (2014) extracted the DNA from the two gut sections using the methodology outlined in Table 4.1. The differences between the DNA extraction methodologies for the NZ and USA samples are also listed in Table 4.1. From here on, these samples are referred to as USA samples.

Table 4.1 | Comparison of the methods used to extract bacterial DNA from the guts of honey bees from New Zealand and United Sates of America.

	NZ	USA
Collection date	Spring (October) 2014	Autumn (October) 2012
Hives assessed	84	2
Samples / hive	1	5
Bees per sample	20	1
Apiaries	21 throughout NZ	1 in Connecticut, USA
Bee age	> 10 days	16 days
Gut section	Crop to rectum	Ileum and rectum
DNA extraction	Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit (Zymo Research Corporation (ZR), California, USA).	Cetyltrimethylammonium bromide
Bead beating	6 m/s for 40 s using a FastPrep®–24 (MP Biomedicals, Seven Hills, Australia).	Multisample bead beater (BioSpec Products), full speed (2 min), ice (1 min), bead-beaten (2 min).
Incubation	None	56°C overnight
Nanodrop	2000c	2000
Sequencing laboratory	New Zealand Genomics Limited (Illumina, Palmerston North, NZ).	Yale Center for Genomic Analysis (Illumina, San Diego, CA, USA).
Hypervariable region	V3V4 computationally trimmed to V4	V4
Sequencing	Illumina MiSeq 2 x 250 bp sequencing (MGS, NZ)	Illumina MiSeq 2 X 250 bp sequencing (Illumina, San Diego, CA, USA).
Range of average reads	32,436–148,889	21,586–49,317
BioProject identifier	To be submitted	PRJNA225925

4.3.1.1 Trimming the V3 and V4 reads for comparative analysis

The V3 reads and some of the V4 reads from the NZ sequences were computationally trimmed using Perl code written by Associate Professor Patrick Biggs (https://github.com/pjbiggs/misc_metagenomics). Figure 4.1 is a schematic diagram of where the NZ V3V4 sequences were trimmed in relation to the USA V4 sequences.

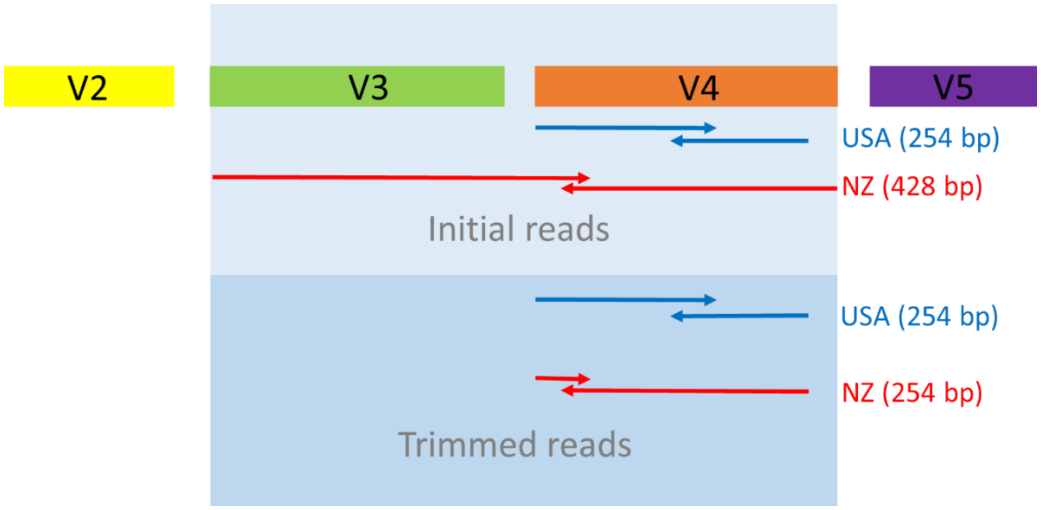


Figure 4.1 | Schematic of how the V3V4 reads of the gut bacteria in New Zealand honey bee samples were trimmed to V4 reads.

The red arrows indicate the NZ reads and the dark blue arrows indicate the USA reads. Hypervariable regions are labelled with a V. The light blue section indicates the initial reads and the dark blue section indicates the trimmed reads.

The trimmed NZ reads were then combined with the USA reads and analaysed in one batch. Visual interpretation indicated that the ASVs of NZ phylotypes differed to those of the USA phylotypes. To determine if this was a trimming issue, or different phylotypes, the ASVs associated with some of the core phylotypes were compared using Geneious 10.0.9 (https://www.geneious.com). It was found that the process of trimming the V3V4 NZ sequences to V4 sequences, resulted in the V4 NZ sequences having two additional nucleotides than the V4 USA sequences. A proportional transformation was used in Geneious 10.0.9 to construct a rooted-tree phylogram of the 16S rRNA V4 gene sequences of gut bacteria isolated from the honey bees in 21 NZ apiaries and the single apiary in Connecticut, USA.

4.3.2 Characterisation of microbial communities

The differences in ASVs for the same phylotypes between the NZ and USA ASVs were compared using Geneious 10.0.9 (Geneious).The unique ASV ID codes from the taxonomy table (for example OTUID: 2b19b72920ddfe62baeb66e339ca1b3d), and two to 15 of the associated nucleotide sequences (FASTA files) for the dominant core phylotypes and some subdominant phylotypes, from both the NZ and USA samples

were loaded into Geneious 10.0.9. All unclassified ASVs were included in the phylotype 'Bacteria'.

The dataset was analysed using the QIIME2 analysis suite, version 2018.2 (Caporaso et al. 2010) (Chapter 2: General Methods 2.8.2 Gene sequence processing and characterisation of microbial communities). The customised 16S rRNA BLAST database was used for gene sequence taxonomic classification (Chapter 2: General Methods 2.8.3). From this database a biological observation matrix (BIOM) was created that contained both the amplicon sequence variants (ASVs) identified from the sequencing of each sample and the associated taxonomic classifications. All ASVs that were unable to be assigned taxonomically to species were assigned to the closest identifiable taxonomic level (if not species then genus, if not genus then family etc.). The phylotypes in the NZ samples were represented by ASVs that differed to those in the USA samples. The sequences were analysed using Geneious 10.0.9 (Geneious).

4.3.3 Statistical analyses

Phylogenetic diversity was measured within a sample (α-diversity), and between samples (β-diversity) using the web-based tool MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017). The filters in MicrobiomeAnalyst were set so the data counts were unfiltered. The dataset was rarefied to its minimum library size (21514) and the data were relativised using total sum but were not transformed.

Alpha-diversity was calculated at the feature level using Kruskal-Wallis pairwise comparisons of four diversity measures: Observed ASVs, Chao1, Shannon, and Simpson.

Beta-diversity for the taxonomic level feature was calculated using the distance methods Bray-Curtis dissimilarity (that uses abundance of each ASV) and Jaccard Index (presence/absence), and the differences between the samples were compared using a permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001). Beta-diversity was displayed as 3-D PCoA plots.

Further data analysis was conducted in R (version 3.5.1) (R Core Team 2018). For all analyses, sequences with a minimum total read composition of < 0.01% prevalence were filtered from the dataset. This low threshold ensured the inclusion of most of the less abundant bacteria in the analysis. The V4 dataset contained 7,919,954 paired-end reads.

To investigate the differences in the number of phylotypes between apiaries, Poisson generalised linear models were used with the number of phylotypes as the response, and region or apiaries as a fixed effect. To explore the relationship between phylotypes and apiaries, the data were visually explored using heat maps, where the response was the mean read composition per replicate. The interaction of the relative abundance of

phylotypes was explored using a linear model (lm) in the R package lme4 (Bates et al. 2014). The assumptions were checked via standard residual plots. The function ‘predictmeans’ was used to plot predicted means to enable comparison between apiaries for each phylotype and significant differences was calculated using an average LSD. An ANOVA was used to determine significant differences among apiaries. The interaction of the relative of phylotypes in relation to apiary was further explored using a nonmetric multidimensional scale (NMDS) plot where the dissimilarity matrix was calculated using the Bray-Curtis dissimilarity method.

4.4 Results

The ASVs associated with the core phylotypes between the NZ and USA samples differed in overall sequence length. This occurred because the sequences were trimmed at slightly different nucleotide positions (further details are outlined in Supplementary material 4.1 and 4.2).

4.4.1 Number of amplicon sequence variants and phylotypes

A total of 7,977,316 paired-end reads were detected in the 94 V4 samples (84 NZ samples and 10 USA samples). This was filtered to 7,919,954 by removing reads with < 0.01% prevalence. The reads were identified as 150 unique ASVs and the number of V4 phylotypes in the NZ samples ranged from 23 to 28, whereas the number of phylotypes in the USA samples was 17 (Table 4.2).

Table 4.2 | Number of unique ASVs from the V4 hypervariable region and the associated range of bacterial phylotypes within the digestive tract of honey bees from NZ and USA.

Apiary	Number of ASVs	Number of phylotypes	Range of phylotypes within region
NL1	78	28	24–28
NL2	78	28	
NL3	68	24	
W1	74	25	25–28
W2	71	26	
W3	88	28	
HB1	80	28	25–28
HB2	74	28	
HB3	71	25	
WN1	65	25	23–25
WN2	55	23	
WN3	70	25	
N1	65	24	23–26
N2	74	23	
N3	74	26	
C1	66	25	25–27
C2	75	27	
C3	67	26	
O1	62	23	23–24
O2	66	23	
O3	58	24	
USA	22	17	17

Apiary locations: Northland (NL1–3), Waikato (W1–3), Hawkes Bay (HB1–3), Whanganui (WN1–3), Nelson (N1–3), Canterbury (C1–3), Otago (O1–3), Connecticut, USA (USA).

The results from the Poisson generalised linear model suggest that there was no significant difference in the number of unique phylotypes among the NZ apiaries (P > 0.96, Chi-squared = 11.26, df = 21).

The ASVs were classified as 30 unique phylotypes (excluding the phylotype Bacteria). Of these, 28 were present in the NZ samples and 20 were present in the USA samples (Table 4.3). Nineteen phylotypes were present in both the NZ and USA samples. A simplified version of a rooted–tree phylogram suggests 14 major phylotype groupings (Figure 4.3); one family (Oscillatoriaceae) and one species (*Limnoraphis robusta*) in the phylum Cyanobacteria, four phylotypes in the phylum Proteobacteria (*S. alvi*, Enterobacteriaceae, *F. perrara*, and *G. apicola*), three in the phylum Proteobacteria (Rhizobiales (including *Bartonella*), Acetobacteraceae (including *Gluconacetobacter*, *Pseudomonas*, and *Acetobacter*) as well as a group of unclassified bacteria from this phylum), and one in each of the phyla Bacteroidetes (Flavobacteriaceae), Tenericutes (*Spiroplasma* spp.), Actinobacteria (*Bifidobacterium spp.*), and Firmicutes (*Lactobacillus* spp.).

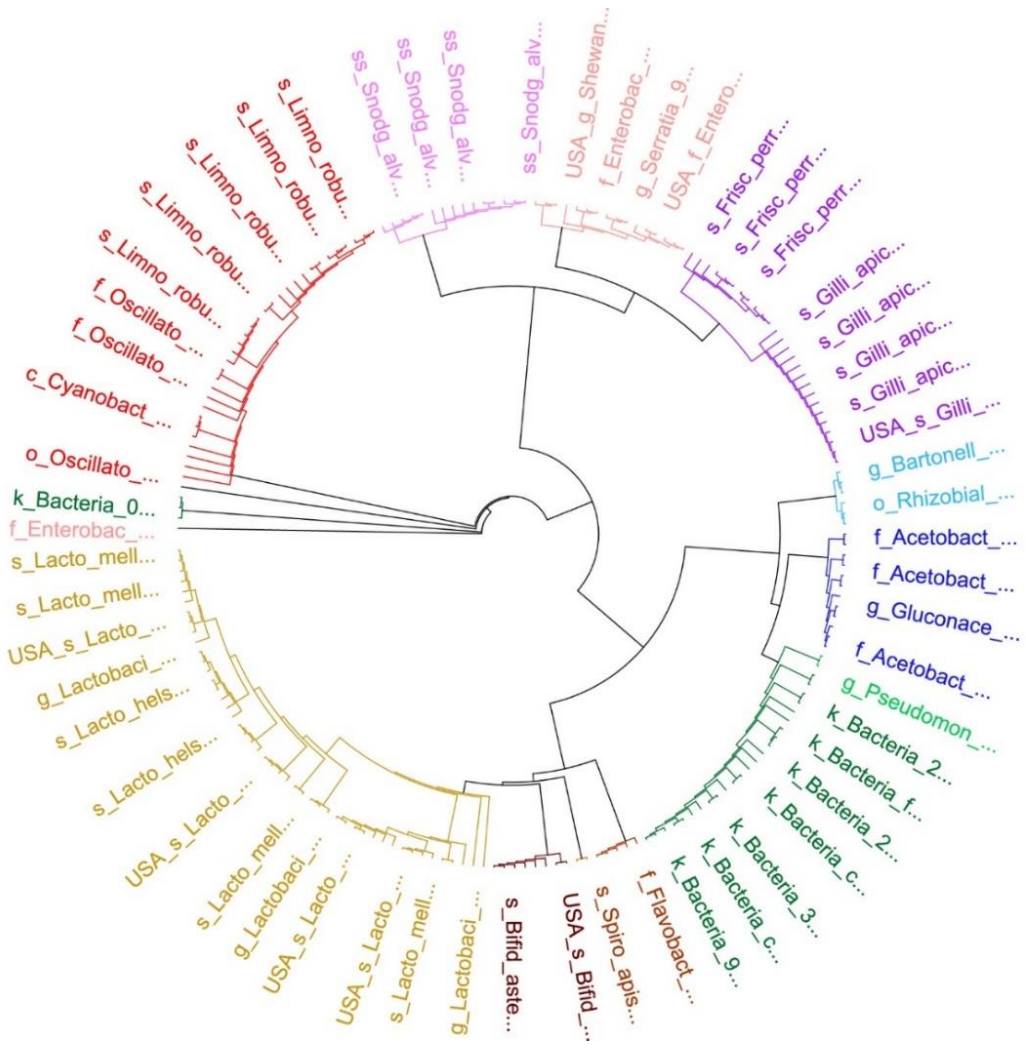


Figure 4.3 | A partial rooted-tree phylogram for the bacteria isolated from the gut of NZ and USA honey bees identified using the V4 hypervariable region of the 16S rRNA genes.

The branches were determined using proportional transformation. The USA sequences have a USA prefix. Phylotype labels: Enterobacteriaceae (f_Enterobac_), Flavobacteriaceae (f_Flavobact_), Oscillatoriaceae (f_Oscillato), *Halomonas spp.* (Halomonas_), *L. robusta* (s_Limno_robu), *S. alvi* (ss_Snodg_alvi), *Shewanella* spp. (Shewanell_), *F. perrara* (s_Frisc_perr), *G. apicola* (s_Gilli_apic), Rhizobiales (Rhizobial_), Acetobacteraceae (Acetobact_), Bacteria (Bacteria), *Bifidobacterium asteroides* (s_Bifid_aste), *Lactobacillus spp.* (g_Lactobaci), *L. apis* (s_Lacto_a), *L. melliventris*. (s_Lacto_me), *Lactobacillus helsingborgensis* (s_Lacto_h).

The complete rooted-tree phylogram indicates that most NZ and USA phylotypes are intermingled and closely connected phylogenetically (Figure 4.4). The first exception was one NZ Enterobacteriaceae ASV (f_Enterobac_) (a proteobacteria) labelled in peach that was positioned on a branch to the left of the plot that differed from the rest of the Enterobacteriaceae ASVs (peach coloured labels). The second was the genus *Halomonas* (a proteobacteria) as it was positioned separately from the rest of the proteobacteria labelled in pink and peach (*S. alvi*, Enterobacteriaceae, *F. perrara*, and *G. apicola*). The USA genus *Shewanella* was positioned within the cluster of NZ and USA ASVs for Enterobacteriaceae and the NZ ASV *Serratia* spp. The 27 unclassified bacterial ASVs (Bacteria labelled in dark green) were only present in the NZ samples. Three of these were closely associated but separated from all other taxonomic groups.

These unclassified NZ bacteria suggest that these ASVs have not been characterised to date. The other 24 unidentified phylotypes were positioned on a branch within the class α-proteobacteria. The genus *Lactobacillus* contained the most ASVs and was closely positioned with the genera *Bifidobacteria* and *Spiroplasma*.

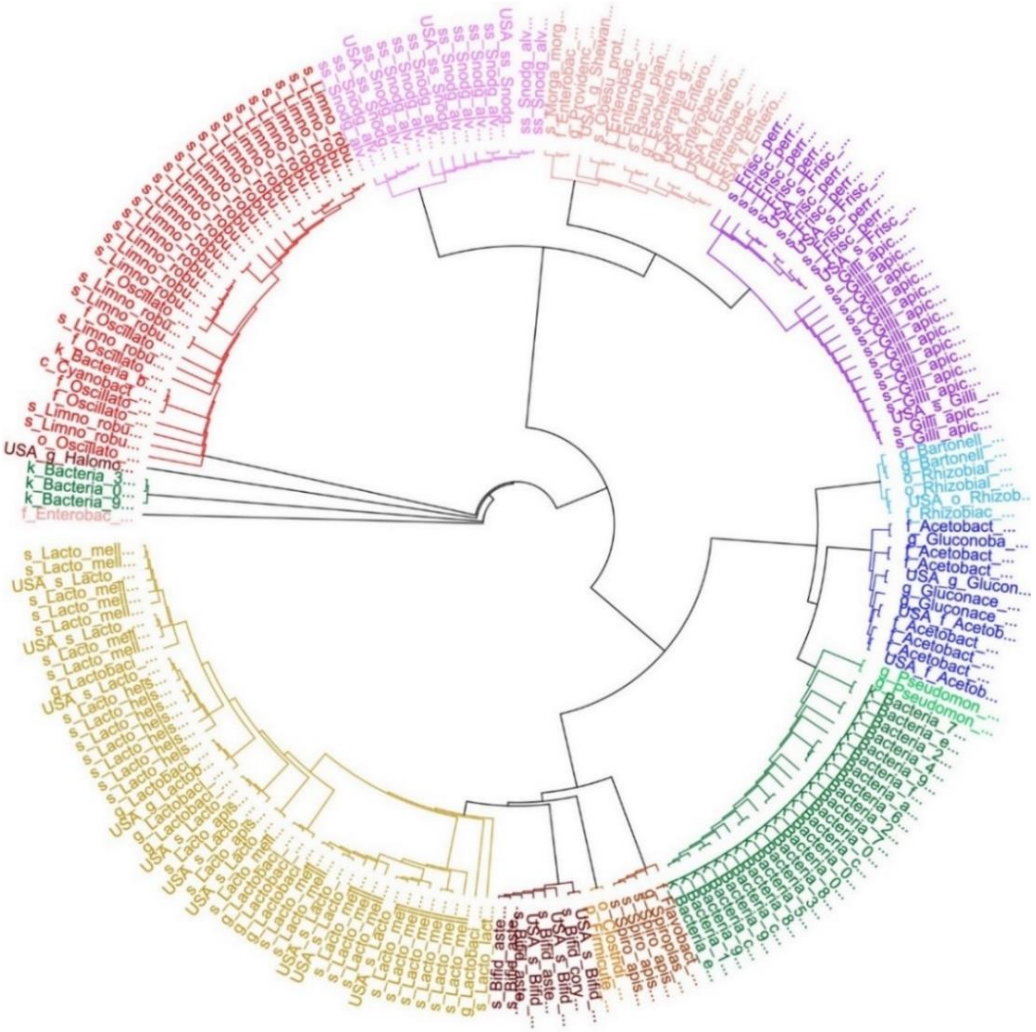


Figure 4.4 | A complete rooted-tree phylogram for the bacteria isolated from the gut of NZ and USA honey bees identified using the V4 hypervariable region of the 16S rRNA genes.

Branches were determined using proportional transformation. The USA sequences have a USA prefix. Phylotype labels: *L. robusta* (s_Limno_robu), *S. alvi* (ss_Snodg_alvi), *Shewanella* spp. (Shewanell_), Enterobacteriaceae (f_Enterobac_), *F. perrara* (s_Frisc_perr), *G. apicola* (s_Gilli_apic), Acetobacteraceae (f_Acetobact), Oscillatoriaceae (f_Oscillato), Gluconoacetobacter (g_Gluconace), Bacteria (Bacteria), *B. asteroides* (s_Bifid_aste), *B. coryneforme* (s_Bifid_cory), *Lactobacillus* spp. (g_Lactobaci), *L. apis* (s_Lacto_apis), *L. melliventris*. (s_Lacto_mell), *Lactobacillus helsingborgensis* (s_Lacto_hels).

A total of 36 phylotypes were identified across the V4 dataset and the V3V4 combined NZ dataset analysed in Chapter 3: Bacteria in the digestive tract of NZ honey bees. Thirty were identified in the V4 dataset compared with 19 in the V3V4 dataset. Six phylotypes in the V3V4 dataset were not identified in the V4 dataset: Cyanobacteria, Proteobacteria, *L. kunkeei*, *E. adhaerens*, *E. coli*, and *S. mirum*. Seventeen phylotypes in the V4 dataset were not identified in the V3V4 combined dataset (Table 4.3): the phylum Firmicutes, the orders Rhizobiales and Oscillatoriales, the family Flavobacteriaceae, the six genera *Gluconacetobacter, Bartonella*, *Halomonas*, *Shewanella*, *Escherichia* and *Spiroplasma*, and the seven species *Bifidobacterium asteroides*, *L. robusta*, *L. apis*, *L. helsingborgensis*, *L. melliventris*, *O. proteus*, and *R. planticola*.

Further analysis of these additional 17 phylotypes shows that the two genera, *Halomonas* and *Shewanella* were not identified in the NZ samples, and their relative abundance in the USA samples was low, despite the sum of the number of reads being high. Nine of the 17 phylotypes were not identified in the USA samples: the order Clostridiales, the family Rhizobiaceae, the genera *Bartonella*, *Escherichia*, *Pseudomonas*, and *Spiroplasma*, and the species *O. proteus*, *S. apis,* and *R. planticola* (Table 4.3). The other six phylotypes were present in both the NZ and USA samples.

Table 4.3 | Number of unique V4 ASVs associated with each phylotype in the V3V4 and V4 datasets.

Bacterial phylotype	V3V4 ASVs in NZ samples	V4 ASVs in NZ samples	V4 ASVs in USA samples
Actinobacteria (P)			
Bifidobacterium coryneforme *****	20	2	2
Bifidobacterium asteroides	–	3	3
Bacteroidetes (P)			
Flavobacteriaceae (F)	–	1	**1**
Cyanobacteria (P)	59		
Oscillatoriales (O)	–	4	**4**
Limnoraphis robusta	–	17	**17**
Firmicutes (P)	–	1	**1**
Lactobacillaceae (F) *Lactobacillus* spp. *****	126	7	7
Lactobacillus apis	–	4	4
Lactobacillus helsingborgensis	–	4	4
Lactobacillus kunkeei	7		
Lactobacillus mellifer	8	2	2
Lactobacillus mellis	12	11	11
Lactobacillus melliventris	–	5	5
Clostridiales (O)	11	1	–
Proteobacteria (P)	13		

Bacterial phylotype	V3V4 ASVs in NZ samples	V4 ASVs in NZ samples	V4 ASVs in USA samples
Alpha – proteobacteria (C)			
Acetobacteraceae (F)	26	7	7
Gluconacetobacter spp. **	–	3	3
Rhizobiales (O)	–	3	3
Bartonellaceae (F) *Bartonella spp.*	–	2	–
Rhizobiaceae (F)	6	1	–
Ensifer adhaerens	11		
Beta – proteobacteria (C)			
Neisseriaceae (F) *Snodgrassella alvi* *	48	12	12
Gamma – proteobacteria (C)			
Halomonas spp.	–	–	1
Pseudomonas spp.	6	2	–
Shewanella spp.	–	–	1
Orbaceae (F)			
Frischella perrara **	23	9	9
Gilliamella apicola *	90	12	12
Enterobacteriaceae (F)	4	6	6
Escherichia spp.	–	1	–
Escherichia coli	4		
Obesumbacterium proteus	–	1	–
Raoultella planticola	–	1	–
Tenericutes (P)			
Spiroplasma spp.	–	1	–
Spiroplasma apis	10	3	–
Spiroplasma mirum	6		
Bacteria (Domain)	9	22	–
Total number of phylotypes (including the domain Bacteria)	**20**	**29**	**21**

The V3V4 dataset was 16S rRNA V3V4 sequence data from the gut of NZ honey bees, and the V4 dataset was V4 sequences from both NZ and USA honey bees. Bacterial phylotypes present in only the NZ V4 samples are shaded in light grey and the phylotypes present only in the USA V4 samples are shaded in darker grey. Phylum (P), class (C), order (O), family (F), genus (spp.).

The heatmap of the means of the number of reads sequenced for each of the apiaries indicate 15 phylotypes were present in all of the NZ and USA apiaries (Figure 4.6). The phylotypes *L. apis* and *G. apicola* were the most abundant and apiary N2 had the most reads. The USA apiary had more *S. alvi* and *Gluconacetobacter* spp. reads than the NZ apiaries. In contrast 18 of the phylotypes were < 0.01% inclusion filter in some apiaries: the phylum Firmicutes, the class Clostridia, the orders Oscillatoriales and Rhizobiales, the families Rhizobiaceae and Flavobacteriaceae, the genera *Bartonella*, *Pseudomonas*, *Spiroplasma*, *Shewanella*, *Halomonas*, *Gluconacetobacter*, and *Escherichia*, and the species *Spiroplasma apis*, *Raoultella planticola*, *Obesumbacterium proteus*, and *L. robusta*. Rhizobiales and *Gluconacetobacter* spp. were detected in all apiaries except O2. Firmicutes, *Bartonella* spp., and *L. robusta* were detected in all apiaries except the USA apiary. Rhizobiaceae was detected in 34 of the NZ colonies from 18 apiaries, but neither of the USA colonies, although, high mean values of Rhizobiales were detected in both USA colonies: USA1 (21873), and USA 2 (5328).

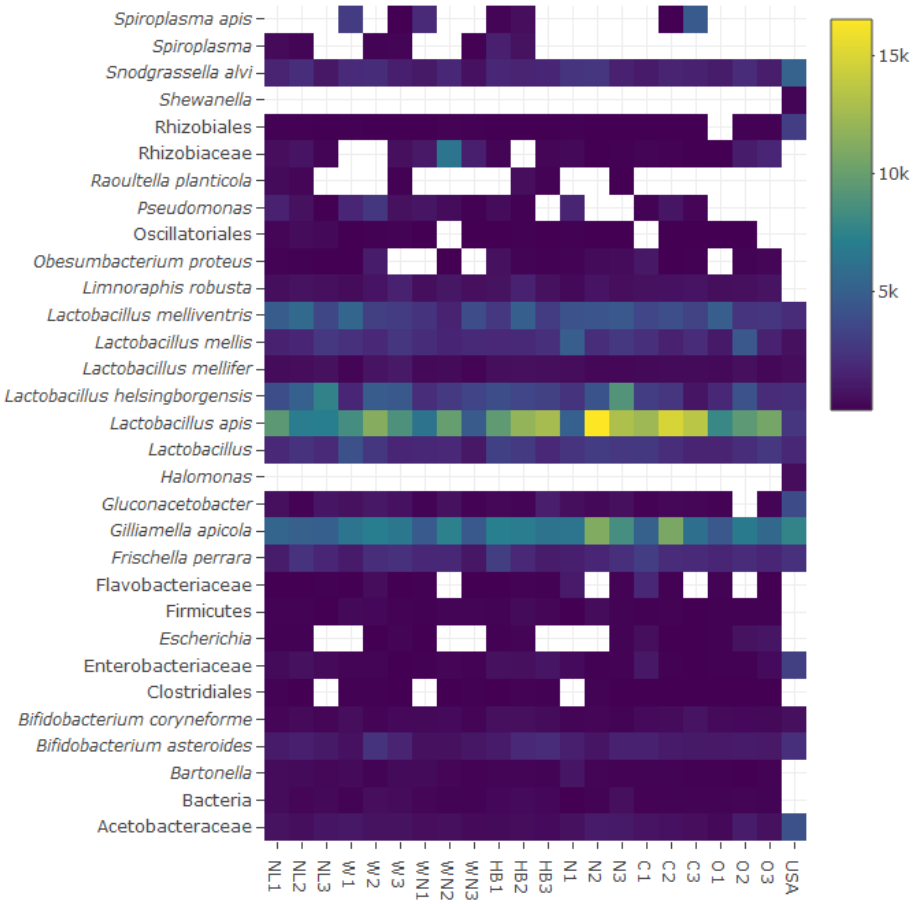


Figure 4.6 | Mean number of bacterial reads for each phylotype in the digestive tract of honey bees from 21 New Zealand apiaries and one USA apiary.

White spaces indicate prevalence < 0.01%. All italicised single phylotypes indicate the genus group for unidentified species e.g. *Lactobacillus* is the genus. Apiary locations: Northland (NL1–3), Waikato (W1–3), Hawkes Bay (HB1–3), Whanganui (WN1–3), Nelson (N1–3), Canterbury (C1–3), Otago (O1–3), Connecticut, USA (USA).

4.4.2 Relative abundance

The results from the linear models suggest significant differences in the relative percent abundance of unique phylotypes among apiaries ($P < 0.001$) (Table 4.5).

Table 4.5 | ANOVA table associated with the interaction of phylotypes in the digestive tract of honey bees from New Zealand and the USA.

Treatment	Sum of squares	DF	F value	*P* value
Apiary	0	21	0	1
Phylotype	84654	30	**741**	**< $2.2X10^{-16}$**
Apiary : Phylotype	6283	630	**2.6**	**< $2.2X10^{-16}$**
Residual	7551	1984		

Calculated using linear model for samples with abundance > 0.01%. Significant differences are bolded.

These differences are further supported by the predicted means plot (Figure 4.7) where a least significant difference (LSD) of > 2.76% suggests a significant difference between the mean relative abundance of phylotypes among apiaries. Differences among apiaries were observed for all dominant core bacteria: *Lactobacillus* spp. including the species *L. mellis* within Firm–4 and *L. apis*, *L. melliventris*, and *L. helsingborgensis* within Firm–5, *G. apicola, S. alvi* and *B. asteroides*. The NZ phylotype *B. coryneforme* was not significantly different. Significant differences were also observed in the less prevalent bacteria: Acetobacteraceae, *F. perrara*, *L. robusta*, Rhizobiaceae, Rhizobiales, and *S. apis.* The USA apiary had significantly more Acetobacteraceae, Rhizobiales, and *S. alvi* and significantly less *G. apicola* and *L. mellis* than all other apiaries. The V4 sequences also identified that apiary N1 had significantly more *L. mellis*.

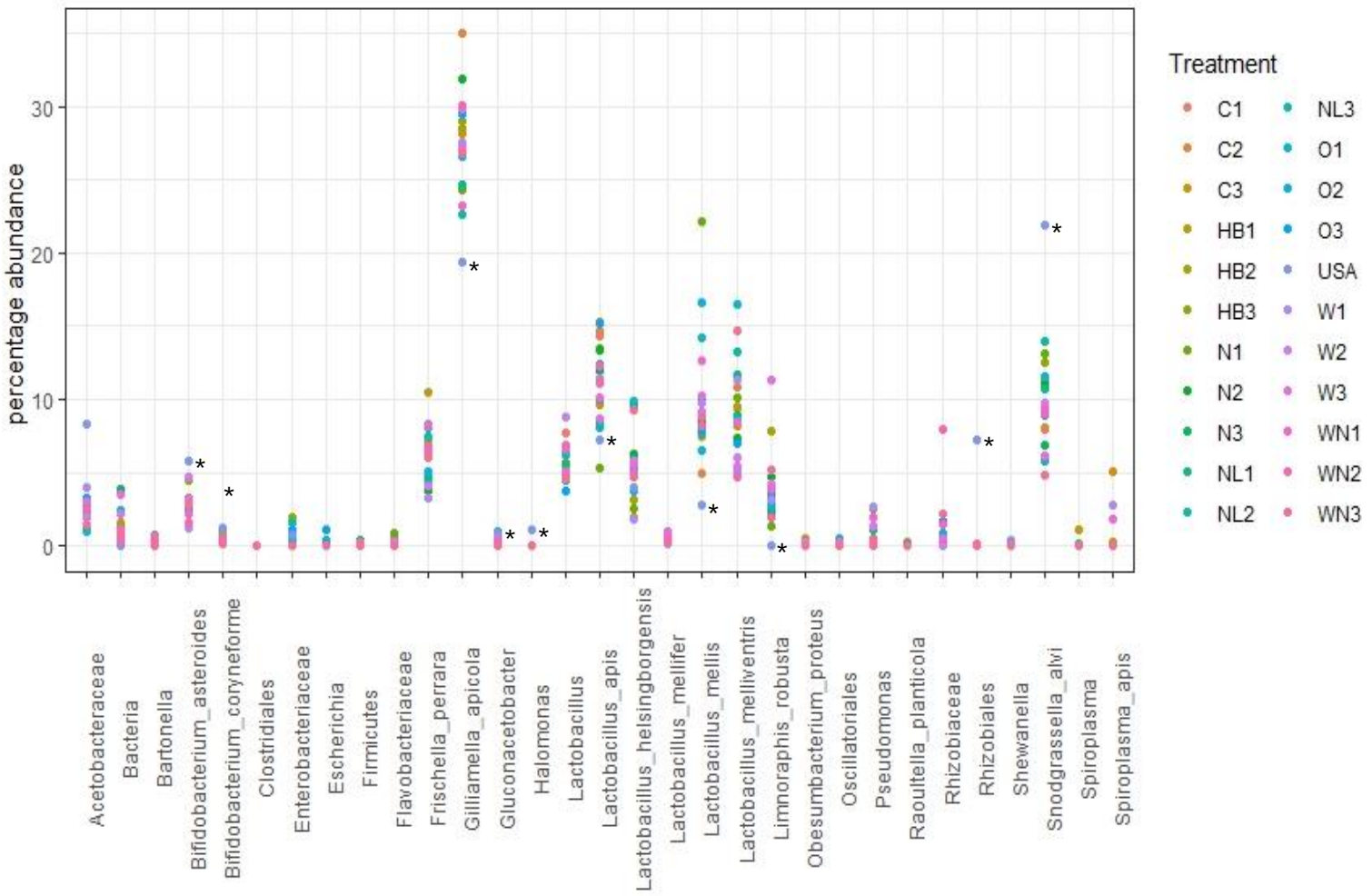


Figure 4.7 | Predicted means of the relative abundance of phylotypes in the digestive tract of honey bees from 21 NZ apiaries and one USA apiary.

Inclusion required > 0.01% prevalence. Predicted mean LSD average = 2.76%. Apiary locations: Northland (NL1–3), Waikato (W1–3), Hawkes Bay (HB1–3), Whanganui (WN1–3), Nelson (N1–3), Canterbury (C1–3), Otago (O1–3), Connecticut, USA (USA). The USA data are identified by an *.

Comparison between the relative abundance of the phylotypes in the NZ apiaries revealed *G*. *apicola* was the most abundant (21.90–34.86%), followed by *L. apis* (4.92–14.88%) in all except three apiaries (NL2, HB1, and N1), then *S. alvi* (4.89–21.85%), *L. mellis* (5.00–21.67%), and *L. melliventris* (4.60–15.79%) (Table 4.6). The relative abundance of the two other phylotypes recognised as dominant core phylotypes (Moran 2015) in the genera *Lactobacillus* (3.69–8.51%) and *Bartonella*, which includes *B. asteroides* (1.23–4.58%) and *B. coryneforme* (0.11–0.81%), were sixth, eleventh and seventeenth, respectively.

In contrast, the top five abundant phylotypes in the USA apiary, were *S. alvi* (21.71%) followed by *G*. *apicola* (16.38%), Acetobacteraceae (8.01%), *F. perrara* (7.87%), and *L. apis* (7.11%) (Table 4.6). The dominant core phylotypes in the genera *Lactobacillus* (4.44%) and *Bartonella* (*B. asteroides* (5.54%), and *B. coryneforme* (1.17%)), were eighth, sixth and thirteenth, respectively.

The relative abundance of the eight phylotypes listed first in Table 4.6 did not differ significantly among the 22 apiaries. However, the descending order of relative abundance for these phylotypes differed between the NZ and the USA apiaries. The NZ apiaries had 2- to 4-fold more *G. apicola* than *L. apis*, compared to the USA apiary. However, *S. alvi* varied as it was similar in abundance to *L*. *apis* in the NZ apiaries, but 3-fold more than *L. apis* and 1.3-fold more than *G. apicola* in the USA apiary.

The relative abundance of the phylotypes in Table 4.6 from Acetobacteraceae onwards, except the Clostridiales and *B. asteroides*, did vary significantly among apiaries. The NZ apiaries were significantly different from the USA apiary for the phylotypes Acetobacteraceae, *B. coryneforme*, and the genera *Halomonas* and *Shewanella,* which were not identified in the NZ colonies. Among the USA samples, four phylotypes were present but their abundance was below the 0.01% inclusion filter: the phyla Firmicutes (1 ASV), the order Oscillatoriales (4 ASVs), the family Flavobacteriaceae (1 ASV), and the species *L. robusta* (17 ASVs). Hence, only 18 USA phylotypes were included in the V4 relative abundance table (Table 4.6).

Table 4.6 | Relative abundance of the V4 phylotypes in the New Zealand and USA samples.

	NL1	NL2	NL3	W1	W2	W3	WN1	WN2	WN3	HB1	HB2	HB3	N1	N2	N3	C1	C2	C3	O1	O2	O3	USA
Gilliamella apicola	24.30	21.90	26.33	27.39	29.49	26.74	22.75	28.88	26.81	28.44	28.42	28.72	23.88	31.64	29.29	27.06	34.86	28.03	26.13	29.41	29.33	16.38
Lactobacillus apis	11.07	7.60	8.35	11.22	10.92	7.55	9.39	11.87	11.07	9.58	10.98	13.33	4.92	13.35	11.08	13.49	14.47	14.88	12.19	9.01	14.78	7.11
Snodgrassella alvi	10.11	13.31	5.67	9.09	9.02	6.10	8.98	8.99	4.48	10.84	10.45	11.56	12.37	10.28	5.03	7.68	7.84	10.21	11.14	8.45	8.66	21.71
Lactobacillus mellis	8.21	7.69	13.37	9.36	9.05	9.69	11.97	7.85	8.52	8.28	7.30	8.14	21.67	8.10	9.92	8.23	5.00	8.48	5.87	16.02	7.54	2.83
Lactobacillus melliventris	11.26	12.53	8.55	10.68	5.70	5.31	8.01	4.62	14.58	7.13	9.26	8.15	9.72	6.84	8.59	9.00	10.59	8.08	15.79	4.60	6.84	4.61
Lactobacillus spp.	5.08	6.54	4.37	8.51	6.49	5.00	6.47	5.74	4.53	5.44	4.55	4.67	5.27	6.40	4.57	7.63	6.64	6.17	5.08	6.68	3.69	4.44
Lactobacillus helsingborgensis	5.73	5.26	9.18	1.72	5.40	5.29	3.80	4.67	8.48	4.22	3.02	3.74	1.93	4.90	8.82	5.02	3.54	1.87	3.45	4.61	4.33	3.27
Acetobacteraceae	2.26	2.12	2.52	3.85	1.85	2.79	2.03	2.23	1.40	1.36	1.22	1.20	2.22	3.03	2.31	2.28	2.09	1.76	0.59	3.92	2.84	8.01
Bifidobacterium asteroides	2.81	2.67	2.24	1.24	4.58	3.00	2.05	1.59	2.68	2.07	3.18	4.42	2.36	1.23	2.68	2.92	2.22	2.41	2.70	2.19	2.80	5.54
Bifidobacterium coryneforme	0.24	0.36	0.24	0.49	0.16	0.25	0.46	0.41	0.26	0.53	0.56	0.36	0.16	0.17	0.11	0.34	0.37	0.81	0.52	0.27	0.42	1.17
Frischella perrara	4.40	7.83	6.85	3.12	3.70	6.21	7.19	6.39	6.00	10.31	6.75	4.35	3.62	3.00	3.95	8.07	5.80	5.40	6.51	4.67	6.08	7.87
Limnoraphis robusta	2.00	1.79	2.66	2.94	3.53	11.30	1.28	1.74	4.94	3.57	6.52	3.43	1.16	4.75	1.74	2.60	2.90	2.47	2.72	2.88	3.40	<
Enterobacteriaceae	0.85	0.91	0.37	0.07	0.17	0.02	0.07	0.28	0.11	0.44	0.31	1.73	0.47	0.03	0.06	0.35	0.05	0.03	0.08	0.09	0.78	1.51
Pseudomonas spp.	4.47	1.37	0.01	2.19	5.43	0.13	2.35	0.26	0.11	0.24	0.07	<	3.21	<	<	0.06	1.77	0.37	<	<	<	<
Rhizobiaceae	0.36	0.49	0.14	<	<	0.37	0.69	1.57	0.59	0.18	<	0.19	0.34	<	0.05	0.19	0.07	0.02	0.03	0.47	0.92	<
Lactobacillus mellifer	0.39	0.44	0.69	0.13	0.71	0.83	0.47	0.45	0.23	0.50	0.42	0.50	0.44	0.20	0.25	0.31	0.39	0.43	0.50	0.54	0.38	0.91
Bartonella spp.	0.48	0.48	0.20	0.27	0.09	0.19	0.55	0.26	0.15	0.15	0.09	0.09	0.74	0.08	0.06	0.07	0.10	0.05	0.06	0.02	0.05	<
Raoultella planticola	0.43	0.27	<	<	<	0.07	<	<	<	<	0.22	0.11	<	<	0.04	<	<	<	<	<	<	<

	NL1	NL2	NL3	W1	W2	W3	WN1	WN2	WN3	HB1	HB2	HB3	N1	N2	N3	C1	C2	C3	O1	O2	O3	USA
Gluconacetobacter spp.	0.65	0.10	0.95	0.41	0.94	0.45	0.15	0.59	0.16	0.20	0.14	0.86	0.34	0.29	0.40	0.09	0.20	0.23	0.10	<	0.33	1.86
Oscillatoriales	0.13	0.46	0.25	0.03	0.06	0.24	0.06	<	0.06	0.04	0.06	0.04	0.05	0.01	0.02	<	0.03	0.04	0.02	0.02	<	<
Clostridiales	0.10	0.02	<	0.04	0.04	0.02	<	0.06	0.04	0.03	0.12	0.04	<	0.05	0.02	0.02	0.02	<	0.02	<	0.02	<
Escherichia	0.05	0.05	<	<	0.02	0.2	0.04	<	<	0.04	0.2	<	<	<	0.07	0.60	0.02	0.04	0.04	0.48	0.97	<
Firmicutes	0.05	0.09	0.02	0.28	0.17	0.07	0.13	0.12	0.11	0.09	0.20	0.15	0.01	0.09	0.06	0.06	0.06	0.03	0.06	0.04	0.06	<
Flavobacteriaceae	1.95	0.04	<	0.02	0.04	0.05	0.97	<	0.18	0.01	0.02	0.08	0.10	<	0.03	<	0.02	0.25	0.02	0.06	<	0.03
Obesumbacterium proteus	0.94	0.01	0.06	0.29	0.08	0.04	0.08	0.29	0.34	0.09	0.04	0.02	<	0.13	0.28	<	0.03	1.23	<	<	0.04	<
Rhizobiales	0.02	0.01	<	0.02	0.01	0.03	0.07	0.01	0.02	0.07	0.08	0.02	<	0.06	0.06	5.61	0.03	0.03	0.03	0.08a	0.11	0.03
Spiroplasma spp.	0.11	0.22	<	<	0.09	0.13	<	<	0.12	1.43	0.55	<	<	<	<	<	<	<	<	<	<	<
Spiroplasma apis	<	<	<	0.43	<	0.01	7.20	<	<	0.31	0.79	<	<	<	<	<	<	10.14	<	<	<	<
Halomonas	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	1.04
Shewanella	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	0.34
Bacteria	1.19	0.87	1.76	0.63	1.92	3.44	0.41	0.33	0.75	1.47	1.86	1.22	0.04	0.45	3.18	0.24	0.59	0.41	0.89	0.60	0.53	<

Relative abundance < 0.01% (<).The phylotypes present only in NZ samples are shaded in light grey and phylotypes only present in USA samples are shaded in darker grey. The p values were generated using ANOVA.

4.4.3 Diversity analysis

The α-diversity of the relative abundance within each sample indicated that the richness of the USA samples was less than the NZ samples and that location significantly influenced richness (Chao1, Observed ASVS), even when evenness was accounted for (Shannon and Simpson) ($P < 0.001$) (Table 4.4, Figure 4.5).

Table 4.4 | Alpha-diversity of the V4 samples using location as the experimental factor.

Diversity measure	*P* value	Kruskal-Wallis statistic
Chao1	2.3177×10^{-7}	43.818
Observed ASVs	2.855×10^{-8}	48.49
Shannon	8.1031×10^{-7}	40.998
Simpson	7.9198×10^{-6}	35.796

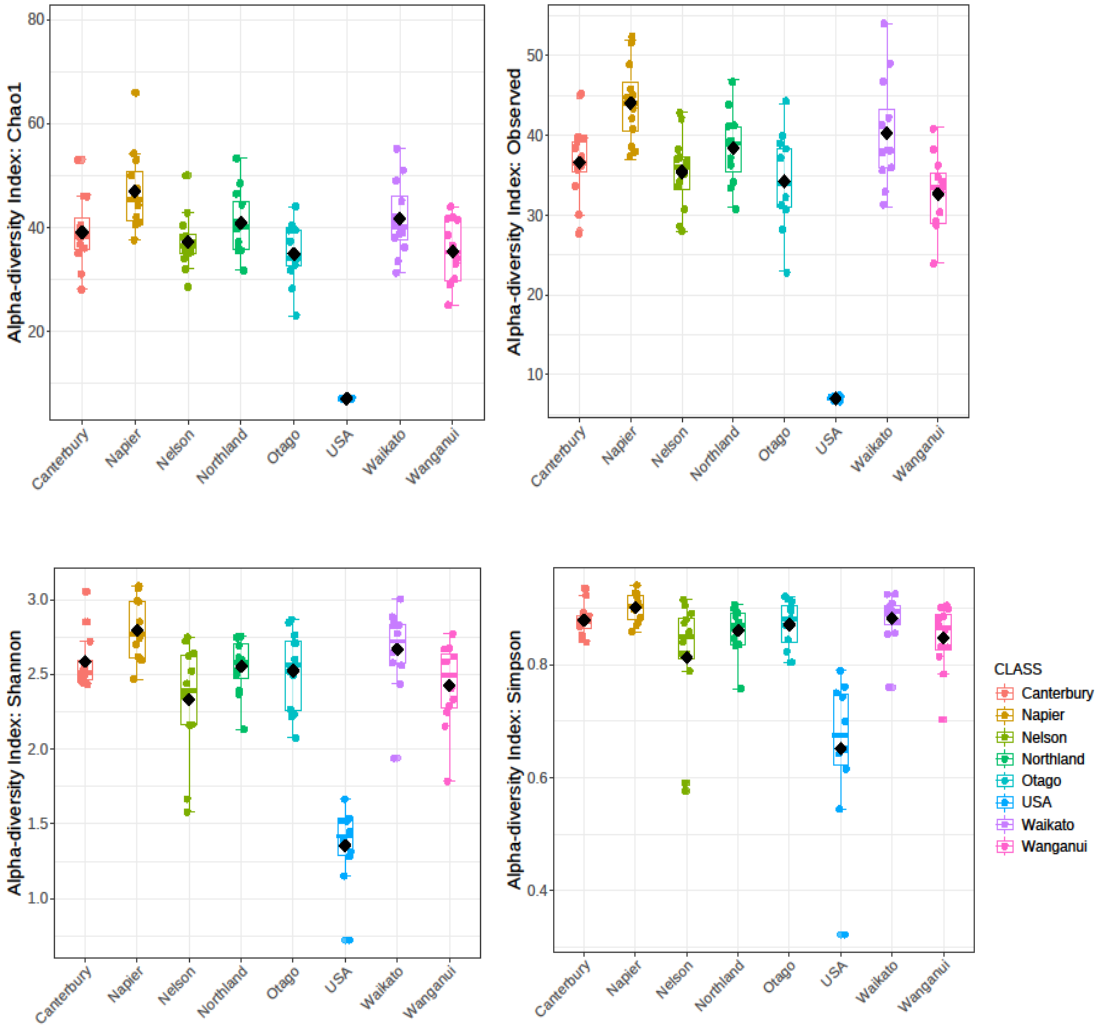


Figure 4.5 | Alpha-diversity measures of honey bee gut bacterial phylotypes from NZ and USA samples.

Location was the experimental factor. Each NZ dot represents data from 20 pooled bees, whereas each USA dot represents an individual bee.

Apiary significantly influenced microbiome diversity between the samples (β-diversity) ($P < 0.001$, R^2 ranged between 0.435 and 0.303 depending on distance method) (Table 4.7), as observed in the 2D PCoA plot where 40.7% of the variation is explained by apiary with the USA apiary separated completely from the NZ apiaries (Figure 4.8). The 3-D PCoA visualisations of the Bray-Curtis and Jaccard distance measures suggest that apiary location explains 47.1% or 31.5% of the community variation, respectively. The USA samples were positioned on the far left of the PCoA plot completely separate from the NZ samples on the right (Figure 4.9). Bacterial communities within the NZ apiaries also appeared to differ as the Napier samples clustered on the left of the PC3 axis but lower on the PC2 axis, whereas the Northland samples clustered higher on the PC2 axis and to the right of the PC3 axis.

Table 4.7 | Beta-diversity using location as the experimental factor.

Distance method	*P* value	*F* value	R^2	PC% Axis 1	PC% Axis 2	PC% Axis 3
Bray-Curtis	< 0.001	9.4	0.4	31.2	9.5	6.4
Jaccard	< 0.001	5.3	0.3	18.6	7.6	5.3

A non-parametric multivariate statistical test (PERMANOVA) was used to compare the affect of apiary on bacterial diversity.

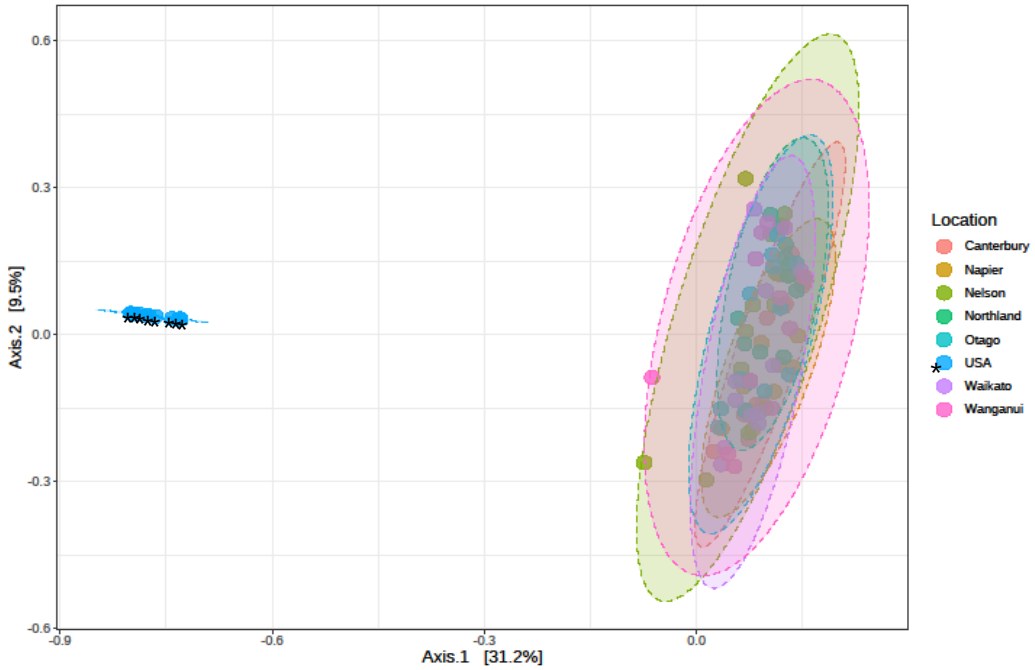
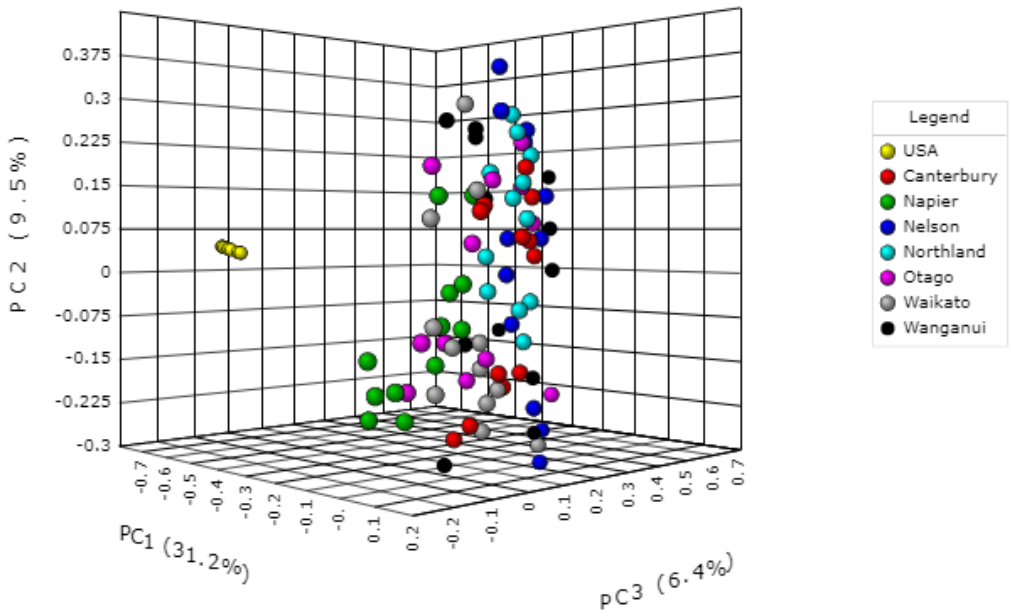
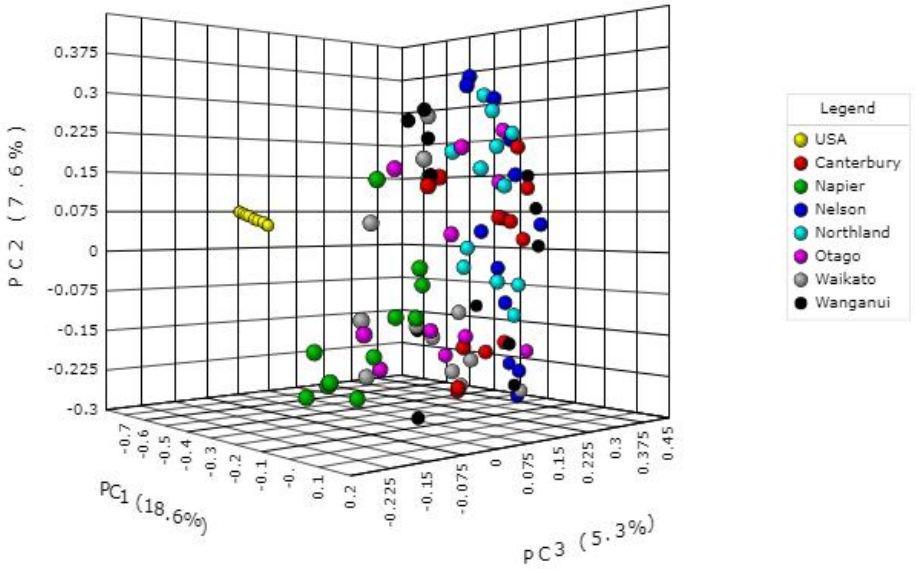


Figure 4.8 | Beta-diversity for bacterial phylotypes within the gut of honey bees located in NZ and the USA.

The PCoA plot used apiary location was the experimental factor and the distance measure was calculated using Bray-Curtis dissimilarity. The colonies were located in eight regions: Northland (NL), Waikato (W), Hawkes Bay (HB), Whanganui (WN), Nelson (N), Canterbury (C), Otago (O), and United States of America (USA).



A | Bray-Curtis Dissimilarity



B | Jaccard Index

Figure 4.9 | Beta-diversity for bacterial phylotypes from the V4 NZ and Powell colonies.

The β–diversity distance measures were: A | Bray-Curtis dissimilarity, and B | Jaccard Index.

4.5 Discussion

The intermingled presence of the dominant core bacteria in all 84 NZ colonies and the two USA colonies in Connecticut, USA not only corroborates the theory that these dominant core bacteria are internationally widespread, but that they have remained stable within an isolated population for over 60 years.

Number of amplicon sequence variants and phylotypes

The richness in the USA samples was less than the NZ samples. This is probably because the number of USA samples was small with just one apiary sampled and just five bees from each of two hives. The five internationally recognised dominant core bacterial phylotypes were present in both the NZ and USA samples: *G. apicola, S. alvi, Lactobacillus* species, and genus *Bifidobacterium*. However, the customised 16S rRNA BLAST database used in this study specifically identified five individual species normally grouped within *Lactobacillus* spp., as well as classifying the core phylotype *Bifidobacterium* to the species *B. asteroides* and *B. coryneforme*. As research in this field continues, I expect to see an increase in data specific to bacterial phylotypes. A possible hypothesis for why the V4 dataset had more phylotypes than the V3V4 dataset is because the V3 hypervariable region is able to identify more bacteria to the genus level, including the numerous species within the genus *Lactobacillus* (McFrederick et al. 2013); the proportion of the highly abundant phylotypes in the V3V4 region may have dwarfed the numerous but less abundant phylotypes (as identified in the V4 dataset) so that they were below the 0.01% inclusion threshold in the V3V4 dataset.

Dominant core bacteria

The number of unique phylotypes did not vary among the apiaries, but the relative abundance of unique phylotypes did differ among regions and apiaries. These differences were significant among the dominant core and subdominant phylotypes. The eight most abundant phylotypes were present in all 84 NZ colonies and the two USA colonies, and their relative abundance differed significantly among the 22 apiaries. The decreasing order of relative abundance of the bacteria in the NZ bee gut samples where *G. apicola* was the most abundant followed by *L. apis* differed from the order in the USA samples where *S. alvi* was the most abundant, followed by *G. apicola*. These data support the growing body of evidence that the five dominant core phylotypes present in the honey bee gut are prevalent worldwide and that they have symbiotic relationships with honey bees (Moran 2015; Anderson and Ricigliano 2017). Currently, these phylotypes are broadly classified in the literature. As sequencing technology continues to develop, coupled with species characterisation research, phylotype classification will become more specific and the symbiotic relationship of each bacterium with bees and bee health will be further recognised.

The variation among the descending order of relative abundance for these phylotypes varied between the NZ and the USA apiaries. *S. alvi* was the dominant bacterium in the USA apiary, whereas it was the second or third abundant in the NZ apiaries. This suggests the gut environment may differ between these populations. Further evidence is that the family Acetobacteraceae is third most abundant in the USA samples but tenth in the NZ samples. The differences may have arisen from the time of year that the samples were collected (Ludvigsen et al. 2015), which in this case was spring for the NZ bees and autumn for the USA bees (Powell et al. 2014), or the age of the bees that were sampled (Martinson et al. 2012). As there was no significant difference in the relative abundance of *F. perrara* between the NZ and USA samples (3–10.31%) and the abundance was < 25%, this indicates the sample bees were not NEWs and potentially older than seven days (Engel et al. 2015). External factors may also have influenced the bacterial composition as they entered the gut, such as diet, pathogens (Maes et al. 2016), or antibiotics (Raymann et al. 2018a), all of which cause dysbiosis and often lead to reduced host function (Anderson and Ricigliano 2017).

The biofilm in the honey bee ileum, the section between the midgut and the rectum, creates an interface between the bee tissue and the environment, i.e. exposure to ingested diet, antibiotics, etc. The core bacterium *S. alvi* is layered on the bee epithelium, overlaid by *G. apicola*, and species within the genus *Lactobacillus* also feature within this biofilm (Anderson et al. 2016). *S. alvi* is associated with protecting bees from from opportunists (Maes et al. 2016), stimulation of adult bee immune response (Kwong et al. 2017), and is displaced by *F. perrara*, *G. apicola*, and hive opportunists *P. apium* and *L. kunkeei* (Anderson and Ricigliano 2017). This displacement is correlated with reduced biofilm function and disruption of bee tissue by *F. perrara* (Engel et al. 2015), and strongly associated with bee development and early mortality (Maes et al. 2016). Analysis of genome-wide libraries indicates that *S. alvi* contains 2,226 protein-coding genes that enable extracellular interactions to form biofilm, for metabolic processes that uptake nutrients, and for stress responses that repair DNA. Of these, 519 are necessary for colonisation and an additional 399 are beneficial for gut colonisation but not essential (Powell et al. 2016).

The variation in the dominance of *S. alvi* observed between the NZ and USA populations may therefore indicate that the biofilm has been compromised, possibly by competition with other bacteria such as *F. perrara* or gut pathogens such as *Nosema* spp. Experimental trials where axenic honey bees are treated with bacteria and challenged with gut pathogens within the laboratory and the hive would assist with the understanding of these interactions (Chapter 7).

Subdominant core bacteria

The occurrence of some phylotypes only present in either the NZ bees or the USA bees, despite there being distantly related phylotypes in the other population, suggests that the phylotypes may have diverged over time. As the last recorded import of USA bees into NZ was in 1880 (Hopkins 1926), and live bee imports into NZ ceased in 1960 (Stevenson et al. 2005), there are at least 60 years over which this divergence may have occurred. A clear example of differences between the two populations is observed in the order Rhizobiales where the distantly related families Rhizobiaceae and Bartonellaceae (specifically *Bartonella* spp.) are present only in the NZ samples. Although *Spiroplasma* spp. are only observed in the NZ samples*, Spiroplasma apis* and *Spiroplasma melliferum* have been identified in previous international studies (Evans and Schwarz 2011).

Whether genetic dysbiosis may have occurred between these two populations, and whether this can be attributed to environmental selection pressure is currently unknown as a longitudinal study of the microbiome and associated metadata have not been conducted. However, open-access storage of sequences in NCBI may provide future opportunities to determine whether external factors cause genetic dysbiosis.

Less prevalent bacteria

The relative abundance of the subdominant and less prevalent phylotypes also varied significantly among apiaries. Nine phylotypes (Clostridiales, Rhizobiaceae, *Bartonella*, *Escherichia*, *Pseudomonas*, and *Spiroplasma*, *O*. *proteus*, *S*. *mirum*, and *R*. *planticola*) were only present in the NZ samples, and two genera, *Halomonas* and *Shewanella* (both γ-proteobacteria)*,* were only present in the USA samples.

The rooted phylogram intermingles the dominant core phylotypes in the NZ and USA samples, as well as the subdominant and less prevalent phylotypes. Neither of the genera *Halomonas* and *Shewanella* were identified in the NZ V4 samples. However, the more broadly associated phylum, Proteobacteria, was present in the NZ ASVs. The USA genus *Halomonas* was positioned separately to all other bacteria in the rooted phylogenetic tree, suggesting this genus may not be related to any within the NZ samples analysed. In contrast the USA genus *Shewanella* was positioned within the cluster of NZ and USA ASVs for Enterobacteriaceae, and this cluster also included the NZ ASV *Serratia* spp. The latter was not identified in the USA samples, suggesting either that the database entry was mislabelled or that NZ species within this family may be related but didn't match the published sequences for this genus sufficiently. This separation may indicate that the NZ honey bees contain *Serratia* spp. (within the family Enterobacteriaceae) that diverged from the USA family Enterobacteriaceae as a result of environmental selection pressure over the past six decades; the duration that NZ honey bees have been isolated from international bee populations (Stevenson et al. 2005). However, more likely explanations are that the abundance of *Shewanella* spp.

may have been below the 0.01% inclusion filter in the NZ samples, or that the presence of *Shewanella* spp. were contamination. In any case NZ bees may provide an interesting model to study selection pressures within related bacterial clusters.

The genera *Halomonas* and *Shewanella* were identified in 'blank' water samples in a study of the developing microbiome throughout the queen rearing process in North Carolina, USA (Tarpy et al. 2015). These genera are salt tolerant bacteria that are common in molecular biology reagents. Therefore, they are likely to be contaminants introduced during the DNA extraction process and hence removed from the analysis as they were considered contaminants. However, the family Halomonadaceae is present in bee bread that adult bees feed to larvae (Anderson et al. 2013), and although the genus *Halomonas* was not identified in extracted gut samples, it was identified in samples from entire bee bodies from four thriving and non-thriving colonies in Ireland (Ribière et al. 2019). *Shewanella profunda* has recently been identified in the honey bee gut and classified using high-throughput mass spectrometry (MALDI-TOF MS Biotyper) (Gasper et al. 2017). The NZ and USA V4 samples were from extracted gut samples, so the presence of the genera *Halomonas* and *Shewanella* in the USA samples may be because these were associated with the honey bee microbiome or they were sample contaminants. However, as the literature associated with this genus, and other less prevalent gut bacteria, is currently limited, this cannot be confirmed. The effect and importance of these less prevalent bacteria should be further verified using epidemiological and host-challenge studies.

The V3 region was trimmed from the NZ sequences to align with the USA sequences. However, the NZ sequences were two nucleotides longer, despite best efforts to prevent this. As the reads were run through DADA2 methodology using QIIME2, exact ASVs were produced. Therefore, the additional two nucleotides may have provided additional information that enabled the eight phylotypes only present in the NZ V4 samples to be classified more specifically. For example, the order Clostridiales in the NZ samples may only have been classified as the phylum Firmicutes in the USA samples, the NZ family Rhizobiaceae and genus *Bartonella* may have been classified in the order Rhizobiales, and the NZ genus *Escherichia* and the two species *O. proteus* and *R. planticola* may have been classified in the family Enterobacteriaceae.

The USA samples showed no evidence of the genera *Pseudomonas* and *Spiroplasma*, or the species *Spiroplasma mirum*, at a higher taxonomic level. This suggests that either these phylotypes may be specific to the NZ samples, or that these opportunists were not detected in the USA samples due to the small sample size. However, they are likely to be present elsewhere as *Pseudomonas oryzihabitans* has recently been identified within the honey bee gut from the Slovak Republic using MALDI–TOF MS Biotyper (Gasper et al. 2017), and the genus *Spiroplasma* was associated with diseased honey bees in France in 1983 and has been identified on

the surface of flowers (Mouches et al. 1983; Mouches et al. 1984). This may therefore be the route of transmission for this phylotype.

V4 dataset compared with the V3V4 dataset from Chapter 3: NZ survey of healthy and sick colonies.

Comparison between different studies may be unreliable as their relative abundance can be influenced by the 16S rRNA hypervariable region being analysed (McFrederick et al. 2013). This NZ study supports this as the relative abundance of core and less prevalent bacteria between the V4 and V3V4 datasets indicates that the genus *Lactobacillus* was not as well represented in the V4 dataset; seventh most abundant, as opposed to being the most abundant phylotype in the V3V4 dataset. The lack of ASVs for several bacteria in the V4 dataset, as opposed to the V3V4 dataset occurs repetitively; respectively, two ASVs of *Pseudomonas* spp. versus six, nine ASVs of *F. perrara* versus 23, and 12 ASVs of *G. apicola* versus 90. These differences in species classification between the two datasets emphasise the importance of comparing phylotypes from datasets that have analysed sequences from the same 16S rRNA hypervariable region(s), and indicate the increased advantage of sequencing both the V3 and V4 hypervariable regions, as opposed to a single region.

The NZ samples from the V4 dataset were a subset of the phylotypes from the NZ V3V4 dataset. It was therefore expected that some V3V4 phylotypes would be absent from the V4 NZ samples, as was the case for six phylotypes (Proteobacteria, Cyanobacteria, *L. kunkeei*, *E. adhaerens*, *E. coli*, and *S. mirum*). This presumably occurred because the V4 region on its own contained less information to distinguish the phylotypes, and it is likely that some of the species may have been assigned to less specific taxonomic levels. For example, *L. kunkeei* may have been assigned to the genus *Lactobacillus*, *E. adhaerens* may have been classified in the order Rhizobiales or included in the family Rhizobiaceae, *E. coli* may have been assigned to the family Enterobacteriaceae, and *S. mirum* may have been included in the genus *Spiroplasma*. Both explanations highlight the importance of comparing datasets using the same sequence information, such as all V3V4 sequences or just V4 sequences. The more specific assignment of the V3V4 phylotypes may also indicate greater classification is achieved using both the 16S rRNA V3 and V4 hypervariable regions.

It was also expected that all NZ V4 phylotypes would be present in the V3V4 dataset. However, 17 NZ V4 phylotypes were not identified in the V3V4 dataset: Firmicutes, Rhizobiales, Oscillatoriales, Flavobacteraceae, *Bartonella*, *Halomonas*, *Shewanella*, *Spiroplasma*, *Gluconacetobacter* spp., *Escherichia* spp., *B. asteroides, L. apis*, *L. helsingborgensis*, *L. melliventris*, *O. proteus*, *R. planticola*, and *L. robusta*. As the V3V4 dataset only had 19 phylotypes the 17 additional phylotypes observed in the V4 dataset may have occurred because they were above the 0.01% inclusion filter as the relative abundance of the dominant core bacteria were lower than those in the V3V4 dataset.

For example, *Lactobacillus* was the most abundant phylotype in the V3V4 dataset (31.0–46.7%), compared with sixth in the V4 dataset (3.69–8.51%). The higher relative abundance of the dominant V3V4 phylotypes means the relative abundance of the less prevalent phylotypes would be lower, and potentially below the 0.01% inclusion filter, excluding then from the V3V4 dataset. A second explanation may be that a longer V3V4 sequence may increase the possibility of their being a different ASV. The target sequences that should classify more specifically due to the additional information may identify with portions of sequences from more organisms, thus the current classifier could not determine which species to classify it, so they were placed in the taxonomic level above. A third option may be the lack of a linear relationship between sequence length and taxonomy in the 16S rRNA sequences.

The absence of the 17 phylotypes in the V3V4 dataset is not likely to be related to the use of different confidence levels (stringency setting) during sequencing as the V4 NZ sequences are a subset of the V3V4 dataset. However, the two datasets may have had different criteria or confidence levels during the computational trimming and bioinformatics analysis. A higher confidence level used in the V4 analysis may have classified the phylotypes less specifically. For example, the phylum Firmicutes in the V4 sequences may be classified more specifically as the genera *Lactobacillus, Bacillus* or *Lachnoclostridium* in the V3V4 analysis. Further examples include the order Rhizobiales which may be classified as the species *Ensifer adhaerens* or included in the family Rhizobiaceae, the genera *Bartonella*, *Halomonas*, and *Shewanella*, may have been included in the phylum Proteobacteria. The genus *Gluconacetobacter* may also be included in the phylum Proteobacteria, or within the family Acetobacteraceae; this is likely as the V3V4 dataset had 32 ASVs associated with Acetobacteraceae. Additionally, the genus *Escherichia*, the species *O*. *proteus*, and *R*. *planticola* identified in the V3V4 sequences may be classified within the Enterobacteraceae family, and *L*. *robusta* and Oscillatoriales may be classified within the phylum Cyanobacteria. The V3V4 dataset had 59 associated ASVs, suggesting that this phylum should be further characterised. *B. asteroides* was not detected in the V3V4 dataset but was present in V4. It is likely that this occurred because the high relative abundance of the core bacteria in the V3V4 dataset meant the relative abundance of *B. asteroides* was below the 0.01% inclusion threshold. If it was present, it is unlikely to be classified as anything other than this bacterium as no broader classification (not even at the phylum level of Actinobacteria) was identified.

Dysbiosis versus adaptation

The difference in the bacterial composition between the NZ and USA bees suggests that specific bacteria may have adapted to their respective local environments or that dysbiosis in the microbiome may have occurred throughout this time. Dysbiosis has been linked to specific diseases and diet in honey bees (Maes et al. 2016), and exposure to antibiotics can reduce the number of bacterial cells in the honey bee gut

(Raymann et al. 2017). Prophylactic antibiotic treatments are used internationally to control bacterial diseases in the honey bee gut; *P. larvae* subspecies larvae is commonly treated with oxytetracycline, a broad-spectrum antibiotic that is active against a wide variety of Gram-positive and Gram-negative organisms. It is illegal to treat colonies with antibiotics in NZ and since the importation of honey bees was prohibited in the 1960s (Stevenson et al. 2005) (excluding an importation of Carniolan bee semen around 2005), the difference in phylotypes was expected.

As the differences observed in this analysis are only possibly related to one potential source of bees imported from California, it is likely that the bacteria within NZ bees may differ from other populations of bees such as those introduced from England and Australia. This is the first opportunity to understand whether bacterial adaptation within the honey bee gut may have occurred over time.

4.6 Conclusion

This V4 analysis is the first attempt at comparing the relatedness of the geographically isolated phylotypes in the NZ honey bee population with an international honey bee population. The intermingled presence of the dominant core bacteria in all 84 NZ colonies and the two USA colonies not only corroborates the theory that these dominant core bacteria are internationally widespread, but that they have remained stable within an isolated population for over 60 years. The latter highlights the importance of the symbiotic relations that these gut bacteria have with honey bees and provides an opportunity to exploit the bee as a model for human health.

Despite this stability of the core bacteria, differences between the NZ and USA populations were observed, and the variation in relative abundance of core and subdominant bacteria between the two countries supports the theory that external factors may cause bacteria to adapt to the gut environment or may cause dysbiosis in the honey bee gut microbiome. Whether the adaptation has occurred in the NZ bees or the USA bees or both is still undetermined, but it clearly shows that the presence of individual phylotypes is less important than what the phylotypes do. Whole genome sequencing would identify the function of potential adaptations on the physiology and health of bee.

The identification of phylotypes present in the gut of NZ honey bees provides a database that can be further researched using species characterisation and epidemiological studies to understand how these changes may have occurred within the NZ honey bee population.

4.7 Where to next

As the USA samples were collected in autumn and the NZ samples were collected in spring, seasonal differences may have influenced the absence of some bacteria. The bacterial composition in the honey bee midgut has been shown to alter throughout the season (Ludvigsen et al. 2015). Whether this occurs in the entire gut will be determined by observing the change in proportion of gut bacterial phylotypes throughout a 12 month period, and thus creating a small honey bee gut dataset for a temperate climate.

4.8 Supplementary material

4.8.1 | Comparison between the bases in the V4 sequences of the core phylotypes in the New Zealand and USA datasets using Geneious 10.0.9.

The ASVs associated with core bacteria from NZ samples differed from the Connecticut samples. For example, an ASV associated with *G. apicola* from a single NZ sequence (ASV identifier: #15: 8b893271247a50f27d01fac627ffa64) was longer than a single USA ASV sequence (ASV identifier: #14: 36aed5b1dc9b5c1a2844e58f2d34b1f5) by one nucleotide. The NZ sequence had an additional nucleotide at the start and at the end of the sequence (Table 4.8.2). The rest of the nucleotides were in the same order between the 2nd and 254th nucleotide positions. Therefore, the USA sequence was a subset of the NZ sequence and both were classified as *G. apicola*.

Further comparisons between several ASVs for each of the core phylotypes support this. Most of the nucleotides in the USA sequences were the same as the NZ sequences but without the nucleotides in position 1 and 255 (*G. apicola S. alvi*, *Lactobacillus spp.*, *Lactobacillus helsingborgensis, F. perrara*, *Bartonella* spp., Enterobacteriaceae). However, there were a couple of exceptions where the USA nucleotides differed from the NZ nucleotides: *L. apis* (nucleotide 41), *L. melliventris* (nucleotide 91), *Bifidobacterium asteroids* (nucleotide 44), Enterobacteriaceae (nucleotide 121), and *Gluconoacetobacter* (nucleotide 40 and 254). The sequences for *Bifidobacterium coryneforme*, Acetobacteraceae, and *Gluconoacetobacter* present in the NZ bees were trimmed so nucleotides 1–19, 1–22, and 1–21 were absent, respectively (Supplementary material 4.8.2).

The sequences were wrapped to fit on the page and the differences between the ASVs are highlighted. The USA samples are identified with _USA. The phylotypes are identified as: *S. alvi* (ss_Snodg_alvi), *Lactobacillus spp.* (g_Lactobaci), *L. apis* (s_Lacto_apis), *L. melliventris*. (s_Lacto_mell), *Lactobacillus helsingborgensis* (s_Lacto_hels), *Bifidobacterium asteroides* (s_Bifid_aste), *Bifidobacterium coryneforme* (s_Bifid_cory), *F. perrara* (s_Frisc_perr), *Bartonella* spp. (g_Bartonell), Acetobacteraceae (f_Acetobact), *Gluconoacetobacter* (g_Gluconace), Enterobacteriaceae (f_Enterobac).

Table 4.8.2. Comparison among the bases in the V4 sequences of the core bacterial phylotypes in the honey bee guts from New Zealand and United Sates of America.

Sequence identifier	V4 sequence
Consensus	
1. s_Gilli_apic_0a0009fc1850341ad51ded04bed23212	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
2. s_Gilli_apic_4a14e2afb132c11d6c750e6b20d241af	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTACT
3. s_Gilli_apic_6636390e3b42f36705ced2232c6b61b1	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
4. s_Gilli_apic_68b504e29fb880d52fd43b843f01cc70	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
5. s_Gilli_apic_6cbb32e6c21dd5bb02c54a8a15085802	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
6. s_Gilli_apic_72284aa919b2b46cce6bafbeebb1e43a	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTGGAGTATT
7. s_Gilli_apic_8b27f0d32ef33ab912b6af4baf761d3f	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
8. s_Gilli_apic_99065fb2e760a05c64867c6c69b76636	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTTAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
9. s_Gilli_apic_a11c1abe2575cb710c0c153b631588a3	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
10. s_Gilli_apic_a67add0b0d6bb7154ddb2ed644818c09	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGTATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
11. s_Gilli_apic_d5c015c365ab7307e2172d4962d255a5	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCTCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
12. s_Gilli_apic_e6f9f0ab9a5fcc26d252e07cd7f08e45	AATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
13. s_Gilli_apic_cf8c4b03428fff81a768649cb0ef5c24	TCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
14. s_Gilli_apic_36aed5b1dc9b5c1a2844e58f2d34b1f5_USA	TACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
15. s_Gilli_apic_8b893271247a50f27df01fac627ffa64	ATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATT
Consensus	
1. s_Gilli_apic_0a0009fc1850341ad51ded04bed23212	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
2. s_Gilli_apic_4a14e2afb132c11d6c750e6b20d241af	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
3. s_Gilli_apic_6636390e3b42f36705ced2232c6b61b1	GTAGAGGAAGGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
4. s_Gilli_apic_68b504e29fb880d52fd43b843f01cc70	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
5. s_Gilli_apic_6cbb32e6c21dd5bb02c54a8a15085802	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGACCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
6. s_Gilli_apic_72284aa919b2b46cce6bafbeebb1e43a	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
7. s_Gilli_apic_8b27f0d32ef33ab912b6af4baf761d3f	GTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCTTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAGGA
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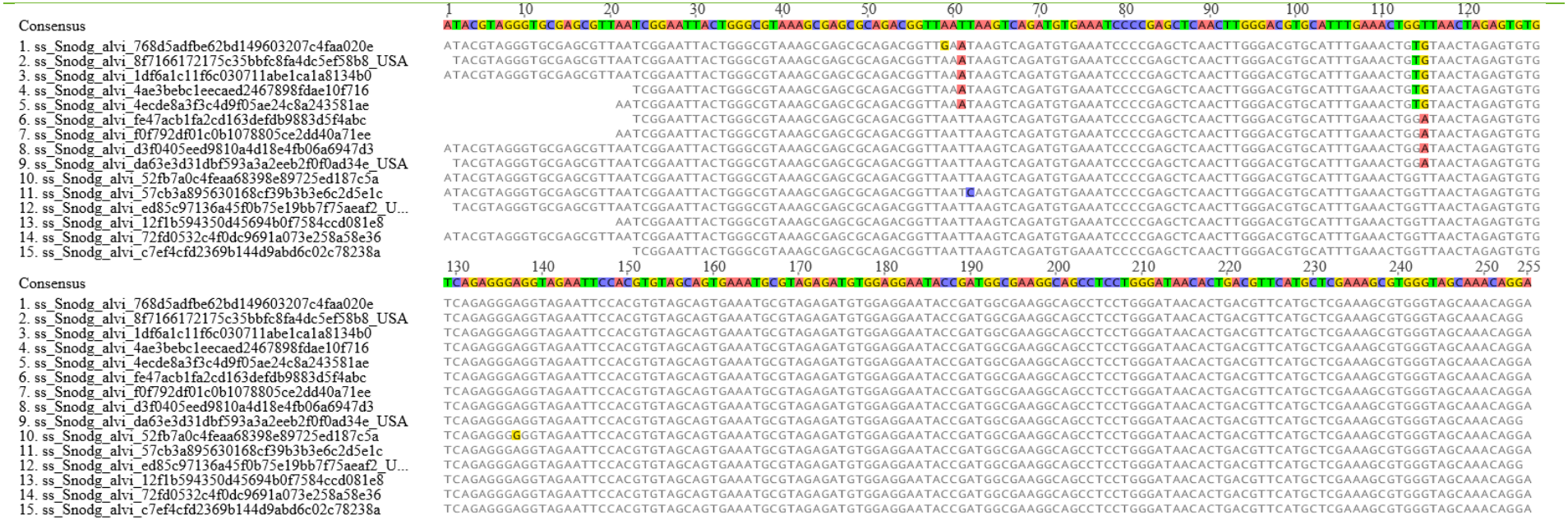

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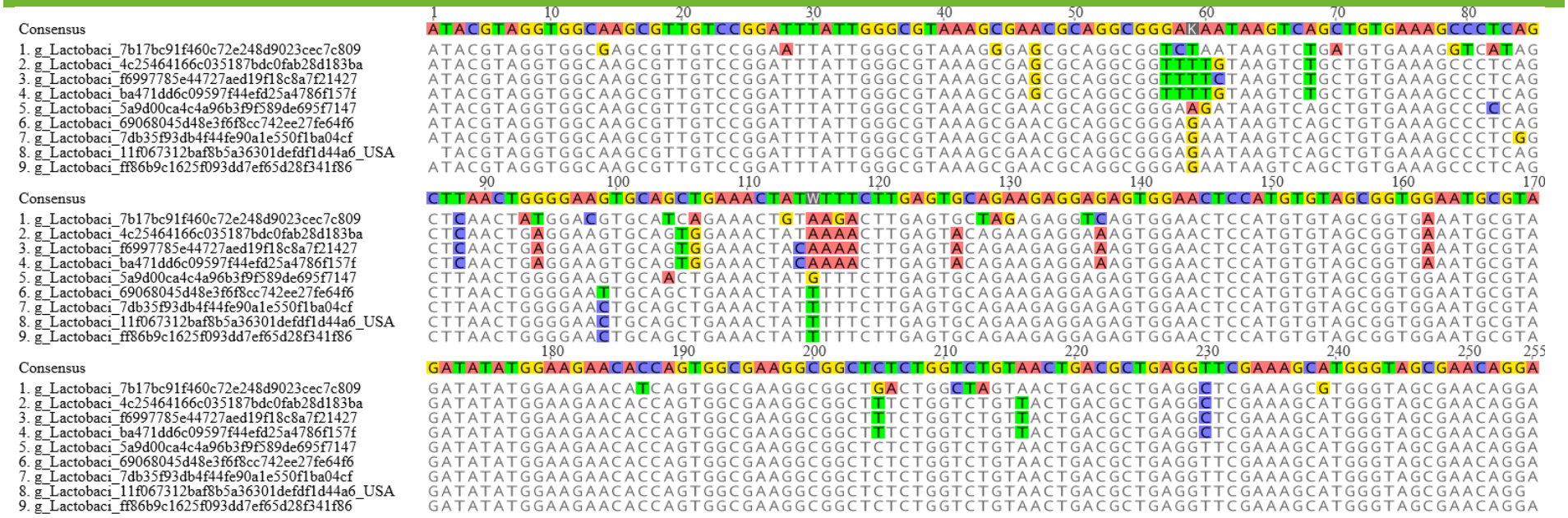

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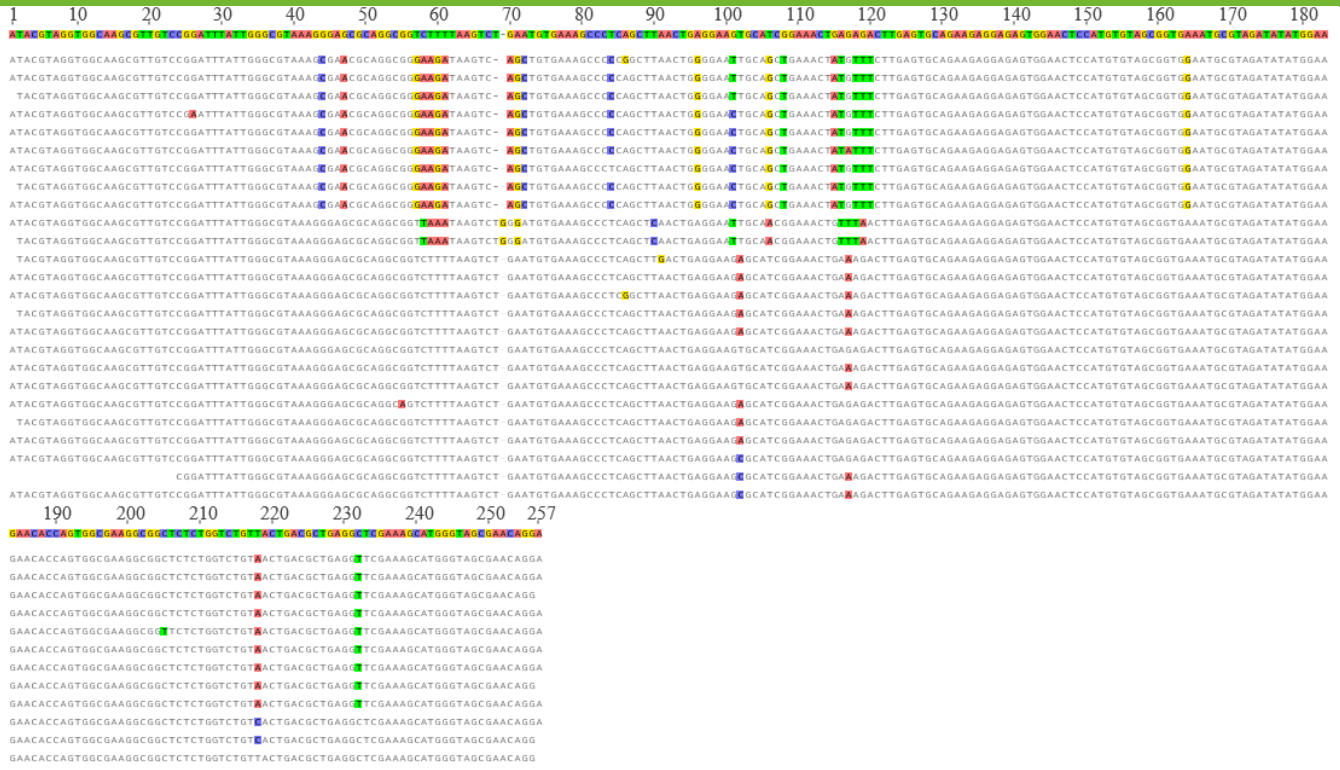

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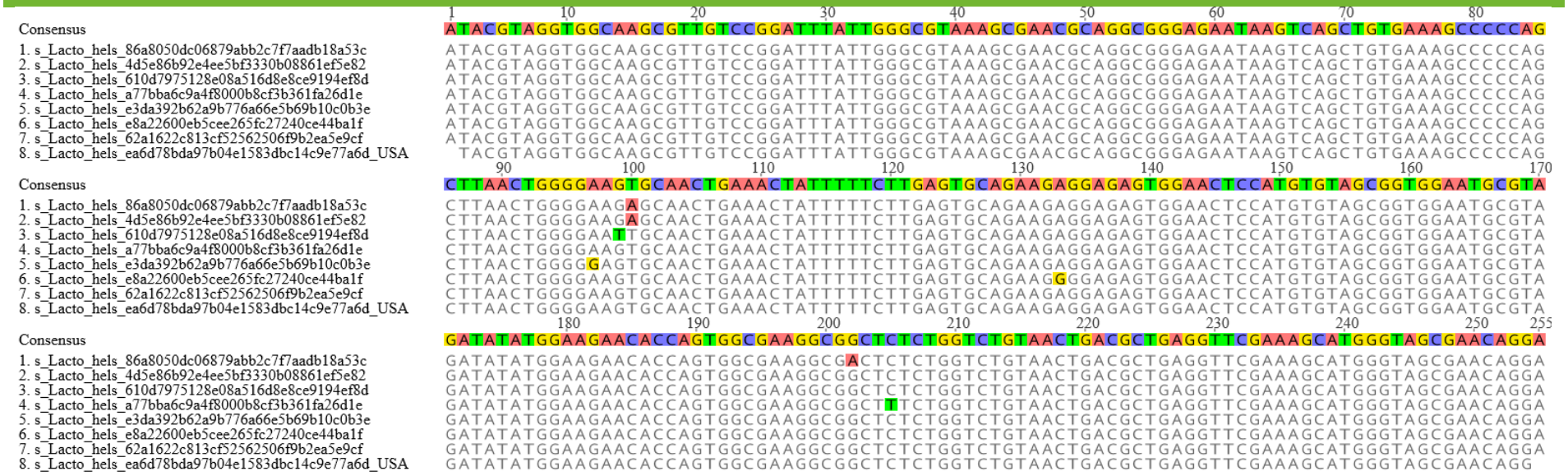

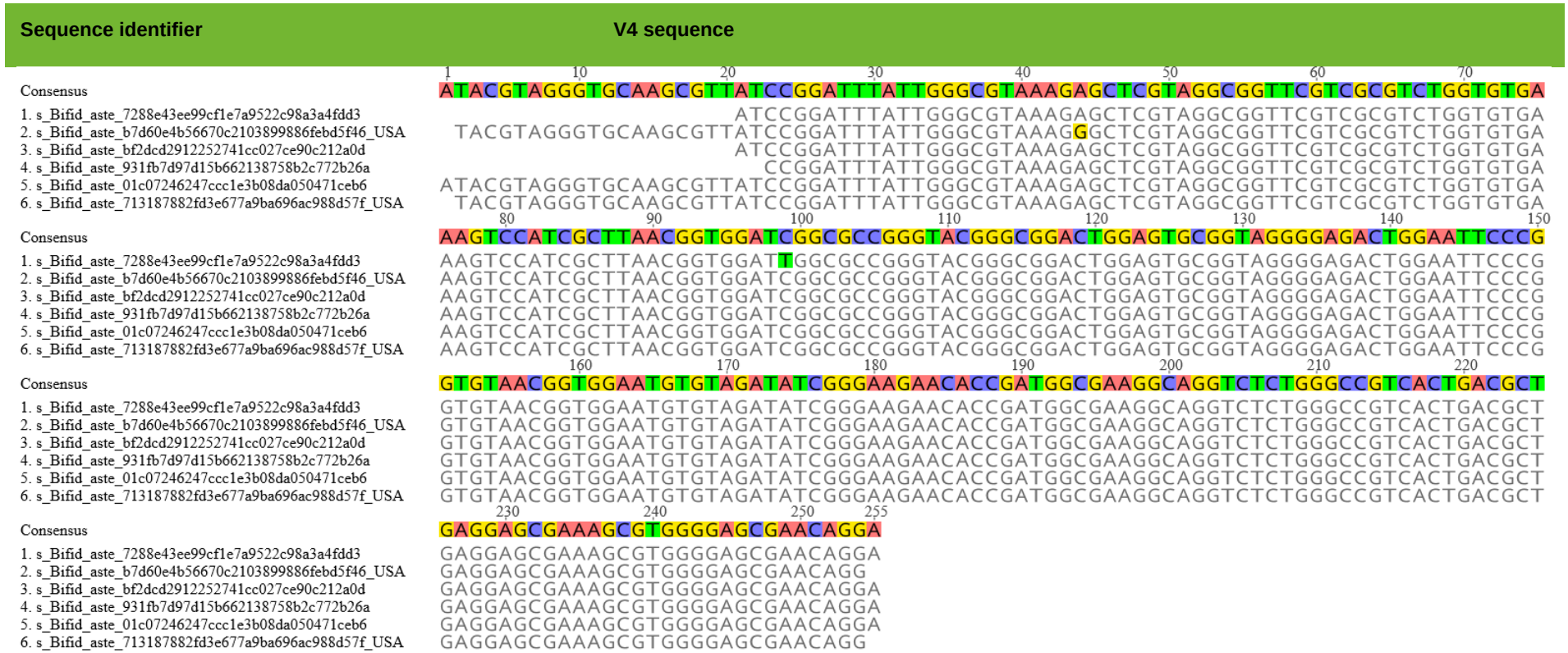
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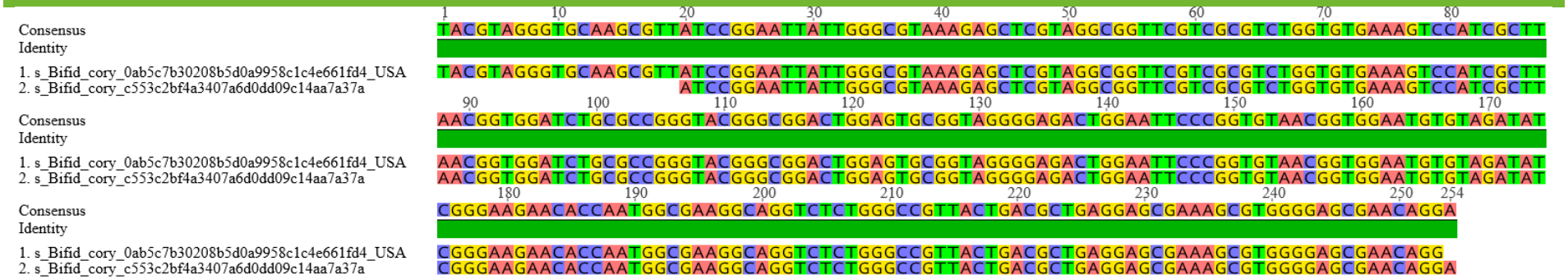

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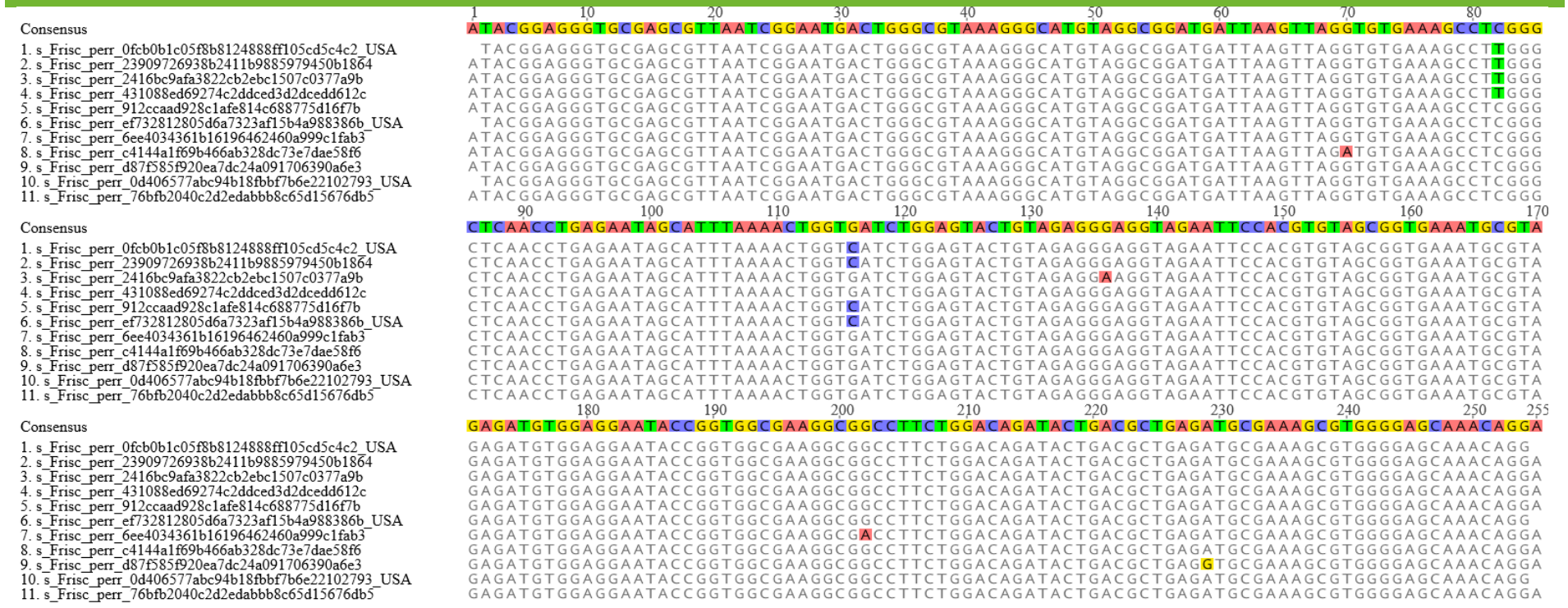

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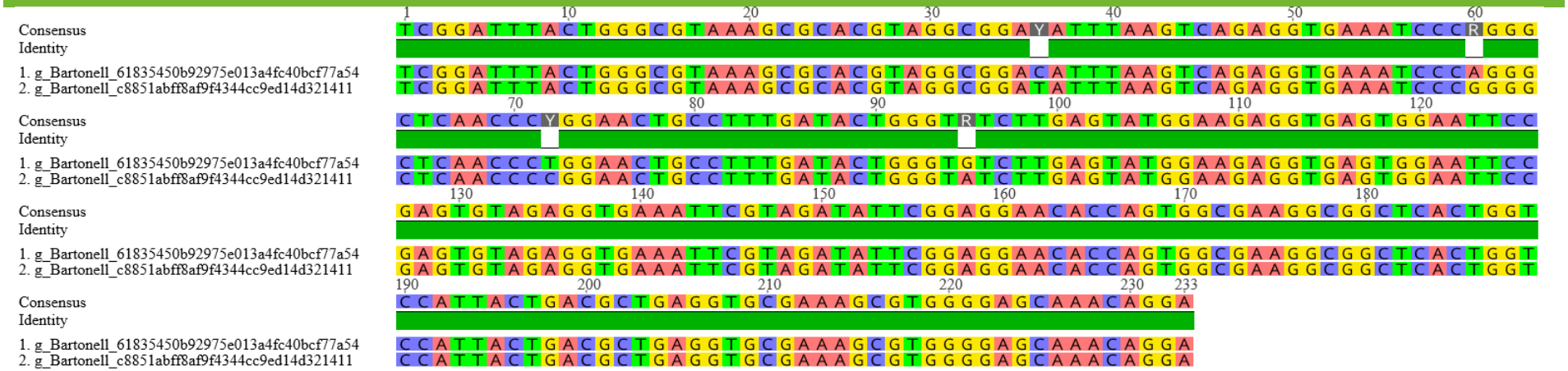

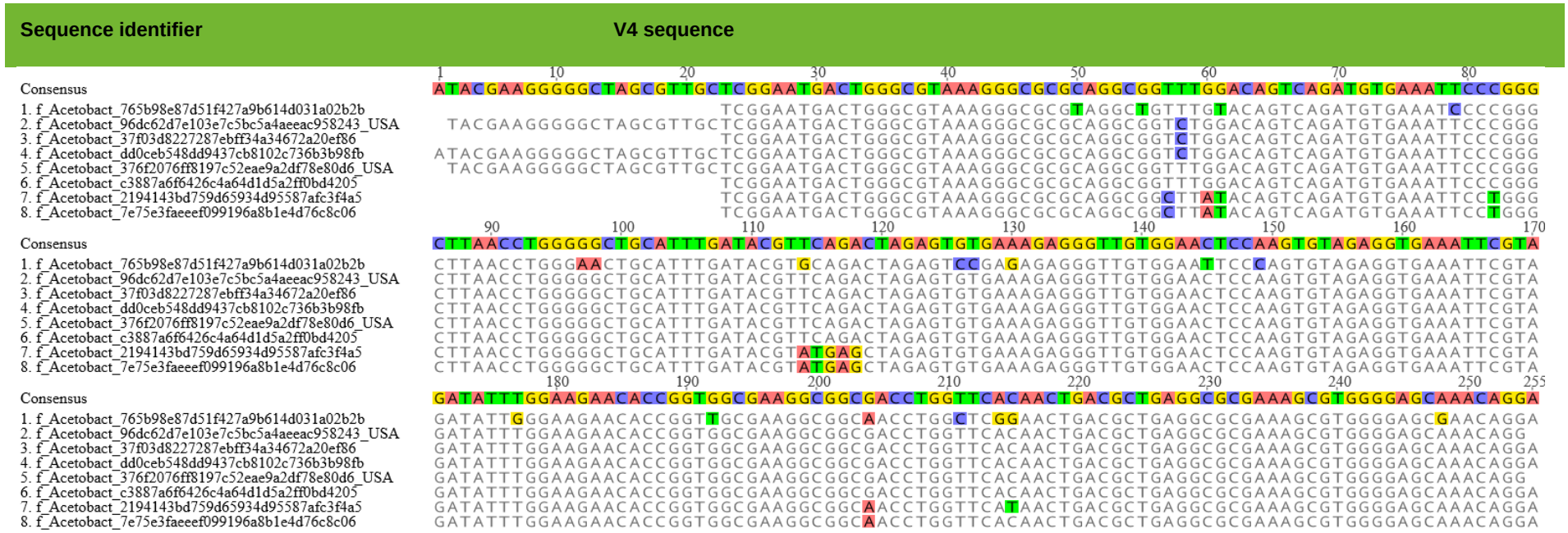
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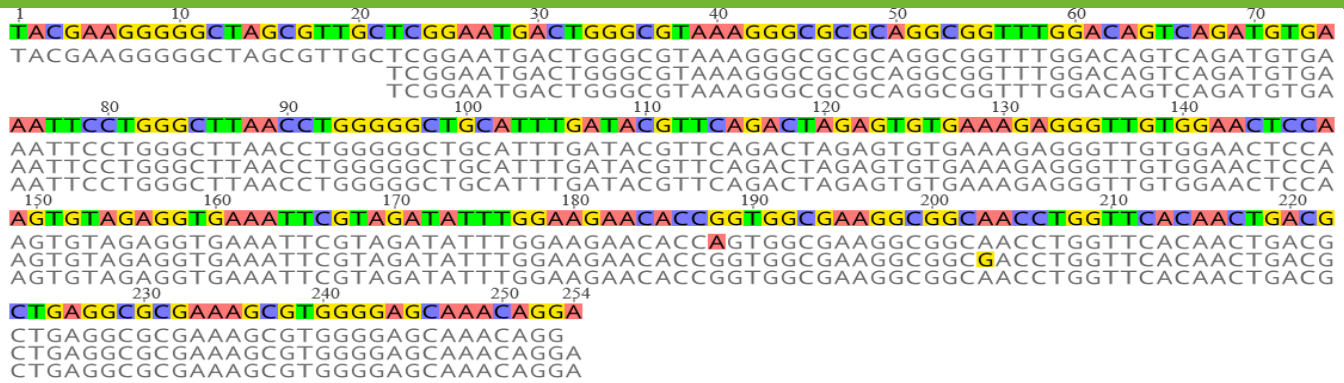
Consensus	
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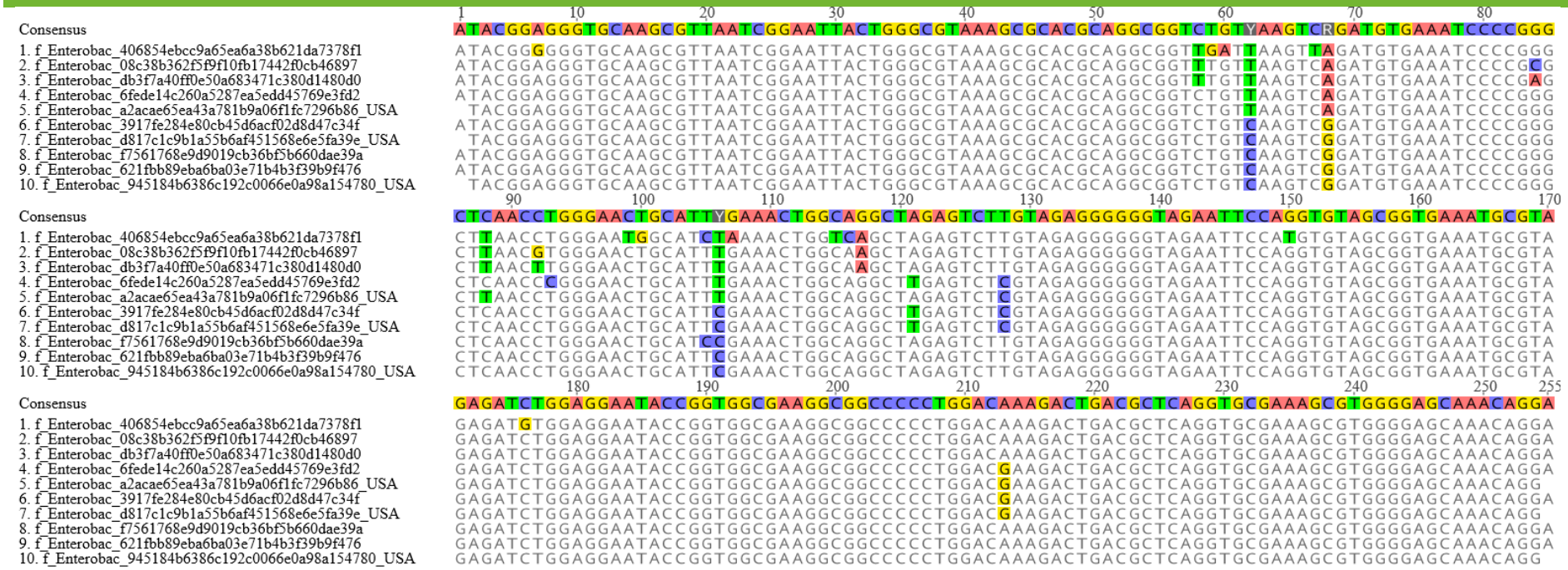


The sequences were wrapped to fit on the page and the differences between the ASVs are highlighted. The USA samples are identified with _USA. The phylotypes are identified as: *S. alvi* (ss_Snodg_alvi), *Lactobacillus spp.* (g_Lactobaci), *L. apis* (s_Lacto_apis), *L. melliventris*. (s_Lacto_mell), *Lactobacillus helsingborgensis* (s_Lacto_hels), *Bifidobacterium asteroides* (s_Bifid_aste), *Bifidobacterium coryneforme* (s_Bifid_cory), *F. perrara* (s_Frisc_perr), *Bartonella* spp. (g_Bartonell), Acetobacteraceae (f_Acetobact), *Gluconoacetobacter* (g_Gluconace), Enterobacteriaceae (f_Enterobac).

Chapter 5

Succession of bacterial phylotypes in the digestive tract of adult honey bees throughout a year

5.1 Abstract

The bacteria within the digestive tract of adult *A. mellifera* from five colonies in a single apiary located at Ruakura, Hamilton, NZ, were identified every three months throughout a year to determine whether the relative abundance of dominant bacteria and the diversity of less prevalent bacteria altered throughout a calendar year. The assessment dates were representative of the four seasons that honey bee colonies encounter in a temperate climate. The diversity of bacterial species in the honey bee gut was assessed using 16S rRNA amplicon sequencing of the V3V4 hypervariable regions. Diversity changed throughout the year as summer bees had the most diverse bacterial composition and spring bees the least. The dominant core phylotypes were present: *Lactobacillus* spp. including *L Lactobacillus mellis*, *Lactobacillus mellifer*, and *Lactobacillus kunkeei*. *Gilliamella apicola*, *Snodgrassella alvi,* and *Bifidobacterium* spp. Of the normally less prevalent but dominant phylotypes, *Frischella perrara* and Acetobacteraceae were present, but *Bartonella apis* and *Parasaccharibacter apium* were absent. This variation is supported by PERMANOVA partitioning as spatial effects were evident, with date of sampling throughout the year contributing the largest component of variation to the overall model and therefore significantly affecting the relative abundance of bacteria within the honey bee gut. The mean relative abundance of core bacteria present did not alter with the time of year when averaged over five colonies. However, the total number of reads for *G. apicola* and *S. alvi* varied among bees from the same colony at the same sample date, and varied among colonies and between assessment dates. No clear seasonal pattern was obvious and this suggests that these species varied in response to the needs of the individual bee host. Five less prevalent phylotypes altered significantly within colonies throughout the year, and among colonies within a season: Rhizobiaceae, *Bacteroides* spp., *Ensifer adhaerens*, *Pseudomonas* spp. and Cyanobacteria. The relative abundance of Rhizobiaceae and *Bacteroides* spp. were highest in the winter assessment. Eleven additional less prevalent phylotypes were only present in the colonies for ≤ 3 assessment dates, indicating that the abundance of less prevalent bacteria changes throughout the year.

5.2 Introduction

The digestive tract of European honey bees (*Apis mellifera* L.) usually contains nine core bacterial phylotypes of which five are consistently dominant and four are less prevalent species. The core bacteria include two phylotypes of *Lactobacillus* (Martinson et al. 2011) belonging to the phylum Firmicutes of which individual species have recently been classified (*Lactobacillus* Firm-4: *L. mellis* and *L. mellifera*, and *Lactobacillus* Firm-5:; *L. apis*, *L. melliventris*, *L. kimbladii*, *L. kullabergensis*, *L. helsinborgensis* (Olofsson et al. 2014; Bonilla-Rosso and Engel 2018). *Snodgrassella alvi* and *Gilliamella apicola* both from the phylum Proteobacteria (Kwong and Moran 2013), and *Bifidobacterium*

from the phylum Actinobacteria (Bottacini et al. 2012). The additional four groups belong to the Proteobacteria phylum and are *Frischella perrara* (Engel et al. 2013b), *Bartonella apis* (Kešnerová et al. 2016), *Parasaccharibacter apium* (Corby-Harris et al. 2014b), and Alpha2.1. The latter two are from the family Acetobacteraceae, and Alpha2.1 is from a *Gluconobacter*-related species group (Martinson et al. 2011).

Although these dominant phylotypes have been observed in multiple localities (Martinson et al. 2011; Moran et al. 2012; Sabree et al. 2012), the consistency of the abundance of the nine core species (of which five are considered dominant) throughout the year has not been well documented. Of the five dominant core bacteria, only *S alvi* and *G. apicola* have been observed to fluctuate between seasons, although only minimally (Ludvigsen et al. 2015). Ludvigsen et al. (2015) sampled the midgut/pyloric region of ten bees from the brood nest of three colonies in Norway each month from spring (May) to autumn (October) 2012 (winter sampling was not conducted). Using both 16S rRNA sequencing and quantitative PCR analysis, they identified the bacterial composition each month for each of three pooled samples. Ludvigsen et al. (2015) found that the relative abundance of *G. apicola* was highest in May and reduced through to October. In contrast *S. alvi* was less abundant in May than October, but lowest in August, and *F. perrara* abundance peaked in August. These bacterial peaks/troughs correlate with changes in the beekeeping season observed in the northern hemisphere in spring (May) and autumn (October). During these times the population within the colony is increasing and decreasing in preparation for nectar availability in summer and winter, respectively, and the bees often require supplementary food such as sucrose, inverted sugar, or high fructose corn syrup. Ludvigsen et al. (2015) therefore proposed that the main drivers of bacterial change in the midgut/pyloric region may be diet and/or environmental exposure. Bacterial variation resulting from these drivers may provide some explanation as to why increased abundance of quality pollens in the landscape increases both health and survival of individual bees and colonies (Smart 2015). The midgut/pyloric region represents < 4% of the entire digestive tract (Martinson et al. 2012; Kwong and Moran 2016a), and is populated predominantly by *S. alvi*, *G. apicola* and *F. perrara*. It is therefore possible that other bacteria that are present within the rest of the gut may also fluctuate throughout the year.

Subtle variation in the less prevalent phylotypes is associated with differences in diet (Chapter 6, (Taylor et al. 2019)), bee age (Martinson et al. 2012; Tarpy et al. 2015), bee caste (Kapheim et al. 2015), and the behavioural tasks they perform (Jones et al. 2018a). Bees that conducted food processing tended to have an increased abundance of *Bartonella apis*, whereas foraging bees showed an increase in *L. kunkeei* (Jones et al. 2018a). As the bees in this study were all the same age, variation in bacterial communities may be associated with the diet or carbohydrate sources that the bees contact. A second study sampled bees from 36 colonies spread across 12 apiaries in two landscape types in southern UK (Jones et al. 2018b). Colonies distant to oilseed

rape (OSR) showed a slight increase in relative abundance of *Lactobacillus kunkeei* and *Bartonella apis*, whereas bees surrounded by OSR displayed an increase in Alpha 2.1, Alpha 2.2 (*Parasaccharibacter* apium) and other less prevalent members of the class α-proteobacteria (Jones et al. 2018b). Environmental landscape was found to explain only 1–6% of the bacterial variation in bees, whereas individual apiaries explained 17–27% of the variability (Jones et al. 2018b).

None of these studies included a time variable so I hypothesise that the number and relative abundance of microbiota within the digestive tract of adult honey bees varies with seasonal changes and this is likely to be because of the variation and availability of food resources. Since the abundance of gut bacteria is highest in the hindgut, as opposed to the midgut/pyloric region (Ludvigsen et al. 2015), and because there is limited information regarding bacterial composition throughout the season, this research compares the diversity of bacteria and the relative abundance of bacteria within the entire digestive tract of adult *A. mellifera* throughout a 12 month period. The timing of the assessment dates represent the four seasons of a temperate climate experienced by honey bees located at Ruakura, Hamilton, NZ.

5.3 Materials and methods

5.3.1 Sites and sampling

Five honey bee colonies were maintained within a single apiary (each colony was within a 10m radius) using standard beekeeping methods at Ruakura Research Centre, PFR, Hamilton, NZ, for the duration of the trial and the year prior. The apiary was located on a farm on the outskirts of Hamilton city where floral resources from both pasture and city gardens were within a 5 km radius). Bayvarol® strips (KVP Pharma + Veterinär Produkte GmbH, Kiel, Germany) were applied to each colony, as per the instructions, to control the external parasite *Varroa destructor*. These products were removed from the colonies two days prior to the first assessment (1 June, 2017). The colonies were also treated with Bayvarol® strips for eight weeks commencing October 2017. Throughout the trial each colony contained a laying queen (queen-right) and were disease-free, as determined by visual inspection. At the start of spring, the colonies all contained 8–10 frames of brood spread through two brood boxes. In late spring, each colony was fitted with an empty honey super (a box of wax frames where the bees store the collected nectar) above the two brood boxes. This was separated from the brood nest by a queen-excluder to restrict the queen's laying within the brood boxes. All five colonies were supplemented with 2 L of 50% sucrose solution in late spring (October) 2017 due to the prolonged periods of rainfall that occurred https://services.metservice.com/towns-cities/hamilton. Although this is standard

beekeeping practice and is required to keep the bees alive, it is unknown whether this practice affects the bacteria in honey bees.

5.3.2 Honey bee sampling

One sample of adult honey bees was collected from each of five colonies, at the start of every third month commencing in winter (1 June) 2017, through spring (1 September), summer (1 December) and autumn (1 March) 2018. The bees were collected from the second frame on the left side of the top brood box, directly in to 99% ethanol and then frozen at -70°C.

5.3.3 DNA extraction and amplification

Twenty bees from each colony from each sampling date were thawed for 3 min and then the digestive tracts were aseptically dissected from crop to rectum into a single clean DNase- and RNase-free 1.5 ml microcentrifuge tube on ice. These pooled samples were stored at -20°C until DNA was extracted using a Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit (Zymo Research Corporation (ZR), California, USA). The quantity of DNA from each pooled sample was assessed using a NanoDrop (Thermo Scientific). The DNA aliquots were frozen at -70°C prior to sending to Massey Genome Service (Massey University, Palmerston North, NZ) for 16S rRNA gene sequencing of the V3V4 hypervariable regions (Kozich et al. 2013) on an Illumina MiSeq platform (Palmerston North, NZ). See Chapter 2: General Methods for additional information regarding honey bee gut extraction (2.4), DNA extraction and quantification (2.5 and 2.6), and Next Generation Sequencing of the 16S rRNA gene with an Illumina MiSeq platform (2.8).

5.3.4 Gene sequence processing and characterisation of microbial communities

The Illumina de-multiplexed fastq sequence data were processed and trimmed to a 0.01 probability of error (an equivalent Phred score of Q20) (Aronesty 2011), then further processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) analysis suite, version 2018.2 (Caporaso et al. 2010; Bolyen et al. 2019). The reads were run through DADA2 methodology in QIIME2 to filter and trim the paired-end sequences, dereplicate them, and filter chimeras to produce exact amplicon sequence variants. To reduce estimation errors due to the different number of sequences per sample, the data were rarefied to 47190, the number of sequences in the smallest sample.

Information in the 16S rRNA BLAST (Basic Local Alignment Search Tool) database from the National Center for Biotechnology Information (NCBI) was customised to make

a new QIIME 2 compatible reference dataset (Chapter 2.8.3) (https://github.com/pjbiggs/16SrRNA_taxonomy) and used to create a Biological observation matrix (BIOM) table that included the Operational taxonomic units used to classify groups of related individuals and their associated taxonomic classification. Any ASVs that were unable to be assigned taxonomically to species were assigned to the closest identified taxonomic level i.e. genus or family.

5.3.5 Statistical analyses

The analysis of the BIOM table was conducted in R (version 3.5.1) (R Core Team 2018). For all analyses, ASVs were included if their minimum total read composition was > 0.01% prevalence across all samples. Phylogenetic diversity was measured using QIIME2 both within a sample (α-diversity), and between samples (β-diversity) (Kuczynski et al. 2012). Alpha-diversity was also characterised using the web-based tool MicrobiomeAnalyst

(https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/faces/docs/AboutView.xhtml) (Dhariwal et al. 2017). Alpha-diversity was calculated at the feature level using Kruskal-Wallis pairwise comparisons of three diversity measures: Shannon, Simpson, and Fisher.

To investigate the differences in the number of phylotypes between assessment dates, a Poisson generalised linear model was used. To explore the relationship between phylotypes, assessment dates, and their effect on relative abundance, a linear mixed effect regression model was applied. Replicate was included as a random effect and model assumptions were checked via standard residual plots. A natural logarithmic transformation was applied for normality assumptions to hold. Post-hoc pairwise comparisons of least-square means were conducted for the 41 phylotypes using Tukey's range test. The natural-logs of percent abundance of individual bacteria with prevalence > 0.5% on a single assessment date, or present on at least two of the assessment dates, were visually compared. A total of 29 species were included.

The relationship between the presence of phylotypes and time of year were visually explored using nonmetric multidimensional scale (NMDS) plots and heat maps. For the NMDS plots, the dissimilarity matrix was calculated using the Bray-Curtis method and for the heat maps the response was the average read per replicate. To compare the effect of seasonal differences on the bacterial community within the honey bee gut, a mixed model permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was conducted using Adonis (Oksanen et al. 2011). The autocorrelation of the bacterial communities within a colony throughout the four assessment dates was accounted for by constraining the permutations within the colonies using the function 'strata'. To visually compare variation in the abundance of bacteria between the colony replicates throughout the year, the total number of reads at each assessment date were

graphed for two core bacteria S. alvi and G. apicola. These two species were selected because they are the only core bacteria that have been observed to fluctuate throughout time (Ludvigsen et al. 2015).

5.4 Results

5.4.1 Identification of phylotypes within the honey bee digestive tract

A total of 1,706,354 read pairs were generated across the samples of all five colonies and throughout the four assessment periods. The reads were clustered in to ASVs and all ASVs < 0.01% prevalence were removed. The remaining 1,694,237 read pairs contained 220 ASVs and these were classified as 41 unique phylotypes (Table 5.1). Of the 41 phylotypes, 25 were classified as unique species, the remaining 16 were identified to phylum, family or genus. The genus Lactobacillus contained 37 ASVs, whereas the three *Lactobacillus* species *L. mellis*, *L. mellifer*, and *L. kunkeei* contained 8, 1, and 1 ASV(s), respectively. The effect of classifying phylotypes in taxonomic levels above genus, such as the genus *Lactobacillus*, is addressed in the discussion. The results from the Poisson generalised linear model suggest that the differences in the number of phylotypes between assessment dates tend towards significance (P = 0.055, Chi Sq = 7.59, degrees of freedom = 3), with the number of phylotypes in the summer samples being 2-fold more than those in the spring samples.

Table 5.1 | Number of unique ASVs and the associated range of phylotypes within the digestive tract of NZ honey bees from colonies assessed throughout the season.

Assessment date	Number of ASVs	Range of phylotypes
Winter	171	17 – 29
Spring	140	16 – 18
Summer	152	16 – 36
Autumn	162	20 – 25

The dominant core phylotypes *G. apicola*, *S. alvi*, and *Bifidobacterium* spp. were present in all samples across all seasons (Figure 5.1). The colony mean of the total raw reads varied across the assessment dates for *S. alvi*, the less prevalent phylotypes Rhizobiaceae and *Pseudomonas*, and the two species *L. mellis* and *L. mellifer*. Using the customised 16S rRNA database (Chapter 2.8.3), the genus *Lactobacillus* and the species *L. mellis*, *L. mellifer*, and *L. kunkeei* were present. *L. apis* were not identified but may have been classified within the genera *Lactobacillus*. Of the four dominant but less prevalent bacteria, *F. perrara* and Acetobacteraceae were present but *B. apis* and

the Acetobacteraceae species *P. apium* were absent. Of the 41 phylotypes, 35 were present in the winter bee samples, 22 were present in the spring bee samples, 39 were present in the summer bee samples, and 35 were present in the autumn bee samples (Figure 5.1). Of the less prevalent phylotypes, 22 were included in further analysis as their relative abundance was > 0.01% on at least one single assessment date or they were present in more than one assessment. Ten of these less prevalent phylotypes were absent from Chapter 3: NZ survey that was conducted in spring. These are the families Flavobacteriaceae and Prevotellaceae, the genus *Bacteroides*, and the species *Bifidobacterium longum*, *Clostridium oroticum*, *Eubacterium tenue*, *Fusobacterium nucleatum*, *Fusobacterium perfoetens*, *Klebsiella oxytoca*, and *Sutterella stercoricanis*.

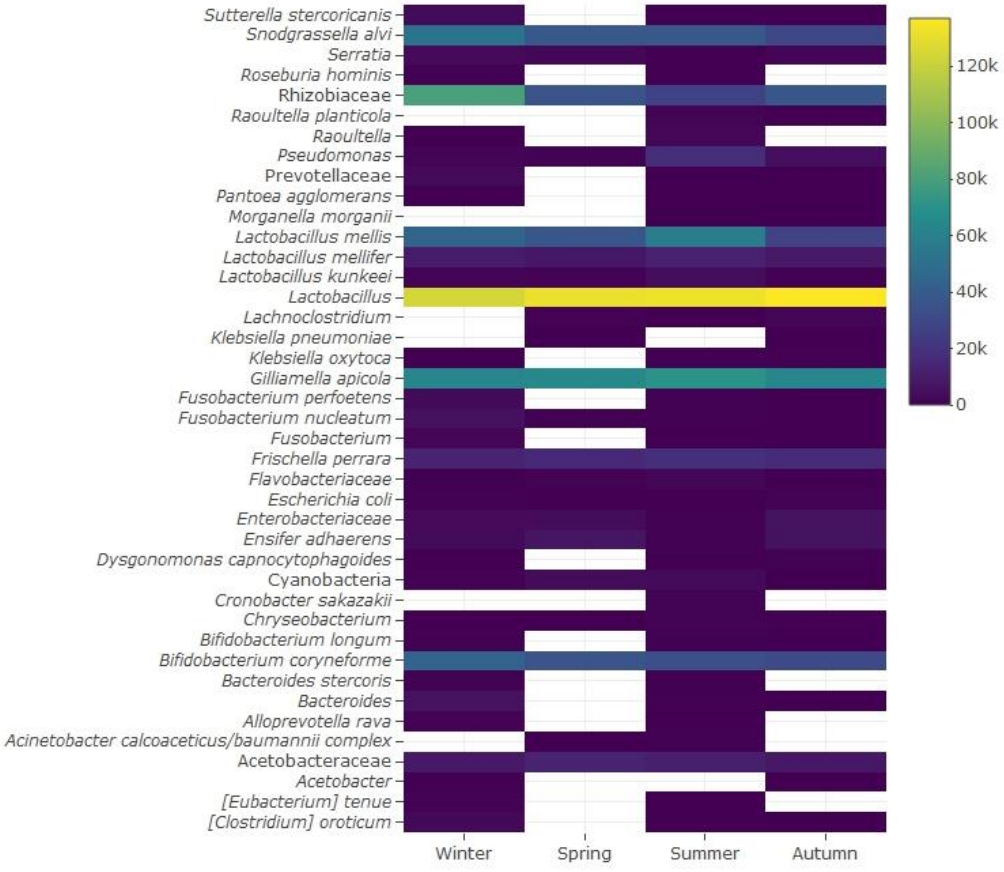
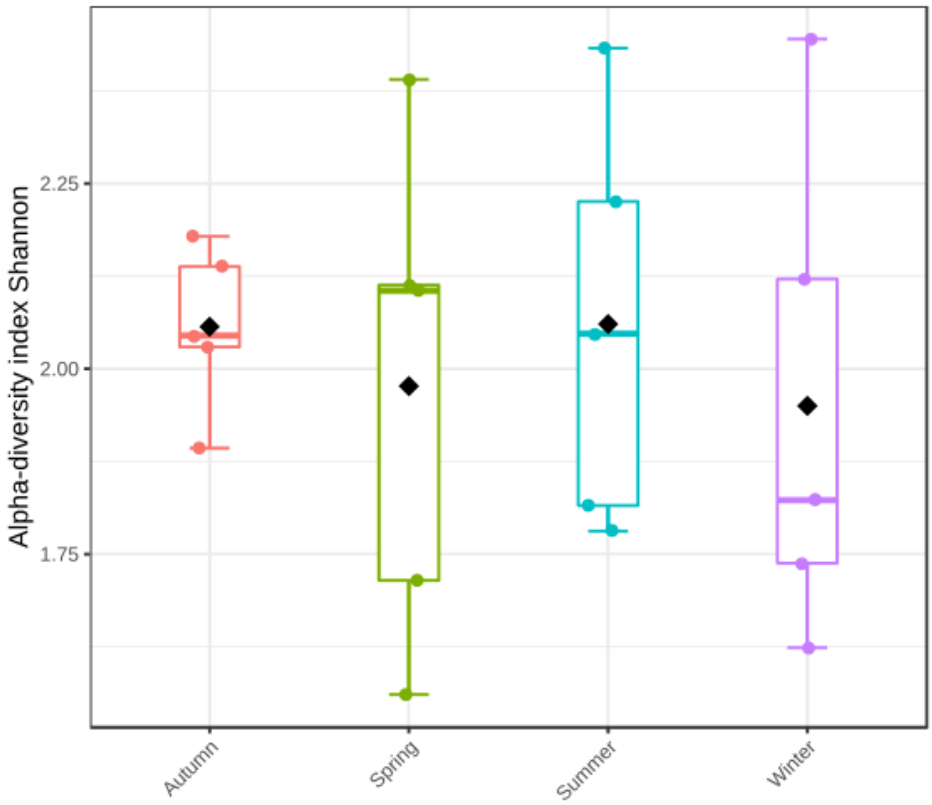


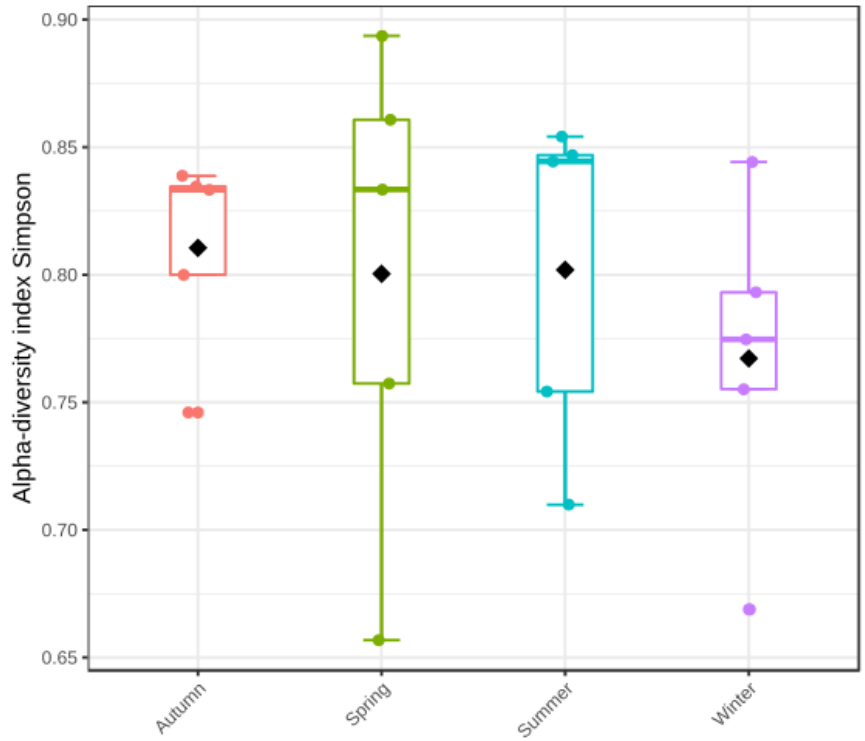
Figure 5.1 | Heatmap of total raw reads of phylotypes > 0.01% prevalent in the digestive tract of honey bees per colony throughout the season.

5.4.2 Diversity analysis

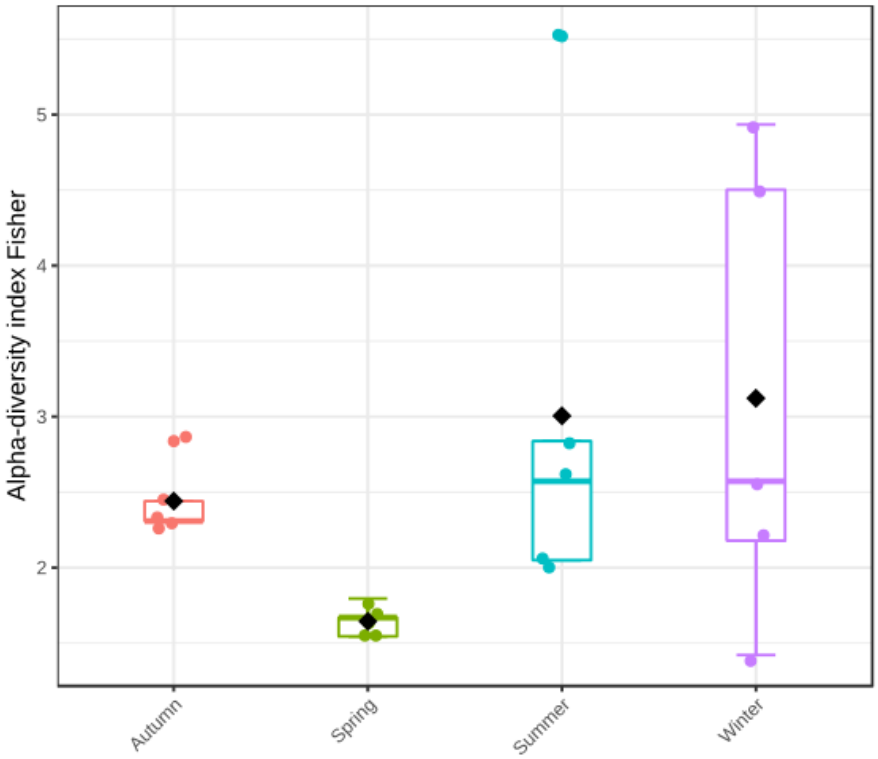
The α-diversity indexes Shannon, Simpson and Fisher, suggest that the diversity of the bacterial communities throughout the year was low and there were no significant differences among the colonies ($P < 0.01$) (Figure 5.2), despite the boxplots suggesting that the most diversity and variation occurred in colonies three and five. The Shannon plot diversity increased in colonies two and three, suggesting that these colonies may include some rarer species in spring and summer.



A | Season | Shannon *P*=0.88, KW statistic 0.69.



B | Season |Simpson *P*=0.74, KW statistic 1.26.



C | Season | Fisher *P*=0.04, KW statistic 8.17.

Figure 5.2 | Alpha-diversity measures represented as boxplots for Shannon (A), Simpson (B), and Fisher (C), indices at species level across all samples.

The sampling dates are on the X-axis and their estimated diversity are on the Y-axis.

5.4.3 Relative abundance

PERMANOVA partitioning showed that spatial effects were evident. The date of sampling throughout the year contributed the largest component of variation to the overall model and therefore significantly affected the relative abundance of bacteria within the honey bee gut ($P < 0.05$) (Table 5.2).

Table 5.2 | PERMANOVA for bacterial communities in the digestive tract of honey bees relative to the time of year.

	Df	Sum Sq	R^2	F	Pr(>F)
Time of year	3	0.183	0.263	1.898	**0.039**
Residual	16	0.513	0.737		
Total	19	0.696	1.000		

Bray-Curtis dissimilarities were used. The analysis was constrained within colonies in relation to time of year that the colonies were sampled. Degrees of freedom (Df), Sum of squares (Sum sq), F value by permutation (F). Boldface indicates statistical significance when $P < 0.05$. P values were based on 999 permutations.

The NMDS plot suggests a weak seasonal pattern in the bacterial community. Spring, summer and autumn treatments clustered together and were separated from the winter treatment along the secondary axis (Figure 5.3). Rhizobiaceae and *Bacteroides* spp. also diverged to the left of axis one and were therefore more closely associated with the winter samples. Rhizobiaceae and *Bacteroides* spp. strongly diverged from *G. apicola*, *L. kunkeei*, and *F. perrara* along axis 1 (Figure 5.3).

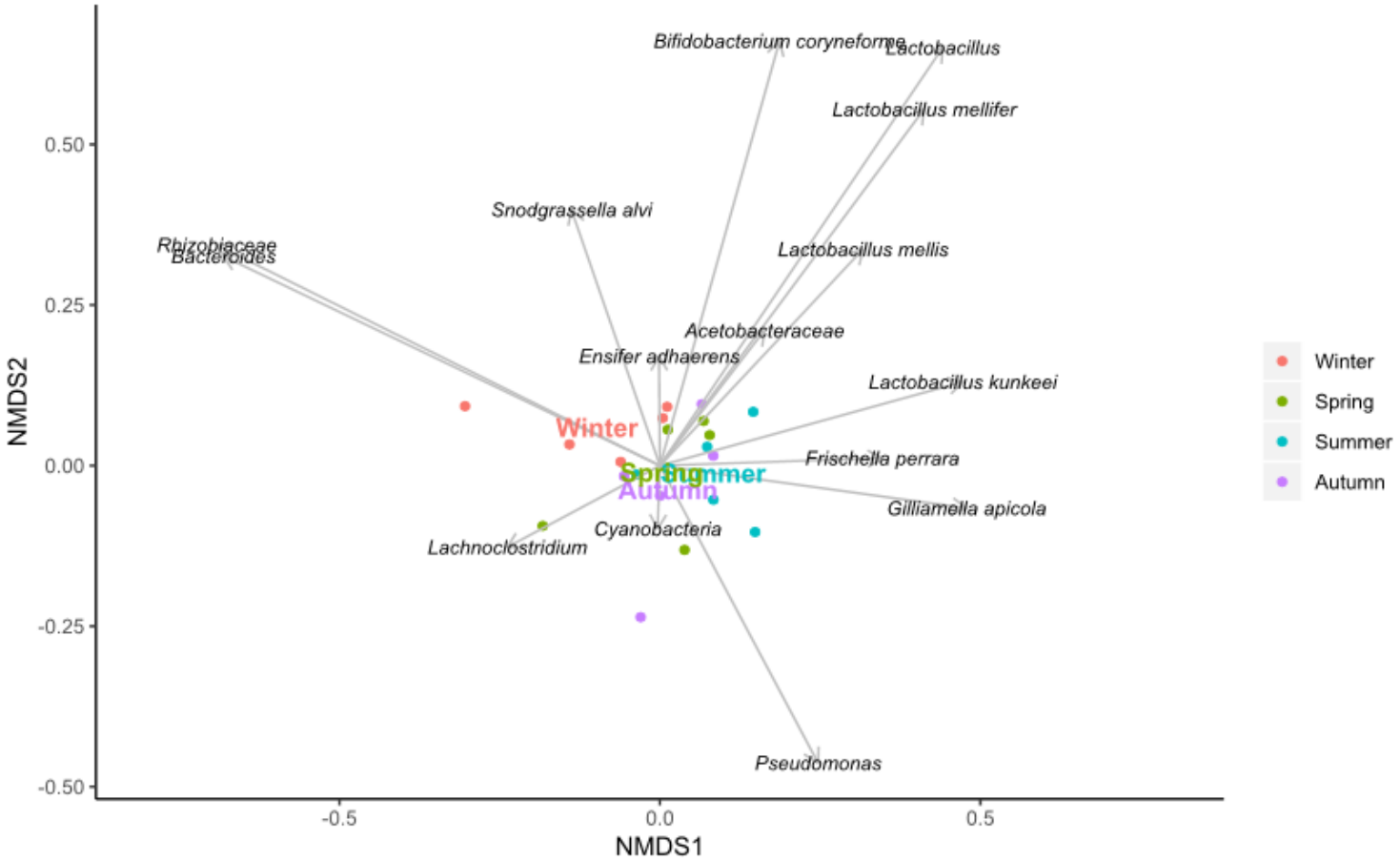


Figure 5.3 | Nonmetric multidimensional scaling plot of relative abundance of bacteria present in the digestive tract of NZ honey bees throughout the year.

Bray-Curtis distances for prevalence > 0.01% total read composition. Solution was reached at a stress level of 0.128.

The percent relative abundance graphed on a log scale (Figure 5.4) indicates the scale of variation where *S. alvi* did not vary among colonies. In contrast, variation among colonies was supported for Rhizobiaceae, *Bacteroides* spp., *E. adhaerens*, *L. kunkeei*, *Pseudomonas*, Cyanobacteria, and the 22 bacteria that were not present in all assessment samples.

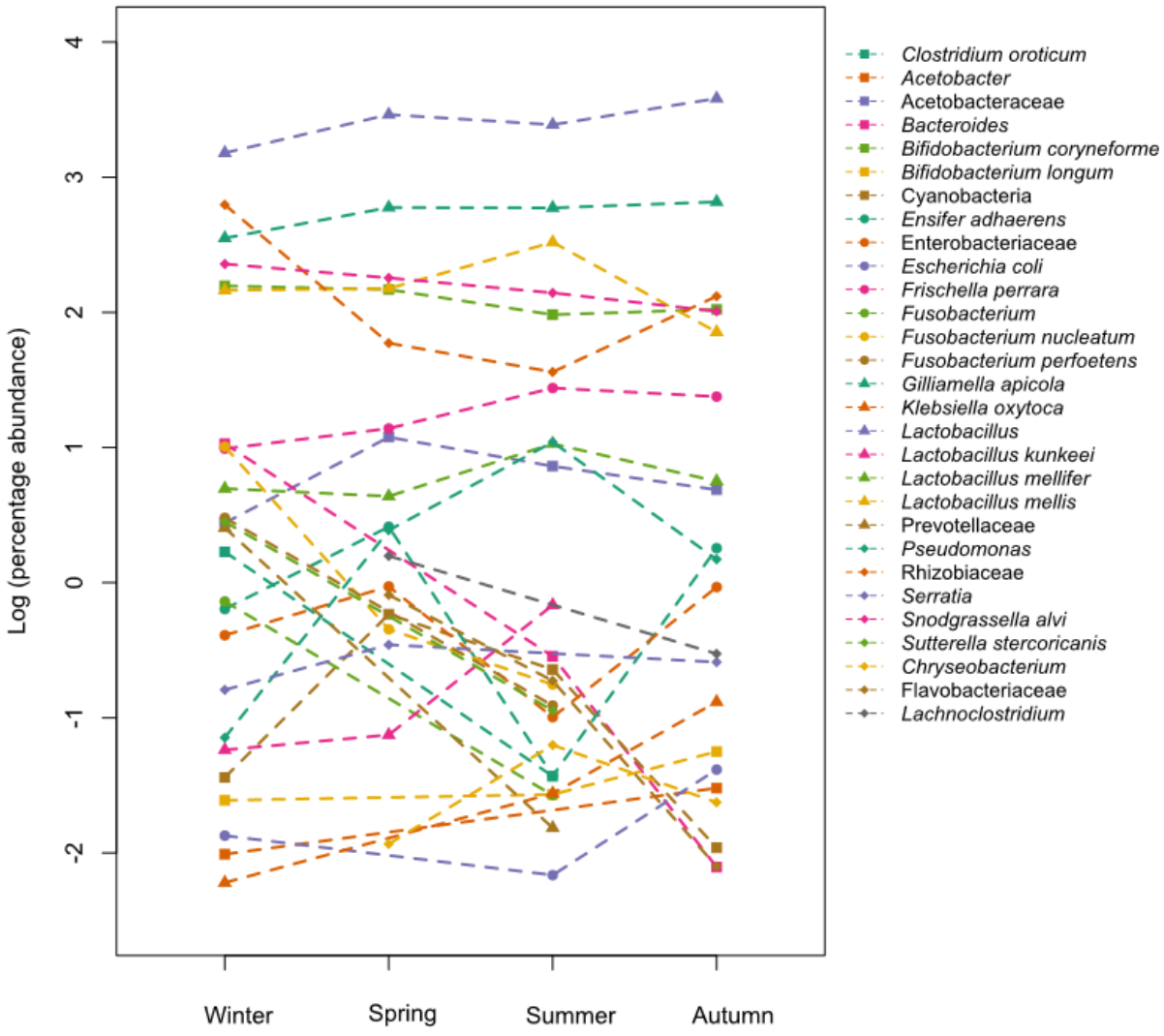


Figure 5.4 | Log transformation of mean relative bacterial abundance in the honey bee digestive tract throughout the year.

Winter (June), spring (September), summer (December), autumn (March).

The analysis of deviance for the linear mixed effect regression model suggests no overall seasonal changes in the number of reads, evidence of significant difference among the relative abundance of phylotypes, and a significant interaction between time of year and phylotype (P < 0.001) (Table 5.3).

Table 5.3 | Analysis of deviance for bacterial communities in the digestive tract of honey bees from five colonies in relation to time of year.

	Chi Sq	Df	Pr(>Chisq)
Time of year	1.55	3	0.67
Phylotype	2124.86	40	**2.2^{-16}**
Time of year : Phylotype	148.35	61	**3.09^{-9}**

Degrees of freedom (Df), Chi-squared test (Chi sq). Boldface indicates statistical significance with $P < 0.05$. The calculation was based on a linear mixed effect regression model using back-transformed abundance data for bacterial communities.

The post-hoc pairwise comparisons show that the core bacteria did not contribute to this variation as their relative abundance remained consistent throughout the year. However, the comparisons did support an interaction with the less prevalent bacteria as the relative abundance of Rhizobiaceae, *Bacteroides* spp., *E. adhaerens* spp., *Pseudomonas* and Cyanobacteria differed significantly with assessment date (P < 0.05) (Table 5.4).

Table 5.4 | Mean relative abundance of the bacterial phylotypes with > 0.5% abundance, or present in at least two assessment dates, in the digestive tract of honey bees throughout a year.

Bacteria	Unique ASVs per treatment	Winter	Spring	Summer	Autumn
Lactobacillus spp.*	18	25.69^{a}	32.38^{a}	30.17^{a}	36.21^{a}
Rhizobiaceae	2	**17.20**^{b}	9.42^{a}	6.16^{a}	9.75^{ab}
*Gilliamella apicola**	15	13.09^{a}	16.26^{a}	16.47^{a}	16.82^{a}
*Snodgrassella alvi**	13	10.96^{a}	9.70^{a}	8.67^{a}	7.49^{a}
*Bifidobacterium coryneforme**	4	9.03^{a}	8.88^{a}	7.48^{a}	7.84^{a}
Lactobacillus mellis	5	9.01^{a}	9.06^{a}	13.23^{a}	7.29^{a}
Frischella perrara	5	2.79^{a}	4.10^{a}	4.37^{a}	4.27^{a}
Lactobacillus mellifer	1	2.06^{a}	1.90^{a}	2.86^{a}	2.19^{a}
Acetobacteraceae	4	1.69^{a}	3.25^{a}	2.48^{a}	2.17^{a}
Bacteroides spp.	1	**1.25**^{b}	<	0.12^{ab}	0.02^{a}
Fusobacterium nucleatum		1.13^{a}	0.14^{a}	0.09^{a}	0.02^{a}
Fusobacterium perfoetens		0.7^{a}	<	0.08^{a}	<
Ensifer adhaerens	1	0.69^{ab}	1.65^{b}	**0.13**^{a}	1.61^{b}
Sutterella stercoricanis		0.69^{a}	<	0.08^{a}	<
Prevotellaceae		0.64^{b}	<	0.03^{a}	<
Serratia spp.		0.62^{a}	0.56^{a}	<	0.38^{a}
Enterobacteriaceae		0.57^{a}	0.87^{a}	< 0.1^{a}	1.39^{a}
Clostridium oroticum		0.55^{a}	<	0.05^{a}	<
Fusobacterium spp.		0.38^{a}	<	0.04^{a}	<
Pseudomonas spp.	3	**0.25**^{a}	0.29^{ab}	3.93^{b}	1.33^{b}
Lactobacillus kunkeei	1	0.22^{a}	0.18^{a}	0.85^{a}	<
Cyanobacteria	1	0.13^{ab}	0.87^{b}	0.67^{ab}	**0.05**^{a}
Acetobacter spp.		0.06^{a}	<	<	0.04^{a}
Bifidobacterium longum		0.05^{a}	<	0.07^{a}	0.06^{a}
Escherichia coli		0.05^{a}	0.01^{a}	0.04^{a}	0.16^{a}
Klebsiella oxytoca		0.02^{a}	<	0.07^{a}	0.08^{a}
Flavobacteriaceae		<	0.19^{a}	0.48^{a}	0.03^{a}
Chryseobacterium spp.		<	0.03^{a}	0.25^{a}	0.18^{a}
Lachnoclostridium spp.	1	<	0.52^{a}	<	0.40^{a}

Boldface indicates statistical significance with $P < 0.05$. These means, identified using Tukey post-hoc comparisons, $\alpha = 0.05$, were back transformed and the dissimilar letters indicate significant differences among treatments. Phylotypes were included if abundance > 0.1% in a single assessment or present in more than one assessment. < indicates means were < 0.01% inclusion threshold. * indicates core bacteria.

The relative abundance of Rhizobiaceae in winter was 2-fold higher than the spring and autumn assessments, and 3-fold higher than the summer assessment. *Bacteroides* spp. in winter was 10-fold higher than the summer assessment, 62-fold higher than the autumn assessment and below the detection level in spring. *E. adhaerens* was present in all four assessment dates but the abundance in summer was 12-fold less than spring and autumn, and 5-fold less in winter. The abundance of *Pseudomonas* in winter was 15-fold less in summer and 5-fold less in autumn. The abundance of Cyanobacteria in the autumn samples was 2-, 17-, and 13-fold less than winter, spring and summer, respectively. The abundance of both Rhizobiaceae and *Bacteroides* spp. were significantly higher in the winter samples, whereas the abundance of *Pseudomonas* was significantly lower in the winter samples and highest in summer. Cyanobacteria abundance was lowest in autumn samples.

Although the total number of reads within the species *G. apicola* and *S. alvi* did not vary across the assessment dates (Figure 5.1), the log graph of the total number of paired end reads indicates the scale of variation that occurs within each colony throughout the four seasons, as well as between the five colonies at each assessment date (Figure 5.5). This variation indicates that five colonies insufficiently identifies the variation that occurs with an apiary. *G. apicola* varied the most in colony 1 (7328 to 18,257), and the largest seasonal variation between colonies is seen in winter (8017 to 18,257). *S. alvi* varied the most in colony 2 (5199 to 13,969), and the largest seasonal variation between colonies is seen in winter (6943 to 13,969).

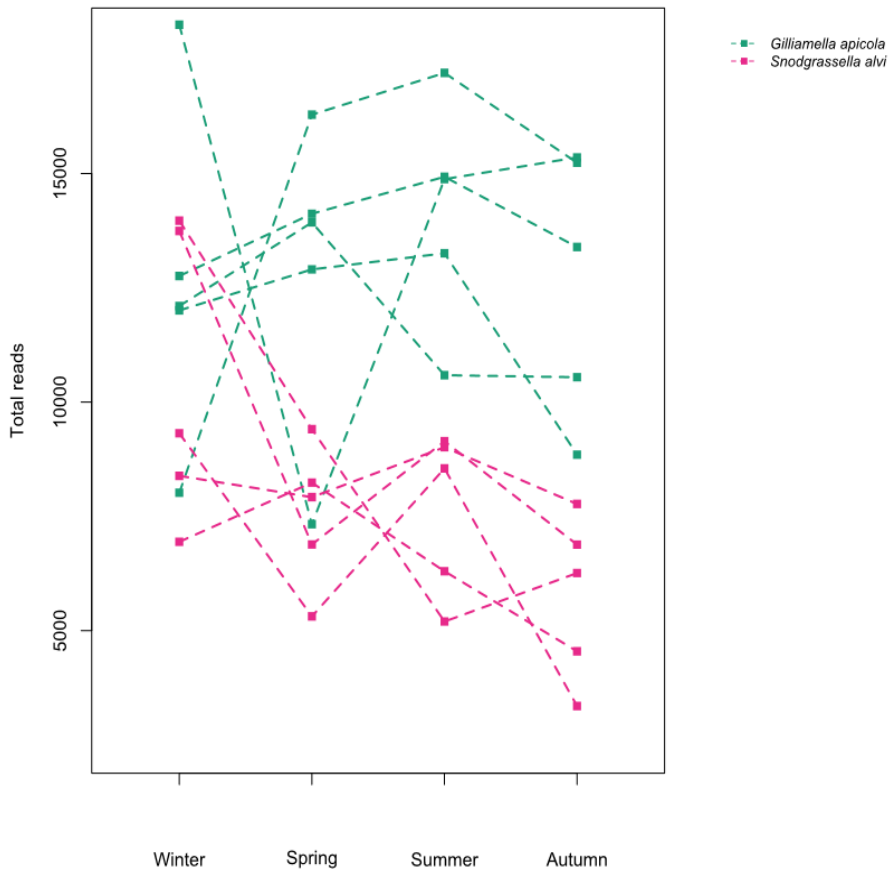


Figure 5.5 | Total of the rarefied paired-end reads of *Gilliamella apicola* and *Snodgrassella alvi* in the digestive tract of honey bees throughout a year for five colonies from a single apiary.

5.5 Discussion

The bacteria within the digestive tract of adult *A. mellifera* from five colonies in a single apiary were identified every three months for a 12 month period to determine whether the bacterial composition changed. The assessment dates represent each of the four seasons that honey bees experience in NZ's temperate climate. The first sampling was conducted in winter to determine the bacterial profile in older bees from which newly emerging bees would acquire their gut bacteria.

All five core phylotypes previously identified (Jones et al. 2018b) were present in this dataset: *Lactobacillus* Firm-4 (*Lactobacillus* spp.), *G. apicola*, *S. alvi*, and *Bifidobacterium*. *L. apis* was not identified. It is possible, that *L. apis* may have been present but only classified to genus. The genus *Lactobacillus* contained 37 unnamed ASVs whereas the three *Lactobacillus* species *L. mellis*, *L. mellifer*, and *L. kunkeei* contained 8, 1, and 1 ASV(s) respectively. This also suggests that the 37 ASVs identified in the *Lactobacillus* genus, represent several species that require further characterisation before analysis at the species level can be conducted. Of the four less prevalent but dominant phylotypes, *F. perrara* and Acetobacteraceae were present in this dataset, but *B. apis* and *P. apium* were not found. The customised NCBI database used in this study to identify sequences was downloaded from the NCBI server in August 2018 (Chapter 2: General Methods 2.8.3) and only identifies ASVs to the specified taxonomic level. It is possible that *B. apis*, which was classified in 2016 (Kešnerová et al. 2016), and *P. apium*, also classified in 2016 (Corby-Harris et al. 2016), may not have been included in the database. If this is the case then *B. apis* may be classified in my study as an unidentified member of the family Rhizobiaceae, and *P. apium* as an unidentified member of the family Acetobacteraceae.

The diversity of bacterial species in the honey bee gut fluctuated with assessment dates throughout the year. Summer bees had the most bacterial diversity with the identification of 36 phylotypes, as well as the largest range of bacteria among the colonies. Autumn and winter bees had similar bacterial diversity with 25 and 29 phylotypes being identified, respectively. In contrast spring bees had the least bacterial diversity with 18 phylotypes. This variation is likely to be linked with seasonal changes as this determines the cyclic, annual development of the colony. NZ's temperate climate means that inclement weather from autumn to spring only slightly limits bee foraging behaviour. However, they still require beekeepers to provide supplementary feed such as sucrose and inverted sugar. However, as the colonies in this trial were fed sucrose after the spring treatment, sucrose is unlikely to be the cause for the reduced diversity in spring but may have caused the increase in summer.

Instead, spring colony growth may be the cause of this reduced diversity. Increased diversity is generally considered to be desirable as diversity can contribute to community resilience against disturbance, mismanagement, and/or degradation (Elmqvist et al.

2003). Additionally, increased microbial diversity is associated with better health as the loss of microbial diversity in humans is often associated with diseases. Examples of this include Crohn's disease (Sha et al. 2013), irritable bowel syndrome (Durbán et al. 2012), and colorectal cancer (Ahn et al. 2013). However, it cannot be assumed that increased bacterial diversity in the honey bee gut is positively associated with bee and/or colony health, as there is no current evidence to support this in bees. It may be possible that spring colony growth resets the bacterial community in the colony as the majority of old winter bees are replaced. These winter bees are, on average, older than spring bees (Fukuda and Sekiguchi 1966), and the increased number of phylotypes may represent a compromised bacterial community that has developed during their extended life. Change in honey bee diet and the associated increase in less prevalent bacteria or the increase in gut pathogens such as *Nosema* may contribute to this increased number of phylotypes in winter by altering the conditions of the bee gut, thus enabling less prevalent bacteria to proliferate. This may also occur in autumn.

NEWs acquire their bacterial communities from other bees (Powell et al. 2014), so newly emerging winter bees will receive a more diverse bacterial community and spring bees will receive a less diverse community. Because bees in spring colonies, on average, are younger, the bacterial community in the bees sampled in spring may have had less time to diversify in comparison to the older winter bees. The high bacterial diversity observed in the early summer bees (December) may result from increased floral resources as nectar diversity and nectar quantity are both high at this time, as supported by the majority of honey being collected at this time in the Waikato region. This summer increase also followed the feeding of sucrose to the colonies in October. Whether this increase in diversity is because the bees forage on food sources that aid the proliferation of these bacteria or the bacteria are present in nectar and can also persist in the crop cannot be determined from these observations and analysis. Further research on the effect of different carbohydrate forms on honey bee gut bacteria is described in Chapter 6.

There was no evidence to suggest that the relative abundance of the dominant core bacteria changed with time of year. This contrasts with evidence presented by Ludvigsen et al. (2015) who did observe small changes in relative abundance of *G. apicola* and *S. alvi* between spring and autumn. However, the plot of the total number of reads of *G. apicola* and *S. alvi*, for each of the five colonies and at each assessment date, suggests that the total number of reads for both bacteria varied within each colony and among colonies throughout each assessment date with no clear pattern. It is therefore likely that the pooled sample of 10 bees, for each of the three colonies used by Ludvigsen et al. (2015), may not have accounted for the normal variation in abundance of core bacteria that occurs within and among bees and their colonies throughout the season. I therefore recommend that no less than 20 bees per colony and five colonies per replicate are used when community amplicon sequencing methods are

employed, as shown in this study. Although changes in the relative abundance of the core bacteria were not observed throughout the year in this study, it is possible that relative abundance may vary between strain types of *G. apicola* and *S. alvi*. However, to determine this, deeper sequencing analyses would be required to compare these strains (Jones et al. 2018a). In contrast to the core bacteria, the relative abundance of five less prevalent phylotypes did alter significantly throughout the year: Rhizobiaceae, *Bacteroides* spp., *E. adhaerens*, Pseudomonas and Cyanobacteria.

The relative abundance of Rhizobiaceae, which resides in the crop, peaked in winter, exceeding the relative abundance of the core bacteria *G. apicola*, *S. alvi*, and *B. coryneforme*. It then receded below the relative abundance of these dominant core bacteria in summer. This increase may have occurred because prolonged feeding regimes of supplementary carbohydrates, such as sucrose and/or invert sugar, are often used to overcome limited food sources during autmn and winter, and sucrose-rich diets have been observed to increase the relative abudance of Rhizobiaceae (Taylor et al. 2019). The increase may also have occurred because the lifespan of worker bees averages > 140 days in winter, compared to 15 to 38 days in summer (Fukuda and Sekiguchi 1966; Graham 1992), possibly altering the conditions within the crop of older bees so the crops are more amenable to proliferation of Rhizobiaceae. The theory that this species is associated with poor bee health and is an early indicator species should be explored further using host challenge studies with gut pathogens, as well as characterisation studies within the laboratory as well as the honey bee gut.

In this study *Bacteroides* spp. was the 10th most abundant phylotype in winter but its abundance was limited in summer and autumn, and negligible in spring. There was a difference of 62-fold across the seasons. The role of this species in honey bees is unknown but as it has been studied in humans perhaps, some parallels can be drawn. *Bacteroides* spp. are obligate anaerobic bacteria usually seen in the distal end of the human gut. Their pathogenicity is limited as they do not form spores and their cell membranes do not contain endotoxin (Actor 2012). However, *Bacteroides* spp. infection does occur in humans after severe trauma in the human gut or abdomen, resulting in the formation of abscesses and fever (Actor 2012). In contrast, some species of *Bacteroides* are versatile carbohydrate and glycan degraders that produce beneficial end products for themselves and their host (Benítez-Páez et al. 2017). *Bacteroides* spp. grown on oxygen-depleted modified Schadler media was found to utilise pectin, a polysaccharide found in the cell wall of plants, to enhance butanoate metabolism that increased butyrate production (Benítez-Páez et al. 2017). Butyrate is a short-chain fatty acid that in humans is a fermentation product of dietary fibre that acts as a main energy source for epithelial cells (Cummings et al. 1987). Butyrate is considered a marker of health in humans (Benedito-Palos et al. 2016) as it is a key metabolite of the genera Lactobacillus and Lachnoclostridium. In honey bees butyrate is not well documented but is present in the haemolymph and is oxidised by flight muscles (George and

Bhakthan 1963). This suggests that butyrate may be absorbed and used by the host rather than being produced by the gut bacteria (Zheng et al. 2017). The presence of the genera *Bacteroides*, *Lactobacillus*, and *Lachnoclostridium* in the honey bee gut suggests that butyrate may be produced in the honey bee gut and the effects of butyrate within the bee should be studied to determine whether these bacteria have a role to play in pollen degradation and/or metabolism of the cell walls.

In NZ's temperate climate, nectar and pollen sources, although limited, are still available through winter. This enables honey bees to forage on dietary fibre such as pectin throughout the entire year. Nectar contains pollen, in which pectin is present in the pollen cell wall. The colonies also normally store pollen for brood food all year around. It is therefore possible that *Bacteroides* spp. may play a role in pectin digestion over winter. Although genome studies suggest some strains of *G. apicola* in the honey bee should also be able to digest pectin (Engel et al. 2012), this is undetermined for Bacteroides. In the absence of dietary fibre in humans, the glycans linked to proteins in the mucus layer of the intestine have been hypothesised to stimulate growth of *Bacteroides* spp. and positively affect gut health (Benítez-Páez et al. 2017). In climates where honey bees overwinter without foraging, their only food sources may be processed carbohydrates such as sucrose, inverted sugar, or high-fructose corn syrup. Unlike honey, none of these supplements contain the protein source of pollen (Zheng et al. 2017). It is unknown whether glycan-stimulation, as observed in humans, occurs in the absence of pollen in honey bees, but it is possible that *Bacteroides* spp. may affect honey bee gut health over winter. Whether this effect is positive or negative is unknown.

There are two possible reasons why *Bacteroides* spp. in worker honey bees may have peaked in winter. The first is that the condition of the honey bee digestive tract may alter with bee age, therefore creating an environment conducive to the growth and stability of the genus *Bacteroides*. The longevity of 'winter' bees exceeds 140 days, which is three times more than the 15- to 38-day life span of bees emerging in spring, summer or autumn (Fukuda and Sekiguchi 1966; Graham 1992). Alternatively, because of nectar paucity or ageing resources stored within the hive in winter, the sources of carbohydrate and protein consumed by the bees in winter may vary from what is consumed throughout the rest of the year. The absence of *Bacteroides* spp. in spring bees correlates with both rapid colony growth and abundant nutritional resources. Egyptian honey has antibacterial effects on *Bacteroides* spp. in human dental infections (Elbagoury and Ramsy 1993), so it is possible that the increased availability of different nectar (dilute honey) sources between spring and autumn may also inhibit the growth of *Bacteroides* spp. These correlations support both theories that an old gut as well as the condition of available resources may affect the growth of *Bacteroides* spp.

At the genus level in humans, the ratio of *Bacteroides* spp. to *Prevotella* spp. was previously thought to relate to food consumption, the former increasing with

consumption of protein and animal fats, and the latter increasing in those consuming carbohydrates (De Filippo et al. 2010; Wu et al. 2011; David et al. 2014; Kovatcheva-Datchary et al. 2015). However, differing species and strains in both of these genera are significantly associated with both of these diets, suggesting that the genera *Bacteroides* and *Prevotella* should not be used to oversimplify associations between diet and microbiota within hosts and that differences must be identified sub-genus (De Filippis et al. 2016). In my analysis, both phylotypes are high in winter but low in spring, summer and autumn which may suggest that both protein and carbohydrates are proliferating these bacteria in winter. As worker bees in NZ consume predominantly carbohydrates containing protein all year, I did not expect either of these two genera to alter throughout the year. Therefore, understanding how bees utilise their primary sources of carbohydrate such as nectar and protein, throughout the year and the metabolites they produce, may provide insight in to whether certain species of bacteria could be used to indicate bee health.

In Chapter 6, the carbohydrate study, *Bacteroides* spp. was absent. However, data were only included in the analysis if they were above the 0.01% prevalence level so it may not have been identified. As the carbohydrate trial was conducted with 13- to 16-day-old bees fed honey or sucrose, they differed from the winter bees sampled in this study which were likely older. Therefore this may have enhanced the growth of *Bacteroides* spp. As this study was established to identify whether gut bacteria change across the seasons, not why the changes have occurred, experimental trials are required to understand the factors involved in these changes.

The fourth least prevalent bacteria, *Pseudomonas*, is a genus of Gram-negative γ-proteobacteria with a large metabolic diversity that enables the colonisation of many niches (Sauer et al. 2002). Some of these species such as *Pseudomonas aeruginosa* form biofilms (Sauer et al. 2002) and others are highly resistant to antibiotics (Livermore 2002). In Apoidea*, Pseudomonas* has been identified in the larval stages of *A. mellifera* and *Bombus terrestris* (Mohr and Tebbe 2006). In this assessment the relative abundance of *Pseudomonas* is highest in summer and lowest in winter. This contrasts with the literature that suggests *Pseudomonas* has not been identified in the gut of adult *A. mellifera* (Audisio et al. 2011). However, *Pseudomonas* has been identified in the midgut of mosquitoes (Dharne et al. 2006). It is possible that the bees in this NZ study may have encountered some form of antibiotic, either natural or synthetic, whilst foraging on nectar in summer to which *Pseudomonas* may have been resistant and some other bacteria were susceptible, thus enabling the increase in relative abundance of *Pseudomonas* spp. This hypothesis could be supported by the decrease in relative abundance of *E. adhaerens* in summer. *E. adhaerens* is a soil bacterium that may possibly be collected by honey bees as they forage and may cause lysis of other bacteria by attaching to them (Rogel et al. 2001).

The fifth bacteria shown to change throughout the season is Cyanobacteria. Species within this phylum are photosynthetic – they produce oxygen from carbon dioxide and are known to produce blooms of blue-green algae in water sources, such as recreational and drinking water sources (Pitois et al. 2000; Chen et al. 2011). These sources are utilised by honey bees so the relative abundance of Cyanobacteria may have peaked in spring as the bees collected water. However, the limited literature regarding the relationship between Cyanobacteria and honey bees precludes more logical theories as to why Cyanobacteria peaked in spring. Interestingly, when the cyanobacteria *Spirulina platensis* was fed to bees in sucrose solution, the colonies showed significant increases in egg laying, disease resistance, flight intensity, honey production and bee bread production (Cebotari et al. 2013). Further research regarding which species of Cyanobacteria were present in the honey bee gut, what influences their proliferation, how they interact with the rest of the gut bacterial community, and which metabolites they produce would enable further understanding of the role that this bacteria plays in the gut of honey bees.

One of the less prevalent bacteria identified in the gut of honey bees in this study, *Bacteroides* spp., was not identified in the NZ regional survey (Chapter 3). However, the former two have been identified elsewhere: *Bacteroides* spp. was previously identified in Massachusetts, USA (Lee et al. 2015), and the family Lachnospiraceae, containing Lachnoclostridium spp., was identified in Beijing, China. The discrepancy in presence of *Bacteroides* spp. is most likely to have occurred because the NZ survey (Chapter3) was conducted in spring, which is when this seasonal study observed the lowest bacterial diversity and when *Bacteroides* spp. were absent. However, Lachnoclostridium spp. was present in the spring and autumn assessments and absent during the winter and summer assessments. As the relative abundance of Lachnoclostridium spp. was < 0.6% throughout the entire year, it may be possible that the relative abundance in the NZ study was below the 0.01% inclusion threshold. This may also be the case for Cyanobacteria. Lachnoclostridium are Gram-positive, obligate anaerobic spore-forming rods, of which some members can ferment plant polysaccharides and produce acetate as a major metabolite from mono- and disaccharides (Yutin and Galperin 2013). Hence, they may be present occasionally to produce energy required by the honey bee when gut conditions change.

5.6 Conclusion

The diversity of bacterial species in the honey bee gut fluctuated throughout the year. Summer bees had the most bacterial diversity (36 phylotypes), as well as the largest range of bacteria among the colonies. Autumn (25) and winter (29) bees had similar bacterial diversity, and spring bees had the least bacterial diversity (18 phylotypes).

It cannot be assumed that increased bacterial diversity in the honey bee gut is positively associated with bee and/or colony health, as there is no current evidence to support this in bees. Winter bees are, on average, older than spring bees (Fukuda and Sekiguchi 1966), and the increased number of phylotypes may represent a compromised core bacterial community, thus enabling the proliferation of less prevalent bacteria in the gut of honey bees that live for extended periods of time. Additionally, changes in diet and reduced opportunity to forage may also be alternative explanations. The relative abundance of Rhizobiaceae, which resides in the crop, peaked in winter, exceeding the relative abundance of the core bacteria *G. apicola*, *S. alvi*, and *B. coryneforme*, and then, receded below the relative abundance of these dominant core bacteria in summer. This suggests that Rhizobiaceae may increase when the core bacteria are compromised.

I suggest that increased colony growth in spring may reset the bacterial community in the colony as the majority of old winter bees are replaced with new bees.

The mean relative abundance of the dominant core bacteria within the honey bee digestive tract did not change throughout the year. However, *G. apicola* and *S. alvi* did vary among individual bee samples from each seasonal time point but with no clear pattern. This suggests that these species may alter in response to occurrences within the gut and this may ultimately aid bee functionality. The variation observed in this chapter will be accounted for throughout this PhD research using adequate sample size. The bacterial diversity and mean relative abundance of less prevalent bacteria, also varied throughout the year. Each trial within this PhD research will therefore be conducted in either spring, summer or autumn, as this will limit the age of sampled bees to < 38 days (Fukuda and Sekiguchi 1966).

5.7 Where to next

The variation of the core and less prevalent bacteria observed throughout the year suggests external factors may be effecting the microbiome in the honey bee gut. The two known major factors that negatively affect bee health are malnutrition and pathogens. Therefore experimental studies will be conducted to determine if these two factors affect the gut bacteria.

Chapter 6

The effect of carbohydrate sources: sucrose, invert sugar and components of mānuka honey, on core bacteria in the digestive tract of adult honey bees (*Apis mellifera*)

Taylor MA, Robertson AW, Biggs PJ, Richards KK, Jones DF, Parkar SG. 2019.

Plos One. 14(12): e0225845.

6.1 Abstract

Bacteria within the digestive tract of adult honey bees are likely to play a key role in the digestion of sugar-rich foods. However, the influence of diet on honey bee gut bacteria is not well understood. During periods of low floral abundance, beekeepers often supplement the natural sources of carbohydrate that honey bees collect, such as nectar, with various forms of carbohydrates such as sucrose (a disaccharide) and invert sugar (a mixture of the monosaccharides glucose and fructose). We compared the effect of these sugar supplements on the relative abundance of bacteria in the gut of bees by feeding bees from a single colony, two natural diets: mānuka honey, a monofloral honey with known antibacterial properties, and a hive diet; and artificial diets of invert sugar, sucrose solution, and sucrose solutions containing synthesised compounds associated with the antibacterial properties of mānuka honey. 16S ribosomal RNA (rRNA)-based sequencing showed that dietary regimes containing mānuka honey, sucrose and invert sugar did not alter the relative abundance of dominant core bacteria after 6 days of being fed these diets. However, sucrose-rich diets increased the relative abundances of three sub-dominant core bacteria, Rhizobiaceae, Acetobacteraceae, and *L. kunkeei*, and decreased the relative abundance of *F. perrara*, all which significantly altered the bacterial composition. Acetogenic bacteria from the Rhizobiaceae and Acetobacteraceae families increased two- to five-fold when bees were fed sucrose. These results suggest that sucrose fuels the proliferation of specific low abundance primary sucrose-feeders, which metabolise sugars into monosaccharides, and then to acetate.

6.2 Introduction

European honey bees (*Apis mellifera* L.) are the primary pollinators of numerous nut, fruit, and vegetable crops, so they play an integral part in global food production (Free 1970; Southwick and Southwick 1992; Morse and Calderone 2000; Gallai et al. 2009). Pollination by honey bee species (*Apis* spp.) and other bee species also ensures reproductive success of uncultivated plants, including those in their native ranges (Morse and Calderone 2000; Gallai et al. 2009; Potts et al. 2010a). In addition to pollination, honey bees also produce economically valuable honey, as well as acting as a source of bee products such as pollen and propolis, the waxy resin collected from leaf buds. All three products are utilised both as food and by the medicinal and dietary-supplement industries. This global utilisation of honey bees has made it important to understand the factors that influence honey bee health. Hive management practices, and the colony's access to adequate nutritional resources, is crucial to colony health. The health and production of a colony is dependent on the location that beekeepers place their hives to forage, the supplementary carbohydrate and protein sources they

feed their bees, and when they do this (Severson and Erickson 1984; Human and Nicolson 2006; Saraiva et al. 2015).

Honey bees require carbohydrate sources that they naturally obtain from nectar. Nectar predominantly consists of water, pollen, and varying proportions of the monosaccharides glucose and fructose, and the disaccharide sucrose (Wykes 1952; White et al. 1962; Chalcoff et al. 2005). Bee-pollinated flowers tend to produce nectar with > 35% sugar and honey bees reduce the moisture content within nectar to about 17% (range 13–24%) resulting in honey with a concentrated mix of sugar comprising of about 69% monosaccharides (approximately 38% fructose and 31% glucose) (Doner 1977), and < 15% disaccharide (sucrose) (White et al. 1962).

The carbohydrates in the honey bee diet may be absorbed by the gut to sustain the bees, or metabolised by gut bacteria before absorption (Baxter et al. 2019) (see Supplementary material at the end of this chapter S.16). However, during winter and spring when nectar can be scarce, and when preparing colonies for winter, beekeepers often feed their bees supplementary carbohydrates. These include sucrose, invert sugar (a mix of glucose and fructose) and high fructose corn syrup (HFCS; a sweetener made from cornflour, in which some glucose has been converted to fructose) (Barker and Lehner 1978; Severson and Erickson 1984; Graham 1992). This additional feeding often protects the bees from malnutrition, which can lead to immune system impairment (Alaux et al. 2010) and increased pesticide susceptibility (Wahl and Ulm 1983). However, extensive feeding of either sucrose or HFCS causes significant differences in gene expression by the honey bee fat body (the nutrient-sensing organ responsible for nutrient storage), including those associated with energy metabolism, and antimicrobial peptide production (Wheeler and Robinson 2014). These epigenomic consequences in honey bees, are very similar to sugar-associated disrupted metabolism seen in vertebrates that are supplemented with either glucose or fructose (Sangüesa et al. 2016).

The function of bacteria residing in the digestive tract of animals, honey bees included, is a rapidly developing field of scientific research that is proving to be fundamental to animal health (Guarner 2005; Colman et al. 2012). A meta-analysis of the composition of gut bacteria in 62 insect species suggest bacterial similarity within the subfamily Apinae, as well as the distinct communities of *A. mellifera* relative to other bees (Colman et al. 2012). This meta-analysis suggests that bacterial community structure in insects may be influenced by diet (Colman et al. 2012). However, as this was not specifically identified for honey bees, and recent research predominantly focusses on the effect of pollen rather than carbohydrate, meaning that not all publications specify the type or amount of supplementary feed consumed (Maes et al. 2016; Jones et al. 2018b; Rothman et al. 2018), the effect of carbohydrate diets on the bacterial composition in the honey bee gut, and how this may influence bee health, has not yet been researched.

The microbiota within the gut of adult worker honey bees contain 8 to 10 core bacterial phylotypes (Kwong and Moran 2016a). These phylotypes are rarely found outside of the honey bee gut and are considered part of the conserved core microbiota, albeit with different relative abundances and being more or less frequently detected (Ludvigsen et al. 2015; Jones et al. 2018b). The dominant core phylotypes consist of two species from the phylum Proteobacteria, *Gilliamella apicola* and *Snodgrassella alvi* (Kwong and Moran 2013); two clusters of species from the phylum Firmicutes, *Lactobacillus* Firm-4; *Lactobacillus* Firm-5 (Babendreier et al. 2007; Martinson et al. 2011); and the species cluster in the phylum Actinobacteria, *Bifidobacterium* (Bottacini et al. 2012). The relative abundances of the remaining core phylotypes are less consistent, and not always detected: *Frischella perrara* (Engel et al. 2013b), *Bartonella apis* (Kešnerová et al. 2016), *Parasaccharibacter apium* (Corby-Harris et al. 2014b), and a *Gluconobacter*-related species group designated Alpha2.1 (Martinson et al. 2011).

The gut has several sections that each contain bacterial populations of different taxonomic compositions (Babendreier et al. 2007). Only a few bacteria reside in the crop and the midgut. These include core species that also reside in the larval gut such as Rhizobiaceae, the nitrogen-fixing bacteria (Yu and Martin 2016), Acetobacteraceae and *Lactobacillus kunkeei* (Vojvodic et al. 2013). The adult ileum is dominated by the non-sugar fermenter *S. alvi* that colonises the gut wall, and the sugar fermenter *G. apicola* that resides in the lumen (Zheng et al. 2017). The distal rectum is dominated by *Lactobacillus* and *Bifidobacterium* (Martinson et al. 2012; Powell et al. 2014).

Bacteria in the honey bee gut are often symbiotic residents, with functions likely to be essential to bee nutrition, digestion, reproduction, and protection against toxins and pathogens (Dillon and Dillon 2004; Engel and Moran 2013b; Lee et al. 2015). Metatranscriptome sequencing has shown that bacteria play several critical roles in metabolising carbohydrate substrates. Some of these bacteria are primary sucrose-feeders, and metabolise sugars into monosaccharides that are further metabolised into acid metabolites such as acetate and lactate that assist with the breakdown of toxic sugars (Lee et al. 2015; Zheng et al. 2016). The gut bacteria thus contribute to the repertoire of enzymes required for carbohydrate digestion (Wang et al. 2015). The bacterial species from the phyla Actinobacteria and the class Bacilli produce several glycoside hydrolases, which in turn break down complex polysaccharides and simple sugars, and also produce peptidases for protein hydrolysis (Lee et al. 2015). In particular, glycoside hydrolase family 32 was found to be linked with sucrose degradation (Lee et al. 2015).

Sucrose solutions and honey are both antibacterial *in vitro* because of osmolytic effects when applied at concentrations ≥ 40% and 10–20% (v/v), respectively (Molan 1992a; Kwakman et al. 2010). The anti-bacterial properties of honey have been attributed to this high sucrose-equivalent concentration ca. 80% (v/v), as well as the presence of

hydrogen peroxide, produced by the enzyme glucose oxidase that the bees add to nectar (Molan 1992a). Mānuka honey, obtained from the plant *Leptospermum scoparium*, comprises ca. 85% sugars, predominantly fructose and glucose, with < 1–15% sucrose (Weston and Brocklebank 1999; Chepulis and Francis 2013; Moniruzzaman et al. 2013). Mānuka honey demonstrates peroxide activity, but methylglyoxal (MGO) is the primary antibacterial compound at concentrations > 0.15 mg/g (Willix et al. 1992; Mavric et al. 2008; Kwakman et al. 2010; Majtan et al. 2012). This was characterised by comparing the bactericidal effects of honey containing high MGO with the effects of sucrose on resistant strains of Gram-negative Gammaproteobacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive organisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faeciumas*) (Kwakman et al. 2010). MGO is derived from the breakdown of dihydroxyacetone (DHA), which is also found in high concentrations in mānuka honey (Mavric et al. 2008; Adams et al. 2009; Atrott et al. 2012). The concentration of MGO in mānuka honey less than one year old is normally between 0.10 and 0.79 mg/g. This can increase to 1.54 mg/g with the breakdown of DHA over the course of a year, or if the honey has been heat treated (Majtan et al. 2012).

Honey bees commonly consume carbohydrates in the form of nectar, honey, sucrose, and invert sugar, but not all carbohydrates are utilised by bees or their microbial residents (Haydak 1970; Wheeler and Robinson 2014). We hypothesise that honeys will affect the diversity and relative abundance of bacteria present in the digestive tract compared with sucrose solutions, and that these effects may be attributed to the differences in the sugar composition in these diets. We used 16S rRNA gene sequencing to investigate the effect of carbohydrate sources on the relative abundance of bacteria present in the digestive tract of caged adult honey bees from a single colony. The effect of two different mānuka honeys (predominantly monosaccharides), were compared with the effect of invert sugar (mix of monosaccharides), sucrose (a disaccharide), and diets containing the mānuka associated chemicals MGO and DHA in sucrose solution. These were also compared with the effects of diet consumed by caged bees in a hive.

6.3 Materials and methods

6.3.1 Honey bee sampling and their treatment diets

A single *A. mellifera* colony, located at The New Zealand Institute for Plant and Food Research Limited (PFR), Hamilton, New Zealand (NZ), was used in this trial to limit the effect of genetic variation. A single frame of black-eyed (18–20 days old) honey bee pupae was selected from a colony in early summer (December 2017) and incubated at 33°C and 65% relative humidity (RH). Throughout a 70-h period, a total of 1050 NEWs, which were < 24 h old were marked on their abdomen with a spot of nail polish, caged and returned to the parent colony for at least seven days. This allowed colonisation of the digestive tract with a full complement of bacteria, as observed by Powell et al. (2014). The bees slowly released themselves from the cages over 24 h as the grass blocking the entrance dehydrated. Ten days after the first marked bees were returned to their colony, 7- to 10-day-old marked bees were recaptured from the colony and ten bees were placed in each plastic queen cage (75 x 30 x 15 mm). It took approximately 4 h to set up the seven diet treatments so replicate cages were allocated to each of the treatments sequentially, one cage per treatment (Figure 6.1). The six modified diets had eight replicates and the hive control diet had five replicates. In total, there were 53 cages of bees (Table 6.1).

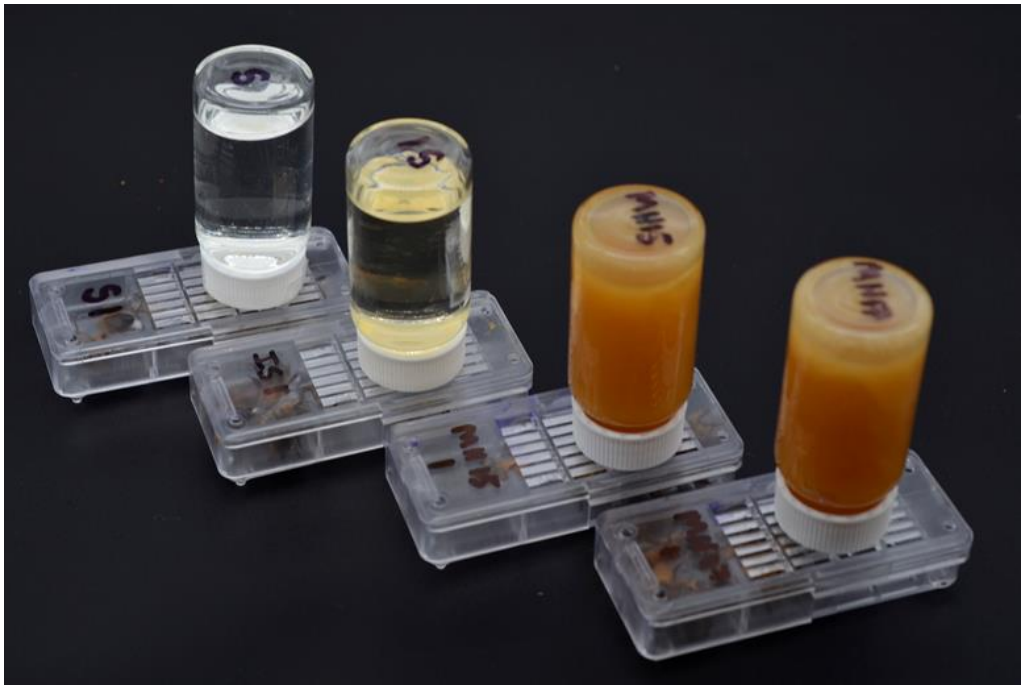


Figure 6.1 | Carbohydrate diets fed to caged honey bees.

Feeding commenced immediately and continued for a duration of 6 days. The control cages (H) were pressed into the wax and honey in a honey frame above the brood nest of the parent hive. The bees consumed the honey *ad libitum* and were likely to have received food from the hive bees. Based on info identified in a pilot feeding trial (Supplementary material 6.6.1), the remaining six treatments were fed to the bees *ad libitum* through gravity feeders and the cages were incubated at 33°C and 65% RH for 6 days. These treatments were refreshed after 3 days. Two treatments were two mānuka honeys harvested by Hikutaia Honey (Opotiki, NZ) from the same apiary, but from different seasons: mānuka honey from the 2015 harvest (MH15, Lot # 112-15), and mānuka honey from the 2017 harvest (MH17, Lot # 49-17). These honeys were extracted from the wax frames at 33°C, and then the honeys were passed through a 1200 µm mesh. Prior to the trial, the honeys were analysed for DHA and MGO by Analytica Laboratories (Hamilton, NZ). Two further treatments were 50% (w/w) sucrose solution mixed with one of two chemically synthesised mānuka components: 1692 mg/kg DHA (Sigma D107204, Lot # MKBS8481V, Sigma-Aldrich, Auckland, NZ) or 745 mg/kg MGO (Sigma M0252, Lot # BCBK5800V, Sigma-Aldrich, Auckland, NZ). The concentrations tested were the maximum concentrations observed in the analysed mānuka honeys (MH15 and MH17) and previously reported in the literature (Mannina et al. 2016). Two more treatments were supplementary carbohydrate solutions used by the beekeeping industry: 67°B invert sugar (IS; NSFGIVB5BULK), and 50% sucrose solution (w/w) (S).

Table 6.1 | Carbohydrate diets fed to caged honey bees.

Treatment	Cage replicates	Diet	Sucrose (%)	MGO (mg/kg)	DHA (mg/kg)
H	5	Hive diet: honey frame above the brood nest	Unknown$^{\Theta}$	_	–
IS	8	20 ml of 67°B bulk invert sugar	0	_	–
S	8	20 ml of 50% sucrose solution	50	_	–
MH15	8	20 g of 100% mānuka honey from 2015	< 1–15 ‡	745	1238
MH17	8	20 g of 100% mānuka honey from 2017	< 1–15 ‡	394	1692
MGO	8	20 ml of 745 mg MGO/kg 50% SS*	~50	745	–
DHA	8	20 ml of 1692 mg DHA/kg 50% SS	~50	_	1692

Θ The hive was not fed supplementary sources of sucrose throughout the spring. ‡ Percent sucrose (w/w) was based on mānuka honey analysis in the literature (Weston and Brocklebank 1999; Chepulis and Francis 2013; Moniruzzaman et al. 2013). * 0.931 ml 40% aqueous MGO + 499.17 ml 50% sucrose. Sucrose solution (SS).

Sixteen days after their emergence as adults, 100% of the caged bees were still alive. At that point, five individuals from each of the 53 cages (a total of 265 bees) were placed in 90% ethanol and stored at –70°C.

6.3.2 DNA extraction, amplification, and 16S rRNA gene sequencing

For each replicate the five stored bees were thawed for three min and then each digestive tract (crop to rectum) was aseptically dissected and pooled into a single DNase- and RNase-free ZR BashingBead™ Lysis Tube (Zymo Research Corporation (ZR), California, USA), in ice, containing 750 µl lysis solution. At this point, the tubes were returned to –70°C until processing as the lysis solution contained a proprietary DNA stabilising agent. The pooling was conducted to ensure homogeneity of the sample extracted, (given that an individual gut sample averaged 26.3 mg such a low biomass would have yielded a low concentration of DNA which may have been insufficient for sequencing), and enabled the inclusion of more biological replicates. The five pooled tracts were processed for DNA extraction using the Zymo Research Quick-DNA™ Fecal/soil Microbe Miniprep kit (Zymo Research Corporation (ZR), California, USA). The samples were homogenised at 6 m/s for 40 seconds using a FastPrep®-24 (MP Biomedicals, Seven Hills, Australia), and then the rest of the ZR protocol was followed. The eluted DNA samples were stored at –70°C prior to being sent on ice by overnight courier to the Massey Genome Service (MGS; Massey University, Palmerston North, NZ) for 16S rRNA gene sequencing of the V3V4 hypervariable region (Kozich et al. 2013).

MGS evaluated the DNA concentration in each sample with Qubit™ 2.0 Fluorometer (ThermoFisher Scientific, NZ) analysis using a dsDNA HS Assay Kit for 12 samples per plate. A PCR reaction was then performed using primers with adaptors: 16Sf_V3 (5' - 3' direction) – CCTACGGGAGGCAGCAG; and 16Sf_V4 (5' - 3' direction) – GTGCCAGCMGCCGCGGTAA (Kozich et al. 2013). The PCR products (c. 420-440 base pairs) were purified to generate a library and their concentrations were analysed using Qubit™. The products were pooled in equimolar concentrations and the concentration and size were confirmed with both Qubit™ and LabChip (PerkinElmer, Waltham, MA, USA) analysis. The PCR products were sequenced with a 250-base paired end run on an Illumina MiSeq™ platform (Illumina Inc.) with version 2 chemistry. Illumina PhiX Control v3 (FC-110-3001) was included as the sequencing control. The resulting sequences are available in the National Center for Biotechnology Information's (NCBI's) Sequence Read Archive (PRJNA531038).

6.3.3 Gene sequence processing and characterisation of microbial communities

A total of 5,127,987 read pairs were detected across all seven treatments and cage replicates. The Illumina de-multiplexed fastq sequence data were processed and trimmed using ea-utils to a 0.01 probability of error, an equivalent Phred score of Q20 (Aronesty 2011), then further processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) analysis suite, version 2018.2 (Caporaso et al. 2010) (https://github.com/PlantandFoodResearch/bioinf_Apis_metabarcoding). The reads were run through DADA2 methodology in QIIME2 to filter and trim the paired-end sequences, dereplicate them, and filter chimeras to produce exact ASVs.

The honey bee microbiome is a relatively new area of research, with new bacterial strains being identified and reclassified frequently. Previous work indicated that some sequences were incorrectly assigned to old nomenclature. To ensure taxonomic classification of honey bee gut bacteria were current, the 16S rRNA BLAST (Basic Local Alignment Search Tool) database was downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) and customised to make a QIIME 2 compatible reference dataset (https://github.com/pjbiggs/16SrRNA_taxonomy). From the dataset a biological observation matrix (BIOM) was created that contained the Operational Taxonomic Units (ASVs) identified from the sequencing of each sample, that matched with the assigned taxonomy. Any ASVs that were unable to be identified taxonomically to species level were assigned to the closest identified taxonomic level.

6.3.4 Statistical analyses

Phylogenetic diversity was measured within a sample (α-diversity), and between samples (β-diversity) using the web-based tool MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017). The data counts were filtered to a minimum of two, as well as a 10% prevalence in the samples. Variance was filtered using a 10% coefficient of variation. To reduce estimation errors that result from the different number of sequences per sample, the data were rarefied to 52880, the number of sequences in the smallest sample. The data were relativised using total sum but were not transformed.

Alpha-diversity was calculated at the feature level using Kruskal-Wallis pairwise comparisons of four diversity measures: Observed ASVs, Chao1, Shannon, and Simpson. β-diversity for the taxonomic level feature was calculated using the distance methods Bray-Curtis dissimilarity (uses abundance of each ASV) and Jaccard Index (presence/absence), and the differences between the samples were compared using a permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001). Beta-diversity was displayed as 3-D PCoA plots.

Further data analysis was conducted in R (version 3.5.1) (R Core Team 2018). For all analyses, sequences with a minimum total read composition of < 0.1% prevalence were filtered from the dataset (the remaining number of reads totalled 4,767,519). To investigate the differences in the number of phylotypes between treatments, a Poisson generalised linear model was used with the number of phylotypes as the response and treatment as a fixed effect. To explore the relationship between phylotypes and treatment, the data were visually explored using heat maps, where the response was the mean read composition per replicate. The interaction of the relative abundance (proportion of total bacterial abundance) of phylotypes was explored using nonmetric multidimensional scale (NMDS) plots. For the NMDS plots, the dissimilarity matrix was calculated using the Bray-Curtis dissimilarity method. A linear mixed effect regression model was performed using the R package lme4 (Bates et al. 2014). Replication was included as a random effect to account for replicate to replicate variability between all phylotypes present within each sample. Assumptions were checked via standard residual plots and a logarithmic transformation was applied. Post-hoc pairwise comparisons of least-square means were carried out using Tukey. The predicted means were back-transformed and dissimilar letters were used to indicate significant differences among treatments. To determine whether carbohydrate diet altered the bacterial community within the gut, a mixed model PERMANOVA (Anderson 2001) was conducted using Adonis2 (Oksanen et al. 2018) to compare the variation in relative abundance between the treatments.

6.4 Results

The 4,767,519 read pairs detected across all seven treatments and cage replicates were clustered into 75 ASVs. ASV sequences were classified as 11 unique phylotypes, of which two were families, one was a genus, and eight were species (Table 6.2). The mean number of ASVs listed in Table 6.2 were similar for each diet treatment but the invert sugar (IS) treatment had the least (69 ASVs). Further analysis of this difference in ASVs revealed no clear pattern, only that the IS treatment had one less ASV for each of five phylotypes (S1 Table) and the Poisson generalised linear model provided no evidence to suggest a difference in the number of phylotypes between treatments (S2 Table). Similarly, the α-diversity analysis indicated that none of the treatments caused a significant influence on the richness (Chao1, Observed ASVs), and this did not change after accounting for evenness (Shannon and Simpson Indices) ($P > 0.05$) (S2 Table).

Table 6.2 | Number of amplicon sequence variants and the associated taxonomic groups within the gut of NZ honey bees.

Diet treatment	Number of ASVs	Number of phylotypes
H	74	11
S	75	11
IS	69	11
MH15	72	11
MH17	72	11
MGO	74	11
DHA	74	11

The bees were sourced from a single hive and fed different carbohydrate diets for 6 days: Hive-fed (H); sucrose (S); invert sugar (IS); 2015 mānuka honey (MH15); 2017 mānuka honey (MH17); methylglyoxal (MGO); dihydroxyacetate (DHA).

The phylotype *Lactobacillus* spp. dominated all the samples with counts 3- to 4-fold higher than all other phylotypes. The 25 ASVs associated with *Lactobacillus* spp. suggests the phylotype contains a lot of genetic diversity (Table 6.3). In comparison, the three species that were identified as *Lactobacillus* species: *L. mellis*, *L. mellifer*, and *L. kunkeei*, contained 8, 1, and 1 ASVs, respectively. *Lactobacillus mellifer* is often included in the phylotype *Lactobacillus* Firm-4. However, this manuscript individually identifies *L. mellifer* and refers to *Lactobacillus* Firm-4 as the phylotype *Lactobacillus* spp.

Table 6.3 | Mean relative abundance for each of the phylotypes in the digestive tract of honey bees fed different carbohydrate diets for 6 days.

Bacterial phylotype	Mean ASVs	H	IS	MH15	MH17	S	MGO	DHA
Lactobacillus* spp.	25	**42.6^{a}** **(25.8-70.4)**	**51.5^{a}** **(34.7-76.5)**	**44.4^{a}** **(29.9-65.9)**	**46.8^{a}** **(31.5-69.6)**	**44.7^{a}** **(30.1-66.4)**	**48.9^{a}** **(32.9-72.7)**	**46.4^{a}** **(31.2-68.9)**
Gilliamella apicola*	13	14.0^{a} **(8.5-23.0)**	11.7^{a} **(7.9-17.4)**	15.1^{a} **(10.2-22.5)**	17.7^{a} **(11.9-26.3)**	**10.1^{a}** **(6.8-15.0)**	10.2^{a} **(6.9-15.1)**	10.1^{a} **(6.8-15.0)**
Lactobacillus mellis	11	11.9^{a} **(7.2-19.7)**	9.3^{a} **(6.3-13.9)**	8.7^{a} **(5.8-12.9)**	7.8^{a} **(5.2-11.6)**	10.5^{a} **(7.0-15.5)**	10.3^{a} **(6.9-15.3)**	9.5^{a} **(6.4-14.1)**
Bifidobacterium coryneforme*	8	8.5^{a} **(5.1-13.9)**	8.0^{a} **(5.4-11.9)**	8.3^{a} **(5.6-12.4)**	7.7^{a} **(5.2-11.5)**	**8.5^{a}** **(5.8-12.7)**	6.8^{a} **(4.6-10.2)**	7.7^{a} **(5.2-11.4)**
Snodgrassella alvi*	5	8.0^{a} **(4.9-13.2)**	6.3^{a} **(4.2-9.3)**	8.0^{a} **(5.4-11.0)**	6.2^{a} **(4.2-9.3)**	**4.8^{a}** **(3.2-7.1)**	5.9^{a} **(3.2-7.1)**	5.2^{a} **(3.5-7.8)**
Ensifer adhaerens	1	1.4^{a} **(0.8-2.2)**	1.2^{a} **(0.8-1.7)**	2.0^{a} **(1.4-3.0)**	2.0^{a} **(1.3-2.9)**	2.4^{a} **(1.6-3.5)**	2.4^{a} **(1.6-3.5)**	2.1^{a} **(1.4-3.1)**
Lactobacillus mellifer*	1	1.5^{a} **(0.9-2.5)**	1.3^{a} **(0.9-2.5)**	1.8^{a} **(1.2-2.7)**	1.5^{a} **(1.0-2.2)**	1.9^{a} **(1.3-2.8)**	1.9^{a} **(1.3-2.8)**	1.8^{a} **(1.2-2.7)**
Frischella perrara	5	5.5^{b} **(3.4-9.1)**	2.7^{ab} **(1.8-4.0)**	4.1^{ab} **(2.8-6.1)**	3.6^{ab} **(2.4-5.4)**	**2.0^{a}** **(1.3-2.9)**	**2.7^{ab}** **(1.8-4.0)**	**2.0^{a}** **(1.4-3.0)**
Rhizobiaceae	4	1.1^{a} **(0.6-1.7)**	0.8^{a} **(0.6-1.2)**	1.4^{a} **(1.0-2.1)**	0.8^{a} **(0.6-1.2)**	**4.3^{b}** **(2.9-6.3)**	**4.1^{b}** **(2.7-6.0)**	**5.0^{b}** **(3.4-7.5)**
Acetobacteraceae	1	1.3^{a} **(0.8-2.2)**	1.5^{a} **(1.0-2.2)**	2.0^{ab} **(1.3-2.9)**	2.5^{ab} **(1.7-3.7)**	**4.1^{b}** **(2.8-6.1)**	3.2^{ab} **(2.2-4.8)**	3.3^{ab} **(2.2-4.9)**
Lactobacillus kunkeei	1	0.3^{ab} **(0.2-0.5)**	0.3^{a} **(0.2-0.4)**	0.2^{a} **(0.1-0.3)**	0.1^{a} **(0.1-0.3)**	**0.8^{b}** **(0.5-1.2)**	0.2^{a} **(0.1-0.3)**	**0.7^{b}** **(0.5-1.1)**

Honey bees from a single hive were fed one of seven carbohydrate diets for six days: Hive-fed (H), invert sugar (IS), 2015 mānuka (MH15), 2017 mānuka (MH17), sucrose (S), methylglyoxal (MGO), and dihydroxyacetate (DHA). The columns of sucrose-rich treatments are shaded in light grey. The back transformed means were identified using Tukey post-hoc comparisons from the linear mixed effect model, $\alpha = 0.05$. The dissimilar letters indicate significant differences among treatment means. Differences are bolded and the phylotypes that changed significantly with diet are shaded in dark grey. The corresponding phylotypes are shaded in medium grey. ASV (Amplicon Sequence Variants). Dominant core bacteria (*).

Although each diet produced very similar gut microbiome diversity and most of the core bacteria were found at similar relative densities across all diets, there is evidence that the proportion of some phylotypes changed in response to diet (Table 6.3, Figure 6.2). The heatmap demonstrates evidence of sucrose treatments (S, MGO, and DHA) affecting the mean composition reads for some of the phylotypes, such as Rhizobiaceae (Figure 6.2). The effect of diet was supported by the Analysis of Deviance for the linear mixed effect regression model where significant interaction between the mean relative abundance of each bacteria within each treatment was evident ($P < 0.001$) (S2 Table).

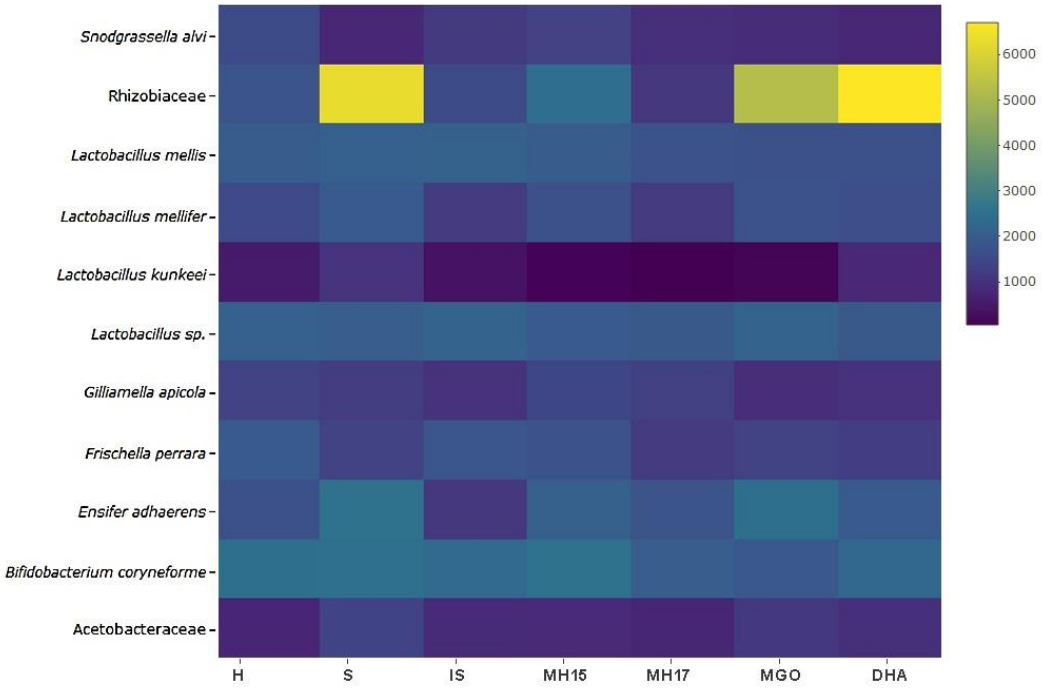


Figure 6.2 | Heatmap of mean composition reads of the bacteria in the honey bee digestive tract fed different carbohydrate diets.

Reads > 0.1% prevalence were included. Honey bees from a single hive were fed one of the following treatments for 6 days: Hive-fed (H), sucrose (S), invert sugar (IS), 2015 mānuka (MH15), 2017 mānuka (MH17), methylglyoxal (MGO), and dihydroxyacetate (DHA).

The effect of the different carbohydrate diets on the phylotypes was further identified with the post-hoc pairwise comparisons where the mean relative abundance of four of the sub-dominant core phylotypes differed significantly ($P < 0.01$) (Table 6.3) (see S1 Table for the mean total abundance for each bacteria within each treatment. The totals in the S1 Table decreased in the same order as Table 6.3): The relative abundance of *F. perrara* was two-fold higher ($P < 0.01$) in the hive control than in the sucrose and DHA treatments. The relative abundance of Rhizobiaceae was 4- to 5-fold higher ($P < 0.01$) in the three sucrose-rich treatments (sucrose, MGO and DHA) than in the four sucrose-poor treatments (H, IS, MH15, MH17). Acetobacteraceae was also 2- to 3-fold higher ($P < 0.01$) in the sucrose treatment than the hive and invert sugar treatments, while the relative abundance of *L. kunkeei* was 2- to 7-fold higher ($P < 0.01$) in the sucrose-rich and DHA treatments compared with the MGO, invert sugar, and mānuka honey treatments. In contrast, the diet treatments did not affect the relative abundance of the five dominant core bacteria (*G. apicola*, *S. alvi*, *Lactobacillus* spp., *Lactobacillus* Firm-5, and *Bifidobacterium*).

The NMDS analysis (Figure 6.3) also suggests that the composition of the microbiome shifted primarily as a function of the sucrose content of the diet. Communities in the sucrose-rich diets (S, MGO, and DHA) were displaced from the sucrose-poor diets (H, IS, MH15, MH17) along axis one of the ordination. The sucrose-rich diets produced communities that converged with a strong representation of Rhizobiaceae, while the sucrose-poor diets tended to increase in *G. apicola*. The relative abundance of *F. perrara* and *L. mellis* tended to move towards the opposite direction on axis two, and thus seemed to be less affected by sucrose content or other contents of the diet.

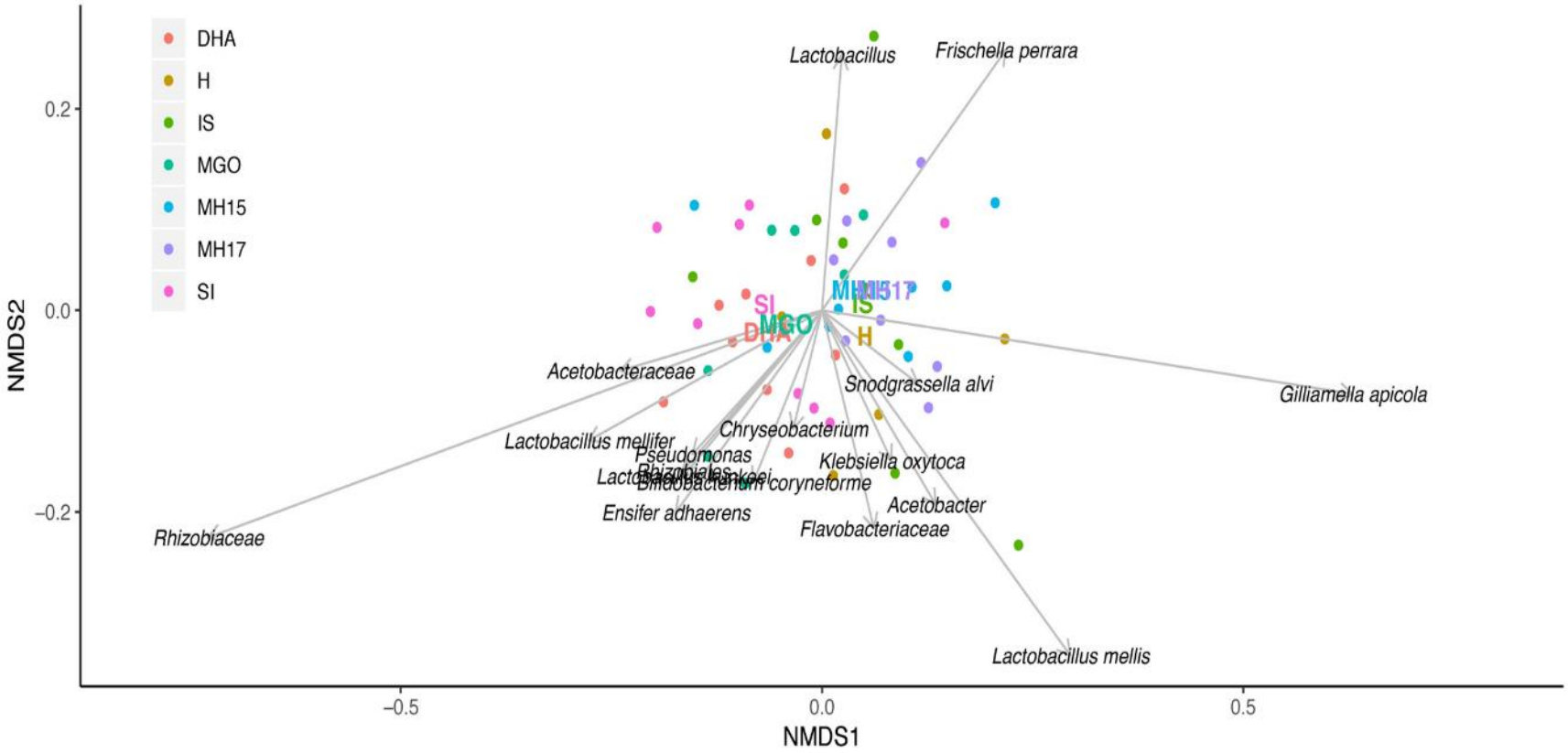


Figure 6.3 | Nonmetric multidimensional scaling plot of relative abundance of bacteria in the digestive tract of honey bees fed different carbohydrate diets.

Total read composition with > 0.1% prevalence was included. Honey bees from a single hive were fed one of the following treatments for 6 days: Hive-fed (H), sucrose (S), invert sugar (IS), 2015 mānuka (MH15), 2017 mānuka (MH17), methylglyoxal (MGO), and dihydroxyacetate (DHA). A solution for the plot was reached at stress level 0.273.

The PERMANOVA confirmed significant differences in community assembly with diet for both distance measures ($P < 0.001$, $R^2 = 0.243$) (Table 6.4).

Table 6.4 | The effect of dietary treatments on the beta-diversity of amplicon sequence variants within the gut of NZ honey bees.

Distance method	P - value	F- value	R squared	Axis 1	Axis 2	Axis 3
Bray-Curtis	< 0.001	1.7153	0.1828	15.8%	8.7%	8.5%
Jaccard	< 0.001	1.4539	0.1594	11.3%	6.5%	6.3%

Honey bees from a single hive were fed one of seven treatments for 6 days. The relative abundance of ASVs were analysed with different distance methods using PERMANOVA.

The PCoA visualisation using Bray-Curtis dissimilarity indicated that the majority of the communities showed separation based on the abundance of sucrose (sucrose, MGO, and DHA), or the limitation of sucrose (H, IS, MH15, MH17) (Figure 6.4) (see S1 Figure for PCoAs based on different distance methods).

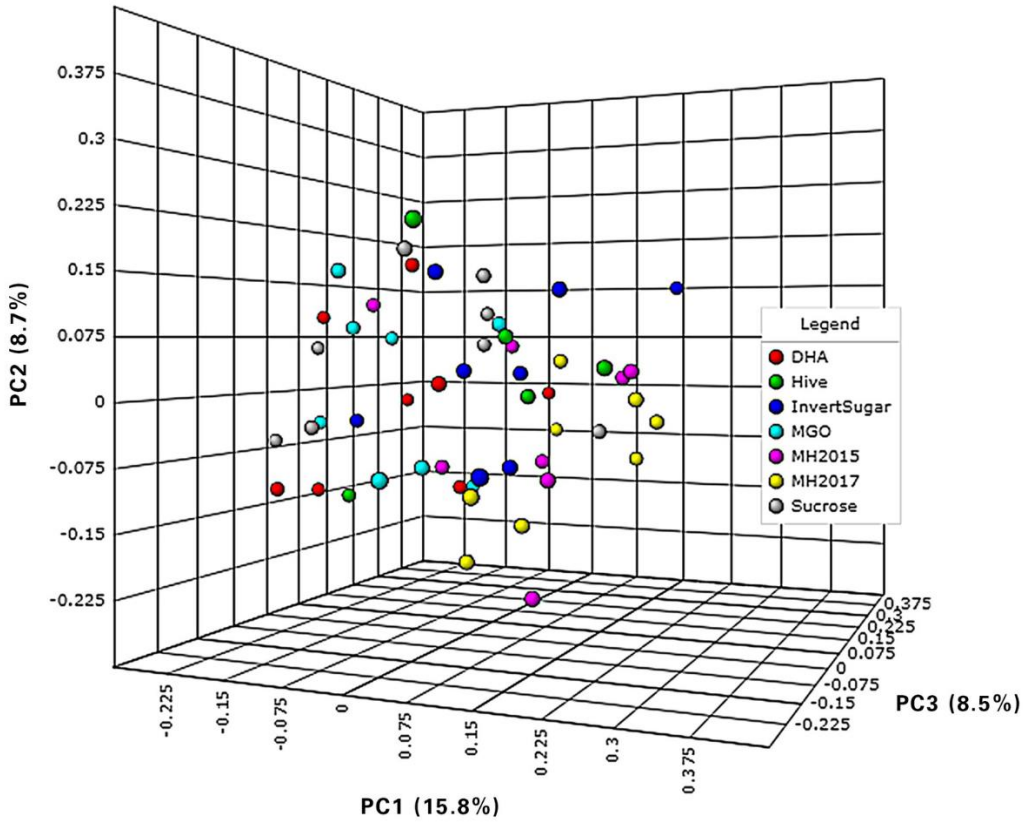


Figure 6.4 | A Principal Coordinates Analysis of the beta-diversity of ASVs within the gut of NZ honey bees.

Honey bees from a single hive were fed one of seven carbohydrate diets for 6 days.

6.5 Discussion

We examined the gut bacteria of adult *A. mellifera* from a single colony after being fed seven different dietary regimes for six days. The effect of carbohydrate composition on the diversity and relative abundance of bacteria present in the digestive tract was determined by comparing the effect of invert sugar (mix of monosaccharides) and two different mānuka honeys (predominantly monosaccharides), with the effect of sucrose (a disaccharide), and diets containing the mānuka associated chemicals MGO and DHA in sucrose solutions. These were all compared to the diet that bees consume within a hive.

There was no evidence of correlation between diet and the relative abundance of the five dominant core bacteria in the digestive tract of *A. mellifera*. However, the sucrose diet altered the relative abundance of some of the sub-dominant core ASVs when compared with the hive control, and a significant shift in the overall composition of the microbiome was observed.

The relative abundance of Rhizobiaceae increased by 4- to 5-fold, Acetobacteraceae increased by 2- to 3-fold, and *L. kunkeei* increased by 2- to 7-fold. In contrast the relative abundance of ASVs from the species *F. perrara* decreased with a sucrose diet by 2-fold. *F. perrara* is associated with scabbing of the epithelial surface in the pylorus, which is potentially due to an immune response in the bees (Engel et al. 2015). All bees were initially exposed to the same hive environment to develop a natural gut microbiome before being fed the specific diet treatment, only the sucrose and DHA treatments appear to have inhibited the proliferation of *F. perrara* and potentially the immune system response.

As both sucrose (Molan 1992a; Kwakman et al. 2008), and mānuka honey are antibacterial (Willix et al. 1992), it was hypothesised that both of these carbohydrate treatments may inhibit the gut bacteria. However, sucrose and mānuka honey appeared to affect the gut bacteria differently as the relative abundances of Rhizobiaceae, Acetobacteraceae, and *L. kunkeei* increased with sucrose but this was not observed in the hive controls or the mānuka honey treatments.

These differences in the sub-dominant core bacteria are further evidence that diet affects the bacterial composition within the digestive tracts of *A. mellifera,* as already seen with different pollen diets and differing environmental landscapes (Maes et al. 2016; Jones et al. 2018b; Rothman et al. 2018). However, as the dominant core bacteria did not alter, we suggest that the biotic factors affecting the honey bee gut microbiome should be discussed more specifically in terms of dominant or sub-dominant core bacteria, as changes seen so far are relatively subtle and seem to mainly effect the less abundant phylotypes.

The relative abundance of the phylotype *Lactobacillus* spp. (Firm-4) was 3- to 4-fold higher than all other phylotypes, across all treatments. This higher relative abundance did not alter with diet, but since the phylotype *Lactobacillus* spp. contained 25 ASVs that were unable to be classified more finely in our study, it is likely to represent several species. This has previously been shown using 16S rRNA gene sequence analyses, and phenotypic and genetic characteristics to isolate seven species of *Lactobacillus* from the lactic acid bacterial community within bees (Olofsson et al. 2014). Of these seven species, only two were identified in our analysis, *L. mellis* and *L. mellifer*, suggesting that additional species may feature within our *Lactobacillus* spp. phylotype, and the effect of diet on these individual bacteria may have been concealed, as some may have increased in relative abundance whilst others decreased.

Rhizobiaceae, Acetobacteraceae, and *L. kunkeei* are major bacterial phylotypes previously identified in the honey bee crop but absent in the mid- and hindguts of nurse and forager bees (Corby-Harris et al. 2014b). The crop and midgut contain < 5% relative abundance of all bacteria in the gut (Martinson et al. 2012), and as expected these bacteria were present in relatively low abundance in our study. This was expected because the digestive tracts of our samples were analysed in their entirety.

In contrast, the dominant core bacteria, which have previously been shown to represent > 94% of the gut bacteria in the mid- and hindgut (Martinson et al. 2012), were relatively abundant. Of these, *G. apicola (Kwong and Moran 2013)*, *S. alvi* (Zheng et al. 2017), *Lactobacillus* spp. (Olofsson and Vásquez 2008; Martinson et al. 2012), and *Bifidobacterium* (Olofsson and Vásquez 2008) are likely to efficiently metabolise sugars to extract energy. We observed no large effects of diet on the relative abundances of these dominant core bacteria, despite the variation of sugar type in the diets. Metagenomics analysis, as compared to 16S RNA sequencing, may have identified changes to the bacterial genes in response to the sugar source.

The Acetobacteraceae are a family of primary feeders that break down the di-, oligo- or poly-saccharides such as sucrose to form mono-saccharides that they then metabolise to form acetate and/or lactate (Kersters et al. 2006; Lee et al. 2015). Acetobacteraceae increase in sucrose-rich environments by establishing symbiotic relationships with insects that feed on sugar-rich diets. They have been observed to aid host nutrition (Crotti et al. 2010), increase larval tissue development in the *Anopheles* mosquito (Mitraka et al. 2013), and are associated with the defective immune genotype causing *Drosophila* gut disease (Ryu et al. 2008). Acetobacteraceae Alpha 2.2, recently described as *Parasaccharibacter apium,* is present in the crop of *A. mellifera* forager bees, as well as their food stores in the hive, and in the larval gut where they presumably metabolise sucrose to generate acetic acid (Corby-Harris et al. 2014b).

Rhizobiaceae are nitrogen-fixing bacteria that may have a pathogenic, symbiotic or saprophytic relationship with the host (Zhou et al. 2013; Geddes and Oresnik 2016). Rhizobiaceae, including the species *Ensifer adhaerens* identified in this trial, are

predominantly sustained on nitrogen-rich food sources normally because of a paucity of carbohydrates in their environment (Zhou et al. 2013). *E. adhaerens* is a soil bacterium (Rogel et al. 2001) that has not previously been identified in the gut of the honey bee. It is possible that *E. adhaerens* was consumed by the bees in this trial if the parent colony had foraged on flowers or water dusted with soil containing this bacterium. The lack of variation in relative abundance of *E. adhaerens* between the treatments suggests that either the bacterium was not affected by diet, or were dead within the gut. The fact that the soil bacterium *E. adhaerens* was present, supports current literature that bees collect bacteria as they forage (McFrederick et al. 2012).

L. kunkeei are acid-resistant, obligate fructophilic bacteria that produce lactic acid, acetic acid and ethanol (Neveling et al. 2012). They are the dominant lactic acid bacteria present in honey, bee-collected pollen, and bee bread. They are also present in royal jelly and the honey bee crop (Anderson et al. 2013; Corby-Harris et al. 2014b; Asama et al. 2015).

Acetobacteraceae is present in larvae and all nurse worker feeding tissue, suggesting bee larvae acquire bacteria from nurse bees (Corby-Harris et al. 2014b). During larval development, the bacteria undergo ecological succession (Vojvodic et al. 2013). For example, the gut of first larval instars of honey bees are dominated by Acetobacteraceae, whereas the fifth instar is dominated by *L. kunkeei* (Vojvodic et al. 2013). Inoculation with Acetobacteraceae by nurse bees may be an important trigger for this microbial succession. Our study suggests that the relative abundance of Acetobacteraceae is influenced by the sucrose content in the honey bee diet, and so we hypothesise that the worker diet may influence the abundance of Acetobacteraceae in honey bee larvae and this may influence larval and/or adult bee mortality.

During the first three days of larval growth in a colony, the larvae consume a carbohydrate-rich diet containing 18% sugar (sucrose and fructose). The sugar content then increases to 45% for the next two days of larval growth before the cells are capped (Rortais et al. 2005). Thus, bacteria with saccharolytic activity, especially invertase, dominate the gut of larvae that are exposed to sucrose-rich diets, and this may explain the increase of Acetobacteraceae and *L. kunkeei* in the gut of adult bees fed the sucrose-rich diets S, MGO, and DHA. Although some isolates of *P. apium* increase larval survival *in vitro* (Corby-Harris et al. 2014b), the effect of increasing saccharolytic activity through the feeding of sucrose-rich diets on bee larval development, the microbiome, and ultimately colony health, is unknown. The key metabolites generated by Acetobacteraceae, such as acetate, may have additional physiological effects in the host other than the recently recognised utilisation of organic acids, such as acetate, pyruvate, and succinate, by *S. alvi* which reduces oxygen in the ileum to generate a more anaerobic atmosphere (Bonilla-Rosso and Engel 2018). The link between the diet of nurse bees that feed larvae, and the associated effect that this may have on adult bee development was not studied in this trial but should be further researched.

The significant increase of Acetobacteraceae in the gut of adult bees after six days of consuming sucrose-rich diets may be directly related to their ability to break down the disaccharide. As the lifespan of a worker bee averages 15–38 days in summer and > 140 days in winter (Fukuda and Sekiguchi 1966; Graham 1992), it is likely that *A. mellifera* colonies may experience prolonged feeding regimes of sucrose during dearth periods, especially in winter. Prolonged feeding of sucrose may potentially cause a resurgence and transmigration of crop-associated residents further along the digestive tract, potentially resulting in changes to the dominant core bacterial composition within *A. mellifera*. While such a bacterial increase may not have any pathogenic implications, an overgrowth of such bacteria may potentially affect the colonisation of the entire microbial community. This overgrowth has been observed in mosquito guts, in which bacterial overgrowth accelerated death (Wei et al. 2017), and in mice, in which infecting agents and chemical triggers induced intestinal inflammation (Lupp et al. 2007). The possibility of bacterial overgrowth in honey bees, and any potential implications should be further investigated.

Once the carbohydrates are used, protein substrates obtained from the host, as well as bacterial metabolites and remnant cell debris, enable the growth of nitrogen-fixing bacteria such as Rhizobiaceae (Yu and Martin 2016). This may explain the increase in Rhizobiaceae observed when the bees in our study were fed sucrose-rich diets. Comparatively, the relative abundance of the dominant core bacteria are likely to remain stable as they are able to utilise other substrates such as nucleosides, flavonoid glycosides, and carboxylic acid (Bonilla-Rosso and Engel 2018) that collectively sustain both the host and bacteria.

Monosaccharides and water are rapidly absorbed across the midgut of honey bees. Glucose, the chief energy source for bees, is absorbed within five min of consumption, whereas sucrose and fructose must be converted to glucose by host enzymes before absorption can occur (Crailsheim 1988). Forager honey bees collect nectar in their crop where invertase (α-glucosidase), the enzyme required for sucrose breakdown, is added (Nicolson and Human 2008) from the hypopharyngeal glands (HG) (Terra and Ferreira 1994; Huang 2010). The HG are most active in nurse bees fed pollen aged 5–15 days as they secrete royal jelly to feed to larvae, which contains protein-rich components and sugar (Free 1961; Brodschneider and Crailsheim 2010). As the bees in our trial were raised in a colony from 1–10 days it is likely the HG were fully developed (Knecht and Kaatz 1990), and it is therefore possible that they were producing invertase, which may catalyse the breakdown of sucrose in the diet to fructose and glucose.

Several strains of *Bifidobacterium asteroides*, previously identified in the crop of forager bees (Olofsson and Vásquez 2008), were not detected in our data. *Bifidobacterium coryneforme*, also previously identified (Olofsson and Vásquez 2008), contributed 7–9% of the gut bacteria in all seven diet treatments, although no response to sucrose was observed. *Bifidobacterium* is infrequent in the crop, frequent in the hindgut, and

proliferates exclusively on pH neutral media (Anderson et al. 2013). This sensitivity to acidic conditions may be why *Bifidobacterium* is found in the hindgut (Anderson et al. 2013), rather than the midgut where acid metabolites are generated by sucrose metabolism (Peng et al. 1985), and thus unaffected by the sucrose treatments.

Although the dominant core bacteria do not require each other to colonise the bee gut, cross-feeding interactions do occur. These interactions may be important for community assembly and its resilience, as illustrated by the large amount of pyruvate produced by *G. apicola*, which is utilised by *S. alvi* (Kešnerová et al. 2017). Similar interactions may also occur among the less abundant members of the community as our results show that the relative abundance of both Acetobactereaceae and *L. kunkeei* increase in the presence of a sucrose-rich diet. Although it is unknown whether the increase of these bacteria was in response to each other, an interaction is likely to have occurred because Acetobacteraceae rapidly metabolise sucrose to generate lactate, glucose and fructose, of which the fructose fuels the growth of *L. kunkeei*, the latter producing both acetate and lactate (Neveling et al. 2012). Cross-feeding interactions may also occur between host and bacteria as the major metabolite of Acetobacteraceae is acetate. Acetate serves as an energy source for the growth of the bees, and it is utilised by the dominant core bacteria, such as *S. alvi*, to fuel respiratory activity (Zheng et al. 2017). In rodents, a build-up of acetate, produced by bacteria fed high calorie diets, decreased the pH of the microbial niche, and this in turn caused feedback inhibition of bacteria (Perry et al. 2016). At this stage it is unknown whether bacteria in the digestive tract of honey bees fed sucrose-rich diets for extended periods may be associated with this type of feedback loop.

The well-documented *in vitro* antibacterial effects of MGO and DHA (its precursor) were not demonstrated in this trial. As MGO is highly reactive, its half life is short in a biological environment (Kalapos 2008) and, therefore, at the time and site of analysis, local concentrations may have been significantly reduced by the time the bees consumed it (Kalapos 2008). Consequently, the MGO may have lost its activity by the time it reached the gastric phase of the digestive tract. MGO may also have denatured in the gut, or perhaps these gut bacteria are simply unaffected by MGO.

Sucrose appears to fuel the rapid proliferation of specific, low-abundance primary feeders such as Rhizobiaceae, as well as Acetobacteraceae and *L. kunkeei*. The major metabolites acetate and lactate that are likely to be produced by these bacteria may have important physiological functions, such as weight gain in honey bees (Lee et al. 2018). Given the distinct effects of the carbohydrates, a metagenomics-based study would have been useful to examine the alterations in the metabolic functionalities of the microbiome. We did consider functional profiling to infer metabolic capabilities. However, none of the computational approaches currently available (Nagpal et al. 2019) were compatible with the customised taxonomic assignation that we used in this study.

In conclusion, we have shown that diet does alter the bacterial composition within the digestive tract of caged adult honey bees. Sucrose-rich diets resulted in the increase of sub-dominant bacteria in the gut of honey bees that produce acetate and lactate metabolites and were associated with significant increases in Acetobacteraceae, Rhizobiaceae, and *L. kunkeei*, compared with those fed the sucrose-poor diets. Sucrose-rich diets were also associated with a significant decrease in *F. perrara*. Further studies are required to understand the long-term effects of these subtle but significant changes in bacterial composition within the honey bee gut that we observed in response to diet, including the effect of increased metabolites and their effect on larval development, dominant core bacterial composition, and ultimately colony health. The effect of supplementary feeding with sucrose, glucose and other carbohydrates on the metabolism of honey bees will be of great interest to the beekeeping industry which routinely practices supplementary carbohydrate feeding.

6.6 Supplementary material

Table S1. Mean total abundance of gut bacteria in NZ honey bees fed different carbohydrate diets for six days.

Treatment	Phylotype	Mean number of unique ASVs	Mean total abundance	SD	Min	Max
DHA	Acetobacteraceae	4	960	727	103	3100
H	Acetobacteraceae	4	738	593	158	2115
IS	Acetobacteraceae	3	861	920	26	2567
MG	Acetobacteraceae	4	1144	853	118	3122
MH15	Acetobacteraceae	4	839	787	107	2515
MH17	Acetobacteraceae	4	740	633	10	2504
S	Acetobacteraceae	5	1390	1059	118	4102
DHA	*Bifidobacterium coryneforme*	4	2262	1853	346	7431
H	*Bifidobacterium coryneforme*	4	2481	1981	312	6686
IS	*Bifidobacterium coryneforme*	3	2322	2047	88	6643
MG	*Bifidobacterium coryneforme*	4	1860	1460	218	5447
MH15	*Bifidobacterium coryneforme*	4	2559	2424	272	9021
MH17	*Bifidobacterium coryneforme*	3	2016	1420	319	5012
S	*Bifidobacterium coryneforme*	4	2498	2103	293	8574
DHA	*Ensifer adhaerens*	1	1938	624	613	2684
H	*Ensifer adhaerens*	1	1716	1478	587	4153
IS	*Ensifer adhaerens*	1	1121	615	386	2309
MG	*Ensifer adhaerens*	1	2470	1351	533	4939
MH15	*Ensifer adhaerens*	1	2091	1024	447	3318
MH17	*Ensifer adhaerens*	1	1799	826	284	2653
S	*Ensifer adhaerens*	1	2558	1045	997	4232
DHA	*Frischella perrara*	5	1251	1729	54	6186
H	*Frischella perrara*	5	1920	2571	50	10127
IS	*Frischella perrara*	4	1825	2601	57	10185
MG	*Frischella perrara*	5	1363	964	50	3458
MH15	*Frischella perrara*	5	1741	1962	7	7127
MH17	*Frischella perrara*	5	1197	1356	15	4024
S	*Frischella perrara*	5	1361	2141	54	9023
DHA	*Gilliamella apicola*	13	1005	1521	8	10546
H	*Gilliamella apicola*	13	1363	1815	20	8639
IS	*Gilliamella apicola*	13	1004	1556	10	11631
MG	*Gilliamella apicola*	13	903	1063	4	5055
MH15	*Gilliamella apicola*	13	1441	2023	30	11663
MH17	*Gilliamella apicola*	13	1323	1965	3	10084
S	*Gilliamella apicola*	13	1243	1853	15	13153
DHA	*Lactobacillus*	25	1880	2888	24	13923
H	*Lactobacillus*	25	2063	2839	9	15211
IS	*Lactobacillus*	25	2170	3224	5	22708
MG	*Lactobacillus*	25	2176	3248	16	19835
MH15	*Lactobacillus*	25	1928	2937	11	21486

Treatment	Phylotype	Mean number of unique ASVs	Mean total abundance	SD	Min	Max
MH17	*Lactobacillus*	25	1893	2844	29	15399
S	*Lactobacillus*	25	2017	2979	20	15304
DHA	*Lactobacillus kunkeei*	1	782	741	25	2078
H	*Lactobacillus kunkeei*	1	519	889	63	2107
IS	*Lactobacillus kunkeei*	1	334	586	13	1650
MG	*Lactobacillus kunkeei*	1	117	101	32	335
MH15	*Lactobacillus kunkeei*	1	93	88	11	245
MH17	*Lactobacillus kunkeei*	1	54	41	23	135
S	*Lactobacillus kunkeei*	1	1033	1050	30	2653
DHA	*Lactobacillus mellifer*	1	1625	628	1122	3033
H	*Lactobacillus mellifer*	1	1529	397	1040	2037
IS	*Lactobacillus mellifer*	1	1200	548	479	2005
MG	*Lactobacillus mellifer*	1	1718	426	1235	2276
MH15	*Lactobacillus mellifer*	1	1690	546	808	2486
MH17	*Lactobacillus mellifer*	1	1177	302	826	1751
S	*Lactobacillus mellifer*	1	1928	339	1567	2482
DHA	*Lactobacillus mellis*	8	1700	1809	10	6902
H	*Lactobacillus mellis*	8	2005	2215	25	10636
IS	*Lactobacillus mellis*	7	2094	2254	9	8127
MG	*Lactobacillus mellis*	8	1679	1892	11	7789
MH15	*Lactobacillus mellis*	6	1979	2282	12	9344
MH17	*Lactobacillus mellis*	7	1727	2212	13	9744
S	*Lactobacillus mellis*	8	2068	2126	3	8015
DHA	Rhizobiaceae	1	6698	5289	310	15273
H	Rhizobiaceae	1	1802	2210	321	5595
IS	Rhizobiaceae	1	1554	2931	55	8716
MG	Rhizobiaceae	1	5230	3827	587	10444
MH15	Rhizobiaceae	1	2454	2982	90	9242
MH17	Rhizobiaceae	1	1119	1444	98	4538
S	Rhizobiaceae	1	6296	6474	1494	18307
DHA	*Snodgrassella alvi*	11	770	1086	1	4773
H	*Snodgrassella alvi*	11	1557	2345	8	10789
IS	*Snodgrassella alvi*	10	1160	1513	4	5733
MG	*Snodgrassella alvi*	11	876	1118	1	6361
MH15	*Snodgrassella alvi*	11	1345	2129	4	10680
MH17	*Snodgrassella alvi*	11	926	1132	3	5629
S	*Snodgrassella alvi*	11	768	1228	2	5961

A treatment is shaded grey when the mean number of ASVs differs from the rest of the treatments.

Table S2. Analysis of Deviance tables and alpha-diversity tables to compare gut bacteria in NZ honey bees fed different carbohydrate diets for six days.

A. Analysis of Deviance Table (Poisson)

Diversity	Df	Deviance	Resid. Df	Resid. Dev
Null			52	0.34768
Treatment	6	0.018121	46	0.32956

Model: poisson, link: log.

B. Analysis of Deviance Table (Type II Wald chi square tests)

Diversity	LR Chisq	Df	Pr(> Chisq)
Treatment	24.214	6	0.0004769***
Phylotype	2338.043	10	< 2.2e-16 ***
Treatment:Phylotype	187.854	60	4.265e-15 ***

Response: log(per.abun). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. Treatments: DHA, H, IS, MG, MH15, MH17, S.

Max LSD	Min LSD	Ave LSD
1.02248	0.56003	0.60358

C. PERMANOVA

	Df	Sum of Squares	R2	F	Pr(> F)
Treatment	6	0.18778	0.24327	2.4647	0.001***
Residual	46	0.58411	0.75673		
Total	52	0.77189	1.0000		

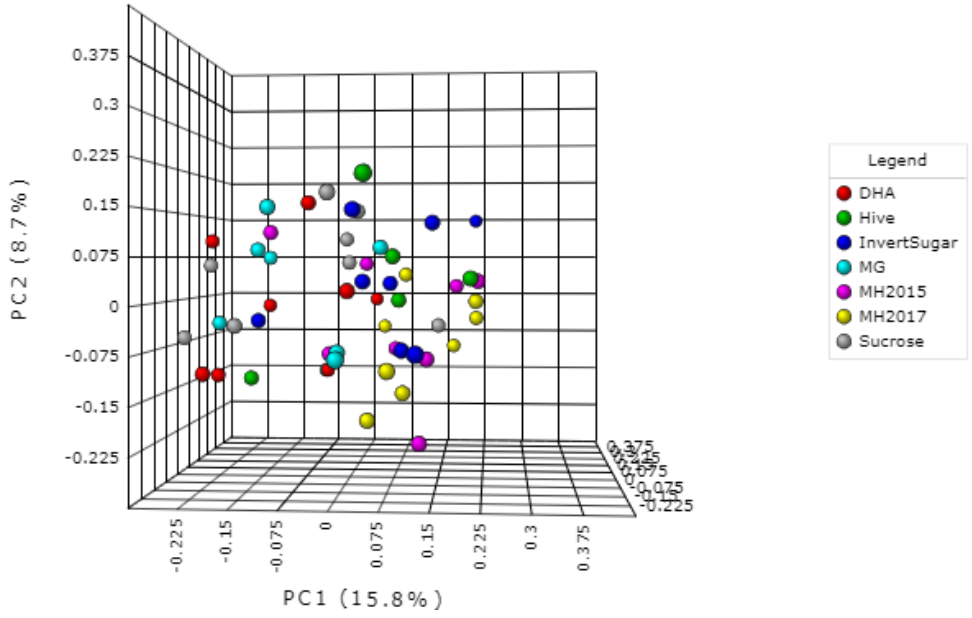
Permutation test for adonis under NA model, marginal effects of terms. Permutation: free, Number of permutations: 999: adonis2(formula = ASVs_dist ~ treatment, data = widerep, method = bray, by = "margin"). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

D. Alpha diversity

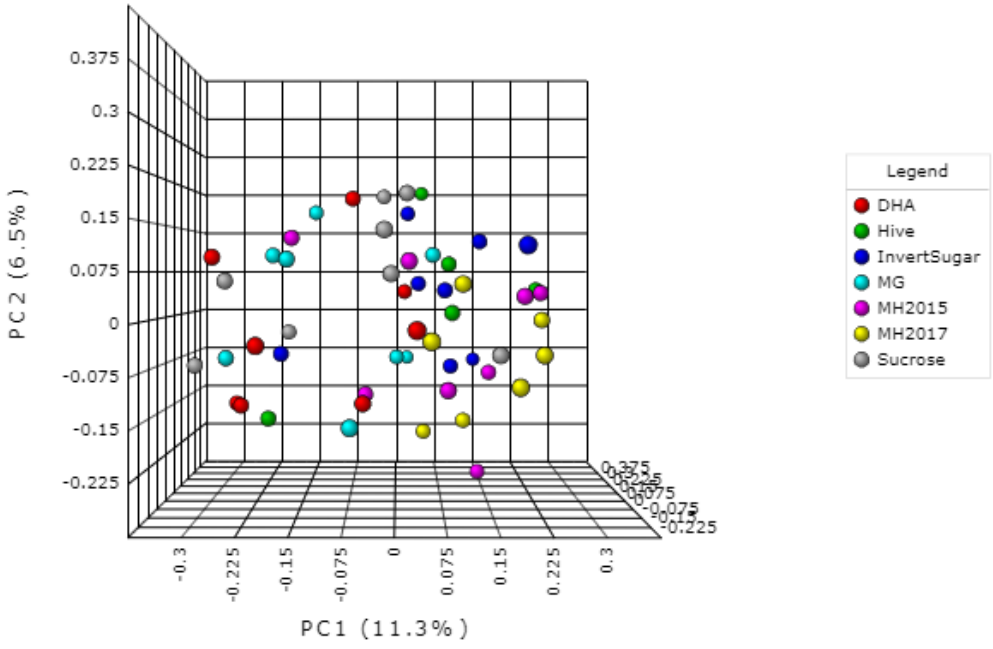
Diversity	P - value	Kruskal-Wallis statistic
Chao1	0.0959	10.675
Observed ASVs	0.0959	10.675
Shannon	0.2515	7.8125
Simpson	0.1261	9.9661

Beta diversity

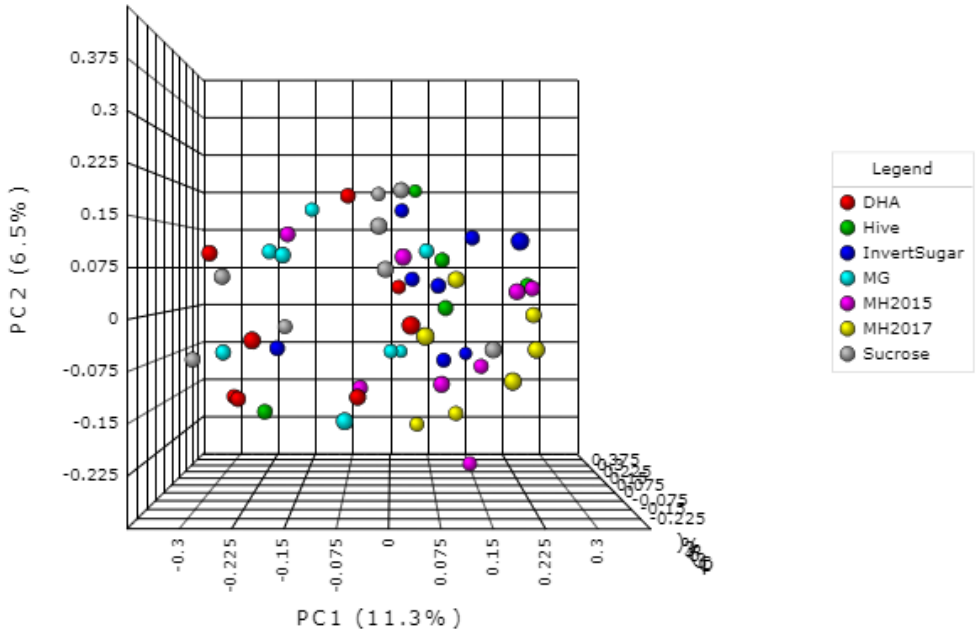
Figures S1 A–C below display the beta-diversity of ASVs within the gut of NZ honey bees sourced from a single hive that were fed different carbohydrate diets for six days. The relative abundance of ASVs were displayed as PCoA plots using different distance matrices.



A. Bray-Curtis PCoA 3-D visualisation of ASVs.



B. Jaccard PCoA 3-D visualisation.



C. Jensen-Shannon PCoA 3-D visualisation.

Figure S1 | Beta-diversity for gut bacteria in NZ honey bees fed different carbohydrate diets for six days, displayed as PCoA 3-D visualisation of ASVs.

A | Bray-Curtis, B | Jaccard, C | Jenson-Shannon.

6.6.1 Pilot trial to determine pollen progression through the digestive tract of worker honey bees

This study enabled me to confirm the minimum feeding duration required to inoculate honey bee workers with gut pathogens and gut bacteria (Chapter 7), or feed varying carbohydrate diets as conducted in Chapter 6. Although the inoculations and diets were presented to the bees in sucrose solution, the bees were also fed pollen which may have slowed the consumption of carbohydrates as the bees were full, or the pollen may have absorbed the sucrose so that it takes longer to digest/move through the gut. A pollen bolus reaches the honey bees anterior midgut 30 min post feeding (Peng et al. 1985) and then moves through the entire midgut over the next 1–3 h (Bailey 1952; Barker and Lehner 1972). As a slowest-progression scenario, this study determined the progression of pollen from the crop to the rectum, using caged honey bees fed pollen mixed with chromic oxide, a water soluble green inorganic compound that has previously been used to study the movement of feed through the digestive tract in weanling pigs (Bruininx et al. 2002).

Methods and materials

Approximately 30 bees in a metal cage (Figure 2.1) without an inverted feeder were group fed 50% sucrose solution (SS) (w/v sugar and water) mixed with apple pollen supplied in an upturned lid. The cage was incubated at 33°C and after 1 h 45 min, the digestive tracts from three bees were extracted. Without the chromic oxide indicator, the crops were yellow or light yellow, the midguts were light brown to dark brown, the hindguts were milky clear, and the rectums were 1 mm thick and clear. It was therefore unclear how far along the gut the pollen had migrated during this time period. To determine if chromic oxide could be used to indicate pollen movement through the gut of adult honey bees, 2 mg of chromic oxide mixed with 1 g pollen and 1 g of 50% SS was fed *ad libitum* to 30 adult bees using an open feeder (Figure 2.12) covered with a 1 mm x 1 mm mesh. The digestive tract of an individual bee was removed and photographed every 30 min for 5 h and then at 6, 24, 48, and 72 h post initial feed time (Figure 2.13).

Figure 6.6.1 | Pollen mixture laced with chromic oxide in open feeders without the mesh cover.

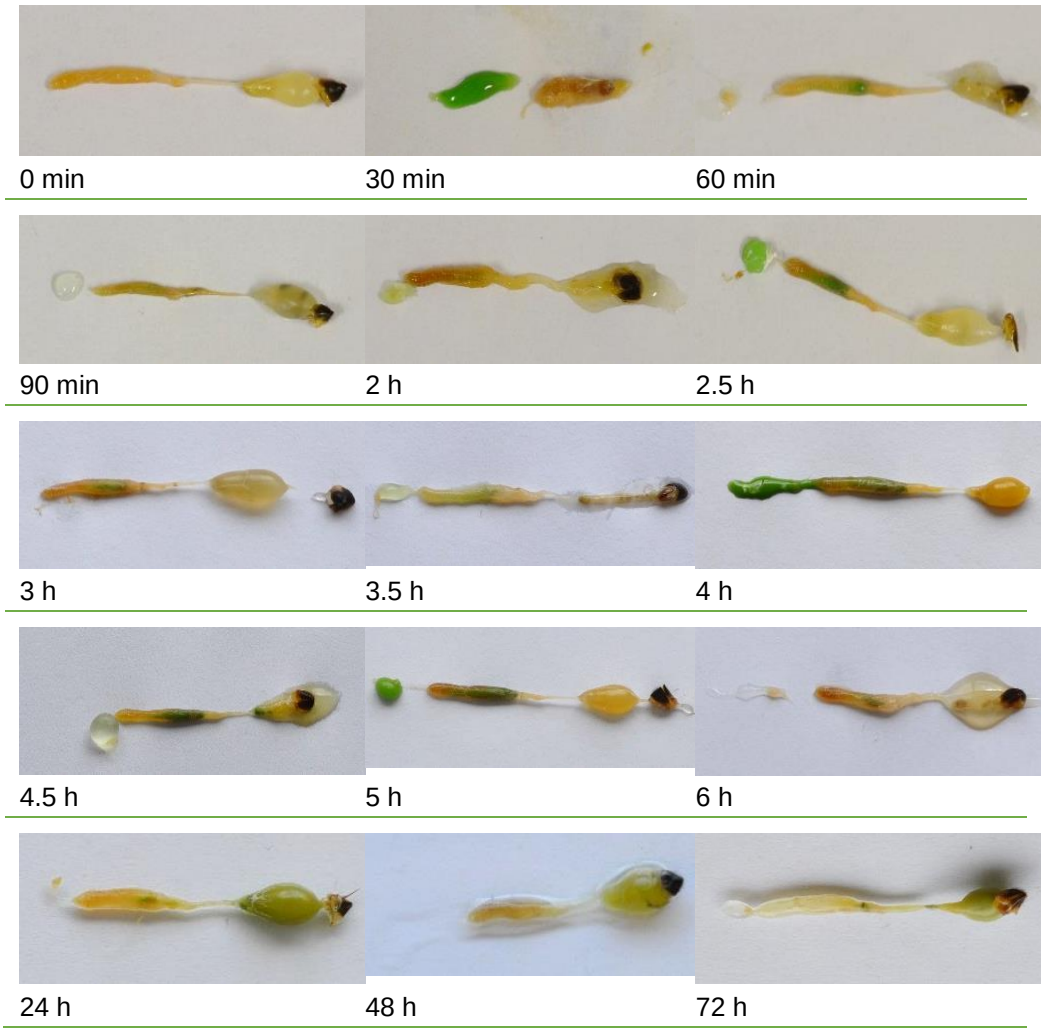


Figure 6.6.2 | The progression of pollen laced with chromic oxide (green) through the digestive tract of adult honey bees throughout 72 hours.

Conclusion

The bolus of pollen mixed with chromic oxide took > 5 h to move from the honey bee crop through to the rectum. The bolus had moved completely into the rectum by 24 h, although residues of chromic oxide still remained in the pylorus. This indicates that an inoculation of 24 h should ensure a treatment has entirely moved through the digestive tract. Therefore, the caged bees in chapter 7 were inoculated using open feeders covered with mesh for a duration of at least 24 h, and the bees in Chapter 6 were fed carbohydrate diets for > 24 h without pollen.

6.7 Statement of contribution

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Michelle Anne Taylor
Name/title of Primary Supervisor:	Professor Alastair Robertson
Name of Research Output and full reference:	
[illegible]	
In which Chapter is the Manuscript /Published work:	Chapter 6
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
I designed the trial, conducted it, extracted the DNA from the bee guts, collected and analysed the sequencing data using bioinformatics and R with assistance to write the code. I designed the manuscript layout, wrote it, submitted it for	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Michelle Anne Taylor Digitally signed by Michelle Anne Taylor Date: 2019.12.19 11:40:25 +13'00'
Date:	19/12/2019
Primary Supervisor's Signature:	Alastair W Robertson Digitally signed by Alastair W Robertson Date: 2019.12.19 11:58:57 +13'00'
Date:	19/12/2019

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019

Chapter 7

The effect of *Nosema apis* spores on *Gilliamella* apicola, the top layer of the ileum biofilm in honey bees

7.1 Abstract

The widespread honey bee pathogen *Nosema apis* is a microsporidian that is associated with winter colony loss and reduced honey yield. As it is not chemically controlled in numerous countries, it is likely to be present in most honey bees. *N. apis* infects the epithelial cells in the ventriculus of adult worker honey bees and these spores pass through the digestive tract and are spread to other bees via a faecal-oral route. The luminal epithelial cells of the ileum, which is downstream from the ventriculus, are lined by a biofilm that is composed of a base layer of *Snodgrassella alvi* that is overlaid by *Gilliamella apicola*. To determine whether *N. apis* infection alters the development of *G. apicola*, the top layer of the biofilm, axenic bees were inoculated with combinations of *G. apicola*, *N. apis,* and crushed honey bee gut. The *N. apis* DNA was quantified from the honey bee gut using qPCR and bacterial composition was determined using 16S rRNA gene sequencing. The results indicate that the presence of *N. apis* does not disrupt the relative abundance of *G. apicola* in inoculated bees, but the lack of normally abundant bacteria or their reduced abundance appears to correlate with an increased abundance of the Rhizobiaceae family and the genus *Serratia*. Additionally, NEWs fed the sterile-sucrose treatment did not appear to be truly devoid of bacteria because after 12 days their guts contained low levels of *G. apicola*, *S. alvi*, *L. apis*, *L. mellis*, *Lactobacillus* spp., *Bifidobacterium* spp., *Serratia* spp., *Acetobacter* spp., Rhizobiaceae, and Cyanobacteria.

7.2 Introduction

The honey bee gut bacterial community may shape the advancement of any one of its members. For example, signs that horizontal gene transfer may occur has been detected among the two commensals *Gilliamella apicola* and *Snodgrassella alvi*; thirty-six *S. alvi* genes had high identity with *G. apicola* genes, eight of which were Rearranged Hot Spot-domain proteins that may play a role in intercellular competition (Kwong et al. 2014). These two species occupy different metabolic niches but form a biofilm in the ileum of the honey bee midgut. *S. alvi*, an oxidiser of carboxylic acids, forms a layer on the luminal epithelial cells of the ileum. This is then overlaid by the saccharolytic fermenter, *G. apicola* (Martinson et al. 2012; Kwong et al. 2014), and interlaced with species within *Lactobacillus* Firm–5 (Anderson et al. 2016). Proliferation of *S. alvi* does not require other bacteria to thrive, suggesting sufficient nutrients can be obtained from the host diet or the host itself (Kwong et al. 2014). It is not known whether *G. apicola*, the most relatively abundant single phylotype in healthy NZ colonies (25–40% of the bacterial community; see Chapter 3), also proliferates in the absence of other resident bacteria, such as the basal layer of the biofilm, *S. alvi*.

The honey bee pathogens *Nosema apis* Zander (Zander 1909) and *N. ceranae* Fries (Fries et al. 1996), are widespread (Bradbear 1988) microsporidia that infect the

epithelial and regenerative cells in the honey bee ventriculus (Fries 1988a; Higes et al. 2019). The spores germinate within 30 min in the gut, penetrate epithelial cells, rapidly multiply to infect neighbouring cells and within 6–10 days generate spores that then burst out of the epithelial cells, pass through the digestive tract and are defecated. The primary source of *N. apis* infection is soiled comb within the hive (Bailey 1953), and the spores can survive in faeces for up to one year (Bailey 1962). *Nosema* disease, also known as nosemosis, is characterised by trembling worker bees, dilated abdomens, brown faecal deposits on the combs and front of hives (Bailey 1967), sick or dead bees outside the colony, and decreased brood production, predominantly in spring (Mattila and Otis 2006). Nosemosis is also associated with winter colony loss and reduced honey yield (Fries 1988b), reduced pollen collection by colonies (Anderson and Giacon 1992), and reduced life span of bees in colonies, despite provision of pollen sources (Mattila and Otis 2006). Although there is no documented evidence, it is suspected that the majority of NZ honey bee colonies contain *N. apis* spores, and this is supported by data from Chapter 3.

Internationally, *N. ceranae* appears to be replacing *N. apis* (Klee et al. 2007), and mixed infections appear more virulent than single species infection (Milbrath et al. 2015). In contrast the small analysis of the NZ sick colonies (Chapter 3) suggests that the recently introduced species *N. ceranae* (Murray and Lester 2015) is still less prevalent than *N. apis* in NZ. The presence of *N. apis* in NZ was first recorded in 1909 (Zander 1909). Fumagillin, a dicyclohexylammonium salt produced by the fungus *Aspergillus fumigatus*, was previously used to control *N. apis*. Detection of residues within honey (Lopez et al. 2008) has led to numerous countries banning the use of products containing Fumagillin. In NZ Fumagillin was used in queen raising colonies but as these colonies do not produce honey, the antibiotic residues of Fumagillin have not been indentified in the human food chain. However, in 2013 Fumidil B™ (active ingredient Fumagillin) was deregistered in NZ and authorisation under the Agricultural Compounds and Veterinary Medicines Act 1997 is now required to use it to control *Nosema* (New Zealand Government 1997). Therefore it is likely that *Nosema* infection is present in most NZ honey bee colonies. This lack of *Nosema* control both in NZ and internationally is likely detrimental to colony health; the epithelial lesions that form within the ventriculus make the bees susceptible to other pathogens, such as viruses (Higes et al. 2007). Honey and pollen yields are also likely to be compromised (Fries 1988b).

Neither species of *Nosema* creates lesions within the ileum or the rectum. However, their spores occur in both of these domains as they progress from the ventriculus, through the ileum, and the rectum (Higes et al. 2019). The effect of these spores on the biofilm (*S. alvi* overlaid by *G. apicola*) as they move through the ileum, in particular the top layer of *G. apicola*, has not been studied. Supplementation with probiotic species of *Lactobacillus* and *Bifidobacterium* is known to benefit bee health (Vásquez et al. 2012) and reduce the level of *N. ceranae* (Baffoni et al. 2016). However, the authors do

not elaborate on the effect of the supplementary bacteria on bee health or the interaction of these bacteria with the native bacterial phylotypes, with or without the presence *of N. ceranae*.

The faecal-oral transmission of *N. apis* in honey bees appears similar to the acquisition pathway of honey bee gut bacteria (Powell et al. 2014). This suggests that NEWs may be inoculated with both gut bacteria and *N. apis* within a similar timeframe. We hypothesise that the colonisation and increase in relative abundance of *G. apicola* is inhibited by *N. apis* infection and/or the lack of the resident bacteria, *S. alvi*. This chapter investigates within-host competition between *N. apis* and core gut bacteria in caged honey bees using a host-challenge trial where axenic bees were inoculated with combinations of (i) the gut slurry (as a source of bacteria that normally colonise the gut), (ii) a strain of *G. apicola* isolated from the guts of NZ honey bees to supplement core bacteria, (iii) *N. apis* isolated from the guts of NZ honey bees as the pathogen to infect the bees, and iv) sucrose solution as a control. The 16S rRNA gene was sequenced from the honey bee gut to characterise the relative abundance of total bacterial communities, in particular, core bacteria, and qPCR analysis enabled the exploration of the effect of *N. apis* and the bacterial community on the biofilm abundance.

7.3 Materials and methods

7.3.1 Preparation of bees and treatments

In May (autumn) 2017, a single frame of sealed brood containing worker bees within 24 hours (h) of emerging (approximately 20 days from egg deposit) was collected from each of four colonies (hives 56, 97, 304 and 355) located at PFR, Ruakura, Hamilton, NZ. Each side of the frame was exposed to UV light for 15 min to kill hive bacteria sensitive to UV. The wax cappings were then removed with a scalpel and the bees were lifted out (Figure 7.1) with forceps and sequentially placed into six cages. Over the next 24 h the bees were incubated at 34°C and ~65% relative humidity (RH) to simulate the temperature in the brood nest and account for the actual emergence date. The temperature was then reduced to 30°C for the following 24 h. During this time the bees continued to develop (Figure 7.2) and commenced walking, enabling them to consume *ad libitum* 50% sterile sucrose solution (SS) scented with lavender from an inverted glass feeder at the top of the cage, and irradiated apple pollen on the base. All SS supplied during the trial was scented with lavender to orient the bees to the feeders.

Figure 7.1 | Honey bees removed from their cells ~24 h prior to the bees naturally emerging.

Figure 7.2 | Honey bees in the final stages of development after being removed from their cells.

The bees in the six cages were anoxiated with CO_2 for 2 min bursts to render the bees unconscious for easy placement of 30 bees into each of 30 metal cages. Each cage had mesh on one side and glass on the other, an absorbent paper towel on the base, and a rubber band to hold the glass in place (Chapter 2: General Methods Figure 2.1). The feeding regime remained the same for an additional 24 h (3 days post removal from the cell). All dead bees and a layer of the paper towel were then removed and the live bees were food-deprived for 2 h. Each cage was then randomly allocated to one of five treatments for 2 h (Table 7.1): Sucrose, *G.apicola*, Gut slurry, *G. apicola* and *N. apis*, and *N. apis*.

Table 7.1 | Bacterial and pathogen treatments supplied to caged axenic honey bees.

Day	Treatment		No. cages	Bees per cage
3 5	Sucrose:	20% sucrose solution (2 h) 50% sucrose solution (24 h)	7	30
3 5	*G. apicola*:	20% sucrose solution + 1.76 x 10^8 *G. apicola* cells (2 h) 50% sucrose solution (24 h)	7	30
3 5	Gut slurry:	20% sucrose solution + honey bee gut slurry(2 h) 50% sucrose solution (24 h)	7	30
3 5	*G. apicola* & *N. apis*:	20% sucrose solution + 1.76 x 10^8 *G. apicola* cells (2 h) 50% sucrose solution + 20,000 *N. apis* spores (24 h)	7	30
3 5	*N. apis*:	20% sucrose solution (2 h) 50% sucrose solution + 20,000 *N. apis* spores (24 h)	7	30

On day 3 of the trial, 600 µl of each treatment was supplied in the base of the cage using a glass feeder (4 mm high with a 16.5mm diameter) covered with mesh (1 mm x 1 mm) and a weighted plastic sleeve (19.1 mm diameter and 10 mm high). The sleeve prevented the bees upturning the feeder (Clinch 1981). Bees in treatments S (sucrose) and N (*Nosema* in sucrose) were fed 20% SS (sucrose solution), bees in treatments G (*G. apicola* in sucrose) and GN (*G. apicola* and *Nosema* in sucrose) were fed a 20% SS containing a concentration of 1.76×10^8 *G. apicola* cells/ml, and bees in treatment GS (*G. apicola* and gut slurry in sucrose) were fed a 20% SS containing gut slurry (6 ml of gut suspension (10 ml sterile water and 10 bee guts (Cantwell 1970)) to 4 ml of 50% SS). After 2 h, the treatment feeders and a layer of the paper towel were removed and the bees were fed 50% SS through gravity feeders until day 5 when the second treatment was administered.

On day 5, the treatments were again supplied in glass feeders for 24 h. Adult workers require 4 mg of usable sugar during a 24 h period (Barker and Lehner 1974), meaning that 1 ml of 50% sucrose solution should adequately sustain 100 workers for 24 h. However, as consumption varies with treatment conditions, 5 ml is the recommended volume of carbohydrate for 100 workers (Williams et al. 2013). To ensure each bee in treatment GN and N consumed *N. apis* spores, all treatment cages were supplied with 1.2 ml of treatment (Table 7.1); treatment bees S, G and GS were supplied with 50% SS, and treatment bees N and NG were supplied with approximately 20,000 *N. apis* spores in 50% SS. The *N. apis* stock solution was quantified on day 5 using a haemocytometer (1/400 mm^2 x 1/10 mm deep, Improved Neubauer, USA) (Cantwell 1970), as detailed in the section below on *N. apis* verification. The 421,500,000 spores/ml stock solution was diluted with an appropriate volume of 50% SS to produce a count of 500,000 spores/ml.

Until day 12, all caged bees were fed straight 50% SS which was replaced every three days, and honey bee mortality was recorded every second day. *Nosema apis* infection is almost fully developed in 10- to 12-day-old honey bees exposed to spores on emergence from the cell (Forsgren and Fries 2010; Huang and Solter 2013). Hence, at day 12, the trial was concluded by removing the abdomens from five bees from each cage. The outside of each abdomen was rinsed in 95% ethanol for five seconds, and the entire gut (crop to rectum) of each bee was aseptically removed by pulling out the stinger and therefore the attached gut. As the bees were fresh, removal of the intact gut from the abdomen did not require dissection. Individual guts were placed immediately into 90 µl of DNA/RNA shield in a sterile ZR BashingBead™ Lysis tube, on ice and frozen at -20°C until further DNA extraction. Sample of the *G. apicola* inoculation (G20), the bee gut slurry inoculation (Beegut), and the *Nosema* inoculation (*Nosema* Inoc) were also stored for DNA extraction.

7.3.2 Gilliamella apicola isolation

To provide the source of *G. apicola* used in this trial, *G. apicola* colonies were isolated from the guts of honey bees from a single hive using basic culture methodology (Brewster 2003). Specifically, 10 bees from hive–32 (Ruakura, PFR) were frozen at -20°C for 30 min, then the guts were aseptically extracted into a 1.5 ml Eppendorf tube (for further details see Chapter 2.4: Dissecting the digestive tract from the honey bee abdomen). A homogenised suspension was made by passing the contents up and down using a sterile 18G blunt needle. A dilution series was established by diluting this suspension with distilled water to produce an expected dilution of ~10^4 bacteria per ml (Table 7.2).

Table 7.2 | Dilution series of honey bee gut suspension.

Tube ID	% Gut concentration	Bee gut volume (µl)	Distilled water (µl)
F1	100	100 of bee gut slurry	0
F2	10	100 of F1	900
F3	1	100 of F2	900
F4	0.1	100 of F3	900
F5	0.01	100 of F4	900

As per the recommended culture conditions for the isolation of *G. apicola* (Kwong and Moran 2013), a 100 µl aliquot of each concentration was spread on plates of Tryptic Soy Agar (TSA; a non-selective, rich media enabling the growth of numerous bacteria) containing 5% sheep blood (Fort Richard Laboratories, Auckland, NZ) (Figures 7.3 and 7.4), and placed in a CO_2 incubator at 5% CO_2 and 35°C. After 48 h the plates were checked for growth and 16 colonies were identified as potential *G. apicola*; the diameter was ~2.5 mm and the colonies were white and smooth (Engel et al. 2013a; Kwong and Moran 2013) (Figure 7.5). Half of each bacterial colony (A) was suspended in 100 µl of sterile Tryptic Soy Broth (TSB), spread on to TSA plates and grown in 5% CO_2, at 35°C for 48 h, or discarded if not verified as *G. apicola*. The other half (B) of the colonies were used to verify the bacteria growing on TSA. They were prepared for qPCR analysis by extracting the DNA. Each was centrifuged in 100 µl of TSB at 1310 rcf, 4°C for 5 min. The supernatant was discarded and each pellet was washed in 500 µl sterile distilled water (DW) by pipetting up and down. The tube was then spun at 1310 rcf, 4°C for 5 min. This wash process was repeated twice. The pellet was re-suspended in 50 µl DW and placed in boiling water for 5 min (*G. apicola* cells are Gram negative (Kwong and Moran 2013); the cell wall is a thin peptidoglycan layer meaning the DNA can be

extracted by boiling). It was then spun at 23369 rcf at 4°C for 60 s to break up the DNA. The supernatant was divided into two aliquots, one of which was frozen at -70°C to keep the stock concentrated and the other was diluted 1:1 with DW. Two µl of the concentrated *G. apicola* DNA was assessed using a Rotor-Gene® 6000 and primers specific to *G. apicola* (forward primer GTATCTAATAGGTGCATCAATT, reverse primer TCCTCTACAATACTCTAGTT (Ludvigsen et al. 2015) (Life Technologies NZ Limited, NZ) using the methodology described in Chapter 2: General Methods 2.7 Quantitative polymerase chain reaction); Activation 95°C for 5 min and 40 cycles of 95°C for 30 s, 60°C for 19 s, 72°C for 60 s. The half A colonies on TSA, verified as *G. apicola* by the half B qPCR, were then grown for 48 h and then the qPCR process to verify *G. apicola* was repeated for 10 colonies, using the DNA extraction discussed above, and a second qPCR amplification cycle was conducted; activation 95°C for 5 min, and 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s. Verified colonies were grown in 1 ml TSB for 2 h, then 10 x 100 µl aliquots were each placed in 7 ml TSB for 48 h. The suspensions were spun down (1310 rcf for 5 min) and then suspended in 3 ml of a glycerol freezing medium (30:70 solution of autoclaved (121°C for 15 min) pre-reduced glycerol plus medium (3 ml glycerol:7ml TSB previously autoclaved and incubated in 5% CO_2 at 35°C for 24–48 h)). The 30% glycerol stocks of the bacteria were then stored in 150 µl aliquots at -70°C. Additional verification of *N. Apis* DNA was conducted by 16S rRNA gene amplicon sequencing (Section 7.3.5).

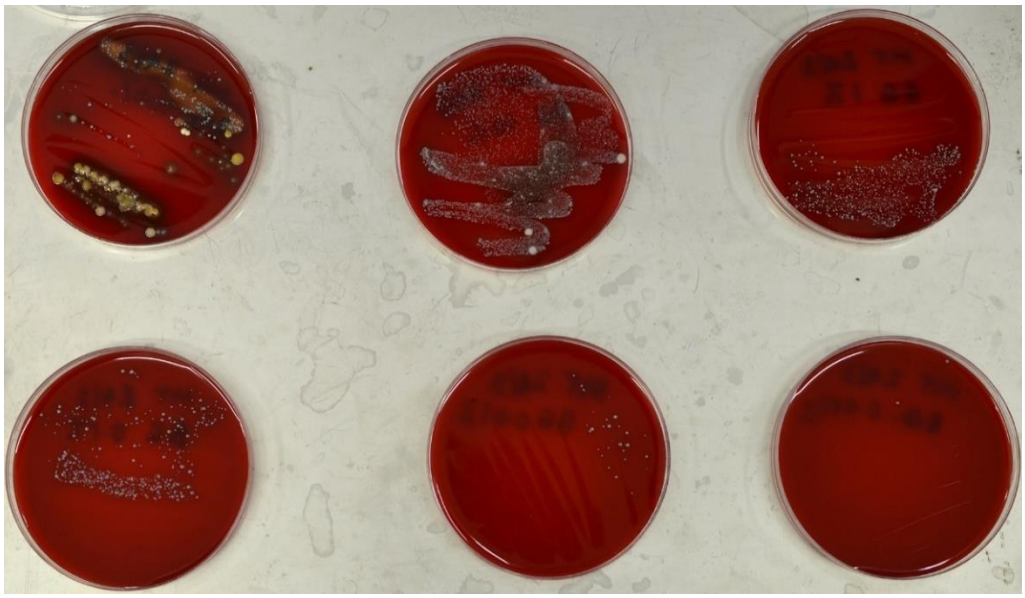


Figure 7.3 | Dilution series of honey bee gut contents spread on TSA plates containing 5% sheep blood.

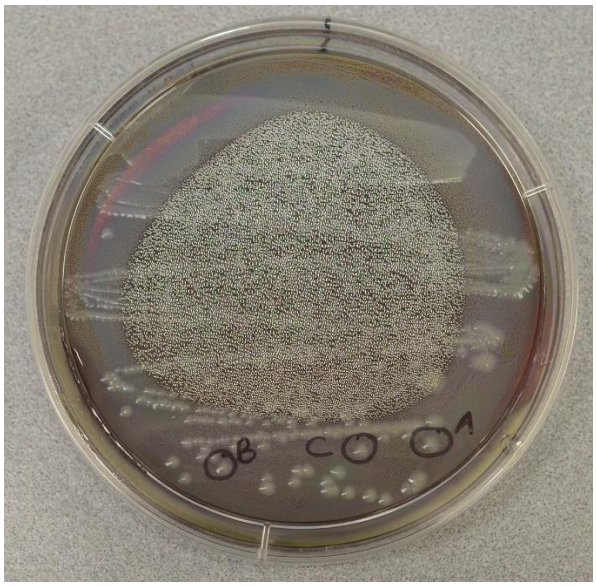


Figure 7.4 | Isolation of potential *Gilliamella apicola* colonies: 48 hours of growth on TSA plates containing 5% sheep blood.

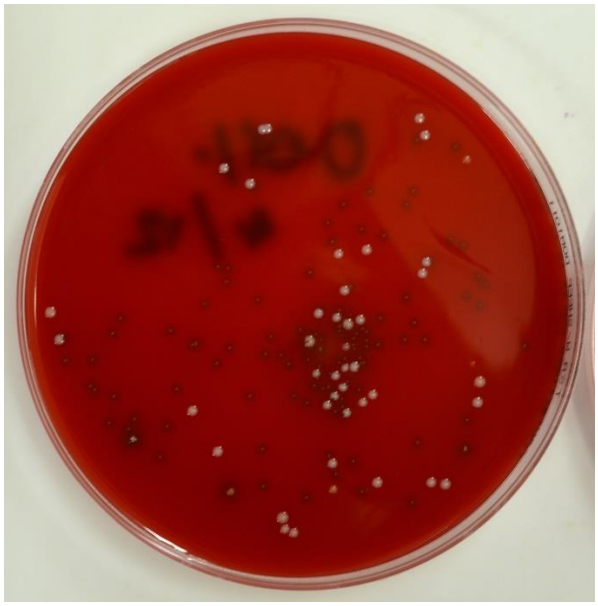


Figure 7.5 | *Gilliamella apicola* colonies isolated and re-plated for a further 48 hours on TSA plates containing 5% sheep blood.

7.3.3 *Gilliamella apicola* proliferation and viability

Two 50 µl aliquots of *G. apicola* previously isolated from honey bees and labelled as samples 1 and 9 were each placed in 10 ml of TSB overnight (14:00 to 09:00 hours). These were then centrifuged at 1310 rcf for 5 min to form fresh pellets that were washed in 1 ml distilled water, combined and centrifuged at 1310 rcf for 2 min. The supernatant was removed and the pellet was re-suspended in 100 ml of 20% sterilised sucrose solution (40ml of 50% sucrose + 60 ml deionised water). The number of spores/ml was determined using the Cantwell (1970) methodology established to count *N. apis* spores. A 10 µl aliquot of a 1:1 solution of Trypan Blue (Sigma-Aldrich, NZ) and the 20% sucrose solution containing *G. apicola* was loaded into a haemocytometer, and the number of spores in 80 of the smallest squares (five squares within the central square –RBC) were counted (80 x 0.00000625 ml). The average total number of spores was 176 (spore count shown in Table 7.3). This was divided by 80 squares, then multiplied by $4x10^7$ ml to determine the number of spores per ml. To account for the volume of Trypan blue used, the number of spores was multiplied by two (the dilution factor): number of spores/ml = (total number spores counted, divided by 80) x $4x10^7$ x 2 = 176 x 500,000 x 2 = 176,000,000 spores/ml.

Table 7.3 | Average spore concentrations identified in *Gilliamella apicola* and *Nosema apis* solutions.

Bacteria	Spores / 80 squares	Average concentration (Spores/ml)
G. apicola 2	17685	176,000,000
Nosema 1	506	253,000,000

7.3.4 Metabolic activity of *Gilliamella apicola*

The metabolic activity of *G. apicola* isolated from NZ honey bees after being frozen in glycerol stock at -70°C was assessed using WST-1. WST-1 is a tetrazolium-based cell proliferation reagent, which in the presence of metabolising bacteria, breaks down in approximately 20 min to 2 h causing a colour change from the red tetrazolium to a yellow formazan product. The colour change is dependent on the amount of bacteria that are in the sample being assessed (http://www.dojindo.com/store/p/141-Microbial-Viability-Assay-Kit-WST.html).

Sixteen bacterial stocks of isolated *G. apicola* previously stored at -70°C in 30% glycerol stocks were assessed using WST-1 (Reference number: 05 015 944 001, Roche Diagnostics, Auckland, NZ). One ml of TSB was added to 50 µl of each stock and

incubated at 37°C in 5% CO_2 for 2 h. A 100 µl aliquot of TSB was added to well A1 of a 96-well plate and then *G. apicola* stocks 1 to 11 were added to wells A2–A12. The samples in row A were repeated in rows B and C. Wells A1, B1, and C1 were the TSB controls with no bacterium. Rows D, E and F contained a 100 µl aliquot of the *G. apicola* stocks 12 to 16 (columns 1 to 5), and stocks 1, 7 and 9, which were either used to make up the *G. apicola* suspensions fed to the bees in this trial or previous pilot trials, were repeated using a 200 µl aliquot (columns 6 to 8) (Table 7.4). Ten µl of WST-1 was added to each cell containing 100 µl, and 20 µl was added to the 200 µl cells. The optical density (OD) of the samples was then determined at 2, 3, 4 and 18 h using a Gen 5™ version 2.01 Spectrophotometer (BioTek Instruments Inc., Vermont, USA) at 460nm. Between reads, the 96-well plate was covered and incubated at 37°C in 5% CO_2. Time points of 2–4 h are considered optimal (by the manufacturer) to discriminate the metabolic activity of viable cells. I included the 18 h time point to identify whether proliferation would still be occurring after this time (most likely contamination), and also to ensure that an obvious change had been observed. A photo log was scored to compare the colour change.

Table 7.4 | The WST-1 plate set up with 16 isolates of NZ *G. apicola*.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TSB	1	2	3	4	5	6	7	8	9	10	11
B	TSB	1	2	3	4	5	6	7	8	9	10	11
C	TSB	1	2	3	4	5	6	7	8	9	10	11
D	12	13	14	15	16	1	7	9				
E	12	13	14	15	16	1	7	9				
F	12	13	14	15	16	1	7	9				

Columns 1–12 and rows A–F partition the well-plate. The 100 µl samples of the *G. apicola* isolates 1–16 are numbered within each well. The three isolates 1, 7 and 9 used to make the *G. apicola* suspension were repeated with 200 µl samples per well (shaded in grey).

7.3.5 *Nosema apis* spore suspension and verification

The infective dose (ID_{50}) of *N. apis* for 50% of a honey bee population is 100 spores / bee and ID100 is 10,000 spores/bee (Fries 1988a). Reliable infection is therefore produced by 10,000 to 33,000 spores per bee but it is common to use doses that are an order of magnitude higher to ensure infection. However, the latter method can cause confusion when determining the final level of infection within the bee, as any non-germinating spores will included in the count (Fries et al. 2013). Each bee in this trial was inoculated with approximately 20,000 *Nosema* spores.

A *N. apis* spore suspension was obtained from 10 forager bees from each of three colonies (hives 32, 190 and 411) located at PFR, Ruakura Research Centre, Hamilton, NZ. The 10 bee abdomens were crushed in 10 ml of sterile deionised water, then mixed thoroughly with an additional 20 ml of water using a sterile 18G needle. DNA was extracted from two 150 µl aliquots of this suspension using a Zymo Research Fecal DNA Miniprep kit (Catalog No D6010, Zymo Research, Irvine, CA, USA) and qPCR was conducted on a Rotorgene 6000 to identify the *Nosema* species. *N. apis* was observed in all three samples but *N. ceranae* was observed in only one of the triplicate samples at a trace level (Supplementary material 2.10.6: Quantitation report for the standard curve production of *Nosema apis* and *Nosema ceranae*). This indicates that *N. apis* was prevalent in the suspension made from the Ruakura hives but *N*. *ceranae* was not. A standard curve was produced to quantify the number of *N. apis* spores present in each bee and to calibrate all further *N. apis* qPCR analysis.

The spore suspension was filtered through a 70 µm sterile nylon Falcon™ cell strainer (Thermo Fisher Scientific, Ref 352350) to remove bee guts and purified by centrifuging the spores at 5860 rcf for five min at room temperature. To produce a *Nosema* suspension with approximately 85% purity (Fries et al. 2013), the supernatant was removed and the spores were washed in 500 µl sterile water. The wash was repeated. After the final supernatant was removed the purified spores were re-suspended in 4 ml of sterile water by vortexing for 5 s. The suspension was frozen at -20°C for 48 h (until day 5 of the trial), then the number of spores were determined (176,000,000 spores/ml, Table 7.3) using the Cantwell (1970) haemocytometer methodology; Number of spores / ml = (total number spores counted / 80) x $4x10^7$ = 506 x 500,000 = 253,000,000 spores / ml. No bacteria was observed.

7.3.6 *Nosema apis* spore viability

N. apis spore viability should have been determined to validate the reliability and reproducibility within the axenic NEWs using the colouration method outlined by Fries et al. (2013). An oversight meant the viability of the spore suspension was not determined but their high viability was anticipated as frozen *N. apis* spores remain viable

for a few years (Bailey and Fernando 1972), and the bees were being inoculated with *N. apis* spores within 48 h of the *N. apis* suspension being produced. Viability of *N. apis* was confirmed as the treatments inoculated with *N. apis*, N and GN, had respectively 16,334-fold and 438,178-fold more *N. apis* spores at the conclusion of the trial than the sucrose control, also exceeding the initial inoculation containing 5.0 x 10^5 spores/ml.

7.3.7 DNA extraction from the honey bee guts

The DNA was extracted from the inoculation suspensions of *G. apicola* and the bee gut slurry in 20% sucrose, as well as two composite bee samples; a composite of 80 µl of lysed bee gut extract from each of the five bees in cage GN1, and a second composite sample from the five bees in cage GN2. The DNA and RNA was also separately extracted from five gut samples from each of the six replicate cages within each of the five treatments. Bees from cage G6 were excluded due to high bee mortality. The total number of samples was therefore 149.

Prior to DNA extraction, 104 ml of 95% ethanol was added to 24 ml of DNA/RNA Wash Buffer, and the lypophilised DNase I was reconstituted at 1 U/µl; 274 µl of DNase/RNase–Free Water was added to DNA Digestion Buffer and gently inverted. The extractions were conducted as per the recommended protocol for the ZymoBIOMICS DNA/RNA Mini Kit (R2002) (Zymo Research, Irvine, CA, USA) (Supplementary material 7.8.2).

The eluted DNA was transferred to 0.2 ml Sapphire qPCR 8-tube strips with optical flat caps (Reference 608281, Lot 16372) (Greiner bio-one, Germany), and stored as 2 x ~50 µl aliquots at -70°C. One was used for DNA analysis using a NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific, NZ) (Chapter 2: General Methods 2.6.2 NanoDrop™ analysis), and qPCR analysis. The other aliquot was sent on ice by overnight courier to the Massey Genome Service (MGS) (Massey University, Palmerston North, NZ) for 16S rRNA gene sequencing of the V3V4 hypervariable region (Kozich et al. 2013) . Further information regarding DNA purification and the library preparation conducted by the MGS is described in Chapter 2: General Methods 2.8: Next Generation Sequencing.

7.3.8 qPCR analysis of *Nosema apis*

To identify the abundance of *N. apis* in the samples, qPCR was conducted using a Rotor Gene 6000 (QIAGEN, Venlo, The Netherlands). Hydrolysis probe assays (TaqMan®) supplied by dnature (Gisborne, NZ) were used to amplify the DNA products specific to *N. apis*. A 2x Mastermix (dnature, Gisborne, NZ) was briefly vortexed and pulse centrifuged. 5 µl of the 2x Mastermix was then combined with 2.5 µl water and 0.5 µl 20x primer probe mix. 8 µl was added to the base of each well and 2 µl of DNA

template was added to the middle left hand-side of the well. The qPCR was run using the protocol recommended by dnature (Gisborne, NZ), and an assay cut-off of < 37 cycles was deemed positive (for further detail see Chapter 2: General Methods section 2.7.5 TaqMan). Each DNA sample, no-template control, and positive *N. apis* control (DNA extracted from the previously identified *N. apis* spore suspension) had three technical replicates.

7.3.9 Gene sequence processing and characterisation of microbial communities

The 16S rRNA sequence data were analysed individually using the QIIME2 analysis suite, version 2018.2 (Bolyen et al. 2019) (Chapter 2: General Methods section 2.8.2 Gene sequence processing and characterisation of microbial communities). The dataset was rarefied to its minimum library size of 12,909 to reduce estimation errors that result from the different number of sequences per sample. The customised 16S rRNA BLAST database outlined in Chapter 2: General methods section 2.8.3) was used to produce a BIOM that contained the ASVs identified from the sequencing of each sample and the associated taxonomic classifications. ASVs that were unable to be assigned taxonomically to species, were assigned to the higher taxonomic level (if not species then genus, if not genus then family etc.).

7.3.10 Statistical analyses

The qPCR data produced for each of the three technical replicates were adjusted to account for dilutions, averaged and the mean concentration of universal bacteria from the bee gut samples were normalised using Log_{10} values. A single-factor analysis of variance, Tukey's 95% confidence interval test and Student's t-tests were conducted.

Phylogenetic diversity was measured within a sample (α–diversity), and among samples (β-diversity) using the web-based tool MicrobiomeAnalyst (Kuczynski et al. 2012; Dhariwal et al. 2017). The filters in MicrobiomeAnalyst were set so the data counts were filtered to a minimum of two and a 10% prevalence in the samples. Variance was filtered using a 10% coefficient of variation. This dataset was rarefied to its minimum library size of 12,909, scaled using total sum, but was not transformed.

Alpha-diversity was calculated at the feature level using Kruskal-Wallis pairwise comparisons of four diversity measures: Observed ASVs, Chao1, Shannon, and Simpson.

Beta-diversity for the taxonomic level of feature was calculated using the distance methods Bray-Curtis dissimilarity (abundance of each ASV) and Jaccard Index (fraction of unique features despite abundance). The differences between the samples were

compared using a permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) and 3-D plots of PCoA were used to present β-diversity.

Further data analysis was conducted in R (version 3.5.1) (R Core Team 2018). For all analyses, sequences with a minimum total read composition of <0.01% prevalence were filtered from the dataset. This low threshold ensured the inclusion of the majority of less abundant bacteria in the analysis.

To investigate the difference in the number of phylotypes between treatments, a Poisson generalised linear model was used with the number of phylotypes as the response and treatment as a fixed effect. To explore the relationship between phylotypes and treatment, the data were visually explored using heat maps, where the response was the sum of the total number of bacterial reads for each phylotype or the mean read composition per replicate. The interaction of the relative abundance (proportion of total bacterial abundance) of phylotypes was explored and a simple linear regression model (lm) was used in the R package lme4 (Bates et al. 2014). Assumptions were checked via standard residual plots and a logarithmic transformation was applied. The package 'predictmeans' v 1.0.1 (Luo et al. 2018) was used to obtain predicted means, the standard errors (SE) and differences (SED) between the means, and to perform pairwise comparisons and permutation tests of the phylotypes in the treatment. The means with a bar of least significant difference (LSD) were plotted for the lm parametric models.

7.4 Results

7.4.1 Identification of the primary bacterium in the *Gilliamella apicola* suspension

The qPCR standard curve for *G. apicola* DNA isolated from NZ honey bees was identified using the *G. apicola*-specific primers. This curve was comparable to that produced by the ATCC wkB1 strain of *G. apicola* where the majority of the DNA product fluoresced at the temperature peak of 85.5°C and a slight shoulder was also observed at 79–80°C (Chapter 2.7.4.2). Coupled with the identification through 16S rRNA amplicon sequencing that *G. apicola* made up 95% of the bacteria in the G and GN inoculums, there is reasonable evidence that the *G. apicola* suspension used to inoculate the bees did in fact contain *G. apicola*.

7.4.2 Metabolic activity in *Gilliamella apicola* aliquots

The metabolic activity of the *G. apicola* isolates were determined using optical density (OD) of the samples (higher the OD the darker the blue) coupled with the colour change observed in the photo log of the samples in the plate wells (red to yellow). Both indicated that all 16 isolates contained bacteria that were able to metabolise WST-1 over the first four hours (Supplementary material 7.8.3.1): The wells A1, B1, and C1 containing the WST-1 controls had low OD readings (< 0.1) for the first 4 h, as supported by the photo log. During the time taken to apply the WST-1 and take the 0 h photo, a colour change was obvious in isolates 8–16, and the three 200 µl samples (D6–8, E6–8, F6–8), thus suggesting the *G. apicola* isolates were metabolising. At the 18 h time point, the controls contained bacteria that were metabolising. It is likely that the bacteria at this time were contaminants and this may have occurred because the plate was not able to be sealed when the OD measurements were determined.

The OD for the three replicates for each isolate was averaged before plotting (Table 7.5). All other samples had a single data point for each time point. The graph indicates that sample 11 had the highest OD throughout the 18 h (Figure 7.6), where OVRFLW indicates an OD > 4. Once this outlier was removed, the OD of all samples increased over the first 4 h, except for the TSB control, indicating *G. apicola* was metabolically active in the wells (Figure 7.7).

Table 7.5 | Average optical density data for *Gilliamella apicola* at each time point.

Sample	0 hours	2 hours	3 hours	4 hours	18 hours
TSB	0.04	0.06	0.06	0.07	0.06
Iso 4	0.29	0.49	0.57	0.71	4
Iso 5	0.21	0.32	0.35	0.39	1.88
Iso 6	0.22	0.34	0.36	0.41	4
Iso 7	0.12	0.26	0.29	0.33	1.03
Iso 8	0.11	0.25	0.28	0.34	0.81
Iso 9	0.12	0.26	0.30	0.34	1.35
Iso 10	0.15	0.31	0.37	0.42	1.06
Iso 11	0.32	1.87	2.50	3.38	4
Iso 12	0.16	0.38	0.46	0.54	4
Iso 13	0.18	0.41	0.47	0.66	2.17
Iso 14	0.17	0.30	0.36	0.46	2.84
Iso 15	0.13	0.18	0.19	0.22	0.53
Iso 16	0.24	0.38	0.44	0.53	2.79
Iso 1: 200 µl	0.19	0.27	0.31	0.36	0.86
Iso 7: 200 µl	0.18	0.26	0.30	0.34	2.09
Iso 9: 200 µl	0.12	0.22	0.25	0.29	0.81

Bacterial isolate is specified using (Iso).

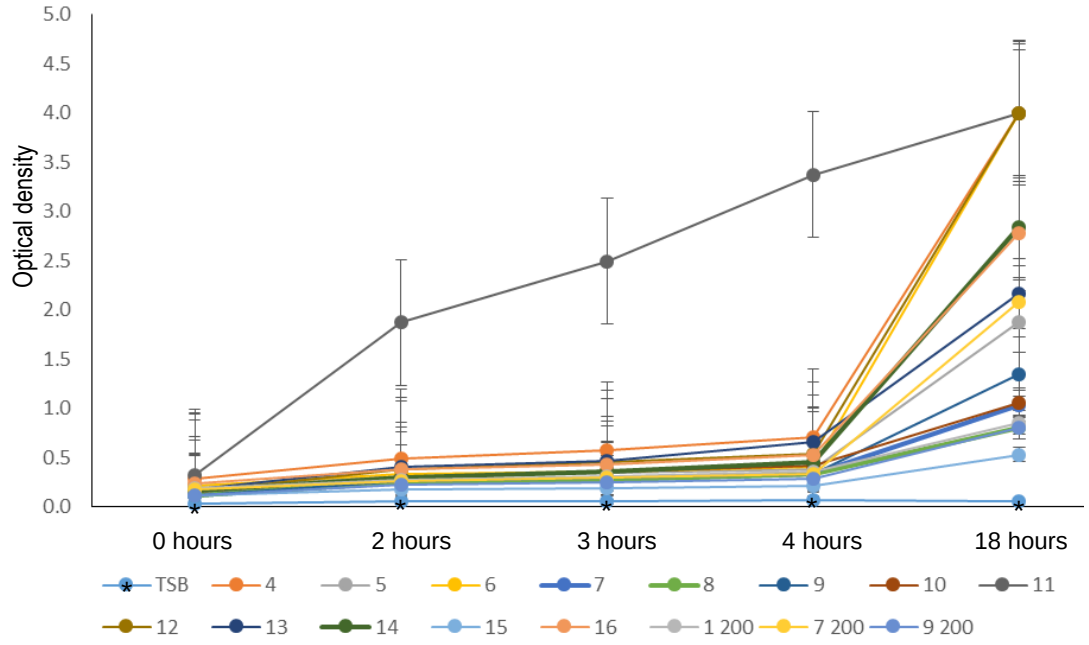


Figure 7.6 | Optical density thoughout an 18 hour period for *Gilliamella apicola* isolated from the honey bee gut.

Optical density was identified using WST-1. Bacterial isolates are identified by number. If 200 µl of an isolate was assessed, the isolate number is followed by 200. The error bars represent the standard deviation of the mean.

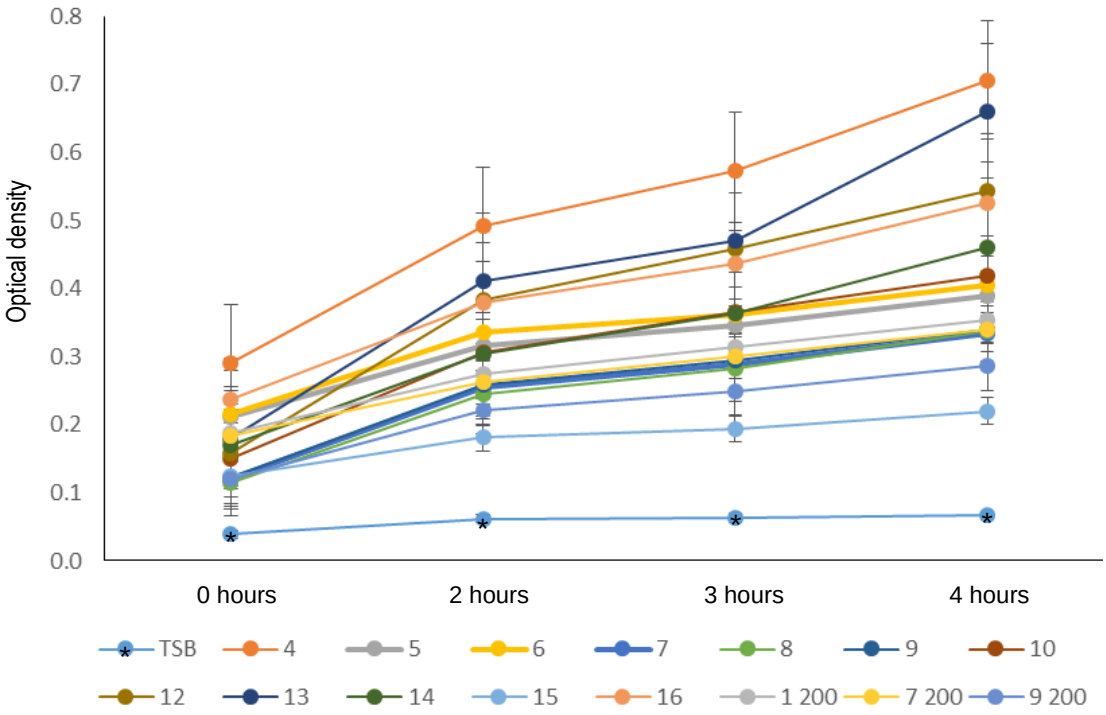


Figure 7.7 Optical density thoughout a four hour period for *Gilliamella apicola* isolated from the honey bee gut.

Optical density was identified using WST-1. The outlier, sample 11, was excluded. Bacterial isolates are identified by number. If 200 µl of an isolate was assessed, the isolate number is followed by 200. The error bars represent the standard deviation of the mean.

7.4.3 Honey bee mortality

Honey bee mortality was between 7 and 20% in the first 72 h for all five treatments and between 10 and 33% at 106 h, except for the *G. apicola* treated bees which had an average mortality of 45% (Figure 7.8). Average bee mortality after 216 h, for all treatments was between 32 and 61%. Enough bees were alive to conduct the qPCR and 16S rRNA sequencing for all cages except cage G6; < 5 bees survived in cage G6 so it was excluded from qPCR analysis and 16S rRNA amplicon sequencing.

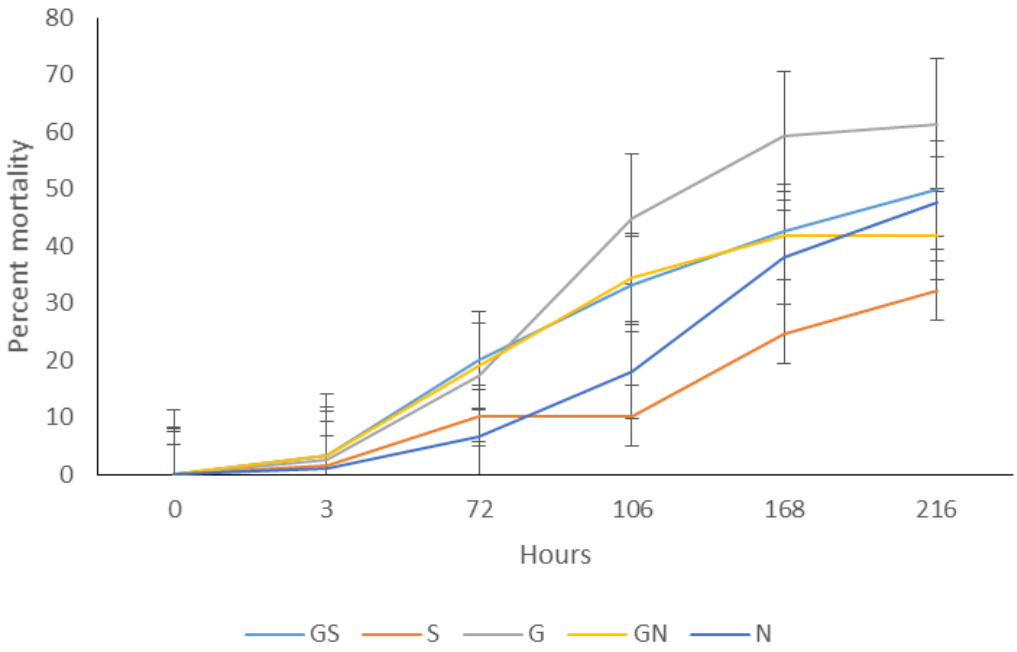


Figure 7.8 | Mortality of caged bees treated with combinations of *Gilliamella apicola* and/or *Nosema apis*. Treatments include combinations of *Gilliamella apicola* (G), sucrose (S) and *Nosema apis* (N).

7.4.4 qPCR analysis of *Nosema apis*

The mean number of *N. apis* spores/ml in the NTC samples was 5.49 x 10^4 (Table 7.6). The number of *N. apis* spores/ml in the *G. apicola* inoculation (G20), the bee gut inoculation (Bee gut), and the *Nosema* inoculation were 4.98 x 10^4, 2.07 x 10^8, and 5.0 x 10^5, respectively. This confirms *N. apis* was relatively absent in the *G. apicola* inoculation but present in the gut slurry and *Nosema* inoculations. This was expected because *Nosema* infection in NZ bees is not chemically controlled, hence it was likely to be present in the bees from which the gut slurry was produced.

Table 7.6 | Mean number of *Nosema apis* spores/ml from the guts of five honey bees inoculated with combinations of *Gilliamella apicola*, *Nosema apis* and gut slurry.

Treatment	Mean spores/ml	Standard deviation
S	4.61×10^5	1.26×10^5
GS	9.7×10^{10}	2.59×10^{10}
G	3.86×10^8	7.01×10^5
GN	2.02×10^{11}	1.03×10^{11}
N	7.53×10^9	1.59×10^{10}
NTC	5.49×10^4	7.15×10^3
G20	4.98×10^4	
Bee gut	2.07×10^8	
Nosema Inoc	5.0×10^5	

The five treatments were sucrose solution (S), *G. apicola* (G), gut slurry (GS), *G. apicola* plus *N. apis* (GN), and *N. apis* (N). No template control (NTC). *G. apicola* inoculation (G20). Bee gut slurry inoculation (Beegut). Nosema inoculation (Nosema Inoc).

The median number of *N. apis* spores from the axenic bees inoculated with just *N. apis* suspension (7.53×10^9 spores/ml) was 16,330–fold more than axenic bees fed just sucrose (4.61×10^5 spores/ml). In contrast, the bees inoculated with *N. apis* contained 13-fold fewer spores than bees inoculated with honey bee gut slurry (9.7×10^{10} spores/ml) (Table 7.6).

The difference in the number of *N. apis* spores/ml among treatment groups was significant ($P < 0.05$) (Table 7.7); graphed on a log scale the GN treatment had the highest mean (Figure 7.9). However, the large variation among the samples within the treatments indicates the GN group was not significantly different from the N or G treatments ($P > 0.05$) (Table 7.7). In contrast the sucrose control had significantly fewer *N. apis* spores/ml than all other treatments ($P < 0.05$). This indicates that the inoculation of *N. apis* spores and the gut bacteria in the slurry were successful. The GS treatment had significantly more ($P < 0.05$) *N. apis* spores/ml than the N treatment.

Table 7.7 | The differences in the number of *N. apis* spores/ml among the guts of honey bees inoculated with combinations of *Gilliamella apicola*, *Nosema apis* and gut slurry.

Treatment comparison	Sum of squares	df	MS	F statistic	*P* value
Between all 5 treatments	7.7×10^{23}	4	1.93×10^{23}	2.76	0.030
N vs GS	1.22 x 1023	1	1.22×10^{23}	11.97	0.001
N vs S	7.94 x 1020	1	7.94×10^{20}	6.48	0.014
S vs GS	1.42 x 1023	1	1.42×10^{23}	14.15	0.004
N vs GN	4.58 x 1023	1	4.58×10^{23}	2.89	0.094
G vs GN	4.52 x 1023	1	4.52×10^{23}	2.61	0.112

ANOVA was used to calculate the differences among groups. Degrees of freedom (df), mean square (MS).

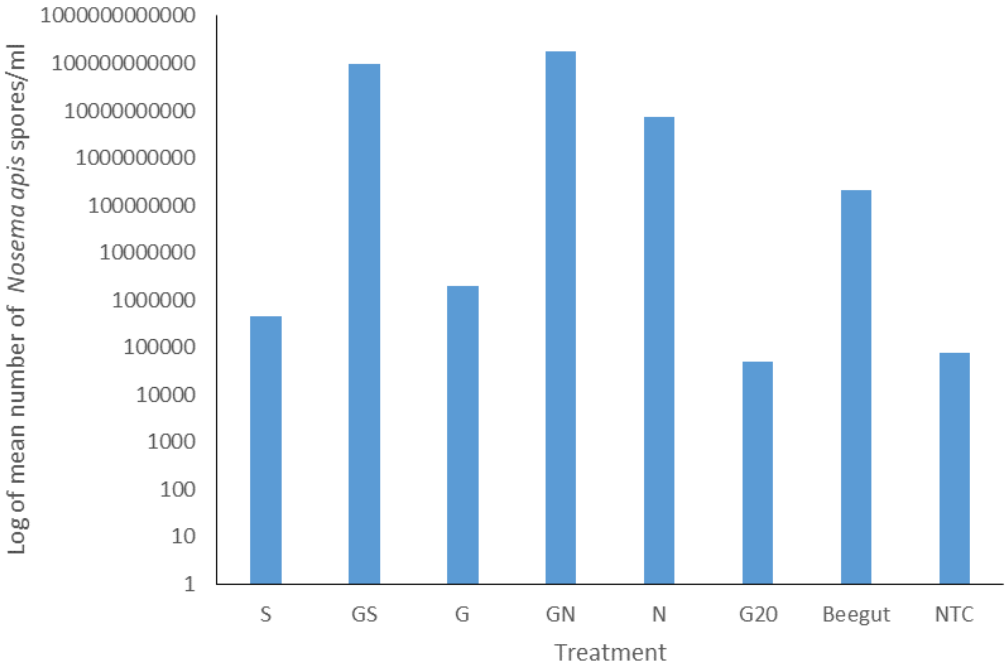


Figure 7.9 | Log of mean number of *Nosema apis* spores in the guts of 12 day old honey bees inoculated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

The five treatments: sucrose solution (S), *G. apicola* (G), gut slurry (GS), *G. apicola* plus *N. apis* (GN), and *N. apis* (N). Honey bee gut slurry inoculation (Beegut). *G. apicola* inoculation (G20).

Twelve days after treatment, *N. apis* DNA from five individual bees in cage GN1 ranged from 8.84×10^4 to 8.3×10^9 with a mean of 2.15×10^9. *N. apis* DNA from bees in cage GN2 ranged from 1.58×10^6 to 2.9×10^{10} with a mean of 1.56×10^{10} (Table 7.8). The composite samples that contained 80 µl of lysed gut extract from each of five bees, had mean *N. apis* concentrations of 1.02×10^9 for GN1 and 1.25×10^{10} for GN2. These are both within the range observed in the individual bees (Table 7.8). The GN1 mean was 2-fold higher than the Comp 1 – GN1 mean, whereas the GN2 mean was similar to that of the Comp 2 – GN2 mean.

Table 7.8 | Mean number of *Nosema apis* spores in the guts of five individual honey bees inoculated with *Gilliamella apicola* and *Nosema apis.* These guts were also pooled into composite samples.

Cage	Bee sample	Mean number of *Nosema apis* spores per ml	Mean of bee replicates
GN1	1	8.84×10^4	
GN1	2	8.30×10^9	
GN1	3	2.47×10^9	
GN1	4	1.18×10^6	
GN1	5	1.47×10^5	2.15×10^9
GN2	1	6.45×10^9	
GN2	2	1.58×10^6	
GN2	3	2.11×10^{10}	
GN2	4	2.11×10^{10}	
GN2	5	2.91×10^{10}	1.562×10^{10}
Comp 1 – GN1	1–5	1.02×10^9	
Comp 2 – GN2	1–5	1.25×10^{10}	

Composite sample of DNA from fives bees from cage GN1 (Comp1). Composite sample of DNA from fives bees from cage GN2 (Comp2).

7.4.5 Number of amplicon sequence variants and phylotypes

The bacterial DNA in the honey bee samples contained 6,197,573 paired reads. The reads were filtered to remove reads with < 0.01% prevalence. Hence, the total number of paired reads analysed in the 149 samples was 5,902,268. This represented 68 ASVs which were classified as 26 unique phylotypes; fifteen phylotypes (47 ASVs) were present in the honey bee gut slurry inoculation, five phylotypes (six ASVs) were present in the *G. apicola* inoculation, and 11 phylotypes (27 ASVs) were present in the composite samples (Table 7.9). The Poisson generalised linear model suggest these phylotype differences among the five treatments were significant ($P < 2.2 \times 10^{-16}$, Chi squared = 192.1, df = 28). The axenic bees that were inoculated with gut slurry had consistently more phylotypes (14–20), whereas the number of phylotypes in axenic bees fed sucrose had a wider spread (8–22). The axenic bees inoculated with *G. apicola* had the least number of phylotypes (5–11) (Table 7.9).

Table 7.9 | The number of unique ASVs and the number of associated phylotypes within the digestive tract of individual NZ honey bees inoculated with combinations of *G. apicola*, *N. apis* and gut slurry.

Sample	Number of replicates	ASVs	Phylotypes	Range in number of phylotypes
S1	5	38	21	8–19
S2	5	53	22	12–22
S3	5	56	24	15–22
S4	5	35	21	8–20
S5	5	27	18	8–17
S6	5	54	23	13–22
G1	5	12	8	5–7
G2	5	19	13	9–11
G3	5	14	11	5–9
G4	5	12	11	5–11
G5	5	11	9	5–9
GS1	5	53	21	16–20
GS2	5	51	20	15–19
GS3	5	54	20	16–18
GS4	5	50	19	14–18
GS5	5	47	17	15–17
GS6	5	52	20	16–18
GN1	5	23	11	6–9
GN2	5	27	13	5–12
GN3	5	27	16	9–13
GN4	5	28	16	7–12
GN5	5	25	13	7–10
GN6	5	23	13	8–12
N1	5	26	17	5–12
N2	5	27	19	10–17
N3	5	28	17	8–12
N4	5	27	15	9–11
N5	5	28	16	9–16
N6	5	26	14	7–10
Beegut	1	47	15	
G20	1	6	5	
Comp	2	27	11	

The five treatments were sucrose solution (S), *G. apicola* (G), gut slurry (GS), *G. apicola* plus *N. apis* (GN), and *N. apis* (N). Composite of the lysed bee guts from five bees in the GN cages (Comp). Honey bee gut slurry inoculation (Beegut). *G. apicola* inoculation (G20).

The heatmap of the sum of the total number of reads for each phylotype shows the dominant core phylotypes: *G. apicola*, *S. alvi*, *Bifidobacterium* spp., and species within the genus *Lactobacillus* were present in all sucrose and gut slurry treated bees (Figure 7.10). *G. apicola* was present in bees from all five treatments, including the sucrose and *N. apis* inoculations where the bees did not receive *G. apicola*. *S. alvi* and *Bifidobacterium* spp. were absent from more than half of the *G. apicola,* and *G. apicola* plus *N. apis* treatment cages, and some of the *N. apis* treatment cages. The highest number of reads were of the family Rhizobiaceae in the *G. apicola* and *G. apicola* plus *N. apis* treatments. The genus *Serratia* in the *G. apicola* treatment also had a high number of reads relative to the rest of the phylotypes.

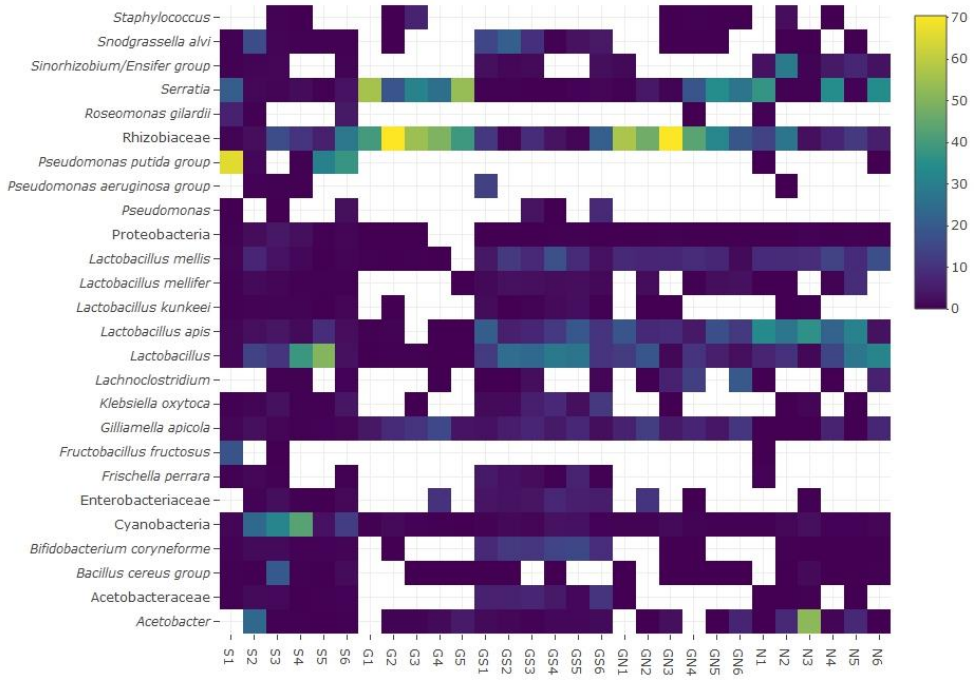


Figure 7.10 | Heatmap of the mean relative abundance for each phylotype in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

White spaces indicate a phylotype < 0.01% abundance. All italicised phylotypes without species classification indicate the genus e.g. *Lactobacillus* is the genus. The treatments were gut slurry (GS1–6), *G. apicola* (G1–5), *G. apicola* and *N. apis* (GN1–6), *N. apis* (N1–6), and sucrose (S1–6).

The plot also indicates several cages that were outliers within a treatment where the dominant phylotypes appear to be less prevalent: S1 (*Pseudomonas putida* group), S4 (Cyanobacteria), S5 (*Lactobacillus* spp.), and N3 (*Acetobacter* spp.). The first three outlier cages were from the sucrose treatment and each of these had only three, four, or five phylotypesrespectively. The fourth was from the *N. apis* treatment and this had five phylotypes. Of these, only *Lactobacillus* spp., *L. apis*, and Rhizobiaceae have so far been identified in healthy NZ honey bees (Chapter 3), suggesting that the phylum Cyanobacteria, the genera *Acetobacter* and *Serratia*, and the species *Fructobacillus*

fructosus, and *Pseudomonas putida*, may only be present in compromised honey bee guts as they may not be suppressed by the normal commensal bacteria.

7.4.6 Relative abundance

The relative abundance of phylotypes in the *G. apicola* inoculum were predominantly *G. apicola* (95%), with 3.7% *Staphylococcus* spp. and 1% Rhizobiaceae (Table 7.10). The bees inoculated with *G. apicola* also had high relative abundance of Rhizobiaceae (38.7–70.4%) and *Serratia* spp. (18.6–56%), as well as the lesser abundant *Acetobacter* spp. (0.4–4.6%), Cyanobacteria (< 0.01–1.4%), and *Lactobacillus apis* (< 0.01–4%). This indicates that these bacteria were resident within the gut of NEWs prior to inoculation.

The gut slurry inoculum contained the dominant core bacteria (Table 7.10): 28.4% *Lactobacillus* spp., 11.3% *G. apicola*, 6.9% *S. alvi*, 12% *L. mellis*, 11.6% *B. coryneforme*, 8.6% *L. apis*, and 2.2% *L. mellifer*. Additionally, 12.2% Rhizobiaceae, 3% Acetobacteraceae, and six phylotypes with < 2% relative abundance were also present. The bacteria in the guts of bees fed the gut slurry inoculum contained all of these species as well as *Pseudomonas aeruginosa* (< 0.01–5.4%), Cyanobacteria (0.1–3%), and *Serratia* (< 0.01–0.8%).

The bacteria in the guts of bees fed sucrose included *Lactobacillus* spp. (0.6–50.4%), *S. alvi* (< 0.01–16.4%), *L. apis* (0.3–8.9%), *L. mellis* (< 0.01–5.7%), Rhizobiaceae (0.5–28.5%), *Acetobacter* (< 0.01–23.7%), Cyanobacteria (0.9–43%), *Serratia* (0.2–16.4%), *Bacillus cereus* (< 0.01–19.9%), *F. fructosus*, (< 0.01–7%), *P. putida* (< 0.01–66%), and *Pseudomonas gilardii* (< 0.01–16.4%) (Table 7.10).

The major bacterium in the guts of the supposedly axenic bees that were inoculated with *N. apis* was *L. apis* (22–36.2%), except for cage N6 where *L. apis* was only 3% and appeared to be replaced by *Lactobacillus* spp. (32.2%). *Lactobacillus* spp. (0.7–32.2%), *L. mellis* (5–16.9%), and Rhizobiaceae (2.5–16.4%) also dominated the bacterial composition (Table 7.10). The remainder of the bacterial species were *G. apicola* (4.4–5.3%), *S. alvi* (one observation 0.1%), Acetobacteraceae (one observation 0.1%), *Acetobacter* (< 0.01–52%), Cyanobacteria (0.9–43%), *Serratia* (0.1–37.3%), *B. cereus* (< 0.01–0.3%), *Lachnoclostridium* spp. (one observation 2.6%), and *P. gilardii* (< 0.01–0.3%), *Staphylococcus* spp. (one observation 1.3%), and Proteobacteria (one observation 0.1%). The presence of these phylotypes indicate that NEWs may not be axenic but that they emerge with a gut bacterial profile, even if the bacterial loading is only miniscule. It is possible that some of the less abundant phylotypes may be contamination from the outside of the gut, as the forceps were not flamed. However, the high relative abundance of *G. apicola* and *S. alvi* suggest that contamination of the samples is unlikely as these species are only present within the gut, rather than the outside where the forceps would have held the sample.

Table 7.10 | Mean relative abundance of phylotypes in the digestive tract of honey bees inoculated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

Treatment	*Lactobacillus**	*Gilliamella apicola*	*Snodgrassella alvi*	*Bifidobacterium coryneforme*	*Frischella perrara*	*Lactobacillus apis*	*Lactobacillus kunkeei*	*Lactobacillus mellifer*	*Lactobacillus mellis*	Rhizobiaceae	Acetobacteraceae	Enterobacteriaceae	*Sinorhizobium/ Ensifer**	*Klebsiella oxytoca*	*Acetobacter**	*Pseudomonas aeruginosa**	Cyanobacteria	*Serratia**
S1	0.6	**0.6**		0.2	0.1	**0.3**	0.1		0.3	0.5				0.1			0.9	16.4
S2	14	**2.5**	16.4	1.6	0.6	**2.5**	0.4	1.2	5.7	2.1	1.3	0.2	0.4	0.5	**23.7**	0.5	**23.6**	0.8
S3	10.7	**0.8**	0.4	1.6		**3.7**	0.4	0.7	2.3	16.5	1.3	2.1	0.6	2.6	0.2	0.2	**31.7**	0.7
S4	**38.3**	**0.3**	0.2	0.2		**1.3**	0.5	0.1	0.8	10.6		0.1			0.1		**43**	1.8
S5	**50.4**	**0.2**	0.1	0.3		**8.9**				5.7	0.1			0.1			2.9	0.2
S6	2.1	**0.8**	0.1	0.5		**2**	0.7	0.1	0.5	**28.5**	0.2	1.3	0.1	3.7	0.2		12.8	2.9
GS1	12.1	**2.6**	14.8	7.9	1.7	20.9	1.7	1.4	4	11.4	6.4	3.4	2.6	1.7	1.6	5.4	0.4	0.1
GS2	**24.8**	**5.4**	**21**	11.8	2.7	**5.8**		2.6	11.5	0.9	6	3.1	0.4	1.5	1		1.3	
GS3	**23.5**	**7.1**	9.4	10.8	1.2	**7.1**	0.6	2.6	8	8.7	7.2	3.5	1.2	5.6	0.4		0.9	
GS4	**28.2**	**4.5**	0.1	**14.2**		**11.8**	1.5	1.9	17.1	3.2	4.1	7.6		1.6	1.1		3	0.1
GS5	**26.9**	**7.5**	2.4	**15.2**	2.7	19.4	2.1	2.1	8.3	0.8	0.9	5.5		2.4	1.1		2.6	0.3
GS6	10.6	**2.3**	4.2	8.9	0.1	**10.4**	0.2	1.1	2.7	**21.1**	10.5	5.1	1.8	11.6	1.7		0.1	0.8
G1		**4**				**0.1**			0.1	**39.7**							0.1	**56**
G2		**8.5**				**0.6**			0.1	**70.4**					0.5		1.4	**18.6**
G3	0.1	**10.5**								**54.5**					0.4		0.3	**31.4**
G4		**15.3**				**4**				**49.4**		8.1			1.7		0.1	**25.3**
G5		**3.2**				**0.1**				**38.7**					4.6			**53.5**
GN1	9.7	**6**				18.4			8.1	**56.9**			0.3				0.5	0.2
GN2	**18.7**	**12.6**				**6.4**		0.7	4.3	**47.2**		6.3					0.6	3.2
GN3	0.9	**4.5**				**8.7**			7.3	**70.2**					2.4		1.6	0.5
GN4	10.8	**6.9**				**3.3**		0.1	8.6	**43.3**							0.6	**18.6**

Treatment	*Lactobacillus**	*Gilliamella apicola*	*Snodgrassella alvi*	*Bifidobacterium coryneforme*	*Frischella perrara*	*Lactobacillus apis*	*Lactobacillus kunkeei*	*Lactobacillus mellifer*	*Lactobacillus mellis*	Rhizobiaceae	Acetobacteraceae	Enterobacteriaceae	*Sinorhizobium/ Ensifer**	*Klebsiella oxytoca*	*Acetobacter**	*Pseudomonas aeruginosa**	Cyanobacteria	*Serratia**
GN5	5.6	**3**				**17**		1.4	5.9	**32.6**							0.2	**34.4**
GN6	2.4	**11.1**				**11.7**		1.1	1.3	18.7					6.9		0.1	27.2
N1	6.8				0.1	**33.9**			5	14			2.3				0.2	37.3
N2	7.8		0.1			**27.6**	0.1		8.4	16.4			29.1		8.1		0.9	0.2
N3	0.7					**36.2**	0.1		5.3	2.5	0.1		0.1	0.2	52		2.4	0.1
N4	14.4	**5.3**				**22**		0.2	14	6.9			1.8		0.6		0.3	34.6
N5	27.9					**31.2**		8.7	8.3	9.2			5.9		7.9		0.4	0.3
N6	32.2	**4.4**				**3**			16.9	5.6			0.6				0.6	34.1
BG	**28.4**	**11.3**	6.9	11.6	1.7	**8.6**	0.7	2.2	12	12.2	3	0.2	0.7	0.5	0.1			
G20		95				**0.1**				1.1								0.1
Comp	18.4	**11.2**				**10.8**		0.6	8.5	**45.7**		3.4					0.2	1.1

The five treatments were sucrose solution (S1–6), gut slurry (GS1–6), *G. apicola* (G1–5), *G. apicola* plus *N. apis* (GN1–6), and *N. apis* (N1–6). Gut slurry inoculum (BG). *G. apicola* inoculum (G20). Composite samples (Comp). Blank spaces indicate relative abundance < 0.01%. *indicates a species group that has not been fully characterised e.g. *Pseudomonas** indicates *Pseudomonas* species group. The relative abundances of major contributors (> 5%) are bolded.

The mixed effects model indicates significant differences in the relative percent abundance of unique phylotypes among the five treatments ($P < 0.01$) (Table 7.11).

Table 7.11 | Analysis of Deviance table (Type II Wald chi-square tests) for the relative abundance of phylotypes in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

Response	Sums of squares	DF	Pr (> Chi-square)
Treatment	153.57	4	2×10^{-16}
Phylotypes	4113.14	25	2×10^{-16}
Treatment: Phylotypes	2235.44	100	2×10^{-16}

Calculated using linear mixed effect regression model with a log transformation.

The predicted means plot of the mean relative abundance of bacterial phylotypes between treatments had a mean least significant difference (LSD) of 0.9 (Figure 7.11). Thus, significant differences were observed within and among treatments for all phylotypes except *F. fructosus*, *Pseudomonas* spp., *Pseudomonas aeruginosa* group, and *Staphylococcus* spp.

The largest differences were observed between *B. coryneforme* where the relative abundances in the G, GN, and N treatments were significantly lower than bees inoculated with gut slurry. The other dominant core bacteria *Lactobacillus* spp., *G. apicola*, and *S. alvi*, also differed as their presence was limited in treatments from which they were excluded.

There was no significant difference between the relative abundance of *G. apicola* in the G, GN and GS treatments, suggesting the presence of *N. apis* did not affect the growth of *G. apicola*.

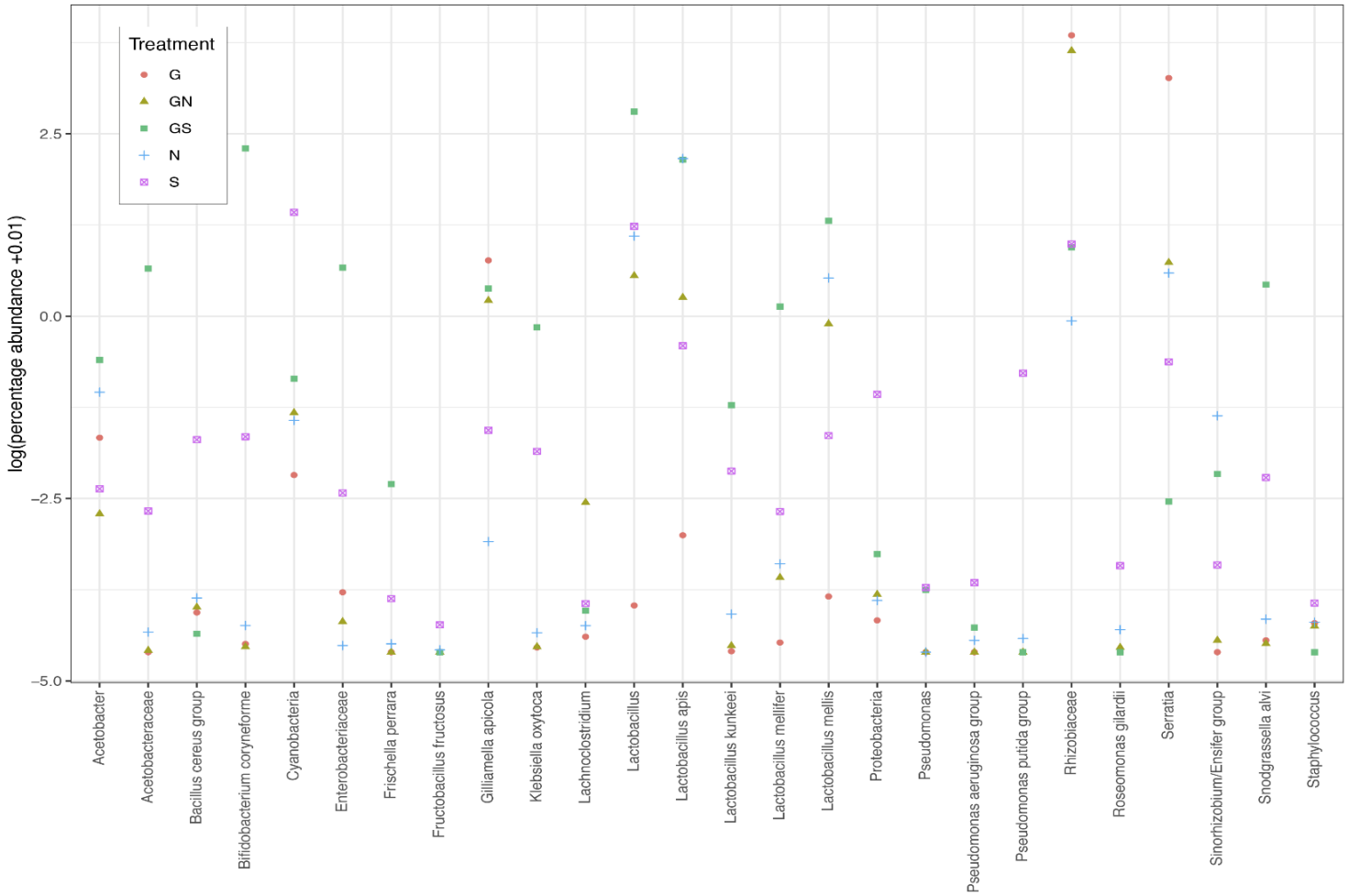


Figure 7.11 | Predicted means of relative abundance of phylotypes in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

Relative abundance is graphed on a log scale. Five treatments: *G. apicola* (G), *G. apicola* and *N. apis* (GN), gut slurry (GS), *N. apis* (N), and sucrose (S). Differences > 0.9 (mean LSD) suggests significance.

7.4.7 Diversity analysis

7.4.7.1 Alpha-diversity

The α-diversity of the relative abundance within each sample indicated that treatment significantly influenced all four indices of richness with bees in the sucrose (S) treatment having the largest diversity and the *G. apicola* (G) treatment having the least diversity (Figure 7.12, Table 7.12). The Chao1 and Observed ASV plots indicate a higher richness in the S treatment; suggesting that the bacteria normally reliant on the metabolites of other bacteria may be able to exist in a sucrose-rich environment. The GS treatment also had high richness but the spread between samples was less than that observed in the S treatment. The Shannon and Simpson diversity plots in combination with the richness diversity plots indicate a wide spread of both richness and evenness of bacterial species in the guts of bees treated with sucrose.

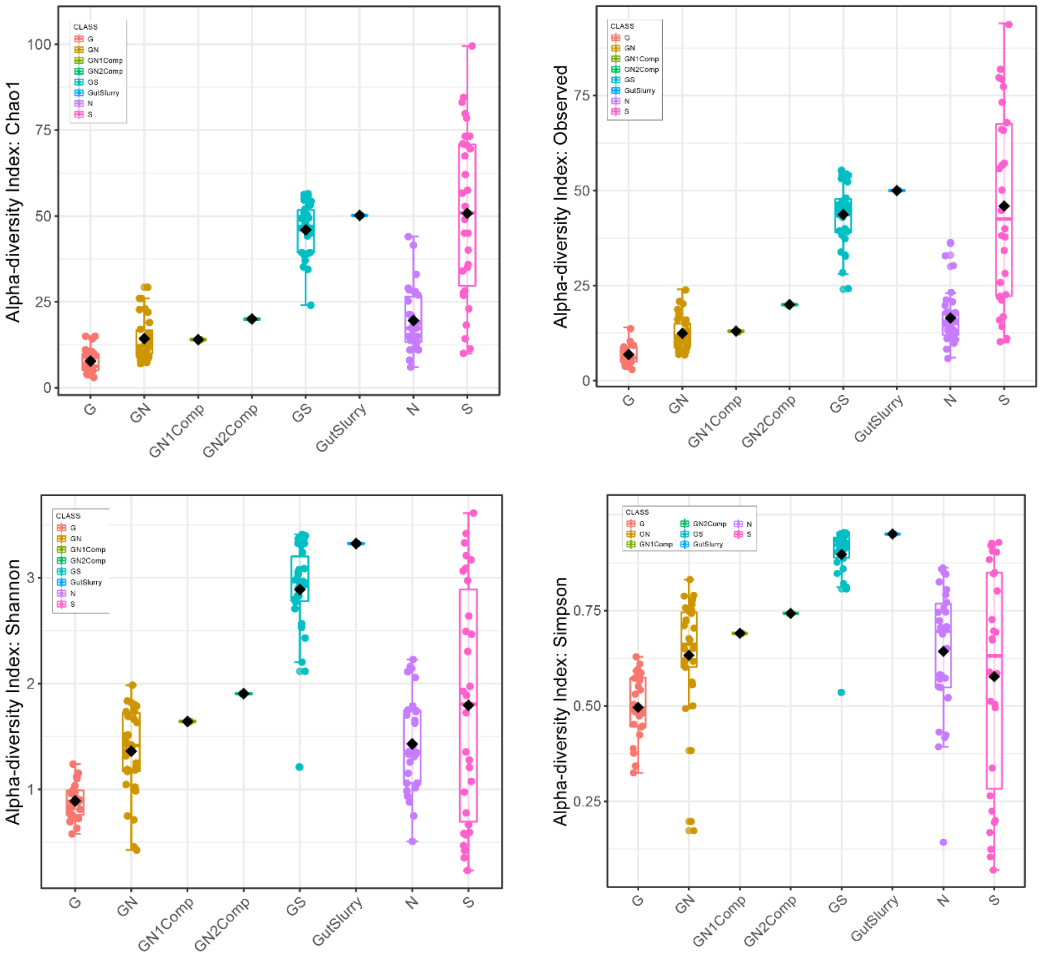


Figure 7.12 | Alpha-diversity measures for honey bees treated with combinations of *Gilliamella apicola, Nosema apis* and gut slurry.

The Observed Index plots the number of observed ASVs. The GN1comp, GN2comp, and GutSlurry points are the inoculations used to treat the bees.

Table 7.12 | Alpha diversity using treatment as the experimental factor.

Diversity measure	*P* value	Kruskal-Wallis statistic
Chao1	5.9762×10^{-20}	106.88
Observed ASVs	5.1158×10^{-21}	111.22
Shannon	4.6317×10^{-13}	72.49
Simpson	1.5388×10^{-12}	69.913

7.4.8 Beta-diversity

The PERMANOVA showed that treatment significantly influenced the honey bee gut microbiome ($P < 0.001$). Dependant on the diversity metrics employed, 52.1% (Bray-Curtis dissimilarity) or 38.2% (Jaccard similarity index) of the variation observed was explained by treatment (Table 7.13, Figure 7.13); the full complement of bacteria in the gut slurry treated bees overlapped with those in the other treatments. However, the overlap with the GN treated bees was only slight, and for the *G. apicola* treated bees the bacterial profile was completely separated.

Table 7.13 | Beta-diversity of the phylotypes in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

Distance method	*P* value	*F* value	R^2	PC% Axis 1	PC% Axis 2	PC% Axis 3	NMDS stress
Bray-Curtis	< 0.001	11.66	0.37	27.9	13.8	10.4	0.19
Jaccard	< 0.001	7.95	0.28	20.1	10.6	7.5	0.19

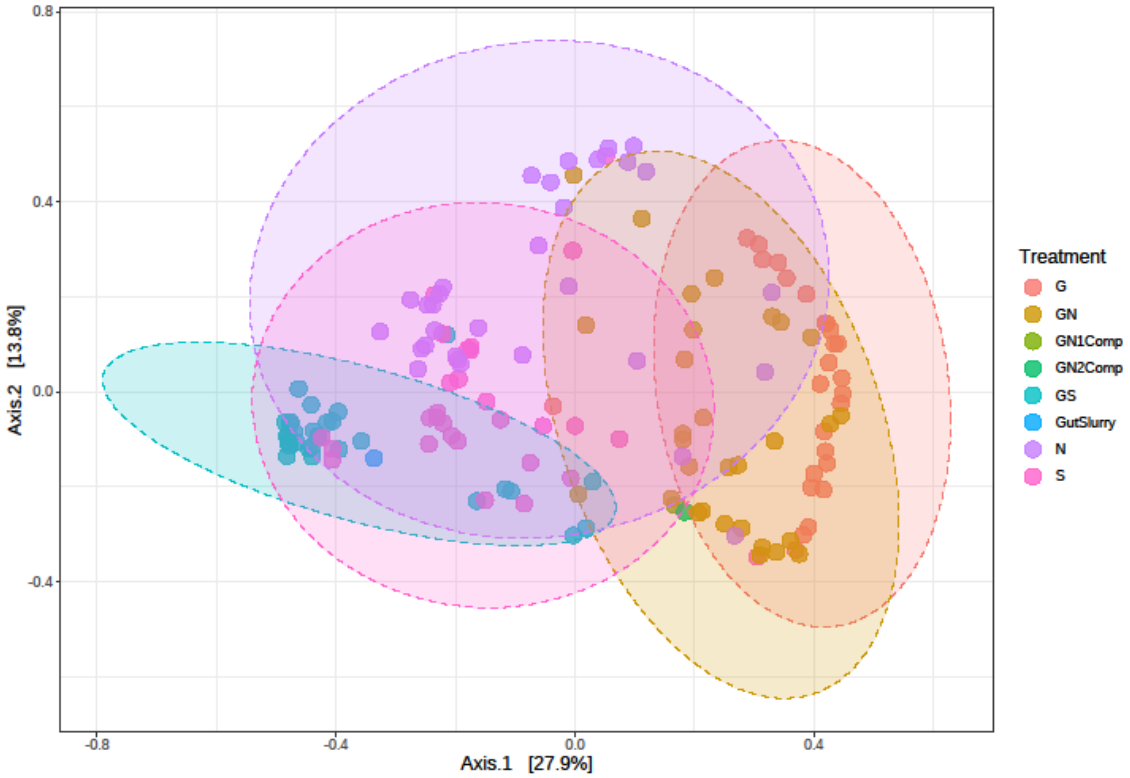
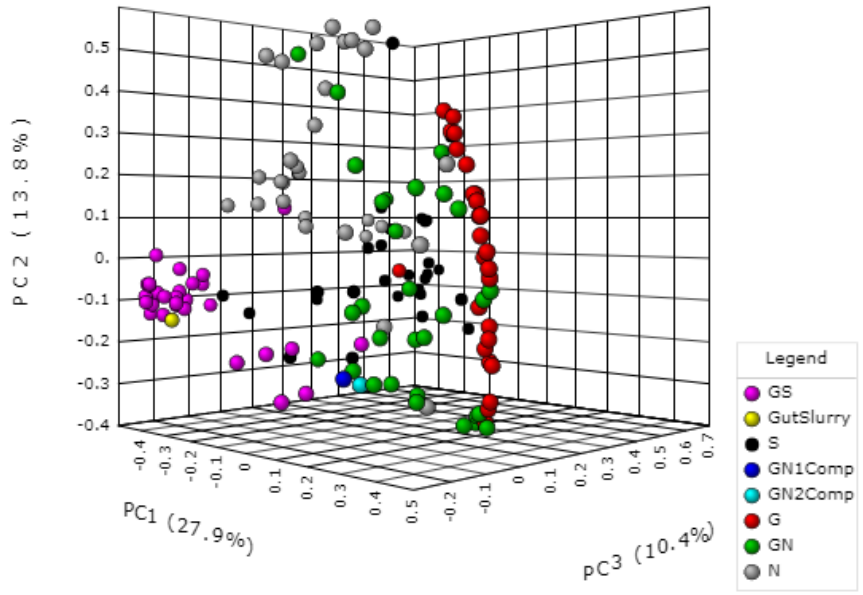


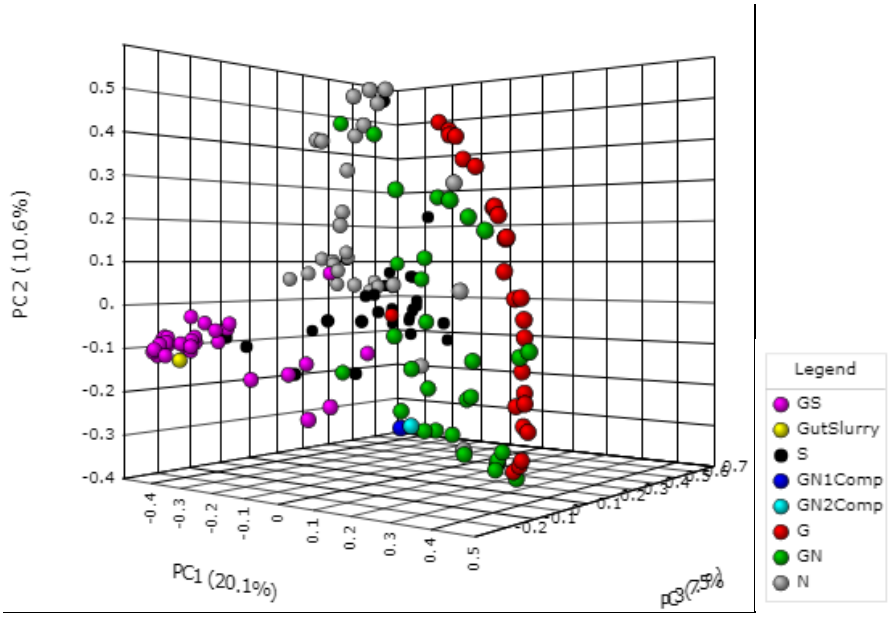
Figure 7.13 | A 3-D PCoA plot of phylotype diversity in the digestive tract of honey bees treated with combinations of Gilliamella apicola, Nosema apis, and gut slurry.

Treatment was the experimental factor calculated using Bray-Curtis dissimilarity. The treatments were: *G. apicola* (G), *G. apicola* and *N. apis* (GN), gut slurry (GS), *N. apis* (N), and Sucrose (S).

The 3-D PCoA visualisations for treatment using Bray-Curtis and Jaccard distance methods indicate the microbiome is altered by treatment (Figure 7.14A–B); the variation in the gut slurry treatment was grouped to the left of axis 1 and towards the base of axis 2, whereas the sucrose treatment was grouped to the left of axis 1, towards the base of axis 2 and to the right of axis 3. The *G. apicola* and the *G. apicola* plus *Nosema* treatments were grouped to the right of axis one and central for axis 3, whereas the *Nosema* treatment was grouped centrally on axis 1, to the top of axis 2.



A | Bray-Curtis



B | Jaccard

Figure 7.14 | Diversity of the phylotypes in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

The treatments were: *G. apicola* (G), *G. apicola* and *N. apis* (GN), gut slurry (GS), *N. apis* (N), and sucrose (S). The distance matrices were A | Bray-Curtis and B | Jaccard.

The NMDS analysis (Figure 7.15) also suggests that the composition of the microbiome shifted primarily as a function of treatment. Bacterial communities in the gut slurry treatment were displaced from those treated with *G. apicola* (G1–6) and sucrose (S1–6) along axis one of the ordination. The bacterial communities in the bees from the sucrose treatment (S1–6) were displaced from those treated with *G. apicola* plus *Nosema* (GN1–6) along axis two of the ordination. The gut slurry treatment converged with a strong representation of Acetobacteraceae and *B. coryneforme,* as well as *K. oxytoca* and *L. kunkeei* while the sucrose treatment had more of the less prevalent bacterium *B. cereus*, as well as Cyanobacteria and Proteobacteria. The GN treatment strongly converged with *G. apicola*, whereas the GS treatment which converged with *B. coryneforme*, diverged from Rhizobiaceae that tended towards the opposite direction on axis one, and thus seemed to be less affected by treatment. This lack of treatment effect may support the theory that Rhizobiaceae occurs when the bacterial composition in the gut is compromised, as all samples were from axenic caged bees that likely had compromised guts.

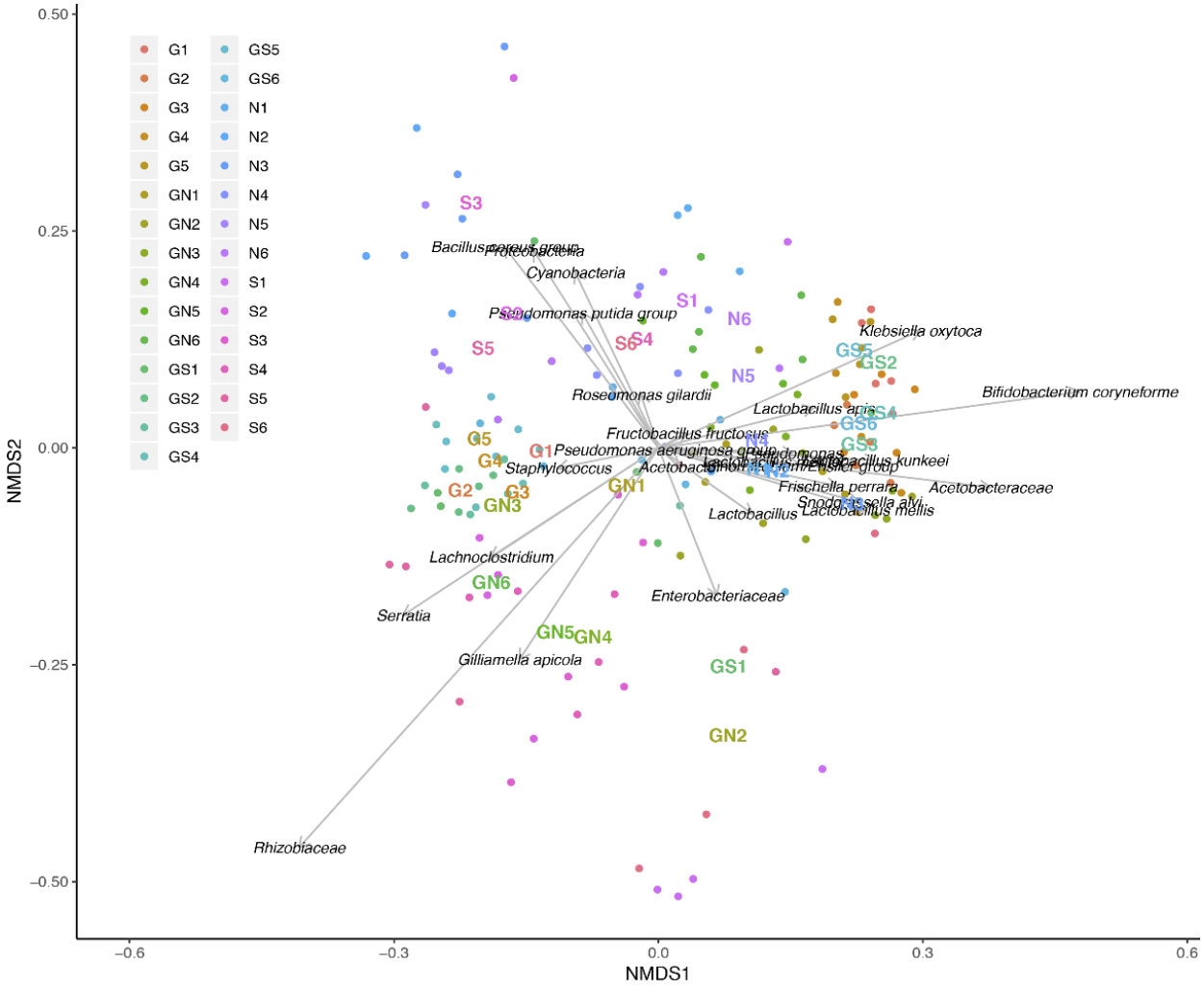


Figure 7.15 | NMDS of relative abundance of phylotypes in the digestive tract of honey bees treated with combinations of *Gilliamella apicola*, *Nosema apis,* and gut slurry.

The treatments were: *G. apicola* (G1–5), *G. apicola* and *N. apis* (GN1–6), gut slurry (GS1–6), *N. apis* (N1–6), and Sucrose (S1–6).

7.5 Discussion

This study investigates within-host competition between the gut pathogen *N. apis*, and core gut bacteria in the gut of caged honey bees. Axenic bees were inoculated with combinations of *G. apicola*, *N. apis*, and gut slurry from hive bees, to test the hypothesis that *N. apis* infection reduces the development and/or relative abundance of *G. apicola*, the coloniser of the outer layer of the ileal biofilm.

Number of amplicon sequence variants and phylotypes

The NGS analysis showed that all treatments were successfully consumed by the honey bees; the high relative abundance of *G. apicola* in the bees inoculated with *G. apicola*, as opposed to the low relative abundance of those not treated with *G. apicola*. This increased proportion of specific bacteria was the same for the bees fed with the gut slurry inoculum.

Gilliamella apicola was present in all five treatments, although it was only observed in one cage in the N treatment. There was no significant difference between the relative abundance of *G. apicola* in the G, GN and GS treatments, suggesting the presence of *N. apis* did not affect the growth/development of *G. apicola*. As *N. apis* resides in the ventriculus where it infects the epithelial and regenerative cells (Fries 1988a; Higes et al. 2019), it was not expected to affect the base layer of the ileal biofilm, which is primarily *S. alvi*. However, the effect of *N. apis* spores on the outer layer of the biofilm was unknown. After erupting out of the epithelial cells, the spores progress from the ventriculus, to the ileum, and through the remainder of the digestive tract (Higes et al. 2019), potentially leaving behind *N. apis* spores in each compartment. These may have aided or inhibited the growth of the saccharolytic fermenter *G. apicola* as the additional spores may have provided *G. apicola* directly with additional carbohydrates, or through the degradation of some of the bee epithelial cells ruptured by *N. apis*, or via indirect immune function. Although the duration of the trial was 12 days, the *N. apis* inoculation was conducted on day 5. This meant there were only 7 days within which the spores could infect, proliferate, and progress through the bee gut. It may therefore be useful to conduct this trial on older bees with a fully developed gut bacterial composition and identify the effect of high levels of *N. apis* infection passing through the digestive tract. Analysis of the individual gut compartments may also identify the bacteria present within each, as well as the response of each bacterium to excessive amounts of epithelial cells and/or *N. apis* spores in each compartment.

The shift in composition of the microbiome, as shown by the NMDS analysis, occurred primarily as a function of treatment, and the diversity metrics suggest treatment explains up to 52% of the variation. The gut slurry treatment converged with a strong representation of Acetobacteraceae and *B. coryneforme,* both frequently present in NZ honey bees (Chapter 3) and diverged from Rhizobiaceae. As the gut slurry was the

positive control, it was intended that the bees would develop a 'close to normal' bacterial composition. Bees in this treatment contained all core dominant bacteria, although the relative abundances of these phylotypes were dissimilar to the ratio observed in healthy bees from a colony (Chapter 3). Despite this, and coupled with the fact that the GS treatment diverged from all other treatments that did not contain high proportions of the dominant core bacteria, it is likely that the GS treatment produced a bacterial composition that more closely approximated a healthy bee gut than the other treatments. This was also supported by the low abundance of Rhizobiaceae in the GS treatment but high abundance in all other treatments. In the G and GN treatments Rhizobiaceae was the most dominant phylotype. This evidence is complementary to that identified in Chapters 3, 4 5 and 6, where Rhizobiaceae increased in honey bee guts with a compromised bacterial composition. The absence of other core bacteria, such as *S. alvi* that was absent from both the G and GN treatments, suggests *S. alvi* acquisition occurs very early in the developmental stage of the bee, and their proliferation indicates presence of adequate carbon substrates, while the normally crop-residing Rhizobiaceae are inhibited.

Axenic bees

The dominant core phylotypes (*G. apicola*, *S. alvi*, *Bifidobacterium* spp., and species within the genus *Lactobacillus*) were present in all sucrose and gut slurry treated bees. The gut slurry treatment supports the literature that gut bacteria can be acquired from nurse bees or hive material (Powell et al. 2014). Although suggested, the literature does not clearly identify whether bees are axenic when they eclose from their wax cell. The presence of bacteria in axenic bees fed the sucrose control and the presence of *G. apicola* in all five treatments (including the sucrose and *N. apis* treatments, albeit at low relative abundance), suggests that NEWs may not be fully axenic when they emerge from their cells. Alternatively, bees may have acquired bacteria from their cages or the environment within the incubator. In this study the sucrose may have provided a substrate for bacteria that would normally be provided by other bacteria or the metabolites they produce. The interaction in the ileum between *G. apicola* and *S. alvi* is an example of this mutualistic behaviour (Anderson and Ricigliano 2017).

The presence of *S. alvi* and *Bifidobacterium* spp. in approximately half of the G and GN treatment cages, and some of the N treatment cages, indicates that core bacteria exist in the honey bee gut when they emerge from the cell. Their limited presence also suggests that the abundance of these bacteria may be inhibited by unfavourable growth conditions, or by the interaction with *G. apicola* or *N. apis*, or that these bacteria require other mutualistic bacteria to proliferate. The presence of *S. alvi* and *Bifidobacterium* spp. in approximately half of the G and GN treatment cages, and some of the N treatment cages, again suggests that core bacteria exist in the honey bee gut when they emerge from the cell. This also suggests that the proliferation of these bacteria may have been inhibited by unfavourable gut conditions, or that the interaction with *G. apicola* or *N. apis*

may have inhibited proliferation, or that these bacteria require other mutualistic bacteria to proliferate.

In bees inoculated with *G. apicola*, the high relative abundance of Rhizobiaceae (38.7–70.4%) and *Serratia* spp. (18.6–56%), and the presence of the less abundant bacteria *Acetobacter* spp. (0.4–4.6%), Cyanobacteria (< 0.01–1.4%), and *Lactobacillus apis* (< 0.01–4%) also suggest that these bacteria are resident within NEWs.

Besides bacteria being present in the gut prior to eclosure, a second possible explanation for the presence of bacteria in axenic bees fed sucrose is that the irradiated pollen contained traces of bacteria. The bacteria in the pollen were not viable as they were irradiated, but the bacterial DNA may still have been identifiable. As the pollen was not analysed, the phylotypes were not identified.

All bees in this trial were removed from the cells prior to emergence to prevent contact with hive ware and other bees, and ultimately the acquisition of gut bacteria (Powell et al. 2014). The negative sucrose control was expected to be somewhat axenic at the end of the trial, similar to the low concentrations of bacteria observed by Powell et al. (2014) after eight days; approximately 1.9×10^6 16S rRNA gene copy numbers. The bees inoculated with the gut slurry (positive control) were expected to have a full complement of bacteria at the end of the trial with approximately 100-fold more bacteria than bees in the sucrose treatment (Powell et al. 2014). This was the case for the GS treatment as the richness in phylotypes was higher than the other treatments. However, the high diversity observed within the sucrose treatment (negative control) was unexpected. As the diversity in these sucrose samples was similar to that of the gut slurry but with a larger spread, some bees contained a diverse bacterial composition and some did not. This acquisition of bacteria may have occurred before or during the trial set-up, or may have resulted from contamination during analysis. The increased diversity may also be explained by the lack of a critical threshold of the core bacteria, resulting in opportunistic colonisation by fast-growing environmental organisms. As the sucrose treatments were sterile and the caged bees had no direct contact with other bees or hive ware, and as all of the samples were processed using the same methodology and at the same time, it is important to note that the gut slurry inoculation likely provided a high abundance of normal commensals and these competitively inhibited the colonisation by the comparatively fewer environmental contaminants, which only colonise if not competitively excluded. This high diversity therefore firstly suggests that the bees were not axenic. Secondly, the high diversity in the sucrose control supports the theory developed in Chapter 5 where increased diversity in the honey bee gut may not be beneficial as diversity appears to be associated with bees that are sick (Chapter 3), bees that are aged such as those that overwinter (Chapter 5), or bees that have been nutritionally challenged (Chapter 6).

The possibility that NEWs contain bacteria is contrary to that observed by Powell et al. (2014) who state that worker bees initially lack bacteria. However, their study supports

the findings in this chapter as they actually did identify bacteria in 8-day-old bees, even if only at a very low abundance (1.9 x 10^6 16S rRNA gene copy numbers). Data in this chapter therefore support the theory that NEWs are not devoid of bacteria. However, as contamination cannot be fully excluded, additional research is required to understand whether NEWs emerge with a limited bacterial composition that may require specific conditions to develop in to a full complement of bacteria.

qPCR analysis

The presence of *N. apis* spores/ml in the *G*. *apicola* inoculation, the bee gut inoculation, and the *Nosema* inoculation confirms *N. apis* was relatively absent in the *G*. *apicola* inoculation but present in the gut slurry and *Nosema* inoculations. The presence of *N. apis* was expected in the gut slurry inoculation because *Nosema* infection in NZ bees is unable to be chemically controlled. Hence, it was likely to be present in the bees used to produce the gut slurry.

The significant difference in the number of *N. apis* spores/ml between treatment groups occurred because the sucrose control had significantly fewer *N. apis* spores/ml than all other treatments, and the GS treatment had significantly more *N. apis* spores/ml than the N treatment ($P < 0.05$).

The presence of *N. apis* in bees inoculated with *N. apis* compared with bees fed sucrose and bees inoculated with *G*. *apicola* and *N. apis* indicates that the methodology used to administer the treatments was successful and that the bees prior to treatment contained minimal *N. apis* infection. As the number of spores in bees inoculated with *N. apis* was significantly lower than that in bees inoculated with honey bee gut slurry, this suggests that the filtered *N. apis* suspension may have contained fewer viable spores than the fresh gut slurry. This probably occurred, despite attempts to limit the loss of spores throughout the verification process, when the 30 ml *N. apis* suspension was passed through a 70 µm sterile filter which was replaced every 10 ml when the filter clogged, thus removing some of the viable spores.

Gilliamella apicola is a facultative anaerobe that produces energy from the aerobic fermentation of carbohydrates. Despite lacking some of the genes required for the TCA cycle, *G. apicola* generates ATP and biosynthetic precursors directly from glycolysis, pentose phosphate pathways, and the Entner-Doudoroff Glucose to Pyruvate pathway (Kwong et al. 2014). In this study the sucrose-rich environments may provide *G. apicola* with sufficient carbohydrate to produce energy, thus enabling its proliferation independent of other bacteria. This is evidenced in their presence within the bees inoculated with *G. apicola*, and both *G*. *apicola* and *N. apis*.

Snodgrassella alvi is an aerobic oxidiser of carboxylic acids (Kwong et al. 2014), so a lack of this bacterium in the ileum may mean less substrate for the reductive cycles and therefore less CO_2 in the digestive tract. As *S. alvi* and *G. apicola* are both facultative anaerobes (Kwong and Moran 2013), they may not be affected. However, this decrease

in CO_2 may influence the atmosphere down the digestive tract inhibiting bacteria that prefer partial or full anaerobic conditions such as the genera *Lactobacillus*, *Frischella,* and *Bifidobacterium*. Interestingly, in the two treatments where *S. alvi* was absent, *Frischella perrara* was also absent, thus suggesting a greater link between these two bacteria than currently understood.

The low abundance of *F. perrara* (0.1–2.7%) in the 12-day-old bees from the gut slurry treatment was unexpected as caged bees fed old protein diets (pollen or bee bread) have higher frequencies of *G. apicola* and *F. perrara* than those fed fresh diets (Maes et al. 2016). Bees in my trial were fed old diets: apple pollen stored at -70°C for 2.5 years. It is possible that the low abundance of *F. perrara* in the treatments was because *G. apicola* was competing with it (Martinson et al. 2012; Powell et al. 2014). However, as *F. perrara* was also low in the N treatment which did not contain *G. apicola,* a further explanation could be acquired through host-challenge trials.

Composite samples

The number of *N. apis* spores from the combined samples of lysed bee guts from five individual bees compared with those within individual samples indicates large variation between individual bees inoculated using these methods. The number of spores in the composite samples fell within the range of the individual samples, suggesting that a composite sample of five bee guts inoculated using these methods may sufficiently indicate *N. apis* numbers. However, the large difference between the individual counts and the count of the composite sample (2.15×10^9) suggests that the presence of some bee guts will conceal the lower concentration in other guts from bees that have consumed a limited amount of the inoculum. For example, the number of *N. apis* spores in bee 1 in cage GN1 was 8.84×10^4, whereas bee 2 contained 8.30×10^9 spores. It is possible that once the gut samples were homogenised, the weightier components may have settled to the base of the tube before the 80 µl was removed for DNA extraction; as the five bee samples were vortexed together before removing the subsamples, the bee samples processed last (~5 min) may have a comparatively lesser number of spores compared with the earlier contributing samples due to the lysis solution. The order of the inclusion was not recorded so this cannot be verified. As only two composite samples were analysed, not enough information is available to make any definitive sampling recommendations. However, if composite samples are used, then these results should only be compared with those collected using similar methodology. Also, to reduce the dilution effect of the lysis solution, bee guts should be pooled directly in to lysis solution, followed by DNA extraction.

To identify the bee-to-bee variation, no less than five bees should be sampled and the number of experimental replicates should provide sufficient power/data. The two composite samples of five individual bees does not provide sufficient data so pooling additional bee guts may provide more accurate information regarding the presence of

N. apis than sampling of individual bee guts. This may also be a more economic and accurate way to understand microbiome composition through 16S rRNA sequencing.

Honey bee mortality

The high bee mortality observed in all five treatments (32–61%) was higher than accepted when adhering to the oral guidelines for testing chemicals on honey bees, as outlined by the Organisation for Economic Co-operation and Development (OECD) (OECD 1998). However, these guidelines recommend conducting trials for 48 h, or 96 h in prolonged trials. As this trial was conducted for 216 h to ensure a full complement of *G. apicola* and *N. apis* had colonised after inoculation (Forsgren and Fries 2010; Powell et al. 2014), this mortality was expected. The high mortality likely occurred because the bees within each treatment cage were combined from the four frames from each of four hives. During this trial I observed worker bees fighting and although the bees from different hives were not differentiated, fighting does not normally occur when bees are caged from the same colony. Although, worker bees do not discriminate among unrelated and related bees from larval phenotypes (Tarpy and Fletcher 1998), and mixed kin groups assembled immediately after emerging learn the composite identity of the group and do not attack bees based on kinship (Breed et al. 1985), I have observed much bee discrimination in the field working with colonies. For example, the transfer of adult workers on frames of brood from one colony to another colony results in better integration when the bees are supplied with a floral scent.

7.6 Conclusions

The pathogen challenge of *N. apis* did not disrupt the development of the relative abundance of *G. apicola*, the outer layer of the biofilm in the honey bee ileum.

The gut slurry inoculation produced a bacterial composition that approximated a healthy bee gut relative to bees inoculated with individual bacteria. The increased abundance of bacterial phylotypes after the bees were inoculated supports the literature that bees can acquire gut bacteria from worker bees.

NEWs do not appear to be axenic when they emerge from their cells as low relative abundance of *G. apicola*, *S. alvi*, *L. apis*, *L. mellis*, *Lactobacillus* spp., *Bifidobacterium* spp., *Serratia* spp., *Acetobacter* spp., Rhizobiaceae, and Cyanobacteria were present.

The lack of abundant bacteria in the honey bee gut correlated with an increase in the opportunistic colonising bacteria Rhizobiaceae and *Serratia*. This is further evidence supporting the evolving theory from Chapters 3, 4, 5 and 6 regarding Rhizobiaceae as an opportunistic bacterium that may be a useful indicator of poor bee health.

The number of *N. apis* spores identified from five individual bees using qPCR indicates large variation among the samples when bees are inoculated using this 'group-fed' methodology. The number of spores in the composite samples fell within the range of the individual samples, suggesting that a composite sample of five bee guts inoculated using these methods may sufficiently indicate *N. apis* numbers.

7.7 Where to next

Compromised honey bee guts appear to have higher relative abundance of the family Rhizobiaceae and the species *Serratia* than those with full complements. This naturally leads to exploring my theory that the composition of the gut microbiome in donor nurse bees influences the microbiome in receiver NEWs. However, as this is the final research Chapter of this PhD, I will need to secure funding to conduct this research.

7.8 Supplementary material

7.8.1 Pilot trial to determine the suitability of inoculating caged honey bees individually and as a group

Caged honey bees can be inoculated individually by hand or as a group using feeders. To ensure the bees become infected after inoculation and so determine the pros and cons of each method, the suitability of inoculating bees individually or as a group was required. The most appropriate inoculation method was then used to conduct the host-challenge study in Chapter 7; the effect of the gut pathogen *N. apis* on the gut bacterium *G. apicola*, the bacterium that forms the top layer of the biofilm in the honey bee ileum.

Methods and materials

In spring (September) 2016, all bees were shaken off two sealed-brood frames (the bees were within 24 h of emerging) from each of two hives (190 and 411) located at PFR in Hamilton, NZ. The frames were incubated at 34°C and 65% relative humidity in ventilated metal carriers (length 390 mm x width 100 mm x height 255 mm). After 24 h approximately 600 NEWs were placed in each of two large cages and fed 50% (w/v) sucrose solution (SS) and irradiated apple pollen *ad libitum*.

At day 3 the temperature was reduced to 30°C for the remainder of the trial to simulate temperatures experienced by bees moving in and out of the brood nest. The bees were then starved for 2–6 h to ensure they consumed the inoculation, and a total of 520 bees were treated with one of six treatments (Table 7.8.1.1):

- Individually inoculated and individually caged bees (IIIC)
- Individually inoculated bees that were then caged as a group (IIGC)
- Bees group-inoculated and group-caged (GIGC)
- Bees individually inoculated with SS and individually caged (IIIC control)
- Bees individually inoculated with SS and caged as a group (IIGC control)
- Bees inoculated with SS as a group and caged as a group (GIGC control).

All bees were anoxiated (put to sleep) with CO_2 for 2 min (Martín-Hernández et al. 2011), either to enable handling of individual bees prior to inoculation, or aid 30 bees to be placed in to each cage allocated to the group treatments. Individually fed bees were inoculated with 5 µl using a pipette tip. The *Nosema*-treated bees were fed SS containing 10,000 spores. The individually inoculated bees were fed 50% SS and pollen *ad libitum* 20 min after they were inoculated to ensure they did not regurgitate the

treatment. The group-fed bees (GIGC and GIGC control) were fed pollen *ad libitum* as soon as the treatment was applied and then 50% SS after 24 h.

Table 7.8.1.1 | Treatment inoculations fed to honey bees individually or as a group.

Treatment	Bees per cage	Cage replicates (cage type)	Total number of bees	Inoculation substance and application method
IIIC	1	20 (queen cage)	20	Individually inoculated with 5 µl 10,000 *N. apis* spores in 50% SS, and individually caged
IIIC Control	1	20 (queen cage)	20	Individually inoculated with 50% SS, and individually caged
IIGC	20	6 (metal cage)	120	Individually inoculated with 5 µl 10,000 *N. apis* spores in 50% SS, and caged in a group
IIGC Control	20	6 (metal cage)	120	Individually inoculated with 5 µl 50% SS, and caged in a group
GIGC	20	6 (metal cage)	120	Group inoculated with 0.4 ml of *N. apis* suspension in 50% SS containing ~200,000 spores, and caged in a group
GIGC Control	20	6 (metal cage)	120	Group inoculated with 50% SS, and group caged

Sucrose solution (SS). *Nosema apis*: *N. apis*.

Mortality was recorded daily for 12 days post inoculation. At the end of the trial the entire digestive tract from each bee was individually dissected and the DNA was extracted from each using the ZR *Quick*-DNA™ Fecal/soil microbe miniprep kit (D6010). The variation in *N. apis* concentration between treatment groups was determined using qPCR, and the results were expressed as the number of gene copies. The data were analysed using Genstat 18th Edition (VSN International 2015); post-hoc pairwise comparisons of least-square means were conducted using Tukey's test to indicate significant differences among treatments. These are shown by dissimilar letters.

Results and discussion

The bee mortality in the individually inoculated and individually caged treatment (IIIC) was significantly higher than that in the associated control group or either of the grouped caged treatments IIGC or GIGC (Figure 7.8.1.1, Table 7.8.1.2); only eight of the 20 bees in the IIIC treatment survived and only 10 of the bees in the IIIC control treatment survived, suggesting that the inoculation method was detrimental to the bees rather than the *Nosema* treatment. The mean number of *N. apis* gene copies was not significantly different between the individually inoculated or group inoculated treatments (Figure

7.8.1.1, Table 7.8.1.2). The GIGC control contained 7-fold less *N. apis* genes, and the IIGC control contained 59-fold less *N. apis* gene copies than the associated treatments.

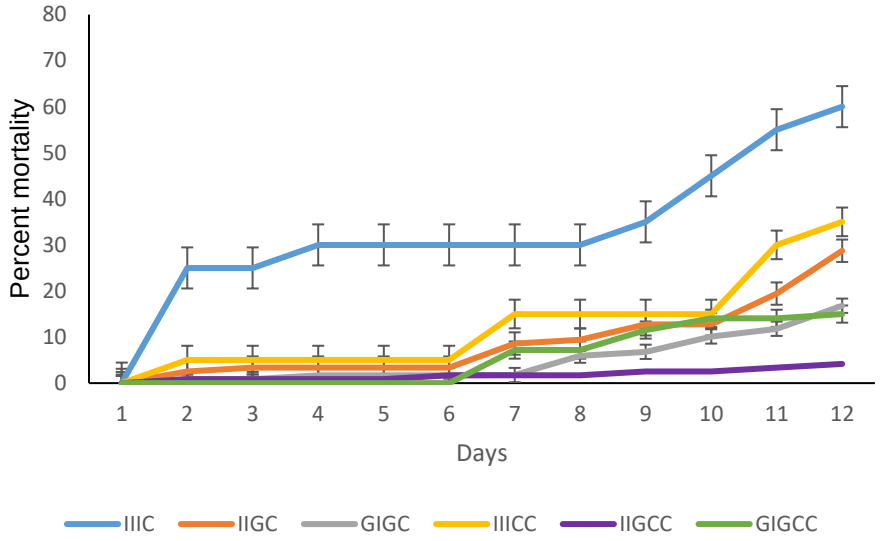


Figure 7.8.1.1 | Honey bee mortality of bees individually inoculated, or group-inoculated with *Nosema apis* spores.

Table 7.8.1.2 | Mean mortality of honey bees after 12 days that were individually inoculated or group-inoculated with *Nosema apis* spores, and the associated number of gene copies.

Treatment	Mean percent mortality	Minimum percent mortality	Maximum percent mortality	*N. apis* gene copies (Mean)	Standard error
IIIC	60	36.1	80.9	20,725,000^{bc}	3,173,002
IIIC Control	35	15.4	59.2	19,231^{a}	
IIGC	28.3	20.5	37.3	22,285,057^{c}	962,179
IIGC Control	4.2	1.4	9.5	376,991^{a}	844,260
GIGC	16.7	10.5	24.6	17,312,500^{b}	897,460
GIGC Control	14.2	8.5	21.7	2,280,882^{b}	888,618

Dissimilar letters are used to indicate significant differences between the mean number of gene copies from each of the treatments.

No difference was observed between *Nosema* counts for the treatments IIIC and GIGC (Table 7.8.1.3, Figure 7.8.1.2). However, there was a significant difference between group caged bees that were inoculated individually or inoculated as a group (GIGCC vs IIGC). The bees in the GIGC treatment were accidentally fed twice the number of *Nosema* spores as those in the IIGC treatment but after the 12 days the *Nosema* spore count was lower.

Table 7. 8.1.3 | Tukeys 95% confidence intervals for differences in the number of *Nosema apis* gene copies in the honey bee gut, 12 days after individual or group-inoculation with *Nosema apis* spores.

Treatment Comparison	Difference	Lower 95%	Upper 95%	Significant
IIICC vs IIGCC	-357760	-7882939	7167418	no
IIICC vs GIGCC	-2261652	-9828579	5305276	no
IIICC vs GIGC	-17293269	-24868746	-9717793	yes
IIICC vs IIIC	-20705769	-32251871	-9159667	yes
IIICC vs IIGC	-22265827	-29906139	-14625514	yes
IIGCC vs GIGCC	-1903891	-5413206	1605424	no
IIGCC vs GIGC	-16935509	-20463220	-13407798	yes
IIGCC vs IIIC	-20348009	-29748507	-10947510	yes
IIGCC vs IIGC	-21908066	-25572936	-18243196	yes
GIGCC vs GIGC	-15031618	-18647531	-11415704	yes
GIGCC vs IIIC	-18444118	-27878070	-9010165	yes
GIGCC vs IIGC	-20004175	-23754023	-16254328	yes
GIGC vs IIIC	-3412500	-12853311	6028311	no
GIGC vs IIGC	-4972557	-8739627	-1205488	yes
IIIC vs IIGC	-1560057	-11052973	7932858	no

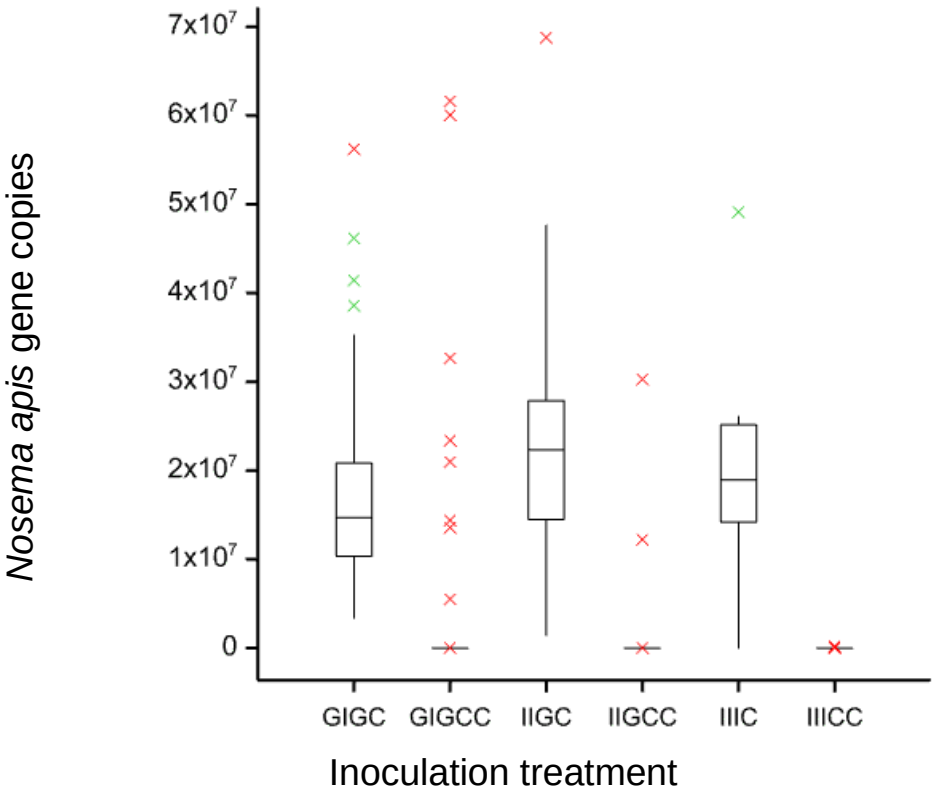


Figure 7.8.1.2 | Mean number of *Nosema apis* gene copies in honey bees 12 days post individual and group inoculation.

The box spans the interquartile range and the horizontal line indicates the median. The whiskers mark the upper and lower fence and extend to the minimum and maximum values. An extreme value greater than 1.5 times the interquartile range is marked as an 'x'.

There are four possible explanations for why *Nosema* spores were observed in some control cages. The first is that there may have been some old bees hiding in the sides of the incubation cages from where the supposed NEWs were selected from. This could be prevented by transferring the frames to new cages once back in the laboratory. The second is that the NEWs were fed on pollen for the first 3 days of life which, although irradiated, may have still contained dead *Nosema* spores. These would not have multiplied during the trial but in the calculation a single spore is multiplied by 50,000. However, this is unlikely to explain the 7 x 10^7 concentrations found in some of the IIGC samples. The third explanation is that the treated bees were accidentally placed in the control cages. If this occurred then it is interesting to note that the presence of *N. apis* in some caged bees did not appear to result in spores being passed to the other bees. For example, the maximum number of positive *N. apis* gene counts observed in one IIGC treatment cage was seven, comparatively, a control cage positive for *N. apis* was more likely to have ≤ 2 positive bee samples. The fourth and most likely explanation for the control bees being positive for *Nosema* is that some of the NEWs would have been in contact with the bee frames for 24 h whereas some would have had < 5 min contact. As the primary transmission of *N. apis* occurs by bees consuming faeces on the frames

(Bailey 1953), the frames have provided a source of inoculation for these early emerging bees. This can be controlled by removing the bees at intervals less than the 24 h, such as every 15 min.

Conclusion

As individual inoculation was no better, bee mortality was higher, and the time to conduct the individual inoculations were extremely lengthy in comparison with group inoculated bees, further inoculation trials of *Nosema* and bacteria were conducted using bees grouped in cages.

7.8.2 Recommended protocol for the ZymoBIOMICS DNA/RNA Mini Kit (R2002)

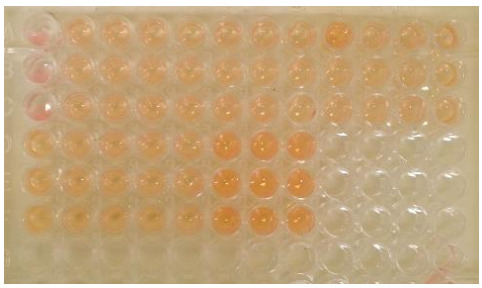
The DNA extractions were conducted as per the recommended protocol for the ZymoBIOMICS DNA/RNA Mini Kit (R2002) (Zymo Research, Irvine, CA, USA):

1. The volume of DNA/RNA shield in the sample tubes was increased from 90 µl to 750 µl and then the samples were homogenised for 40 s with a high-throughput cell disrupter, FastPrep®-24 (MP Biomedicals, Seven Hills, Australia), at 6 m/s.
2. ZR BashingBead™ Lysis Tube containing the gut samples were centrifuged for 1 min at ≥ 10,000 *g*.
3. 400 µl of supernatant was transferred to a new collection tube (CT) and 400 µl of DNA/RNA Lysis Buffer were mixed well.
4. The sample was transferred to a Spin-Away™ Filter in a new CT and centrifuged at ≥10,000 *g* for 30 s.
5. The flow-through was saved for RNA purification and the Spin-Away™ Filter was transferred to a new CT. 400 µl of DNA/RNA Prep Buffer was added to the column and centrifuged at ≥ 10,000 *g* for 30 s. The flow-through was discarded.
6. 700 µl of DNA/RNA Wash Buffer was added to the column and centrifuged at ≥ 10,000 *g* for 30 s. The flow-through was discarded.
7. 200 µl of DNA/RNA Wash Buffer was added to the column and centrifuged at ≥ 10,000 *g* for two min to ensure complete removal. The column was carefully transferred into a new microcentrifuge tube.
8. 100 µl of DNase/RNase–Free Water was added directly to the column matrix and left to stand for five min before being centrifuged at ≥ 10,000 *g* for 30 s to elute the DNA from the column.
9. The base of a Zymo-Spin™ IV-HRC Spin Filter, was snapped off, and the filter was inserted in to a collection tube (CT), and centrifuged at exactly 8000 *g* for 3 min. The flow- through was discarded. 400 µl of DNase/RNase–Free Water was added to the filter and centrifuged at ≥ 8000 *g* for two min.
10. The eluted DNA was transferred to a Zymo-Spin™ IV-HRC Spin Filter in a new microcentrifuge tube and centrifuged at ≥ 8000 *g* for one min.

7.8.3 Metabolic activity of *Gilliamella apicola* isolates

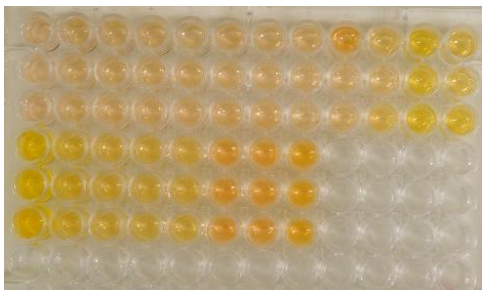
The metabolic acitivity of the *G. apicola* isolates were determined using optical density (OD) of the samples (higher the OD the darker the blue) coupled with the colour change observed in the photo log of the samples in the plate wells (red to yellow). Both indicate that all 16 isolates contained bacteria that were able to metabolise WST-1 over the first four hours (Figure 7.8.3.1): The wells A1, B1, and C1 containing the WST-1 controls had low OD readings (< 0.1) for the first 4 h, as supported by the photo log. Even during the time taken to apply the WST-1 and take the 0 h photo, a colour change was obvious in isolates 8–16, and the three 200 µl samples (D6–8, E6–8, F6–8), thus suggesting the *G. apicola* isolates were viable. After 18 h, all samples, including the TSB control, were yellow compared with the TSB WST-1 control at 0 h. This colour change suggests that bacteria within the wells were metabolising. As the suspension was not sequenced at the 18 h time point, the specific bacteria that were proliferating cannot be identified. However, as the controls contained bacteria that were metabolising, it is likely that the bacteria were contaminants. Contamination may have occurred because the plate was unable to be sealed when the OD measurements were determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.039	0.047	0.046	0.047	0.046	0.046	0.047	0.047	0.047	0.047	0.047	0.047
B	0.039	0.049	0.048	0.047	0.046	0.047	0.047	0.047	0.047	0.047	0.047	0.047
C	0.047	0.048	0.047	0.047	0.290	0.212	0.215	0.121	0.114	0.123	0.151	0.322
D	0.047	0.047	0.047	0.049	0.300	0.225	0.217	0.117				
E	0.047	0.047	0.047	0.047	0.296	0.219	0.206	0.123				
F	0.158	0.180	0.170	0.125	0.116	0.119	0.126	0.119				
G												



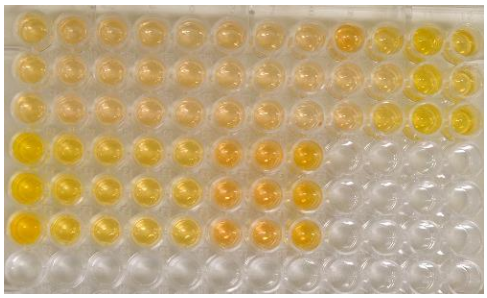
a | At 0 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.050	0.047	0.046	0.047	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046
B	0.074	0.049	0.048	0.046	0.046	0.047	0.047	0.047	0.047	0.047	0.047	0.047
C	0.047	0.048	0.047	0.047	0.492	0.317	0.337	0.255	0.246	0.259	0.306	1.874
D	0.047	0.047	0.047	0.046	0.497	0.316	0.317	0.260				
E	0.046	0.046	0.047	0.047	0.490	0.326	0.286	0.239				
F	0.384	0.411	0.304	0.181	0.151	0.181	0.186	0.167				
G												



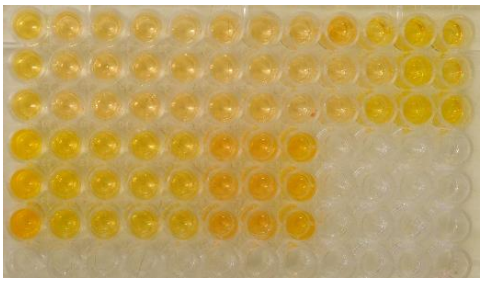
b | After 3 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.049	0.046	0.046	0.047	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046
B	0.078	0.049	0.048	0.047	0.046	0.047	0.047	0.047	0.047	0.047	0.047	0.047
C	0.047	0.048	0.047	0.047	0.573	0.346	0.361	0.288	0.282	0.295	0.366	2.495
D	0.047	0.047	0.047	0.049	0.571	0.361	0.348	0.295				
E	0.046	0.047	0.047	0.047	0.576	0.380	0.351	0.269				
F	0.458	0.470	0.363	0.194	0.161	0.199	0.204	0.182				
G												



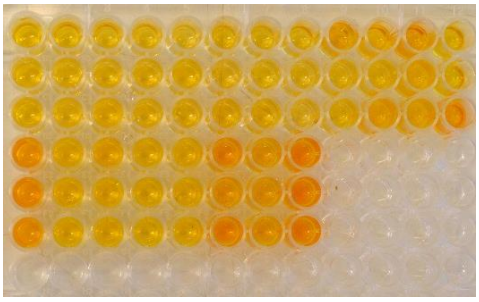
c | After 3 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.054	0.046	0.045	0.046	0.046	0.045	0.044	0.044	0.048	0.047	0.046	0.045
B	0.079	0.048	0.048	0.046	0.046	0.047	0.048	0.047	0.048	0.047	0.047	0.045
C	0.046	0.048	0.046	0.047	0.706	0.389	0.406	0.334	0.338	0.340	0.419	3.375
D	0.047	0.047	0.047	0.046	0.698	0.404	0.391	0.343				
E	0.045	0.045	0.047	0.047	0.708	0.427	0.393	0.313				
F	0.544	0.661	0.460	0.220	0.173	0.233	0.237	0.203				
G												



d | After 4 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.048	0.045	0.044	0.046	0.045	0.045	0.045	0.045	0.050	0.049	0.045	0.046
B	0.078	0.048	0.047	0.045	0.046	0.047	0.046	0.045	0.048	0.049	0.048	0.046
C	0.045	0.055	0.044	0.047	OVRFLW	1.880	OVRFLW	1.031	0.807	1.352	1.059	OVRFLW
D	0.046	0.046	0.046	0.044	OVRFLW	0.967	1.985	0.899				
E	0.045	0.046	0.047	0.045	OVRFLW	1.013	3.695	1.052				
F	OVRFLW	2.171	2.837	0.531	0.354	0.598	0.577	0.481				
G												



e | After 18 hours.

Figure 7.8.3.1 | Viability of *Gilliamella apicola* using WST-1 throughout 18 hours. On the left the optical density is recorded for each well at the specified time point and the associated photo log is on the right.

Chapter 8

Synthesis

8.1 Key findings of this thesis

The aim of this doctoral research was to characterise the composition and relative abundance of core gut bacteria in NZ honey bees, and to examine the effect of environmental/external factors that influence the bee host and how these affect bacteria in the honey bee gut. The response of the less dominant bacteria as potential indicators of poor health in honey bees was also a secondary focus.

After setting the scene in ***Chapter 1*** and defining the methodology in ***Chapter 2*****,** ***Chapter 3*** establishes the first characterisation of the bacterial community in the gut of NZ honey bees. Bees from 21 apiaries spread throughout seven NZ regions contained 27 unique phylotypes with > 0.01% relative abundance. These included the five dominant core bacteria identified in bees internationally. Eight phylotypes were only present in colonies deemed sick by beekeepers, two of which have potential as indicators of poor bee health: the genera *Serratia* and *Acetobacter*. The family Rhizobiaceae was also a promising indicator as it was present in 100% of the sick colonies but only 27.5% of the healthy colonies from the same apiaries. The gut pathogen *Nosema apis* was also associated with each sick colony but *Nosema ceranae* was only present in one colony from the Nelson region. This suggests that the observations of the beekeepers may have been consistently in response to *N. apis* but not *N. ceranae*. To date the newly introduced *N. ceranae* has not outcompeted *N. apis* in NZ, as has occurred internationally (Paxton et al. 2007). The environmental measures associated with each apiary appeared to influence the bacterial composition, in particular bees foraging on 'Native bush' and 'mānuka' were distinct from those foraging on introduced floral resources.

Throughout the analysis in ***Chapter 3***, it became clear that assimilating reliable taxonomic information for recently characterised bacteria within the honey bee gut was problematic. I sought to develop a customised 16S rRNA BLAST database compatible with QIIME2. This was achieved in combination with my supervisor, Associate Professor Patrick Biggs.

NZ honey bees have been isolated from international bees since 1960 (Stevenson et al. 2005). ***Chapter 4*** compares the gut bacterial composition of NZ bees with the gut composition in honey bees from Connecticut, USA. The intermingled presence of the dominant core bacteria in all 84 NZ colonies and the two USA colonies not only corroborates the theory that these dominant core bacteria are internationally widespread, but that they have remained stable within an isolated population for over 60 years. This highlights the importance of the symbiotic relations that these gut bacteria have with honey bees and provides an opportunity to exploit the bee as a model for human health. This chapter reveals that genera *Shewanella* and *Halomonas* were present only in the USA samples and nine phylotypes were present only in the NZ samples. This variation indicates that dysbiosis may have occurred or that the

phylotypes may have systemically adapted to NZ conditions to result in a unique NZ bee bacterial fingerprint. The path that led to this variation is unclear but this creates a model system for studying divergent bacterial populations within a host. Furthermore, given that we only conducted this study to identify the differences between bacteria in two geographically isolated honey bee populations, metagenomics studies were not conducted. Since the functional and metabolic capacity of the phylotype (i.e. what they do) is more important than the community composition (i.e. who they are), further studies are required to fully understand and characterise the effect of the geographic isolation on the health of NZ honey bees.

In the bee literature this is the first example showing that the different hypervariable regions alter the number of ASVs and the relative abundances observed for the same DNA. This highlights the importance for comparing data from DNA extracted using similar methodologies to advance this field of research.

Chapter 5 identified that the gutbacterial composition of bees from five hives varied throughout a 12 month period. The bacterial composition in summer bees was the most diverse, autumn and winter bees had lesser diversity, and spring bees had the least diversity. This may suggest that the increased bee population in spring may result in a cleansing of less prevalent bacteria for the year ahead. On average, the relative abundance of the five dominant core bacteria did not alter throughout the year. However, the relative abundance of *G. apicola* and *S. alvi* did alter within individual bees throughout the year suggesting that these species may alter their abundance in response to occurrences within the gut and this may ultimately aid bee functionality. The variation in bacterial composition observed in this chapter was used to guide the sample size and age structure of bees for the remaining experimental studies; a minimum of 20 bees were used in each pooled sample and the trials used only spring bees, or summer bees, or autumn bees, as this limited the age of the sampled bees (< 38 days). The relative abundance of the less prevalent phylotypes altered throughout the seasons with Rhizobiaceae abundance peaking in winter when bees often have elevated pathogen levels due to their increased longevity. In particular Rhizobiaceae exceeded the relative abundance of all dominant core phylotypes, except *Lactobacillus* spp. This evidence adds weight to my theory that Rhizobiaceae may be a useful early indicator of poor bee health. Further studies that characterise the effects of these inconsistently present bacteria would determine the validity of this cleansing theory.

In ***Chapter 6*** I showed that supplementary carbohydrates often fed to bees during dearth periods, such as winter are not equivalent food sources to honey or nectar. Sucrose-rich diets increased the relative abundances of three sub-dominant core bacteria, Rhizobiaceae, Acetobacteraceae, and *Lactobacillus kunkeei*, and decreased the relative abundance of the core species *Frischella perrara*. In combination, these significantly altered the bacterial composition. Acetogenic bacteria from the Rhizobiaceae and Acetobacteraceae families increased two- to five-fold when bees

were fed sucrose, suggesting that sucrose fuels the proliferation of specific low-abundance primary sucrose-feeders. This increase in organic acids that fuel host metabolism has been shown to impact host physiology and body weight in other studies with honey bees.

The effect of the gut pathogen *N. apis* on the development of *G. apicola*, the outer layer of the biofilm in the luminal surface of the honey bee ileum, was explored in ***Chapter 7***. *N. apis* did not disrupt the development of *G. apicola*. The gut slurry used to inoculate NEWs in this chapter increased the number of bacterial phylotypes in these NEWs, thus supporting the limited literature that NEWs acquire gut bacteria from older worker bees. This study also confirms that NEWs are not axenic when they emerge from their cells as their guts contain low levels of *G. apicola*, *S. alvi*, *L. apis*, *L. mellis*, *Lactobacillus* spp., *Bifidobacterium* spp., *Serratia* spp., *Acetobacter* spp., Rhizobiaceae, and Cyanobacteria. Finally, this chapter also identified a correlation between the lack of abundant bacteria in the honey bee gut with an increase in the opportunistic colonising bacteria Rhizobiaceae and Serratia. This is further evidence in support of the evolving theory from Chapters 3, 4, 5 and 6 that Rhizobiaceae is an opportunistic bacteria that may be a useful indicator of poor bee health.

8.2 Future research opportunities

Although the trials within this PhD research were intended to be conducted sequentially to modify the lines of enquiry as required, the time required to understand and code the bioinformatics to produce these results prohibited this. The trials were therefore conducted concurrently. However, the data from these chapters provide powerful evidence of association among bacteria in the honey bee gut and bee health. Similarly, bee diet influences the gut bacteriome and metabolism, demonstrating the inter-kingdom dependencies that are key to microbial survival and host health.

We are in the initial stages of understanding the intricacies of the bacterial community within the honey bee gut, and how gut conditions could be manipulated to support bacteria associated with healthy bees. My thesis contributes to this picture by showing that the presence of several less prevalent bacteria, particularly Rhizobiaceae, occur consistently in the gut of honey bees with compromised gut bacteria. Rhizobiaceae was present in bees not only deemed sick by beekeepers, or in old winter bees, but in bees challenged by the supplementary feed sucrose (as opposed to those fed honey), as well as the gut pathogen *N. apis*. This suggests the Rhizobiaceae family may be opportunistic bacteria that occur when the gut composition is compromised. This concept could be developed further as a useful early indicator of poor colony health.

To date, most experimental studies regarding gut bacteria are conducted using individual or caged honey bees. However, this may not always reflect what transpires within a commercial-sized colony. The collaborative nature of the colony, the

consumption of propolis containing antibiotic properties, and the self-removal by sick bees may partially explain why entire colonies do not succumb to challenge trials. The effects of external factors, such as food resources and pathogen loading on less prevalent gut bacteria, including Rhizobiaceae, should be conducted using commercial-sized colonies. Further studies should also be conducted to determine the conditions that favour and inhibit the proliferation of less prevalent bacteria, and their potential for ensuring bee health.

The effect of supplementary feeding with sucrose, glucose and other carbohydrates on the metabolism of honey bees is as yet unknown, but will be of great interest to the beekeeping industry which routinely practises supplementary carbohydrate feeding. Compounding the effects of supplementary sugars on bee gut bacteria and metabolism is the lack of nutrients which are abundantly present in natural honey. This lack of nutrients may therefore reduce the health of honey bees and potentially their resilience to pathogens. Sucrose inhibits bacteria proliferation *in vitro* so potentially the feeding of sucrose solutions throughout winter and in spring may impact the bacterial profile within colonies, possibly altering the digestion of nutrients.

The role of bacteria in the digestion of pollen is of interest academically, as well as in practice as beekeepers regularly feed pollen supplements at significant cost, yet adult workers may not be able to fully utilise them. The association between nutrient utilisation and honey bees should therefore be further studied.

References

Actor JK. 2012. Elsevier's Integrated Review: Immunology and Microbiology (2nd ed.). Philadelphia, Pennsylvania, United States of America: Elsevier Saunders.

Adams CJ, Manley-Harris M, Molan PC. 2009. The origin of methylglyoxal in New Zealand mānuka (*Leptospermum scoparium*) honey. Carbohydrate Research. 344(8): 1050-3.

Ahn J-H, Hong I-P, Bok J-I, Kim B-Y, Song J, Weon H-Y. 2012. Pyrosequencing analysis of the bacterial communities in the guts of honey bees *Apis cerana* and *Apis mellifera* in Korea. Journal of Microbiology. 50(5): 735-745.

Ahn J, Sinha R, Pei Z, Dominianni C, Wu J, Shi J, Goedert JJ, Hayes RB, Yang L. 2013. Human gut microbiome and risk for colorectal cancer. Journal of the National Cancer Institute. 105(24): 1907-1911.

Aizen MA, Harder LD. 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current Biology. 19(11): 915-918.

Alaux C, Brunet J-L, Dussaubat C, Mondet F, Tchamitchan S, Cousin M, Brillard J, Baldy A, Belzunces LP, Le Conte Y. 2010. Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). Environmental Microbiology. 12(3): 774-782.

Alaux C, Crauser D, Pioz M, Saulnier C, Le Conte Y. 2014. Parasitic and immune modulation of flight activity in honey bees tracked with optical counters. Journal of Experimental Biology. 217(19): 3416-3424.

Alippi AM, Reynaldi FJ. 2006. Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources. Journal of Invertebrate Pathology. 91(3): 141-146.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25(17): 3389-3402.

Amdam GV, Hartfelder K, Norberg K, Hagen A, Omholt SW. 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? Journal of Economic Entomology. 97(3): 741-747.

Ament SA, Corona M, Pollock HS, Robinson GE. 2008. Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. Proceedings of the National Academy of Sciences. 105(11): 4226-4231.

Anderson DL, Giacon H. 1992. Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. Journal of Economic Entomology. 85(1): 47-51.

Anderson DL, Trueman JWH. 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Experimental & Applied Acarology. 24(3): 165-189.

Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, Walton A, Jones BM, 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): 83125.

Anderson KE, Rodrigues PA, Mott BM, Maes P, Corby-Harris V. 2016. Ecological succession in the honey bee gut: shift in *Lactobacillus* strain dominance during early adult development. Microbial Ecology. 71(4): 1008-1019.

Anderson KE, Ricigliano VA. 2017. Honey bee gut dysbiosis: a novel context of disease ecology. Current Opinion in Insect Science. 22: 125-132.

Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology. 26(1): 32-46.

Antúnez K, Martín-Hernández R, Prieto L, Meana A, Zunino P, Higes M. 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environmental Microbiology. 11(9): 2284-2290.

Aronesty E. 2011. ea-utils: Command-line tools for processing biological sequencing data; https://expressionanalysis.github.io/ea-utils/.

Arredondo D, Castelli L, Porrini MP, Garrido PM, Eguaras MJ, Zunino P, Antúnez K. 2018. *Lactobacillus kunkeei* strains decreased the infection by honey bee pathogens *Paenibacillus larvae* and *Nosema ceranae*. Beneficial Microbes. 9(2): 279-290.

Arrese EL, Soulages JL. 2010. Insect fat body: energy, metabolism, and regulation. Annual Review of Entomology. 55: 207-225.

Asama T, Arima TH, Gomi T, Keishi T, Tani H, Kimura Y, Tatefuji T, Hashimoto K. 2015. *Lactobacillus kunkeei* YB 38 from honeybee products enhances IgA production in healthy adults. Journal of Applied Microbiology. 119(3): 818-826.

Atrott J, Haberlau S, Henle T. 2012. Studies on the formation of methylglyoxal from dihydroxyacetone in manuka (*Leptospermum scoparium*) honey. Carbohydrate Research. 361: 7-11.

Atwal A, Goyal N. 1971. Infestation of honeybee colonies with *Tropilaelaps*, and its control. Journal of Apicultural Research. 10(3): 137-142.

Audisio CM, Torres MJ, Sabaté DC, Ibarguren C, Apella MC. 2011. Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut. Microbiological Research. 166(1): 1-13.

Audisio M, Benítez-Ahrendts M. 2011. *Lactobacillus johnsonii* CRL1647, isolated from *Apis mellifera* L. bee-gut, exhibited a beneficial effect on honeybee colonies. Beneficial Microbes. 2(1): 29-34.

Babendreier D, Joller D, Romeis J, Bigler F, Widmer F. 2007. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. Federation of European Microbiological Societies: Microbiology Ecology. 59(3): 600-610.

Baffoni L, Gaggìa F, Alberoni D, Cabbri R, Nanetti A, Biavati B, Di Gioia D. 2016. Effect of dietary supplementation of *Bifidobacterium* and *Lactobacillus* strains in *Apis mellifera* L. against *Nosema ceranae*. Beneficial Microbes. 7(1): 45-51.

Bailey L. 1952. The action of the proventriculus of the worker honeybee, *Apis mellifera* L. Journal of Experimental Biology. 29: 310-326.

Bailey L. 1953. The transmission of *Nosema* disease. Bee World. 24(9): 171-172.

Bailey L. 1962. Bee diseases. Rep Rothamstad exp Stan: 16–161.

Bailey L. 1963. The pathogenicity for honey-bee larvae of microorganisms associated with European foulbrood. Journal of Insect Pathology. 5(2): 198-205.

Bailey L, Gibbs A, Woods R. 1964. Sacbrood virus of the larval honey bee (*Apis mellifera* Linnaeus). Virology. 23(3): 425-429.

Bailey L. 1967. *Nosema apis* and dysentery of the honeybee. Journal of Apicultural Research. 6(3): 121-125.

Bailey L. 1969. The multiplication and spread of sacbrood virus of bees. Annals of Applied Biology. 63(3): 483-491.

Bailey L, Fernando E. 1972. Effects of sacbrood virus on adult honey bees. Annals of Applied Biology. 72(1): 27-35.

Baker H. 1982. Chemical constituents of nectar in relation to pollination mechanisms and phylogeny. Biochemical Aspects of Evolutionary Biology: 131-171.

Balvočiūtė M, Huson DH. 2017. SILVA, RDP, Greengenes, NCBI and OTT—how do these taxonomies compare? BMC genomics. 18(2): 114.

Bamrick JF. 1967. Resistance to American foulbrood in honey bees: VI. Spore germination in larvae of different ages. Journal of Invertebrate Pathology. 9(1): 30-34.

Barker R, Lehner L. 1972. A look at honey bee gut functions. American Bee Journal. 112: 336-338.

Barker RJ, Lehner Y. 1974. Influence of diet on sugars found by thin-layer chromatography in thoraces of honey bees, *Apis mellifera* L. Journal of Experimental Zoology. 188(2): 157-164.

Barker RJ, Lehner Y. 1978. Laboratory comparison of high fructose corn syrup, grape syrup, honey, and sucrose syrup as maintenance food for caged honey bees. Apidologie. 9(2): 111-116.

Bates D, Mächler M, Bolker B, Walker S. 2014. Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67(1): 1-48.

Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM. 2019. Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. mBio. 10(1): e02566-18.

Beetsma J. 1985. Feeding behaviour of nurse bees, larval food composition and caste differentiation in the honey bee (*Apis mellifera* L.). In: Holldobler B, Lindauer, M., ed. Fortschritte der Zoologie Experimental Ecology. Netherlands: Wageningen University and Research. p. 407-410.

Belal M, Al-Mariri A, Hallab L, Hamad I. 2013. Detection of *Cronobacter* spp.(formerly *Enterobacter sakazakii*) from medicinal plants and spices in Syria. The Journal of Infection in Developing Countries. 7(02): 082-089.

Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, Pérez-Sánchez J. 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (Sparus aurata). Aquaculture. 454: 8-18.

Benítez-Páez A, Gómez del Pulgar EM, Sanz Y. 2017. The glycolytic versatility of Bacteroides uniformis CECT 7771 and its genome response to oligo and polysaccharides. Frontiers in cellular and infection microbiology. 7: 383.

Biavati B, Vescovo M, Torriani S, Bottazzi V. 2000. Bifidobacteria: history, ecology, physiology and applications. Annals of Microbiology. 50(2): 117-132.

Biavati B, Mattarelli P. 2006. The family Bifidobacteriaceae. Prokaryotes: Springer. p. 322-382.

Biesmeijer JC, Roberts S, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers A, Potts S, Kleukers R, Thomas C. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science. 313(5785): 351-354.

Bishop GH. 1961. Growth rates of honey bee larva. Journal of Experimental Zoology. 146(1): 11-20.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F. 2019. Reproducible, interactive, scalable and

extensible microbiome data science using QIIME 2. Nature Biotechnology. 37(8): 852-857.

Bonilla-Rosso G, Engel P. 2018. Functional roles and metabolic niches in the honey bee gut microbiota. Current Opinion in Microbiology. 43: 69-76.

Botías C, Anderson DL, Meana A, Garrido-Bailón E, Martín-Hernández R, Higes M. 2012. Further evidence of an oriental origin for *Nosema ceranae* (Microsporidia: Nosematidae). Journal of Invertebrate Pathology. 110(1): 108-113.

Botías C, Martín-Hernández R, Barrios L, Meana A, Higes M. 2013. *Nosema* spp. infection and its negative effects on honey bees (*Apis mellifera iberiensis*) at the colony level. Veterinary Research. 44(1): 25.

Bottacini F, Milani C, Turroni F, Sánchez B, Foroni E, Duranti S, Serafini F, Viappiani A, Strati F, Ferrarini A. 2012. *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect gut. Plos One. 7(9): e44229.

Boucher Y, Douady CJ, Papke RT, Walsh DA, Boudreau MER, Nesbo CL, Case RJ, Doolittle WF. 2003. Lateral gene transfer and the origins of prokaryotic groups. Annual Review of Genetics. 37(1): 283-328.

Bradbear N. 1988. World distribution of major honeybee diseases and pests. Bee World. 69(1): 15-39.

Bradley T. 1985. The excretory system: structure and physiology. In Gilbert LI, Kerkut GA (ed), Regulation: digestion, nutrion, excretion Pergamon Press, Oxford, Uniuted Kingdom: 421-462.

Breed MD, Butler L, Stiller TM. 1985. Kin discrimination by worker honey bees in genetically mixed groups. Proceedings of the National Academy of Sciences. 82(9): 3058-3061.

Brewster JD. 2003. A simple micro-growth assay for enumerating bacteria. Journal of Microbiological Methods. 53(1): 77-86.

Broderick NA, Raffa KF, Goodman RM, 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.

Brodschneider R, Crailsheim K. 2010. Nutrition and health in honey bees. Apidologie. 41(3): 278-294.

Brown P, Robertson T. 2018. New Zealand Colony Loss Survey Report – 2017. New Zealand: Ministry for Primary Industries, https://www.mpi.govt.nz/growing-and-harvesting/honey-and-bees/bee-colony-loss-survey/

Brown SP, Cornforth DM, Mideo N. 2012. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends in Microbiology. 20(7): 336-342.

Bruininx E, Binnendijk G, Van der Peet-Schwering C, Schrama J, Den Hartog L, Everts H, Beynen A. 2002. Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs. Journal of Animal Science. 80(6): 1413-1418.

Brune A, Emerson D, Breznak JA. 1995. The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Applied and Environmental Microbiology. 61(7): 2681-2687.

Brune A. 1998. Termite guts: the world's smallest bioreactors. Trends in Biotechnology. 16(1): 16-21.

Budge GE, Adams I, Thwaites R, Pietravalle S, Drew GC, Hurst GD, Tomkies V, Boonham N, Brown M. 2016. Identifying bacterial predictors of honey bee health. Journal of Invertebrate Pathology. 141: 41-44.

Butchart SH, Walpole M, Collen B, Van Strien A, Scharlemann JP, Almond RE, Baillie JE, Bomhard B, Brown C, Bruno J. 2010. Global biodiversity: indicators of recent declines. Science. 328(5982): 1164-1168.

Calderone NW. 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992–2009. Plos One. 7(5): e37235.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods. 13(7): 581.

Cantwell G. 1970. Standard methods for counting *Nosema* spores. American Bee Journal. 110: 222-223.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 7(5): 335.

Cariveau DP, Powell JE, Koch H, Winfree R, Moran NA. 2014. Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). The ISME Journal.

Casteels-Josson K, Zhang W, Capaci T, Casteels P, Tempst P. 1994. Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. Journal of Biological Chemistry. 269(46): 28569-28575.

Cebotari V, Toderaş I, Buzu I, Rudic V. 2013. The role of "Apispir+ Zn" biostimulator in increasing of productivity of *Apis mellifera* bee colonies. University of Agricultural Sciences and Veterinary Medicine Iasi Scientific Papers Series Animal Science. 59(18): 103-107.

Cerenius L, Lee BL, Söderhäll K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology. 29(6): 263-271.

Chakravorty S, Helb D, Burday M, Connell N, Alland D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods. 69(2): 330-339.

Chalcoff VR, Aizen MA, Galetto L. 2005. Nectar concentration and composition of 26 species from the temperate forest of South America. Annals of Botany. 97(3): 413-421.

Chen C-Y, Yeh K-L, Aisyah R, Lee D-J, Chang J-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresource technology. 102(1): 71-81.

Chen Y, Evans JD. 2007. Historical presence of Israeli acute paralysis virus in the United States. American Bee Journal. 147(12): 1027-1028.

Chen Y, Evans JD, Smith IB, Pettis JS. 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. Journal of Invertebrate Pathology. 97(2): 186-188.

Chenoweth M, Somerville G, Krause D, O'Reilly K, Gherardini F. 2004. Growth characteristics of *Bartonella henselae* in a novel liquid medium: primary isolation, growth-phase-dependent phage induction, and metabolic studies. Applied and Environmental Microbiology. 70(2): 656-663.

Chepulis L, Francis E. 2013. The glycaemic index of mānuka honey. e-SPEN Journal. 8(1): e21-e24.

Chittka A, Chittka L. 2010. Epigenetics of royalty. Plos Biology. 8(11): e1000532.

Chuang Y-M, Tseng S-P, Teng L-J, Ho Y-C, Hsueh P-R. 2006. Emergence of cefotaxime resistance in *Citrobacter freundii* causing necrotizing fasciitis and osteomyelitis. Journal of Infection. 53(3): 161-163.

Clinch P. 1981. Rapid methods for orally dosing adult honey bees, individually or in groups, without anaesthetic. New Zealand Journal of Experimental Agriculture. 9(1): 27-30.

Colman DR, Toolson EC, Takacs-Vesbach C. 2012. Do diet and taxonomy influence insect gut bacterial communities? Molecular Ecology. 21(20): 5124-5137.

Corby-Harris V, Pontaroli AC, Shimkets LJ, Bennetzen JL, Habel KE, Promislow DE. 2007. Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. Applied and Environmental Microbiology. 73(11): 3470-3479.

Corby-Harris V, Maes P, Anderson KE. 2014a. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. Plos One. 9(4): e95056.

Corby-Harris V, Snyder LA, Schwan MR, Maes P, McFrederick QS, Anderson KE. 2014b. Origin and effect of Acetobacteraceae Alpha 2.2 in honey bee larvae and description of *Parasaccharibacter apium*, gen. nov., sp. nov. Applied and Environmental Microbiology: 2043-14.

Corby-Harris V, Snyder L, Meador C, Naldo R, Mott B, Anderson K. 2016. *Parasaccharibacter apium*, gen. nov., sp. nov., improves honey bee (Hymenoptera: Apidae) resistance to *Nosema*. Journal of Economic Entomology. 109(2): 537-543.

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P-L, Briese T, Hornig M, Geiser DM. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 318(5848): 283-287.

Cozmuta AM, Bretan L, Cozmuta LM, Nicula C, Peter A. 2012. Lead traceability along soil-melliferous flora-bee family-apiary products chain. Journal of Environmental Monitoring. 14(6): 1622-1630.

Crailsheim K. 1988. Intestinal transport of sugars in the honeybee (*Apis mellifera* L.). Journal of insect physiology. 34(9): 839-845.

Crailsheim K. 1990. The protein balance of the honey bee worker. Apidologie. 21(5): 417-429.

Crailsheim K. 1991. Interadult feeding of jelly in honeybee (*Apis mellifera* L.) colonies. Journal of Comparative Physiology B. 161(1): 55-60.

Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, Sacchi L, Bourtzis K, Mandrioli M, Cherif A. 2010. MiniReview: Acetic acid bacteria, newly emerging symbionts of insects. Applied and Environmental Microbiology. 76(21): 6963-6970.

Crotti E, Balloi A, Hamdi C, Sansonno L, Marzorati M, Gonella E, Favia G, Cherif A, Bandi C, Alma A. 2012. Microbial symbionts: a resource for the management of insect-related problems. Microbial Biotechnology. 5(3): 307-317.

Cummings J, Pomare E, Branch W, Naylor C, Macfarlane G. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 28(10): 1221-1227.

Currie RW, Pernal SF, Guzmán-Novoa E. 2010. Honey bee colony losses in Canada. Journal of Apicultural Research. 49(1): 104-106.

Dahle B. 2010. The role of *Varroa destructor* for honey bee colony losses in Norway. Journal of Apicultural Research. 49(1): 124-125.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 505(7484): 559.

Day S, Beyer R, Mercer A, Ogden S. 1990. The nutrient composition of honeybee-collected pollen in Otago, New Zealand. Journal of Apicultural Research. 29(3): 138-146.

de Boer R, Peters R, Gierveld S, Schuurman T, Kooistra-Smid M, Savelkoul P. 2010. Improved detection of microbial DNA after bead-beating before DNA isolation. Journal of Microbiological Methods. 80(2): 209-211.

De Filippis F, Pellegrini N, Laghi L, Gobbetti M, Ercolini D. 2016. Unusual sub-genus associations of faecal Prevotella and Bacteroides with specific dietary patterns. Microbiome. 4(1): 57.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences. 107(33): 14691-14696.

de Groot AP. 1953. Protein and amino acid requirements of the honeybee (*Apis mellifica* L.). In: 67th Communication of the Research Institute for Animal Husbandry ed. Utrecht: Laboratory of Comparative Physiology.

DeGrandi-Hoffman G, Chen Y, Huang E, Huang MH. 2010. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). Journal of insect physiology. 56(9): 1184-1191.

Delfinado MD, Baker EW. 1961. *Tropilaelaps*, a new genus of mite from the Philippines (Laelaptidae (s. Lat.]: Acarina). Fieldiana Zoology. Chicago: Chicago Natural History Museum. p. 53-56.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Research. 45(W1): W180-W188.

Dharne M, Patole M, Shouche YS. 2006. Microbiology of the insect gut: tales from mosquitoes and bees. Journal of biosciences. 31(3): 293-295.

Dillon R, Dillon V. 2004. The gut bacteria of insects: nonpathogenic interactions. Annual Reviews in Entomology. 49(1): 71-92.

Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM, Furlan M, Desnues C, Haynes M, Li L. 2008. Functional metagenomic profiling of nine biomes. Nature. 452(7187): 629.

Disayathanoowat T, Young JPW, Helgason T, Chantawannakul P. 2012. T-RFLP analysis of bacterial communities in the midguts of *Apis mellifera* and *Apis cerana* honey bees in Thailand. Federation of European Microcrobiological Societies: Microbiology Ecology. 79(2): 273-281.

Dively GP, Embrey MS, Kamel A, Hawthorne DJ, Pettis JS. 2015. Assessment of chronic sublethal effects of imidacloprid on honey bee colony health. Plos One. 10(3): e0118748.

Doner LW. 1977. The sugars of honey—a review. Journal of the Science of Food and Agriculture. 28(5): 443-456.

Downey DL, Winston ML. 2001. Honey bee colony mortality and productivity with single and dual infestations of parasitic mite species. Apidologie. 32(6): 567-575.

Durbán A, Abellán JJ, Jiménez-Hernández N, Salgado P, Ponce M, Ponce J, Garrigues V, Latorre A, Moya A. 2012. Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome. Environmental microbiology reports. 4(2): 242-247.

Egert M, Wagner B, Lemke T, Brune A, Friedrich MW. 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Applied and Environmental Microbiology. 69(11): 6659-6668.

Eiri DM, Suwannapong G, Endler M, Nieh JC. 2015. *Nosema ceranae* can infect honey bee larvae and reduces subsequent adult longevity. Plos One. 10(5): e0126330.

El Sanousi S, El Sarag M, Mohamed SE. 1987. Properties of *Serratia marcescens* isolated from diseased honeybee (*Apis mellifera*) larvae. Microbiology. 133(1): 215-219.

Elbagoury EFA, Ramsy S. 1993. Antibacterial action of natural honey on anaerobic bacteroides. Egyptian Dental Journal. 39(1): 381-386.

Elmqvist T, Folke C, Nyström M, Peterson G, Bengtsson J, Walker B, Norberg J. 2003. Response diversity, ecosystem change, and resilience. Frontiers in Ecology and the Environment. 1(9): 488-494.

Emsen IM. 2007. A different and safe method of split thickness skin graft fixation: Medical honey application. Burns. 33(6): 782-787.

Endo A, Irisawa T, Futagawa-Endo Y, Takano K, du Toit M, Okada S, Dicks LM. 2012. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. International Journal of Systematic and Evolutionary microbiology. 62(3): 500-504.

Engel MS. 1999. The taxonomy of recent and fossil honey bees (Hymenoptera: Apidae; *Apis*). Journal of Hymenoptera Research. 8(2): 165-196.

Engel P, Martinson VG, Moran NA. 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 RR, Koga R, Kwong WK, McFrederick QS, Moran NA. 2013a. Standard methods for research on *Apis mellifera* gut symbionts. Journal of Apicultural Research. 52(4): 1-24.

Engel P, Kwong WK, Moran NA. 2013b. *Frischella perrara* gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honeybee, *Apis mellifera*. International Journal of Systematic and Evolutionary Microbiology. 63: 3646-3651.

Engel P, Moran NA. 2013a. Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. Gut Microbes. 4(1): 60-65.

Engel P, Moran NA. 2013b. The gut microbiota of insects–diversity in structure and function. Federation of European Microbiological Societies: Microbiology Reviews. 37(5): 699-735.

Engel P, Bartlett KD, Moran NA. 2015. The bacterium *Frischella perrara* causes scab formation in the gut of its honeybee host. mBio. 6(3): e00193-15.

Engel P, Kwong WK, McFrederick Q, Anderson KE, Barribeau SM, Chandler JA, Cornman RS, Dainat J, de Miranda JR, Doublet V. 2016. The bee microbiome: Impact on bee health and model for evolution and ecology of host-microbe interactions. mBio. 7(2): e02164-15.

Evans JD, Lopez DL. 2004. Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology. 97(3): 752-756.

Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R. 2009. Colony collapse disorder: a descriptive study. Plos One. 4(8): e6481.

Evans JD, Schwarz RS. 2011. Bees brought to their knees: microbes affecting honey bee health. Trends in Microbiology. 19(12): 614-620.

Fahrenholz L, Lamprecht I, Schricker B. 1989. Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. Journal of Comparative Physiology B. 159(5): 551-560.

Ferreira L, Sánchez-Juanes F, García-Fraile P, Rivas R, Mateos PF, Martínez-Molina E, González-Buitrago JM, Velázquez E. 2011. MALDI-TOF mass spectrometry is a fast and reliable platform for identification and ecological studies of species from family Rhizobiaceae. Plos One. 6(5): e20223.

Forsgren E, Fries I. 2010. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. Veterinary parasitology. 170(3-4): 212-7.

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

Fox R. 2013. The decline of moths in Great Britain: a review of possible causes. Insect Conservation and Diversity. 6(1): 5-19.

Free J. 1961. Hypopharyngeal gland development and division of labour in honey-bee (*Apis mellifera* L.) colonies. Proceedings of the Royal Entomological Society of London Series A, General Entomology, vol 36. Wiley Online Library. p. 5-8.

Free JB. 1970. Insect Pollination of Crops. London, New York: Academic Press.

Free JB. 1993. Insect pollination of crops (2nd ed.). UK: Academic Press.

Frias BED, Barbosa CD, Lourenço AP. 2016. Pollen nutrition in honey bees (*Apis mellifera*): impact on adult health. Apidologie. 47(1): 15-25.

Fridman S, Izhaki I, Gerchman Y, Halpern M. 2012. Bacterial communities in floral nectar. Environmental Microbiology Reports. 4(1): 97-104.

Fries I. 1988a. Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. Apidologie. 19(3): 319-328.

Fries I. 1988b. Comb replacement and *Nosema* disease (*Nosema apis* Z.) in honey bee colonies. Apidologie. 19(4): 343-354.

Fries I, Feng F, da Silva A, Slemenda SB, Pieniazek NJ. 1996. *Nosema ceranae* n. sp.(Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). European Journal of Protistology. 32(3): 356-365.

Fries I, Chauzat M-P, Chen Y-P, Doublet V, Genersch E, Gisder S, Higes M, McMahon DP, Martín-Hernández R, Natsopoulou M. 2013. Standard methods for *Nosema* research. Journal of Apicultural Research. 52(1): 1-28.

Fukuda H, Sekiguchi K. 1966. Seasonal change of the honeybee worker longevity in Sapporo, North Japan, with notes on some factors affecting the life-span. Japanese Journal of Ecology. 16(5): 206-212.

Fukuda H, Ohtani T. 1977. Survival and life span of drone honeybees. Population Ecology. 19(1): 51-68.

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

Gary NE. 1992. Activities and behaviour of honey bees. In: Graham JM, ed. The hive and the honey bee, revised edition. Hamilton, Illinois: Dadant & Sons. p. 269-372.

Gasper J, Terentjeva M, Kántor A, Ivanišová E, Kluz M, Kačániová M. 2017. Identification of *Apis mellifera* gut microbiota with MALDI-TOF MS Biotyper. Scientific Papers: Animal Science & Biotechnologies. 50(1): 192-196.

Geddes BA, Oresnik IJ. 2016. The mechanism of symbiotic nitrogen fixation. In: The Mechanistic Benefits of Microbial Symbionts, Advances in Environmental Microbiology, vol 2. Cham. Switzerland: Springer International Publishing

Geneious http://www.geneious.com. Version 10.0.9.

George J, Bhakthan N. 1963. *In vitro* lipase activity and the oxidation of butyrate by the honey bee flight muscle. Journal of insect physiology. 9(3): 311-315.

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.

Gilliam M, Valentine DK. 1976. Bacteria isolated from the intestinal contents of foraging worker honey bees, *Apis mellifera*: the genus *Bacillus*. Journal of Invertebrate Pathology. 28(2): 275-276.

Gilliam M, Buchmann S, Lorenz B, Schmalzel R. 1990. Bacteria belonging to the genus *Bacillus* associated with three species of solitary bees. Apidologie. 21(2): 99-105.

Gilliam M. 1993. Chalkbrood control. Asian Apiculture, Wicwas Press, Cheshire, USA: 589-595.

Gilliam M. 1997. Identification and roles of non-pathogenic microflora associated with honey bees. Federation of European Microbiological Societies: Microbiology Letters. 155(1): 1-10.

Gmeinbauer R, Crailsheim K. 1993. Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. Journal of insect physiology. 39(11): 959-967.

Good AP, Gauthier M-PL, Vannette RL, Fukami T. 2014. Honey bees avoid nectar colonized by three bacterial species, but not by a yeast species, isolated from the bee gut. Plos One. 9(1): e86494.

Goodwin R, Taylor M, McBrydie H, Cox H. 2005. Base levels of resistance to common control compounds by a New Zealand population of *Varroa destructor*. New Zealand Journal of Crop and Horticultural Science. 33(4): 347-352.

Goodwin RM, Taylor MA. 2008. Control of varroa: A guide for New Zealand Beekeepers. New Zealand: H & A Design & Print.

Gordon R, Haynes W, Pang CH-N. 1973. The genus *Bacillus*. Washington, USA: Department of Agriculture.

Goulson D, Nicholls E, Botías C, Rotheray EL. 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347(6229).

Graham JM. 1992. The hive and the honey bee: Dadant & Sons.

Grange JM, Davey RW. 1990. Antibacterial properties of propolis (bee glue). Journal of the Royal Society of Medicine. 83: 159-160.

Guarner F. 2005. The intestinal flora in inflammatory bowel disease: normal or abnormal? Current Opinion in Gastroenterology. 21(4): 414-418.

Guzmán-Novoa E, Eccles L, Calvete Y, Mcgowan J, Kelly PG, Correa-Benítez A. 2010. *Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada. Apidologie. 41(4): 443-450.

Hamdi C, Balloi A, Essanaa J, Crotti E, Gonella E, Raddadi N, Ricci I, Boudabous A, Borin S, Manino A. 2011. Gut microbiome dysbiosis and honeybee health. Journal of Applied Entomology. 135(7): 524-533.

Hammes WP, Hertel C. 2006. The genera *Lactobacillus* and *Carnobacterium*. The prokaryotes: Springer. p. 320-403.

Harrison JM. 1986. Caste-specific changes in honeybee flight capacity. Physiological Zoology. 59(2): 175-187.

Haydak MH. 1968. Nutrition des larves d'abeilles. In: R. C, ed. Traité de biologie de l'abeille. Paris: Masson et Cie. p. 302-333.

Haydak MH. 1970. Honey bee nutrition. Annual Review of Entomology. 15(1): 143-156.

Heath L. 1982. Development of chalk brood in a honeybee colony: a review. Bee World. 63(3): 119-130.

Herbert E, Bickley W, Shimanuki H. 1970. The brood-rearing capability of caged honey bees fed dandelion and mixed pollen diets. Journal of Economic Entomology. 63(1): 215-218.

Hermans SM, Buckley HL, Lear G. 2018. Optimal extraction methods for the simultaneous analysis of DNA from diverse organisms and sample types. Molecular Ecology Resources. 18(3): 557-569.

Higes M, García-Palencia P, Martín-Hernández R, Meana A. 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). Journal of Invertebrate Pathology. 94(3): 211-217.

Higes M, García-Palencia P, Urbieta A, Nanetti A, Martín-Hernández R. 2019. *Nosema apis* and *Nosema ceranae* tissue tropism in worker honey bees (*Apis mellifera*). Veterinary Pathology: 0300985819864302.

Highfield AC, El Nagar A, Mackinder LC, Laure M-LN, Hall MJ, Martin SJ, Schroeder DC. 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology. 75(22): 7212-7220.

Hleba L, Petrová J, Cubon J, Chlebo R, Kántor A, Kacániová M. 2014. Identification and antibiotic resistance profile of Enterobacteriaceae species and *Lactobacillus* spp. isolated from honey bees (*Apis mellifera*) digestive tract. The Journal of Microbiology, Biotechnology and Food Sciences. 3(1): 304-307.

Hoffmann JA. 2003. The immune response of *Drosophila*. Nature. 426(6962): 33-38.

Hopkins I. 1926. Practical beekeeping: being the 6th Edition of the Australasian Bee Manual: Whitcombe & Tombs.

Huang W-F, Bocquet M, Lee K-C, Sung I-H, Jiang J-H, Chen Y-W, Wang C-H. 2008. The comparison of rDNA spacer regions of *Nosema ceranae* isolates from different hosts and locations. Journal of Invertebrate Pathology. 97(1): 9-13.

Huang W-F, Solter LF. 2013. Comparative development and tissue tropism of *Nosema apis* and *Nosema ceranae*. Journal of Invertebrate Pathology. 113(1): 35-41.

Huang Z. 2010. Honey bee nutrition. American Bee Journal. 150(8): 773-776.

Human H, Nicolson SW. 2006. Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. *davyana* (Asphodelaceae). Phytochemistry. 67(14): 1486-1492.

Illumina. 2017. An introduction to Next-Generation Sequencing Technology.

Jack RW, Tagg JR, Ray B. 1995. Bacteriocins of Gram-positive bacteria. Microbiological Reviews. 59(2): 171-200.

Jay S, Jay D. 1993. The effect of kiwifruit (*Actinidia deliciosa* A Chev) and yellow flowered broom (*Cytisus scoparius* Link) pollen on the ovary development of worker honey bees (*Apis mellifera* L). Apidologie. 24(6): 557 - 563.

Jay SC. 1963. The development of honeybees in their cells. Journal of Apicultural Research. 2(2): 117-134.

Jeyaprakash A, Hoy MA, Allsopp MH. 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.

Johnson BR. 2008. Within-nest temporal polyethism in the honey bee. Behavioral Ecology and Sociobiology. 62(5): 777-784.

Jones J, Fruciano C, Marchant J, Hildebrand F, Forslund S, Bork P, Engel P, Hughes W. 2018a. The gut microbiome is associated with behavioural task in honey bees. Insectes Sociaux. 65(3): 419-429.

Jones JC, Fruciano C, Hildebrand F, Al Toufalilia H, Balfour NJ, Bork P, Engel P, Ratnieks FL, Hughes WO. 2018b. Gut microbiota composition is associated with environmental landscape in honey bees. Ecology and Evolution. 8(1): 441-451.

Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. 2011. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 60(5): 631-637.

Kakumanu ML, Reeves AM, Anderson TD, Rodrigues RR, Williams MA. 2016. Honey bee gut microbiome is altered by in-hive pesticide exposures. Frontiers in Microbiology. 7: 1255.

Kalapos MP. 2008. The tandem of free radicals and methylglyoxal. Chemico-biological interactions. 171(3): 251-271.

Kandler O. 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek. 49(3): 209-224.

Kapheim KM, Rao VD, Yeoman CJ, Wilson BA, White BA, Goldenfeld N, Robinson GE. 2015. Caste-specific differences in hindgut microbial communities of honey bees (*Apis mellifera*). Plos One. 10(4): e0123911.

Keller I, Fluri P, Imdorf A. 2005. Pollen nutrition and colony development in honey bees: part 1. Bee World. 86(1): 3-10.

Kersters K, Lisdiyanti P, Komagata K, Swings J. 2006. The family acetobacteraceae: the genera *acetobacter*, *acidomonas*, *asaia*, *gluconacetobacter*, *gluconobacter*, and *kozakia*. The prokaryotes: Springer. p. 163-200.

Kešnerová L, Moritz R, Engel P. 2016. *Bartonella apis* sp. nov., a honey bee gut symbiont of the class Alphaproteobacteria. International Journal of Systematic and Evolutionary Microbiology. 66(1): 414-421.

Kešnerová L, Mars RAT, Ellegaard KM, Troilo M, Sauer U, Engel P. 2017. Disentangling metabolic functions of bacteria in the honey bee gut. Plos Biology. 15(12): e2003467.

Khan KA, Ansari MJ, Al-Ghamdi A, Nuru A, Harakeh S, Iqbal J. 2017. Investigation of gut microbial communities associated with indigenous honey bee (*Apis mellifera jemenitica*) from two different eco-regions of Saudi Arabia. Saudi Journal of Biological Sciences. 24(5): 1061-1068.

Killer J, Kopečný J, Mrázek J, Rada V, Benada O, Koppová I, Havlík J, Straka J. 2009. *Bifidobacterium bombi* sp. nov., from the bumblebee digestive tract. International Journal of Systematic and Evolutionary Microbiology. 59(8): 2020-2024.

Killer J, Dubná S, Sedláček I, Švec P. 2014. *Lactobacillus apis* sp. nov., from the stomach of honeybees (*Apis mellifera*), having an *in-vitro* inhibitory effect on the causative agents of American and European foulbrood. International Journal of Systematic and Evolutionary Microbiology. 64(1): 152-157.

Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P. 2007. Widespread dispersal of the microsporidian *Nosema*

ceranae, an emergent pathogen of the western honey bee, *Apis mellifera*. Journal of Invertebrate Pathology. 96(1): 1-10.

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences. 274(1608): 303-313.

Klein AM, Steffan–Dewenter I, Tscharntke T. 2003. Fruit set of highland coffee increases with the diversity of pollinating bees. Proceedings of the Royal Society B: Biological Sciences. 270(1518): 955-961.

Kleinschmidt G, Kondos A. 1976. Influence of crude protein levels on colony production. Australasian Beekeeper. 80: 251–257.

Knecht D, Kaatz H. 1990. Patterns of larval food production by hypopharyngeal glands in adult worker honey bees. Apidologie. 21(5): 457-468.

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

Koch H, Schmid-Hempel P. 2011b. Bacterial communities in central European bumblebees: low diversity and high specificity. Microbial Ecology. 62(1): 121-133.

Koch H, Schmid-Hempel P. 2012. Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. Ecology Letters. 15(10): 1095-1103.

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

Koeniger G, Koeniger N, Fabritius M. 1979. Some detailed observations of mating in the honeybee. Bee World. 60(2): 53-57.

Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, Hallen A, Martens E, Björck I, Bäckhed F. 2015. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. Cell metabolism. 22(6): 971-982.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology. 79(17): 5112-5120.

Kucharski R, Maleszka J, Foret S, Maleszka R. 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science. 319(5871): 1827-1830.

Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. 2012. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Current Protocols in Microbiology. 27(1): 1E.5.1-1E.5.20.

Kurstjens S, Hepburn H, Schoening F, Davidson B. 1985. The conversion of wax scales into comb wax by African honeybees. Journal of Comparative Physiology B. 156(1): 95-102.

Kuzina LV, Peloquin JJ, Vacek DC, Miller TA. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). Current Microbiology. 42(4): 290-294.

Kwakman PH, Van den Akker JP, Güçlü A, Aslami H, Binnekade JM, de Boer L, Boszhard L, Paulus F, Middelhoek P, te Velde AA, Vandenbroucke-Grauls CM, Schultz MJ, Zaat SA. 2008. Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. Clinical Infectious Diseases. 46(11): 1677-82.

Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, Zaat SA. 2010. How honey kills bacteria. Federation of American Societies: Experimental Biology. 24(7): 2576-2582.

Kwong WK, Moran NA. 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. International Journal of Systematic and Evolutionary Microbiology. 63: 2008-2018.

Kwong WK, Engel P, Koch H, Moran NA. 2014. Genomics and host specialization of honey bee and bumble bee gut symbionts. Proceedings of the National Academy of Sciences. 111(31): 11509-11514.

Kwong WK, Moran NA. 2015. Evolution of host specialization in gut microbes: the bee gut as a model. Gut Microbes. 6(3): 214-220.

Kwong WK, Moran NA. 2016a. Gut microbial communities of social bees. Nature Reviews Microbiology. 14(6): 374-384.

Kwong WK, Moran NA. 2016b. *Apibacter adventoris* gen. nov., sp. nov., a member of the phylum Bacteroidetes isolated from honey bees. International Journal of Systematic and Evolutionary Microbiology. 66(3): 1323-1329.

Kwong WK, Mancenido AL, Moran NA. 2017. Immune system stimulation by the native gut microbiota of honey bees. Royal Society Open Science. 4(2): 170003.

Lai KK. 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults — Case reports and a review of the literature. Medicine. 80: 113-122.

Lambert B, Kersters K, Gossele F, Swings J, De Ley J. 1981. *Gluconobacters* from honey bees. Antonie Van Leeuwenhoek. 47(2): 147-157.

Langstroth LL, Dadant CP. 1922. Langstroth, on the Hive & Honey Bee: American bee journal.

Law CJ, Maloney PC, Wang D-N. 2008. Ins and outs of major facilitator superfamily antiporters. Annual Review of Microbiology. 62: 289-305.

Lee FJ, Rusch DB, Stewart FJ, Mattila HR, Newton IL. 2015. Saccharide breakdown and fermentation by the honey bee gut microbiome. Society for Applied Microbiology: Environmental Microbiology. 17(3): 796-815.

Lee FJ, Miller KI, McKinlay JB, Newton IL. 2018. Differential carbohydrate utilization and organic acid production by honey bee symbionts. FEMS Microbiology Ecology. 94(8): fiy113.

Lim HC, Chu C-C, Seufferheld MJ, Cameron SA. 2015. Deep sequencing and ecological characterization of gut microbial communities of diverse bumble bee species. Plos One. 10(3): e0118566.

Livermore DM. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clinical Infectious Diseases. 34(5): 634-640.

Lopez MI, Pettis JS, Smith IB, Chu P-S. 2008. Multiclass determination and confirmation of antibiotic residues in honey using LC-MS/MS. Journal of Agricultural and Food Chemistry. 56(5): 1553-1559.

Ludvigsen J, Rangberg A, Avershina E, Sekelja M, Kreibich C, Amdam G, Rudi K. 2015. Shifts in the midgut/pyloric microbiota composition within a honey bee apiary throughout a season. Microbes and Environments. 30(3): 235.

Ludvigsen J, Porcellato D, L'Abée-Lund TM, Amdam GV, Rudi K. 2017. Geographically widespread honeybee-gut symbiont subgroups show locally distinct antibiotic-resistant patterns. Molecular Ecology. 26(23): 6590-6607.

Ludvigsen J, Porcellato D, Amdam GV, Rudi K. 2018. Addressing the diversity of the honeybee gut symbiont *Gilliamella*: description of *Gilliamella apis* sp. nov., isolated from the gut of honeybees (*Apis mellifera*). International Journal of Systematic and Evolutionary Microbiology. 68: 1762-1770.

Luo D, Ganesh S, Koolaard J. 2018. Package 'predictmeans'. See https://CRANR-projectorg/package=predictmeans.

Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. 2007. Host-mediated inflammation disrupts the intestinal microbiota and

promotes the overgrowth of Enterobacteriaceae. Cell Host and Microbe. 2(2): 119-129.

Lyapunov YE, Kuzyaev R, Khismatullin R, Bezgodova O. 2008. Intestinal enterobacteria of the hibernating *Apis mellifera mellifera* L. bees. Microbiology. 77(3): 373-379.

Maciel-Vergara G, Jensen AB, Eilenberg J. 2018. Cannibalism as a possible entry route for opportunistic pathogenic bacteria to insect hosts, exemplified by *Pseudomonas aeruginosa*, a pathogen of the giant mealworm *Zophobas morio*. Insects. 9(3): 88.

Maes PW, Rodrigues PA, Oliver R, Mott BM, Anderson KE. 2016. Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and *Nosema* disease in the honeybee (*Apis mellifera*). Molecular Ecology. 25(21): 5439-5450.

Majtan J, Klaudiny J, Bohova J, Kohutova L, Dzurova M, Sediva M, Bartosova M, Majtan V. 2012. Methylglyoxal-induced modifications of significant honeybee proteinous components in mānuka honey: possible therapeutic implications. Fitoterapia. 83(4): 671-677.

Maleszka R. 2008. Epigenetic integration of environmental and genomic signals in honey bees: the critical interplay of nutritional, brain and reproductive networks. Epigenetics. 3(4): 188-192.

Malone L, Giacon H, Newton M. 1995. Comparison of the responses of some New Zealand and Australian honey bees (*Apis mellifera* L) to *Nosema apis* Z. Apidologie. 26(6): 495-502.

Malone LA, Pham-Delègue M-H. 2001. Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* sp.). Apidologie. 32(4): 287-304.

Mannina L, Sobolev AP, Coppo E, Di Lorenzo A, Nabavi SM, Marchese A, Daglia M. 2016. Antistaphylococcal activity and metabolite profiling of mānuka honey (*Leptospermum scoparium* L.) after *in vitro* simulated digestion. Food and Function. 7(3): 1664-1670.

Manning R. 2001. Fatty acids in pollen: a review of their importance for honey bees. Bee World. 82(2): 60-75.

Markowitz VM, Kyrpides NC. 2007. Comparative genome analysis in the integrated microbial genomes (IMG) system. Comparative Genomics Methods in Molecular Biology™: Humana Press. p. 35-56.

Martín-Hernández R, Botías C, Barrios L, Martínez-Salvador A, Meana A, Mayack C, Higes M. 2011. Comparison of the energetic stress associated with experimental *Nosema ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*). Parasitology Research. 109(3): 605-612.

Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA. 2011. A simple and distinctive microbiota associated with honey bees and bumble bees. Molecular Ecology. 20(3): 619-628.

Martinson VG, Moy J, Moran NA. 2012. Establishment of characteristic gut bacteria during development of the honeybee worker. Applied and Environmental Microbiology. 78(8): 2830-2840.

Mattila H, Otis G. 2006. Effects of pollen availability and *Nosema* infection during the spring on division of labor and survival of worker honey bees (Hymenoptera: Apidae). Environmental Entomology. 35(3): 708-717.

Mattila HR, Otis GW. 2007. Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecological Entomology. 32(5): 496-505.

Mattila HR, Rios D, Walker-Sperling VE, Roeselers G, Newton IL. 2012. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. Plos One. 7(3): e32962.

Maurizio A. 1954. Pollen nutrition and vital processes in the honey bee. Landw Jb Schweiz. 68: 115-182.

Mavric E, Wittmann S, Barth G, Henle T. 2008. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. Molecular Nutrition and Food Research. 52(4): 483-9.

McFrederick QS, Wcislo WT, Taylor DR, Ishak HD, Dowd SE, Mueller UG. 2012. Environment or kin: whence do bees obtain acidophilic bacteria? Molecular Ecology. 21(7): 1754-1768.

McFrederick QS, Cannone JJ, Gutell RR, Kellner K, Plowes RM, Mueller UG. 2013. Specificity between lactobacilli and hymenopteran hosts is the exception rather than the rule. Applied and environmental microbiology. 79(6): 1803-1812.

McGregor SE. 1976. Insect pollination of cultivated crop plants. United States Department of Agriculture: Agricultural Research Service.

McMenamin AJ, Genersch E. 2015. Honey bee colony losses and associated viruses. Current Opinion in Insect Science. 8: 121-129.

McMullan JB, Brown MJ. 2005. Brood pupation temperature affects the susceptibility of honeybees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). Apidologie. 36(1): 97-105.

McOrist AL, Jackson M, Bird AR. 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. Journal of microbiological methods. 50(2): 131-139.

Michener CD. 2007. The bees of the world. Baltimore, Maryland: The Johns Hopkins University Press; 2nd ed.

Micheu S, Crailsheim K, Leonhard B. 2000. Importance of proline and other amino acids during honeybee flight. Amino Acids. 18(2): 157-175.

Milbrath MO, van Tran T, Huang W-F, Solter LF, Tarpy DR, Lawrence F, Huang ZY. 2015. Comparative virulence and competition between *Nosema apis* and *Nosema ceranae* in honey bees (*Apis mellifera*). Journal of Invertebrate Pathology. 125: 9-15.

Mitraka E, Stathopoulos S, Siden-Kiamos I, Christophides GK, Louis C. 2013. *Asaia* accelerates larval development of *Anopheles gambiae*. Pathogens and Global Health. 107(6): 305-311.

Mitsuoka T. 1992. The human gastrointestinal tract. The Lactic Acid Bacteria. 1: 69-114.

Mohr KI, Tebbe CC. 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.

Molan PC. 1992a. The antibacterial activity of honey 2. Variation in the potency of the antibacterial activity. Bee World. 73(2): 59-76.

Molan PC. 1992b. The antibacterial activity of honey 1. The nature of the antibacterial activity. Bee World. 73(1): 5-28.

Molan PC. 2006. The evidence supporting the use of honey as a wound dressing. The International Journal of Lower Extremity Wounds. 5(1): 40-54.

Moniruzzaman M, Sulaiman SA, Khalil MI, Gan SH. 2013. Evaluation of physicochemical and antioxidant properties of sourwood and other Malaysian honeys: a comparison with mānuka honey. Chemistry Central Journal. 7(1): 138.

Moore AJ, Breed MD, Moor MJ. 1987. The guard honey bee: ontogeny and behavioural variability of workers performing a specialized task. Animal Behaviour. 35(4): 1159-1167.

Moran NA, Hansen AK, Powell JE, Sabree ZL. 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. Plos One. 7(4): e36393.

Moran NA. 2015. Genomics of the honey bee microbiome. Current Opinion in Insect Science. 10: 22-28.

Moritz B, Crailsheim K. 1987. Physiology of protein digestion in the midgut of the honeybee (*Apis mellifera* L.). Journal of insect physiology. 33(12): 923-931.

Morse RA, Calderone NW. 2000. The value of honey bees as pollinators of US crops in 2000. Bee Culture. 128(3): 1-15.

Mouches C, Bove J, Tully J, Rose D, McCoy R, Carle-Junca P, Garnier M, Saillard C. 1983. *Spiroplasma apis*, a new species from the honey-bee *Apis mellifera*. Annales de l'Institut Pasteur / Microbiologie. 134(3): 383-397.

Mouches C, Bové J, Albisetti J. 1984. Pathogenicity of *Spiroplasma apis* and other *spiroplasmas* for honey-bees in southwestern France. Annales de l'Institut Pasteur / Microbiologie. 135(1): 151-155.

Mukherjee K, Twyman RM, Vilcinskas A. 2015. Insects as models to study the epigenetic basis of disease. Progress in Biophysics and Molecular Biology. 118(1-2): 69-78.

Murray ZL, Lester PJ. 2015. Confirmation of *Nosema ceranae* in New Zealand and a phylogenetic comparison of *Nosema* spp. strains. Journal of Apicultural Research. 54(2): 101-104.

Muszynska J, Leznicka B. 1992. Acidity of the rectum content in worker bees. Pszczelnicze Zeszyty Naukowe (Poland). 36: 109-114.

Nagpal S, Haque MM, Singh R, Mande S. 2019. iVikodak–A platform and standard workflow for inferring, analyzing, comparing, and visualizing the functional potential of microbial communities. Frontiers in Microbiology. 9: 3336.

Nappi A, Christensen B. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochemistry and Molecular Biology. 35(5): 443-459.

Naug D. 2009. Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biological Conservation. 142(10): 2369-2372.

Nelson JA, Sturtevant AP, Lineburg B. 1924. Growth and feeding of honeybee larvae Illinois, USA: United States Department of Agriculture.

Neumann P, Carreck NL. 2010. Honey bee colony losses. Journal of Apicultural Research. 49(1): 1-6.

Neveling DP, Endo A, Dicks LM. 2012. Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hives. Current Microbiology. 65(5): 507-515.

New Zealand. 1998. Biosecurity (National American Foulbrood Pest Management Plan) Order 1998. SR 1998/260. Reprint 2 March 2018 ed. Wellington, N.Z.: http://www.legislation.govt.nz/regulation/public/1998/0260/latest/whole.html.

New Zealand Government. 1997. Agricultural Compounds and Veterinary Medicines Act 1997. New Zealand: http://www.legislation.govt.nz/act/public/1997/0087/latest/DLM414577.html.

New Zealand Government. 1998. Biosecurity Order 1998 (National American Foulbrood Pest Management Plan) SR 1998/260. Reprint 2 March 2018 ed. Wellington, New Zealand: http://www.legislation.govt.nz/regulation/public/1998/0260/latest/whole.html.

New Zealand Government. 2017. Ministry for Primary Industries 2017 Apiculture Montioring Programme. New Zealand: Ministry for Primary Industries, https://landusenz.org.nz/wp-content/uploads/2018/03/2017-Apiculture-monitoring-report-FINAL.pdf.

New Zealand Government. 2018. Ministry for Primary Industries 2018 Apiculture Monitoring Programme New Zealand: Ministry for Primary Industries, https://www.mpi.govt.nz/dmsdocument/34329/direct.

Nicolson SW. 1998. The importance of osmosis in nectar secretion and its consumption by insects. American Zoologist. 38(3): 418-425.

Nicolson SW, Human H. 2008. Bees get a head start on honey production. Biology Letters. 4(3): 299-301.

OECD. 1998. OECD Guideline for the testing of chemicals No. 213: Honey bees, acute oral toxicity test https://www.ibacon.com/your-study-type/terrestrial-ecotoxicology/bees/oecd-213214-honey-bees-acute-oral-and-acute-contact.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2011. vegan: Community ecology package. R package version: 117-118.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Peter Solymos, Henry M, Stevens H, Szoecs E, Wagner H. 2018. Vegan: Community Ecology Package. R package version 2.5-3.

Ollerton J, Erenler H, Edwards M, Crockett R. 2014. Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. Science. 346(6215): 1360-1362.

Ollerton J. 2017. Pollinator diversity: distribution, ecological function, and conservation. Annual Review of Ecology, Evolution, and Systematics. 48: 353-376.

Olofsson TC, Vásquez A. 2008. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Current Microbiology. 57(4): 356-363.

Olofsson TC, Alsterfjord M, Nilson B, Butler È, Vásquez A. 2014. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov.,

Lactobacillus melliventris sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera*. International Journal of Systematic and Evolutionary Microbiology. 64: 3109-3119.

Olsen GJ, Woese CR, Overbeek R. 1994. The winds of (evolutionary) change: breathing new life into microbiology. Journal of Bacteriology. 176(1): 1.

Paxton RJ, Klee J, Korpela S, Fries I. 2007. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie. 38(6): 558-565.

Peng Y-S, Nasr M, Marston JM, Fang Y. 1985. The digestion of dandelion pollen by adult worker honeybees. Physiological Entomology. 10(1): 75-82.

Percival MS. 1961. Types of nectar in angiosperms. New Phytologist. 60: 235-281.

Pernal SF, Currie RW. 2000. Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L). Apidologie. 31(3): 387-410.

Perry RJ, Peng L, Barry NA, Cline GW, Zhang D, Cardone RL, Petersen KF, Kibbey RG, Goodman AL, Shulman GI. 2016. Acetate mediates a microbiome–brain–β-cell axis to promote metabolic syndrome. Nature. 534(7606): 213.

Peters W, Kalnins M. 1985. Aminopeptidases as immobilized enzymes on the peritrophic membranes of insects. Entomologia Generalis. 11(1-2): 25-32.

Pitois S, Jackson M, Wood B. 2000. Problems associated with the presence of cyanobacteria in recreational and drinking waters. International Journal of Environmental Health Research. 10(3): 203-218.

Pollock J, Glendinning L, Wisedchanwet T, Watson M. 2018. The madness of microbiome: attempting to find consensus “best practice” for 16S microbiome studies. Applied and Environmental Microbiology. 84(7): e02627-17.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE. 2010a. Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution. 25(6): 345-353.

Potts SG, Roberts SP, Dean R, Marris G, Brown MA, Jones R, Neumann P, Settele J. 2010b. Declines of managed honey bees and beekeepers in Europe. Journal of Apicultural Research. 49(1): 15-22.

Powell JE, Martinson VG, Urban-Mead K, Moran NA. 2014. Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. Applied and Environmental Microbiology. 80(23): 7378-7387.

Powell JE, Leonard SP, Kwong WK, Engel P, Moran NA. 2016. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. Proceedings of the National Academy of Sciences. 113(48): 13887-13892.

R Core Team. 2018. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing: ISBN 3-900051-07-0: URL http://www.R-project.org.

Rada V, Machova M, Huk J, Marounek M, Dušková D. 1997. Microflora in the honeybee digestive tract: counts, characteristics and sensitivity to veterinary drugs. Apidologie. 28(6): 357-365.

Ramsey SD, Ochoa R, Bauchan G, Gulbronson C, Mowery JD, Cohen A, Lim D, Joklik J, Cicero JM, Ellis JD. 2019. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. Proceedings of the National Academy of Sciences. 116(5): 1792-1801.

Rangberg A, Diep DB, Rudi K, Amdam GV. 2012 Paratransgenesis: an approach to improve colony health and molecular insight in honey bees (*Apis mellifera*)? Integrative and Comparative Biology. 52(1): 89-99.

Raymann K, Shaffer Z, Moran NA. 2017. Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. Plos Biology. 15(3): e2001861.

Raymann K, Bobay LM, Moran NA. 2018a. Antibiotics reduce genetic diversity of core species in the honeybee gut microbiome. Molecular Ecology. 27(8): 2057-2066.

Raymann K, Coon KL, Shaffer Z, Salisbury S, Moran NA. 2018b. Pathogenicity of *Serratia marcescens* strains in honey bees. mBio. 9(5): e01649-18.

Reddy MR, Reddy KS, Chouhan YR, Bee H, Reddy G. 2017. Effective feather degradation and keratinase production by *Bacillus pumilus* GRK for its application as bio-detergent additive. Bioresource Technology. 243: 254-263.

Reynaldi F, De Giusti M, Alippi A. 2004. Inhibition of the growth of *Ascosphaera apis* by *Bacillus* and *Paenibacillus* strains isolated from honey. Revista Argentina de Microbiologia. 36(1): 52.

Reynaldi FJ, López AC, Albo GN, Alippi AM. 2003. Differentiation of *Ascosphaera apis* isolates by rep-PCR fingerprinting and determination of chalkbrood incidence in Argentinean honey samples. Journal of Apicultural Research. 42(4): 68-76.

Ribière C, Hegarty C, Stephenson H, Whelan P, O'Toole PW. 2019. Gut and whole-body microbiota of the honey bee separate thriving and non-thriving hives. Microbial Ecology. 78: 195-205.

Rinderer TE. 1988. Evolutionary aspects of the Africanization of honey-bee populations in the Americas. In: G. R. Needham, R. E. Page, M. Delfinado-Baker, Bowman CE, eds. Africanized honey bees and bee mites. Chichester, England Ellis Horwood. p. 13-28.

Ritz BW, Gardner EM. 2006. Malnutrition and energy restriction differentially affect viral immunity. The Journal of Nutrition. 136(5): 1141-1144.

Roberts WC. 1944. Multiple mating of queen bees proved by progeny and flight tests. Gleanings in Bee Culture. 72(6): 225-59.

Robinson GE, Page RE, Strambi C, Strambi A. 1989. Hormonal and genetic control of behavioral integration in honey bee colonies. Science. 246(4926): 109-112.

Rogel MA, Hernández-Lucas I, Kuykendall LD, Balkwill DL, Martinez-Romero E. 2001. Nitrogen-fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. Applied and Environmental Microbiology. 67(7): 3264-3268.

Rortais A, Arnold G, Halm M-P, Touffet-Briens F. 2005. Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie. 36(1): 71-83.

Ross MG, Russ C, Costello M, Hollinger A, Lennon NJ, Hegarty R, Nusbaum C, Jaffe DB. 2013. Characterizing and measuring bias in sequence data. Genome Biology. 14(5): R51.

Rothman JA, Carroll MJ, Meikle WG, Anderson KE, McFrederick QS. 2018. Longitudinal effects of supplemental forage on the honey bee (*Apis mellifera*) microbiota and inter-and intra-colony variability. Microbial Ecology. 76(3): 814-824.

Roulston T, Cane JH. 2000. Pollen nutritional content and digestibility for animals. Plant Systematics and Evolution. 222(1-4): 187-209.

Rowley AF, Powell A. 2007. Invertebrate immune systems–specific, quasi-specific, or nonspecific? The Journal of Immunology. 179(11): 7209-7214.

Rucker RR, Thurman WN, Burgett M. 2012. Honey bee pollination markets and the internalization of reciprocal benefits. American Journal of Agricultural Economics. 94(4): 956-977.

Rueppell O, Bachelier C, Fondrk MK, Page Jr RE. 2007. Regulation of life history determines lifespan of worker honey bees (*Apis mellifera* L.). Experimental Gerontology. 42(10): 1020-1032.

Runckel C, Flenniken ML, Engel JC, Ruby JG, Ganem D, Andino R, DeRisi JL. 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. Plos One. 6(6): e20656.

Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M, Lee W-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science. 319(5864): 777-782.

Sabaté DC, Carrillo L, Audisio MC. 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Research in Microbiology. 160(3): 193-199.

Sabree ZL, Hansen AK, Moran NA. 2012. Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees. Plos One. 7(7): e41250.

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

Sangüesa G, Shaligram S, Akther F, Roglans N, Laguna JC, Rahimian R, Alegret M. 2016. Type of supplemented simple sugar, not merely calorie intake, determines adverse effects on metabolism and aortic function in female rats. American Journal of Physiology-Heart and Circulatory Physiology. 312(2): H289-H304.

Santo Domingo J, Kaufman M, Klug M, Holben W, Harris D, Tiedje J. 1998. Influence of diet on the structure and function of the bacterial hindgut community of crickets. Molecular Ecology. 7(6): 761-767.

Saraiva MA, Zemolin APP, Franco JL, Boldo JT, Stefenon VM, Triplett EW, de Oliveira Camargo FA, Roesch LFW. 2015. Relationship between honeybee nutrition and their microbial communities. Antonie van Leeuwenhoek: 1-13.

Sartor RB. 2008. Therapeutic correction of bacterial dysbiosis discovered by molecular techniques. Proceedings of the National Academy of Sciences. 105(43): 16413-16414.

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of bacteriology. 184(4): 1140-1154.

Schlüns H, Moritz RF, Neumann P, Kryger P, Koeniger G. 2005. Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. Animal Behaviour. 70(1): 125-131.

Schmickl T, Crailsheim K. 2004. Costs of environmental fluctuations and benefits of dynamic decentralized foraging decisions in honey bees. Adaptive Behavior. 12(3-4): 263-277.

Schmidt JO, Thoenes SC, Levin M. 1987. Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. Annals of the Entomological Society of America. 80(2): 176-183.

Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Applied and Environmental Microbiology. 69(10): 6018-6024.

Seeley TD, Tarpy DR. 2007. Queen promiscuity lowers disease within honeybee colonies. Proceedings of the Royal Society of London B: Biological Sciences. 274(1606): 67-72.

Servin AL. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. Federation of European Microbiological Societies: Microbiology Reviews. 28(4): 405-440.

Severson D, Erickson E. 1984. Honey bee (Hymenoptera: Apidae) colony performance in relation to supplemental carbohydrates. Journal of Economic Entomology. 77(6): 1473-1478.

Sha S, Xu B, Wang X, Zhang Y, Wang H, Kong X, Zhu H, Wu K. 2013. The biodiversity and composition of the dominant fecal microbiota in patients with inflammatory bowel disease. Diagnostic microbiology and infectious disease. 75(3): 245-251.

Sikorowski P, Lawrence A. 1994. Microbial contamination and insect rearing. American Entomologist. 40(4): 240-253.

Smart MD. 2015. The influence of mid-continent agricultural land use on the health and survival of commercially managed honey bee (*Apis mellifera* L.) colonies. Unpublished Ph.D thesis, University of Minnesota.

Snowdon JA, Cliver DO. 1996. Microorganisms in honey. International Journal of Food Microbiology. 31(1): 1-26.

Southwick EE, Southwick L. 1992. Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. Journal of Economic Entomology. 85(3): 621-633.

Spivak M, Gilliam M. 1998. Hygienic behaviour of honey bees and its application for control of brood diseases and Varroa: Part II. Studies on hygienic behaviour since the Rothenbuhler era. Bee World. 79(4): 169-186.

Staley JT, Konopka A. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annual Reviews in Microbiology. 39(1): 321-346.

Standifer L. 1967. A comparison of the protein quality of pollens for growth-stimulation of the hypopharyngeal glands and longevity of honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). Insectes Sociaux. 14(4): 415-425.

Stevenson M, Benard H, Bolger P, Morris R. 2005. Spatial epidemiology of the Asian honey bee mite (*Varroa destructor*) in the North Island of New Zealand. Preventive Veterinary Medicine. 71(3): 241-252.

Stokstad E. 2007. Puzzling decline of US bees linked to virus from Australia. Science. 317(5843): 1304-1305.

Svetlana J, Dobrila J-D, Lj V. 2003. *Citrobacter freundii* as a cause of disease in fish. Acta Veterinaria. 53(5-6): 399-410.

Taber III S. 1954. The frequency of multiple mating of queen honey bees. Journal of Economic Entomology. 47(6): 995-998.

Tao K, Long Z, Liu K, Tao Y, Liu S. 2006. Purification and properties of a novel insecticidal protein from the locust pathogen *Serratia marcescens* HR-3. Current Microbiology. 52: 45-49.

Tarpy DR, Fletcher DJ. 1998. Effects of relatedness on queen competition within honey bee colonies. Animal Behaviour. 55(3): 537-543.

Tarpy DR, Mattila HR, Newton IL. 2015. Development of the honey bee gut microbiome throughout the queen-rearing process. Applied and Environmental Microbiology. 81(9): 3182-3191.

Taylor MA, Goodwin RM, McBrydie HM, Cox HM. 2007. Destroying managed and feral honey bee (*Apis mellifera*) colonies to eradicate honey bee pests. New Zealand Journal of Crop and Horticultural Science. 35(3): 313-323.

Taylor MA, Robertson AW, Biggs PJ, Richards KK, Jones DF, Parkar SG. 2019. The effect of carbohydrate sources: sucrose, invert sugar and components of mānuka honey, on core bacteria in the digestive tract of adult honey bees (*Apis mellifera*). Plos One. 14(12): e0225845.

Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME, Bergoin M. 2004. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Applied and Environmental Microbiology. 70(12): 7185-7191.

Terra WR, Ferreira C. 1994. Insect digestive enzymes: properties, compartmentalization and function. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 109(1): 1-62.

Tews I, Vincentelli R, Vorgias CE. 1996. N-Acetylglucosaminidase (chitobiase) from *Serratia marcescens*: gene sequence, and protein production and purification in *Escherichia coli*. Gene. 170: 63-67.

Todd JH, De Miranda JR, Ball BV. 2007. Incidence and molecular characterization of viruses found in dying New Zealand honey bee (*Apis mellifera*) colonies infested with *Varroa destructor*. Apidologie. 38(4): 354-367.

Toth AL, Kantarovich S, Meisel AF, Robinson GE. 2005. Nutritional status influences socially regulated foraging ontogeny in honey bees. Journal of Experimental Biology. 208(24): 4641-4649.

Tu S, Qiu X, Cao L, Han R, Zhang Y, Liu X. 2010. Expression and characterization of the chitinases from *Serratia marcescens* GEI strain for the control of *Varroa destructor*, a honey bee parasite. Journal of Invertebrate Pathology. 104(2): 75-82.

Tysset C, Durand C. 1968. Contribution to the study of the intestinal microbism of healthy worker bees (*Apis mellifica*): reckoning and study of the constitutive groups (1968). Bulletin Technique Apicole. 11(2): 107-118.

vanEngelsdorp D, Hayes Jr J, Underwood RM, Pettis J. 2008. A survey of honey bee colony losses in the US, fall 2007 to spring 2008. Plos One. 3(12): e4071.

VanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y. 2009. Colony collapse disorder: a descriptive study. Plos One. 4(8): e6481-e6481.

Vasquez A, Olofsson TC. 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, Olofsson TC, Sammataro D. 2009. A scientific note on the lactic acid bacterial flora in honeybees in the USA – A comparison with bees from Sweden. Apidologie. 40(3): 417-417.

Vásquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, Olofsson TC. 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. Plos One. 7(3): e33188.

Ventura M, O'flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, Van Sinderen D, O'toole PW. 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nature Reviews Microbiology. 7(1): 61.

Verstraete W. 2007. Microbial ecology and environmental biotechnology. The ISME Journal. 1: 4-8.

Visavadia BG, Honeysett J, Danford MH. 2008. Manuka honey dressing: An effective treatment for chronic wound infections. British Journal of Oral and Maxillofacial Surgery. 46(1): 55-56.

Vojvodic S, Rehan SM, Anderson KE. 2013. Microbial gut diversity of Africanized and European honey bee larval instars. Plos One. 8(8): e72106.

von Planta A. 1888. Ueber den futtersaft der bienen. Zeitschrift für Physiologische Chemie. 12(4): 327-354.

VSN International 2015. Genstat for Windows 18th Edition. VSN International, Hemel Hempstead, UK: https://www.vsni.co.uk.

Wahl O, Ulm K. 1983. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. Oecologia. 59(1): 106-128.

Wang M, Zhao W-Z, Xu H, Wang Z-W, He S-Y. 2015. *Bacillus* in the guts of honey bees (*Apis mellifera*; Hymenoptera: Apidae) mediate changes in amylase values. European Journal of Entomology. 112(4): 619-624.

Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature. 450(7169): 560-565.

Watanabe ME. 1994. Pollination worries rise as honey bees decline. Science. 265: 1170.

Webster TC, PENG YS, Duffey SS. 1987. Conservation of nutrients in larval tissue by cannibalizing honey bees. Physiological Entomology. 12(2): 225-231.

Wegener G, Tschiedel V, Schlöder P, Ando O. 2003. The toxic and lethal effects of the trehalase inhibitor trehazolin in locusts are caused by hypoglycaemia. Journal of Experimental Biology. 206(7): 1233-1240.

Wei G, Lai Y, Wang G, Chen H, Li F, Wang S. 2017. Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. Proceedings of the National Academy of Sciences. 114(23): 5994-5999.

Welker N, Campbell LL. 1967. Comparison of the α-amylase of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Journal of Bacteriology. 94(4): 1131-1135.

Weston RJ, Brocklebank LK. 1999. The oligosaccharide composition of some New Zealand honeys. Food Chemistry. 64(1): 33-37.

Wheeler MM, Robinson GE. 2014. Diet-dependent gene expression in honey bees: honey vs. sucrose or high fructose corn syrup. Scientific Reports. 4: 5726.

White GF. 1906. Bacteria of the Apiary. Technical series (United States Bureau of Entomology); no 14.

White JW, Riethof ML, Subers MH, Kushnir I. 1962. Composition of American honeys Technical Bulletin No. 1261 Agricultural Research Service, United States Department of Agriculture, Washington, DC.

Williams GR, Alaux C, Costa C, Csaki T, Doublet V, Eisenhardt D, Fries I, Kuhn R, McMahon DP, 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.

Willix DJ, Molan PC, Harfoot CJ. 1992. A comparison of the sensitivity of wound-infecting species of bacteria to the antibacterial activity of manuka honey and other honey. Journal of Applied Bacteriology. 73: 388-394.

Winfree R, Williams NM, Dushoff J, Kremen C. 2007. Native bees provide insurance against ongoing honey bee losses. Ecology Letters. 10(11): 1105-1113.

Winston ML. 1991. The Biology of the Honey Bee. United States of America: Harvard University Press.

Woyciechowski M, Moroń D. 2009. Life expectancy and onset of foraging in the honeybee (*Apis mellifera*). Insectes Sociaux. 56(2): 193-201.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science. 334(6052): 105-108.

Wu M, Sugimura Y, Takaya N, Takamatsu D, Kobayashi M, Taylor D, Yoshiyama M. 2013. Characterization of bifidobacteria in the digestive tract of the Japanese honeybee, *Apis cerana japonica*. Journal of Invertebrate Pathology. 112(1): 88-93.

Wu Y, Zheng H, Corona M, Pirk C, Meng F, Zheng Y, Hu F. 2017. Comparative transcriptome analysis on the synthesis pathway of honey bee (*Apis mellifera*) mandibular gland secretions. Scientific Reports. 7(1): 4530.

Wykes GR. 1952. An investigation of the sugars present in the nectar of flowers of various species. New Phytologist. 51: 210-215.

Xu B, Wang Y, Zhu S, Zhou H, Gou C, Dong W, Wang Y, Gao Y, Ma H. 2019. Transcriptional profiling reveals the molecular bases of immune regulation in *Apis mellifera* in response to *Ascosphaera apis* infection. Entomological Research. 49(1): 26-34.

Yacobson B, Elad D, Rosenthal K, Kmer I, Slovecky I, Efrat H. 1991. A recent chalkbrood outbreak in Israel: attempts at therapeutic intervention. American Bee Journal. 131: 786.

Yoshiyama M, Kimura K. 2009. Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus larvae*, the causal agent of American foulbrood. Journal of Invertebrate Pathology. 102(2): 91-96.

Yu R-Y, Martin WF. 2016. Symbiotic Associations: All About Chemistry. In: Hurst C, ed. The Mechanistic Benefits of Microbial Symbionts: Springer, Cham. p. 3-11.

Yun J-H, Lee J-Y, Hyun D-W, Jung M-J, Bae J-W. 2017. *Bombella apis* sp. nov., an acetic acid bacterium isolated from the midgut of a honey bee. International Journal of Systematic and Evolutionary Microbiology. 67(7): 2184-2188.

Yutin N, Galperin MY. 2013. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environmental Microbiology. 15(10): 2631-2641.

Zaghloul O, Mourad A, El MK, Nemat F, Morsy M. 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Communications in Agricultural and Applied Biological Sciences. 70(4): 703-714.

Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim M-S, Blanot D, Oh B-H, Ueda R, Mengin-Lecreulx D, Lemaitre B. 2006. The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. Immunity. 24(4): 463-473.

Zander E. 1909. Tierische parasiten als krankenheitserreger bei der biene. Münchener Bienenzeitung. 31: 196-204.

Zhai Q, Li T, Yu L, Xiao Y, Feng S, Wu J, Zhao J, Zhang H, Chen W. 2017. Effects of subchronic oral toxic metal exposure on the intestinal microbiota of mice. Science Bulletin. 62(12): 831-840.

Zheng H-Q, Gong H-R, Huang S-K, Sohr A, Hu F-L, Chen YP. 2015. Evidence of the synergistic interaction of honey bee pathogens *Nosema ceranae* and *Deformed wing virus*. Veterinary Microbiology. 177(1): 1-6.

Zheng H, Nishida A, Kwong WK, Koch H, Engel P, Steele MI, Moran NA. 2016. Metabolism of toxic sugars by strains of the bee gut symbiont *Gilliamella apicola*. MBio. 7(6): e01326-16.

Zheng H, Powell JE, Steele MI, Dietrich C, Moran NA. 2017. Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. Proceedings of the National Academy of Sciences. 114(18): 4775-4780.

Zheng H, Steele MI, Leonard SP, Motta EV, Moran NA. 2018. Honey bees as models for gut microbiota research. Lab Animal. 47(11): 317-325.

Zhou G-c, Wang Y, Zhai S, Ge F, Liu Z-h, Dai Y-j, Yuan S, Hou J-y. 2013. Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium *Ensifer adhaerens* strain TMX-23. Applied Microbiology and Biotechnology. 97(9): 4065-4074.