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Gut microbial communities and pathogens infection in New Zealand bumble bees (*Bombus terrestris*, Linnaeus, 1758)

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ABSTRACT

The gut microbiome is an important component of bee health. Previous research around the globe indicated that bee gut microbiome can be affected by the presence of pathogens. We surveyed for the presence of three specific pathogens in populations of the buff-tailed bumble bee, *Bombus terrestris* (Linnaeus, 1758), across New Zealand. The pathogen *Crithidia bombi* was the most prevalent and widespread pathogen across the studied sites, with prevalence ranging from 30 to 100% of the bees examined. *Nosema bombi* was, however, only found in North Island sites. The *Deformed wing virus* was detected in bumble bees at all the sites except one (Twizel in the South Island) with prevalence ranging from 0 to 60%. The *B. terrestris* gut microbiome and the associated pathogens from two contrasting locations were studied. Bacteria such as *Snodgrassella alvi* and Lactobacillales were observed. We also found that infections with *C. bombi* were associated with more diverse, distinct gut microbiome perhaps indicating disruptions of gut microbe communities that contribute to impair bumble bees' health.

Introduction

Bumble bees and honey bees are important natural pollinators and therefore key to food production (Goulson 2010). However, together with the global entomofauna, they experience population declines (Goulson 2019; Goulson et al. 2008). Increased mortality of bees around the world has been linked to several interacting factors such as exposure to pesticides, pathogens and lack of food (Goulson et al. 2015). The *Deformed wing virus* (DWV), the trypanosomatid *Crithidia bombi* and the microsporidium *Nosema bombi* are important pathogens observed to affect bumble bee fitness elsewhere around the globe (Cameron & Sadd 2020). Pathogens such as *Crithidia* spp. live within the bumble bee mid-gut and are known to be associated with disrupted bee gut microbial communities (Motta et al. 2018). Similarly, *Nosema* spp. has also been associated with changes in the gut bacteriome (Hubert et al. 2017). The DWV does not specifically reside in the bee gut. However, variation in the bee gut microbiota has been associated with tolerance to DWV infection in honey bees (Dosch et al. 2021). The prevalence of these pathogens can vary between landscapes and regions. Variation between areas in floral resources, for example, appears to alter pathogen loads within bumble bees (DeGrandi-Hoffman & Chen 2015; Dolezal & Toth 2018; McNeil et al. 2020). Declines in pathogen communities with latitude have also been observed (McNeil et al.

2020). Some of this spatial variation in bumble bee infection rates can be correlated with pathogen infection in other hosts such as honey bees (Fürst et al. 2014).

The gut microbiome can play a key role in an organism's general health. Bees rely on a healthy gut microbiome to promote growth and defence against pathogens, although the precise functions of gut bacteria are often difficult to pinpoint (Engel et al. 2012). In bumble bees, colony fitness has been associated with variation in the bacteriome and pathogen defence (Koch & Schmid-Hempel 2011; Cariveau et al. 2014). The bee gut microbiome also aids other functions such as digestion (Engel et al. 2012; Lee et al. 2015). However, only a few bacterial taxa are consistently found in bee guts (Kwong & Moran 2016).

We surveyed the presence and prevalence of three specific pathogens *C. bombi, N. bombi,* and DWV in the buff-tailed bumble bee, *Bombus terrestris*, at six sites around New Zealand. The buff-tailed bumble bee was first introduced into New Zealand for pollination purposes in 1885 but has since spread widely throughout New Zealand (Donovan 2007). In a second part of the analysis, we used 16S amplicon sequencing to characterise variation in community composition of gut microbiome in relation to locality and pathogen infection. This analysis was performed at two contrasting locations.

Materials and methods

Sampling and nucleic acids extraction

We sampled between 6 and 16 individual bumble bee foragers (i.e. all female individuals) per site from seven locations across New Zealand in October and November 2019 (Figure 1). Our sampling approach consisted of opportunistically collecting live foragers at each study site while they were foraging on flowers. Consequently, we did not ascertain the relatedness of the bees or if they were from the same or different nests. Bees were subsequently frozen in ethanol at −80°C. Bumble bee guts were dissected, and the DNA and RNA were simultaneously extracted using GENEzol plant DNA reagent (Geneaid Biotech, Taiwan) as follows. First, the guts were homogenised in 2
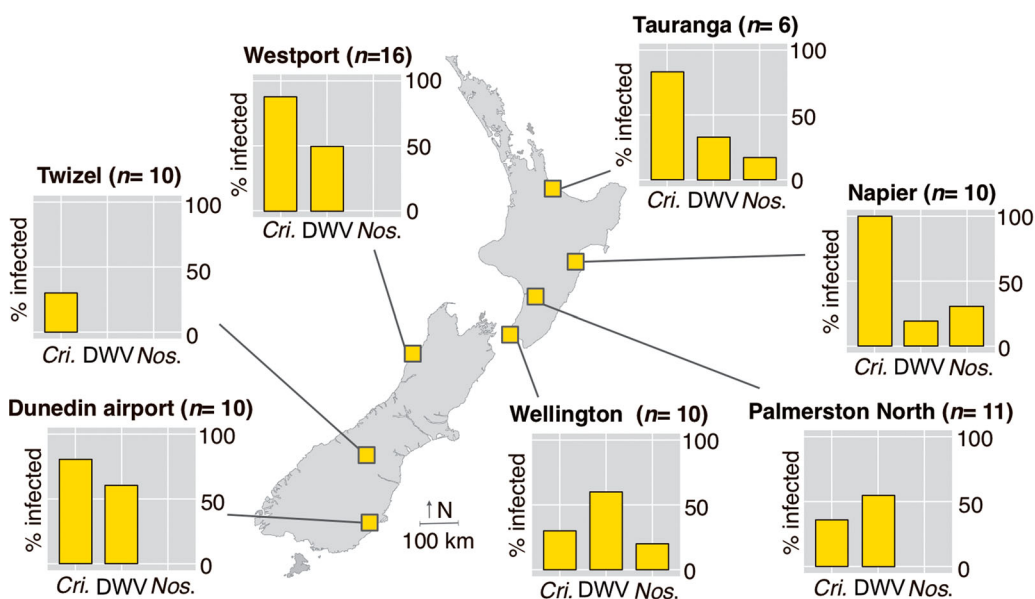
Figure 1. Prevalence of *Nosema bombi* (Nos.), *Crithidia bombi* (Cri.) and *Deformed wing virus* (DWV) in bumble bee foragers across NZ. The prevalence is based on the presence or absence of the pathogen in individual foragers caught on plants at each sample site (*n* = 6–16 bees at each sampling location).

mL tubes containing 1 mL GENEzol, 5 μL β-mercaptoethanol (Sigma Aldrich, USA), and two stainless-steel beads in a Precellys Evolution homogeniser (Bertin Instruments, France). Chloroform-isoamyl alcohol mixture (Sigma Aldrich) was used to extract nucleic acids, which were then precipitated using isopropanol (Sigma Aldrich) and washed with 70% (v/v) ethanol. DNA and RNA concentrations were measured by absorbance on a NanoPhotometer platform (Implen, Germany).

Screening of specific pathogens using PCR

We used Polymerase Chain Reaction (PCR) assays for the detection of *C. bombi*, *N. bombi* and DWV with specific conditions developed for each pathogen. Each PCR reaction contained 0.4 μM of each primer, 12.5 μL TaqMan Red Mix (Bioline, UK), and 200 ng sample DNA made to 25 μL total volume with nuclease-free water. For *C. bombi*, we used the published primers CB-SSUrRNA-F2 (CTTTTGACGAACAACTGCCCTATC) and CB-SSUrRNA-B4 (AACCGAACG-CACTAAACCCC) (Schmid-Hempel & Tognazzo 2010). Cycling conditions were: 95°C for 5 min; 40 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min; 72°C for 10 min and holding at 4°C. *Nosema bombi* was detected using published primers Nbombi-SSU-Jf1 (CCATG-CATGTTTTTGAAGATTATTAT) and Nbombi-SSU-Jr1 (CATATATTTTTAAAATATGAAA-CAATAA) (Klee et al. 2006) with reaction mixes the same as for the *C. bombi* PCR. Cycling conditions were: 95°C for 4 min; 45 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min; 72°C for 4 min and holding at 4°C. *Deformed wing virus* was detected using Reverse Transcriptase (RT)-PCR. For the RT step 500 ng sample RNA was made to 16 μL with water and mixed with 4 uL SuperScript IV VILO (Invitrogen/ThermoFisher Scientific, USA). Reactions proceeded at 25°C for 10 min, 50°C for 10 min, 85°C for 5 min and holding at 4°C. The DWV PCR utilised F15 primer (TCCATCAGGTTCTCCAATAACGGA) and B23 (CCACCCAAATGCTAACTCTAAGCG) (Yue & Genersch 2005). Each reaction contained 2.5 μL cDNA. Cycling conditions were: 95°C for 1 min; 35 cycles of 95°C for 15 s, 54°C for 15 s, 72°C for 30 s; 72°C for 5 min and a hold step at 4°C.

Gut microbiome analysis (16S amplicon sequencing)

Total DNA extracts were dried on DNAStable (Biomatrica, USA) following the manufacturer's instructions and shipped at room temperature to BGI's laboratory (Hong Kong). DNA quality was checked by BGI using Qubit (Invitrogen, USA), with all samples qualifying for rDNA amplification (i.e. amount was greater than 50 ng and there was limited degradation). Sequencing of 250 paired-end reads was carried on Illumina HiSeq 2500. Processing of raw data included removal of reads with a Phred score <20 over a 30 base pair sliding window, removal of resulting reads less than 75% of their original length, removal of adapter-contaminated reads and removal of reads containing ambiguous bases as well as low complexity reads (i.e. with 10 consecutive identical bases). Clean data can be downloaded from the NCBI SRA repository at http://www.ncbi.nlm.nih.gov/bioproject/814717 under the accession number PRJNA814717.

Overall, we obtained $222,182 \pm 31,445$ clean reads per sample (mean ± sd). Taxonomic ranks were assigned to representative sequence using the *Ribosomal Database Project (RDP) Naïve Bayesian Classifier* v.2.2 and tags were clustered into OTUs at 97% sequence similarity using the software *USEARCH* v.7. Chimeras were also filtered out using *UCHIME* v.4.2.4. Available information in the databases resulted in some OTUs not being identified to the species level. In *R* v.4.0.5, we used a custom script to compute the relative abundance of bacteria OTUs using the total number of reads in each sample. To focus on the most abundant taxa, we assigned low abundance OTUs (i.e. comprising less than 1% of the total read count in each sample) to a 'Minor taxa' category.

To compare gut microbial diversity, we employed a PERMANOVA approach based on Jaccard measure of relative abundance using the *adonis* function in the vegan *R* package (Oksanen et al. 2020). We specified the model with 999 permutations using microbial community as

response variable, and location as well as infection with *C. bombi*, *N. bombi* and DWV as inter-acting factors.

Results

Prevalence of *C. bombi*, *N. bombi* and deformed wing virus

Critihida bombi was the most prevalent pathogen in our sample set. It was present in all studied sites, with prevalence ranging from 30% to 100% (see Table S1). *Nosema bombi* was, however, only found in North Island sites, where prevalence ranged from 0% to 30%. The *Deformed wing virus* was detected in all sites except for Twizel in the South Island with prevalence ranging from 0 to 60% (Figure 1). We also unexpectedly observed bumble bees with substantial infections of the phoretic stage, heteromorphic deutonymphs (hypopi) of the mite *Kuzinia laevis* (Dujardin) in the samples from Palmerston North. Some individual bees hosted more than 30 mites.
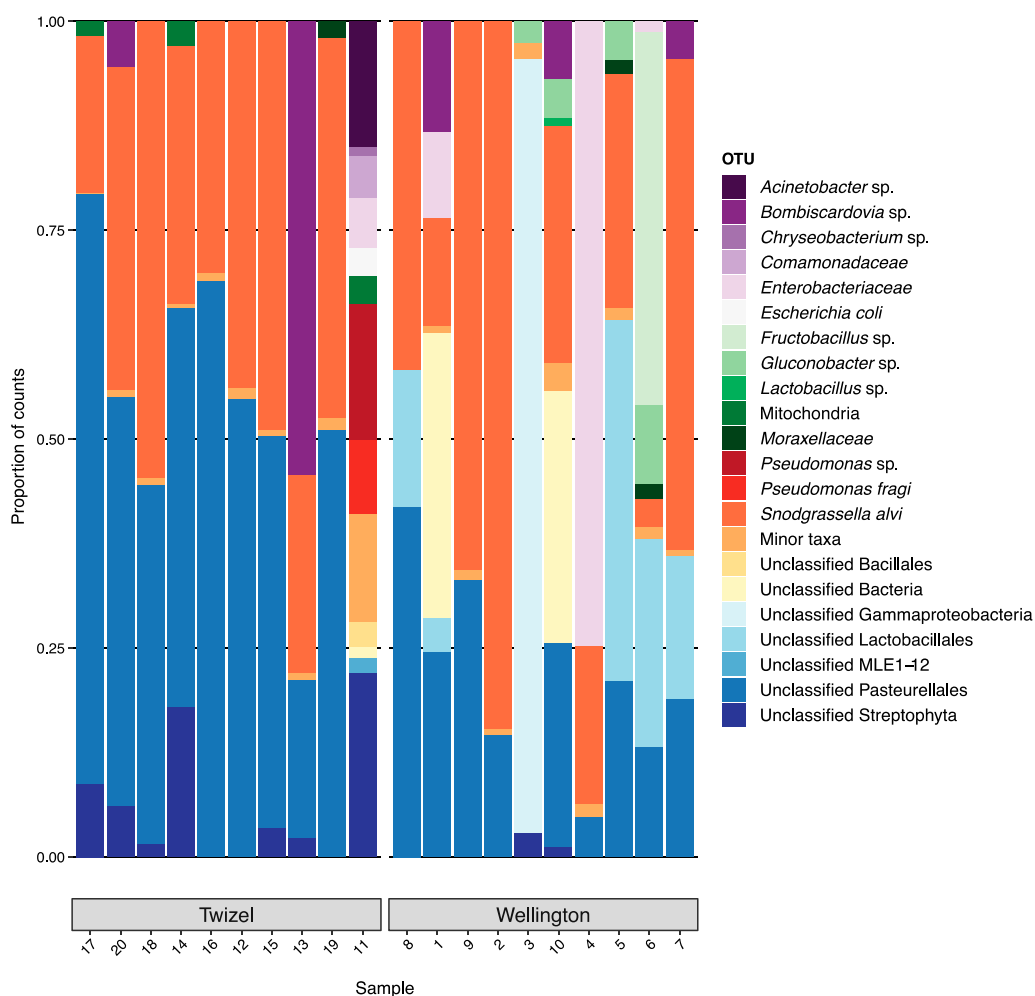
Figure 2. Frequency plot showing the proportion of reads assigned to Operational Taxonomic Units (OTUs) using BLASTn of 16S amplicon sequences. Gut samples were collected from bumble bee foragers in Wellington (North Island) and Twizel (South Island). Taxa representing less than 1% of all the reads within and across samples were labelled as 'Minor taxa'.

Table 1. Frequency of the most abundant bacteria in bumble bee gut samples from Wellington (North Island). Taxa present are shown in bold.

Wellington samples	1	2	3	4	5	6	7	8	9	10
Acinetobacter sp.	0	0	0	0	0	0	0	0	0	0
***Bombiscardovia* sp.**	0.13	0	0	0	0	0	0.04	0	0	0.07
Chryseobacterium sp.	0	0	0	0	0	0	0	0	0	0
Comamonadaceae	0	0	0	0	0	0	0	0	0	0
Enterobacteriaceae	0.1	0	0	0.75	0	0.01	0	0	0	0
Escherichia coli	0	0	0	0	0	0	0	0	0	0
***Fructobacillus* sp.**	0	0	0	0	0	0.45	0	0	0	0
***Gluconobacter* sp.**	0	0	0.03	0	0.05	0.1	0	0	0	0.05
***Lactobacillus* sp.**	0	0	0	0	0	0	0	0	0	0.01
Mitochondria	0	0	0	0	0	0	0	0	0	0
Moraxellaceae	0	0	0	0	0.02	0.02	0	0	0	0
Pseudomonas sp.	0	0	0	0	0	0	0	0	0	0
Pseudomonas fragi	0	0	0	0	0	0	0	0	0	0
Snodgrassella alvi	0.13	0.85	0	0.19	0.28	0.03	0.59	0.42	0.66	0.28
Minor taxa	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0	0.01	0.03
Unclassified Bacillales	0	0	0	0	0	0	0	0	0	0
Unclassified Bacteria	0.34	0	0	0	0	0	0	0	0	0.3
Unclassified Gammaproteobacteria	0	0	0.93	0	0	0	0	0	0	0
Unclassified Lactobacillales	0.04	0	0	0	0.43	0.25	0.17	0.16	0	0
Unclassified MLE1-12	0	0	0	0	0	0	0	0	0	0
Unclassified Pasteurellales	0.25	0.15	0	0.05	0.21	0.13	0.19	0.42	0.33	0.24
Unclassified Streptophyta	0	0	0.03	0	0	0	0	0	0	0.01

Characterisation of B. terrestris *gut microbiome*

We found a limited number of bacteria consistently present in *B. terrestris* guts (Figure 2; Tables 1 and 2). *Snodgrassella alvi* was found in all but two samples, ranging from 0 to 86% of all reads (mean ± sd: 36 ± 21% of the total microbiome). The second most abundant OTU was unclassified Pasteuralles detected in all but two samples (34 ± 21% of the total microbiome). Lactobacilliales were found in six samples, with the proportion of microbiome ranging from 1% to 43% of the total microbiome. Enterobacteriaceae bacteria were found in four samples with the proportion of

Table 2. Frequency of the most abundant bacteria in bumble bee gut samples from Twizel (South Island). Taxa present are shown in bold.

Twizel samples	11	12	13	14	15	16	17	18	19	20
***Acinetobacter* sp.**	0.15	0	0	0	0	0	0	0	0	0
***Bombiscardovia* sp.**	0	0	0.54	0	0	0	0	0	0	0.05
***Chryseobacterium* sp.**	0.01	0	0	0	0	0	0	0	0	0
Comamonadaceae	0.05	0	0	0	0	0	0	0	0	0
Enterobacteriaceae	0.06	0	0	0	0	0	0	0	0	0
Escherichia coli	0.03	0	0	0	0	0	0	0	0	0
Fructobacillus sp.	0	0	0	0	0	0	0	0	0	0
Gluconobacter sp.	0	0	0	0	0	0	0	0	0	0
Lactobacillus sp.	0	0	0	0	0	0	0	0	0	0
Mitochondria	0.03	0	0	0.03	0	0	0.02	0	0	0
Moraxellaceae	0	0	0	0	0	0	0	0	0.02	0
***Pseudomonas* sp.**	0.16	0	0	0	0	0	0	0	0	0
Pseudomonas fragi	0.09	0	0	0	0	0	0	0	0	0
Snodgrassella alvi	0	0.44	0.24	0.31	0.49	0.3	0.19	0.55	0.45	0.39
Minor taxa	0.13	0.01	0.01	0.01	0.01	0.01	0	0.01	0.02	0.01
Unclassified Bacillales	0.03	0	0	0	0	0	0	0	0	0
Unclassified Bacteria	0.01	0	0	0	0	0	0	0	0	0
Unclassified Gammaproteobacteria	0	0	0	0	0	0	0	0	0	0
Unclassified Lactobacillales	0	0	0	0	0	0	0	0	0	0
Unclassified MLE1-12	0.02	0	0	0	0	0	0	0	0	0
Unclassified Pasteurellales	0	0.55	0.19	0.48	0.47	0.69	0.71	0.43	0.51	0.49
Unclassified Streptophyta	0.22	0	0.02	0.18	0.03	0	0.09	0.01	0	0.06

microbiome ranging from 1-75% of the total microbiome. *Bombiscardovia sp.* was found in five samples, and its proportion in the gut microbiome when present ranged from 4% to 54% of the total microbiome. *Streptophyta* bacteria were detected in nine samples at relatively low proportions (1–22% of the total microbiome), likely reflecting plant material ingested by the bumble bees. Similarly, mitochondrial genes were detected in most samples and do not reflect gut microbiome. Some other taxa were present in small amounts and in only a few samples: *Gluconobacter* sp. was found in four samples (3-10% of the total microbiome) and unclassified Moraxellaceae were found in three samples (2% total microbiome). Unclassified bacteria were found in three samples (1–34% of total microbiome). *Fructobacilus sp.* was found in one sample only (sample 6) and represented 45% of the total microbiome. One sample stood out by its unusual gut microbial community: *Acinetobacter* sp., *Chryseobacterium* sp., unclassified Comamonadaceae, *Escherichia coli*, *Pseudomonas* sp., *Pseudomonas fragi*, unclassified Bacilliales and unclassified MLE1-12 (uncultured taxa) were found in one Twizel sample only (i.e. sample 11).

Factors associated with gut microbiome composition

When including all samples in the analysis, we found a significant effect of location ($0.001 < p < 0.01$) as well as significant effects of the presence of *N. bombi* and *C. bombi* (all $0.01 < p < 0.05$). We also found significant interactions between infection by both *N. bombi* and *C. bombi* and the interaction between DWV and *C. bombi* (all $0.01 < p < 0.05$; Table 3). However, the results related to *N. bombi* have to be taken with caution as only two samples tested positive. Non-metric multidimensional analysis shows that *C. bombi* infection appeared to be associated with more distinct gut microbiomes (Figures 3 and 4). Furthermore, more pronounced clustering in Twizel samples indicates more similar communities within Twizel than within Wellington (Figures 3 and 4).

Discussion

Our results demonstrate the presence of three important pathogens of *B. terrestris* in New Zealand. We found the parasitic trypanosomatid, *C. bombi*, across all studied locations, indicating widespread presence and common infection in New Zealand. Infection rates were >80% in four out of six collection sites, although more sampling would have been appropriate to gain better estimate of infection prevalence, as it can vary greatly over time and location (Schmid-Hempel et al. 2019). *Crithidia bombi* is normally considered as a commensal organism, although it can also be a cause of increased mortality when combined with other stressors (Brown et al. 2003, 2000; Schmid-Hempel et al. 2019). The parasitic fungi *N. bombi* spread in North America has been shown to be correlated with *Bombus* spp. declines (Cameron et al. 2016). It has been previously observed in the North Island of New Zealand (McIvor & Malone 1995) where it has so far not been demonstrated to influence colony productivity (Fisher & Pomeroy 1989). We did not find evidence for the presence of

Table 3. PERMANOVA results based on Jaccard dissimilarities using abundance data for bacterial community structure including all samples, in relation to sampling location (City), and infection with *Nosema bombi*, *Deformed Wing virus* and *Crithida bombi*.

|	Df	Sums of Squares	Means of squares	F-value	R^2	P-value
City	1	0.53236	0.53236	6.1230	0.17326	0.001***
NOS	1	0.38322	0.38322	4.4076	0.12472	0.017*
DWV	1	0.08165	0.08165	0.9391	0.02657	0.498
CRI	1	0.27444	0.27444	3.1564	0.08932	0.031*
City:CRI	1	0.19535	0.19535	2.2468	0.06358	0.057
NOS:CRI	1	0.31164	0.31164	3.5844	0.10143	0.023*
DWV:CRI	1	0.33757	0.33757	3.8825	0.10986	0.010**
Residuals	11	0.95640	0.08695		0.31126	
Total	18	3.07263	1.00000			

Note: NOS = *N. bombi*; DWV = *Deformed Wing Virus*; CRI = *C. bombi*.
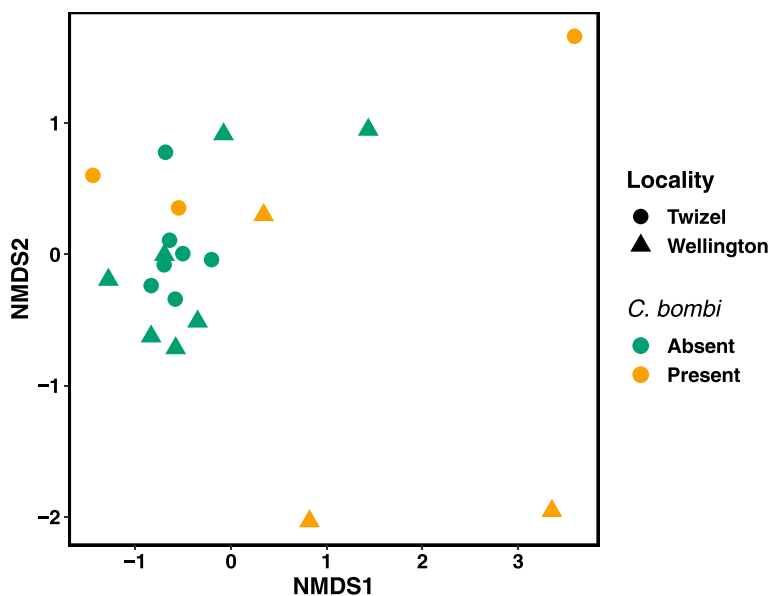
Figure 3. Non-metric multi-dimensional scaling analysis showing higher dispersion of Wellington samples (triangles) as well as *Crithidia bombi*-infected samples (in yellow). Dissimilarity matrix of gut bacteria communities was constructed using the Jaccard distance. Gut samples were collected from bumble bee foragers in Wellington (North Island) and Twizel (South Island), and microbiome data was obtained using 16S amplicon sequencing.

N. bombi in the South Island, although it is possible that this pathogen could be found with additional sampling. However, infection rates of bumble bees by *N. bombi* can also exhibit a positive correlation with temperature and humidity (Sharma et al. 2021). Perhaps *N. bombi* is not tolerant of cooler temperatures, as we have observed in other insect pathogens in New Zealand (Lester & Bulgarella 2021), or it is possible that this pathogen has not yet dispersed to the South Island. *Bombus terrestris* was first introduced in New Zealand via the South Island in 1885 (Gurr 1972) and a lack of opportunity to disperse to the South Island seems unlikely as bee movements between islands have not been restricted. We observed DWV as a common pathogen of *B. terrestris*. The only previous
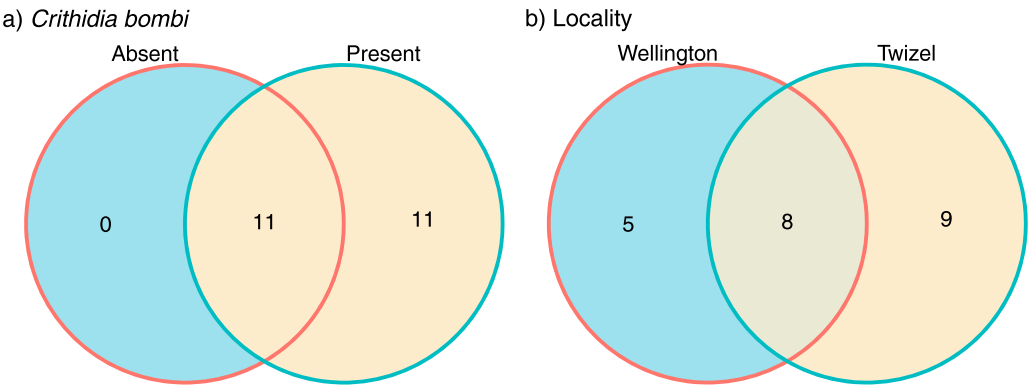
Figure 4. Venn diagrams showing gut microbiome diversity associated with (a) *Crithidia bombi* infection and (b) locality. Infections with *C. bombi* is associated with increased gut microbiome diversity. Higher diversity of taxa in Twizel was driven by the hyperdiverse sample 11 (*C. bombi*-infected), which contained eight taxa only found in Twizel. Gut samples were collected from bumble bee foragers in Wellington (North Island) and Twizel (South Island), and microbiome data was obtained using 16S amplicon sequencing.

viral screening of New Zealand bumble bees did not report DWV detection, although a small number of bees were assayed (Dobelmann et al. 2020). DWV affects bumble bees in a similar way to how it affects honey bees, causing wing deformities (Genersch et al. 2006).

The gut microbiota in bees is highly variable depending on species, age, caste and season, and is a determining factor in health (Kwong & Moran 2016). In our dataset, we found in most samples *Snodgrassella alvi* and *Bombiscardovia* sp., bacteria previously identified as a core honey bee gut bacterium (Moran et al. 2012). Unfortunately, many reads could only be identified down to class or order, making detailed analysis of these taxa difficult. Nevertheless, orders such as Pasteurellales and Lactobacillales are frequently detected in bee microbiome studies (Moran 2015). Disruptions of the bees gut microbiome can be associated with a shift towards more opportunistic environmental bacteria, including Entorobacteriaceae and other Gammaproteobacteria (Kwong & Moran 2016; Moran et al. 2012; Sabree et al. 2012). We observed a significant effect of the presence of *N. bombi* and *C. bombi* on the gut microbiota of bumble bees. Both *N. bombi* and *C. bombi* can alter the gut microbial communities of *B. terrestris*, with infections by these pathogens associated with increase in microbial diversity (Koch et al. 2012). In our study, samples infected with *C. bombi* often exhibited more diverse and distinct gut microbiome. Pathogens such as tryponosomatids can indeed disrupt gut microbiomes (Cariveau et al. 2014; Kwong & Moran 2016). Furthermore, higher microbiota diversity has been shown to be associated with decreased resistence to *C. bombi* (Näpflin & Schmid-Hempel 2018).

Sampling location also significantly affected bumble bee gut microbiota. Temperature can mediate the effects of these intestinal symbionts on bumble bee gut pathogens (Palmer-Young et al. 2018). With our sampling design, we do not know what aspect of location is specifically influencing the gut microbiota. The two sites will have differed in many aspects including temperature, food availability, types of vegetation, agrochemical usage and the abundance and diversity of other pollinators. Overall, small sample size and low number of sampling locations are limiting the interpretation of our results. Further research would be required to disentangle the influence of these factors on the bee gut microbiota and health.

The presence of high populations of the mite *K. laevis* was observed in Palmerston North. This species is thought to be primarily phoretic on *B. terrestris*, where they are carried as deutonymps (OConnor 1988). Populations of *B. terrestris* invading Tasmania, which were assumed and likely to be from New Zealand, were found to have high infection loads of this mite (Allen et al. 2007). Interestingly, contrary to our findings in New Zealand the incipient bumble bee populations in Tasmania were apparently free from pathogens including *C. bombi* and *N. bombi,* and displayed no wing deformities that might be associated with DWV (Allen et al. 2007). More recent work on the Tasmanian *B. terrestris* has also failed to find DWV, but has found these bumble bees to be infected with both the *Kashmir bee virus* and *Sacbrood virus* (Fung et al. 2018).

Our work demonstrates that bumble bees in New Zealand do suffer infections by pathogens associated with honey bees including DWV and *Kashmir bee virus* (Dobelmann et al. 2020), as well as their own specific pathogens. Apicultural practices involving honey bees, including their introduction and movement, can give rise in repeated spillover events and substantially influence the presence of pathogens such as DWV in bumble bees (Fürst et al. 2014). We show that parasites such as *C. bombi* are associated with increased variation in gut microbiome and the presence of more diverse taxa. Our study provides preliminary results on variation in bumble bee pathogens and the gut microbial communities in New Zealand.

Supplementary data

Table S1. Infection status of bumble bee foragers for *Crithidia bombi*, *Nosema bombi* and Deformed wing virus detected using PCR.

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Disclosure statement

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