Degree project

UNIVERSITY OF SKÖVDE

16S Nanopore sequencing of *Lactobacillus* spp. in *Apis mellifera*, and investigation of their bacteriocin activity

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Abstract

Apis mellifera is the most common honeybee species in the world. In recent years, there have been several reports of declines in wild, and domesticated populations. Central to honeybee health are the mutualistic relationships they have with their intestinal microbiome. The *Lactobacillus* species living in their digestive tract assist with nutrient digestion and pathogen protection. The aim of this study was to investigate which *Lactobacillus* spp. were present in the intestines of subspecies *A. mellifera mellifera*, and *A. mellifera ligustica*, and if they were able to inhibit growth of the pathogen *Melissococcus plutonius*. The 16S rRNA gene was amplified from gDNA extracted from two complete intestines per sample, in a PCR reaction with barcoded primers. Fragments were then analyzed with nanopore sequencing. In vitro assays of catalase-treated cell-free supernatants from *Lactobacillus* cultures were set up against living cultures of *M. plutonius* on KBHI agar plates and liquid broth media, in two experiments. The same seven *Lactobacillus* species previously found in honeybees were confirmed to be present in the bees of this study. The ratio of species was different between individual samples, which supports earlier findings suggesting the variation is dependent on factors such as individual health, food source, and sampling season. Liquid broth in vitro assay resulted in no inhibition of early growth phase, while the last cell count measure at 24 h, recorded statistically significant difference in mean values between *A. apinorum* and negative control ($p<0.001$). Further research is needed to investigate optimum conditions for inhibition.

List of abbreviations

PCR – Polymerase Chain Reaction

DNA - DeoxyriboNucleic Acid

gDNA – Genomic DeoxyriboNucleic Acid

NGS – Next Generation Sequencing

BHI media – Brain Heart Infusion media

KBHI media – Brain Heart Infusion media with potassium dihydrogen phosphate

BT kit – Dneasy blood and Tissue kit (Qiagen)

ZB kit – Zymobiomics DNA miniprep kit (Zymo research)

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1 Introduction

1.1 Honeybees

Bees are important pollinators in nature and commercial food production. They boost productivity in the agricultural sector which is highly dependent on bee pollination, where it has been determined that about a third of the world's food production depends on bee pollination of crops (Chapman et al., 2019; Eilers et al., 2011). The honeybees, which make up a large part of the pollination occurring especially in commercial applications, belong to the genus *Apis* (Klein et al., 2007). While there are eight surviving species of honeybees, the most common are the domesticated species *Apis mellifera* (western honey bee) and *Apis cerana* (estern honey bee) used for several thousand years for honey production (Gisder & Genersch, 2017). Their importance has led to increasing concern in recent years since decline of both domesticated and wild populations of pollinators has been reported (Allen-Wardell et al., 1998; Allsopp et al., 2008). Although sources agree on a general pollinator decline, there is no single reason, but a combination of different factors. Decline of natural habitats in favor of monocultural farming, biodiversity decline and the use of pesticides are a few reasons leading to lower populations of insects and animals that are natural pollinators (Vanbergen & Initiative, 2013). The diet of honeybees specifically consists of carbohydrates collected from the nectar of flowering plants or honeydew secreted by plants or insects. Nectar consists of water, and the sugar species glucose, fructose and sucrose (Taylor et al., 2019). The water content of the nectar is reduced by the bees to around 17%. To produce honey, the reduced nectar or honeydew is mixed with pollen in the honeystomach, where it is exposed to enzymes that break down the pollen and the sugars, making nutrients more readily available for digestion. The bees then regurgitate the nectar solution into the honeycomb, and use their wings to evaporate the water out of the nectar. When enough water has evaporated, the honeycomb is sealed with wax secreted from glands located in their abdomen (Crane, 1991; Taylor et al., 2019).

1.2 Honeybee microbiome

It is not yet fully understood how bees digest proteins and carbohydrates, but an increasing amount of studies are investigating the mutualistic relationships of the honeybees and their microbiome and how the intestinal bacteria aid in digestion (Kwong & Moran, 2016; Martinson et al., 2012; Tarpy et al., 2015). Research has shown that *A. mellifera* harbors intestinal microbiomes of low taxonomic complexity, where in the workers of the hive, the microbiome is dominated by bacteria belonging to less than ten phylotypes. The phylotypes are composed of bacteria sharing more than 97% identity in the 16S rRNA sequence (Kwong & Moran, 2016; Martinson et al., 2011; Moran et al., 2012; Raymann & Moran, 2018). For bacterial identification, the 16S rRNA gene has been the standard choice for a long time. The gene contains regions of high variety between species, while other highly conserved regions are useful for attaching primers which will work across most bacterial genera (Johnson et al., 2019; Kembel et al., 2012; Langille et al., 2013). *Lactobacillus* is one of the most dominant genera of bacteria found in honeybees, together with *Bifidobacterium*, *Acetobacteraceae*, *Snodgrassella,* and *Gilliamella* respectively (Corby-Harris et al., 2014).

1.2.1 Mutualistic *Lactobacillus* spp.

Positive probiotic effects associated with members of the *Lactobacillus* genus are their ability to inhibit growth of pathogenic species. Several different mechanisms are used to achieve this, for example by producing high amounts of lactic acid to reduce the pH level and thus limiting the growth of pathogens needing a neutral or basic enviroment (Aiba et al., 1998; Bernet-Camard et al., 1997). A number of *Lactobacillus* spp. also produces hydrogen peroxide since they lack heme, and can therefore not reduce oxygen to water by utilizing the cytochrome system . Instead flavoproteins are utilized to convert the oxygen to H_2O_2 (Eschenbach et al., 1989; Hawes et al., 1996). Other studies has documented bacteriocin production in some *Lactobacillus* spp., which acts specifically towards bacterial species known to be pathogenic, belonging to the genera *Staphylococcus*, *Shigella*, *Listeria*, *Klebsiella*, *Salmonella*, *Pseudomonas* and *Enterobacter.* Additionally, these bacteriocins do not affect other probiotic species such as other members in the *Lactobacillus* genera or *Bifidobacterium* species (Bernet-Camard et al., 1997). In relation with honeybees specifically, multiple studies has been showing promising results of *Lactobacillus* species inhibitory effect against the common pathogenic bacterial species *Paenibacillus larvae* and *Melissococcus plutonius,* which causes american foulbrood and european foulbrood, respectively, of which the latter is being examined in this study (Arredondo et al., 2018; Forsgren et al., 2010; Yoshiyama et al., 2013).

1.3 Next generation sequencing

Identification of bacterial species has been presented with difficulties in the past, using methods such as pyrosequencing that comes with certain restrictions (Liu et al., 2007). With new sequencing methods referred to as Next Generation Sequencing (NGS), even genomic data can be sequenced and analyzed (Liu et al., 2012). One of the most widely used NGS methods is the second generation Illumina sequencing, which sequences fragments of 5-300 bp. The problem when sequencing long genomes is that these small fragments have to be "knitted" together in an estimated orientation to construct a genome, which can cause problems especially with repetitive regions (Heydari et al., 2019; Prosperi & Salemi, 2012). With third generation sequencing tools such as nanopore sequencing, complex samples can be analyzed without complicated preparation (Bleidorn, 2016; Jain et al., 2016). What is unique with nanopore sequencing compared to other methods such as Illimuna, is that reads of >100 kb is possible (Jain et al., 2018). It works by first adding barcodes to the DNA or RNA strands prepared for sequencing. Depending on which kit is being used it works differently. For the 16S barcoding kit (SQK-16S024), barcodes are attached to the primers, giving each sample its own "identity". It is therefore possible to sequence multiple samples in a single sequencing run, since the nanopore device will register the barcode attached to the fragment passing through the pore (Menegon et al., 2017; Pomerantz et al., 2017). A sequencing adapter is then attached to the barcoded fragment, which connects to the membrane bound nanopore (Figure 1). While the fragment is being transported through the pore, a -180 mV current is running through the pore, disrupting the ionic current, and the sequencing unit is measuring the signal trace to register which nucleotide is passing through (Lu et al., 2016). The data is then processed in the MinKNOW software. Output files

are in FAST5, and there is an option to convert the files to FASTQ in MinKNOW, called basecalling. The basecalling can also be completed with for example Guppy software. Conversion to FASTQ files are needed for further data analysis with EPI2ME or other softwares of choice (Wick et al., 2019).

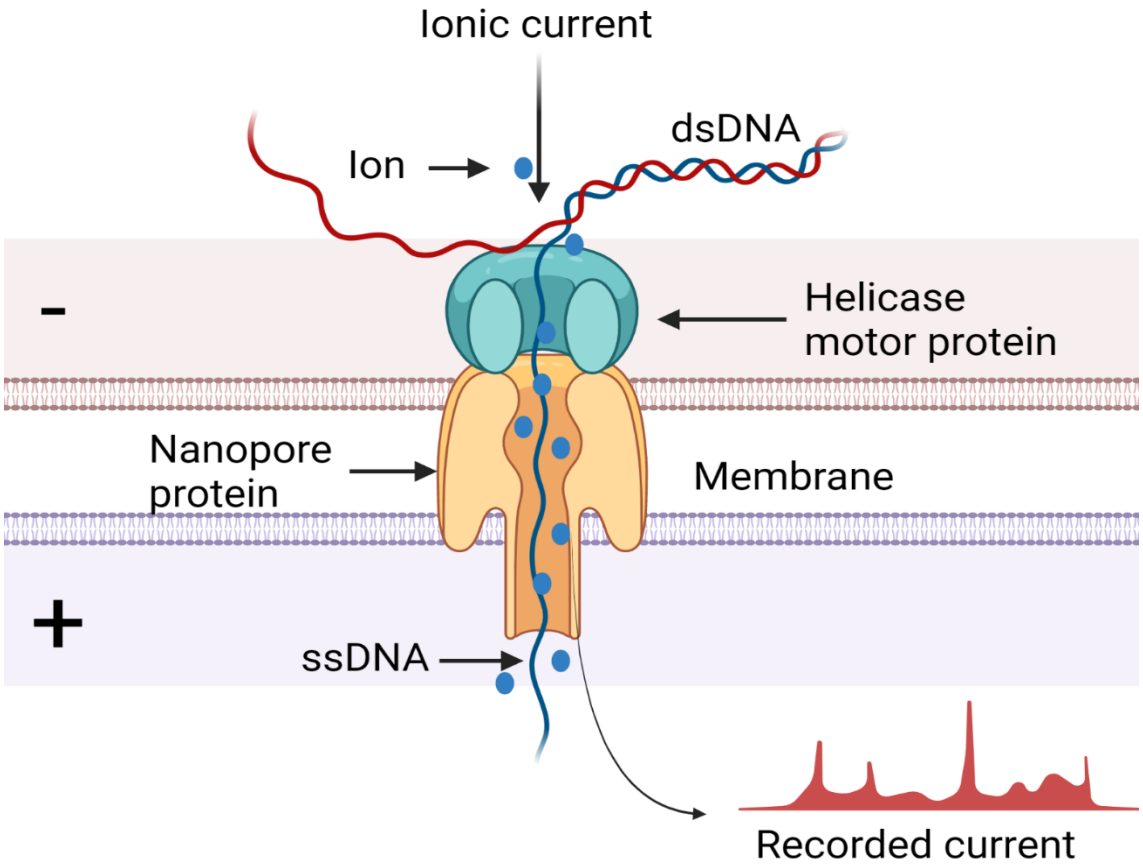

Figure 1. Simplification of a nanopore protein complex. The sample dsDNA is attached to the helicase motor protein, which connects to the nanopore with an adapter sequence. The helicase motor protein splits the dsDNA and transfers a strand of ssDNA through the nanopore, while the sequencing device measures the ionic current disruption signal. Each nucleotide will affect the current differently, and a sequence can be assembled in the MinKNOW software.

The benefit of using Illumina platform is a higher accuracy on sequencing results. To be taken into account when comparing previous studies of Illumina and nanopore sequencing, is when the study was conducted. Both technologies are constantly being improved and updated to increase read accuracy. A study from 2019 compared the mentioned technologies on whole genome sequencing of Hepatitis B, and reported that in 11.5% of the positions of three full genome reads, the nanopore sequencing data differed from the sequences gathered with Illumina. Since the Illumina data had fewer variants of the virus in

the analysis, the conclusion of the study was that the difference was likely due to nanopore sequencing errors (McNaughton et al., 2019). Raw sequencing data from single cell transcriptome sequencing gathered in 2020 reported accuracy of 93% for nanopore sequencing reads (Lebrigand et al., 2020). A 2019 study on hemoglobin transcripts, the median accuracy for the study was 94% on the reads generated by the Oxford Nanopore Sequencing (Byrne et al., 2019). The consensus seems to be that error rates range between 6-15% for nanopore sequencing, and ~0.1% for Illumina MiSeq version (Jain et al., 2017; Loman et al., 2012; Nicholls et al., 2019; Winand et al., 2019).

2 Aim

The aim of this study was to develop a methodology to determine which species of *Lactobacillus* spp. are present in the honeybee *A. mellifera* subspecies: *A. mellifera ligustica*, and *A. mellifera mellifera*. Two different DNA extraction kits were evaluated, and the third generation NGS technology Oxford Nanopore Sequencing was applied to sequence the whole 16S gene by using commercial 27F and 1492R primers for accurate bacterial identification. The second part of the project was to evaluate the ability of *Lactobacillus* spp. to inhibit *M. plutonius* through bacteriocin inhibition by filter disc growth test, and liquid broth growth experiment.

Research questions for the project:

Do the *Lactobacillus* species present in the honeybees produce inhibitory agents acting towards the honeybee pathogen *M. plutonius*?

Do the same species of *Lactobacillus* dominate in the intestines of different subspecies of *A. Mellifera*?

Are there specific *Lactobacillus* species present in one of the subspecies of *A. Mellifera* that are missing in others?

Hypothesis:

H_1: The two subspecies of *A. Mellifera* harbors a different composition of *Lactobacillus* species in their intestines

H_0: The two subspecies of *A. Mellifera* do not harbor a different composition of *Lactobacillus* species in their intestines.

3 Materials and method

3.1 Dissection

Tools used for dissecting bees and extracting intestines (Figure G appendix) were submerged in a 2.7% sodium hypochlorite solution for ≥10 minutes. The sodium hypochlorite was then washed by submerging each tool subsequently in three seperate 50 mL falcon tubes of ultra-pure water. Worker bees from four different sources in Uddevalla, Sweden were prepared in this experiment. The tools were disinfected between each sample collection, with freshly prepared tubes of 2.7% sodium hypochlorite. The honeybees were collected from colonies two months before the start of the experiment, which were in winter hibernation at the time. The bees were then frozen at -20°C. Thawing of the bees were done in room temperature, and then surface disinfected in a 1% sodium hypochlorite solution prepared with ultra-pure H_2O. 1 mL/bee of 1% sodium hypochlorite was added to a 15 mL falcon tube together with max five bees in the same tube. The tube was then vortexed at a low setting for 2 minutes. Washing off the chlorine solution was then immediately done by submerging each bee in three separate 15 mL falcon tubes containing ultra-pure H_2O. Dissection was then performed by first carefully removing the legs and wings. The two spiracles closest to the stinger were cut open on both sides, and the head carefully removed. With a pincer, the entire intestine was then removed by grasping the outside of the bottom spiracle and carefully separating it from the abdomen. Two full intestines were collected from each sample. The intestines were collected in 1.5 mL eppendorf tubes before DNA-extraction.

3.2 DNA extraction

Two extraction kits were tested in this study to determine the difference in effectiveness and DNA integrity post extraction of intestinal honeybee samples. The intestinal extraction amount was two intestines per colony of subspecies. However, isolated *Lactobacillus* spp. cultures and control cultures from Culture Collection University of Gothenburg (CCUG) (Table A, appendix) were isolated with Zymobiomics DNA miniprep kit only. After extraction with both kits, the samples were measured with Qubit™ 1X dsDNA High Sensitivity (HS) kit in a Qubit fluorometer 4 (Thermo Fisher Scientific) to determine DNA concentration. DS-11 spectrophotometer (DeNovix) was used to measure contamination/purity levels. The agarose gel was prepared with a 1X TAE buffer, 1X GelRed (Biotium) and 1% w/v agarose. The DNA extractions were then loaded in gel wells together with Gel Loading Dye, Purple (6X) (New England Biolabs) according to the protocol, Protocol for All DNA Loading Dyes (B7024). The fragments were compared with a 1 kb DNA Ladder (New England Biolabs) and a 100 bp DNA Ladder respectively (New England Biolabs). Voltage was set to 90V, for 60 minutes. The gel was then viewed in a Gel Doc EZ Gel Documentation System using Image lab (v.6.0.1) program.

3.2.1 Zymobiomics DNA miniprep kit (ZB kit)

The intestinal samples were prepared according to protocol with the exception of step 1. 750 µl of ZymoBIOMICS™ Lysis Solution was added to the prepared 1.5 mL eppendorf sample tubes and vortexed to dissolve the sample in the lysis solution in order to get the

maximum amount of sample content out of the tubes. The contents were then transferred to the ZR BashingBead™ Lysis Tubes and process continued according to protocol. 72h cultures of *Lactobacillus* spp. controls and isolated samples were added to a 2 mL eppendorf tube and centrifuged for 5 min at 5000 RCF. The supernatant was then removed before dissolving samples in 750 µl of ZymoBIOMICS™ Lysis Solution, which was transferred to the ZR BashingBead™ Lysis Tubes and the process continued according to protocol. In step 2, the bead beater device used for this experiment were Disruptor Genie (Scientific Industries), set for 40 minutes. The sample was eluted in 100 µl of ZymoBIOMICS™ DNase/RNase Free Water.

3.2.2 DNeasy blood and tissue kit (Qiagen) (BT kit)

Intestinal samples previously collected in 1.5 mL eppendorf tubes were suspended in 180 µl buffer ATL. Samples were then lysed with 50 mg of acid washed glass beads in a TissueLyser LT (Qiagen) according to Tissuelyser handbook – p. 21, excluding step 1. After, the extraction was completed according to the DNeasy blood & tissue handbook – p. 30-33. In step 2, tubes containing intestinal samples were incubated at 56°C in a shaking incubator at 220 rpm for 1.5 h. Samples were eluted in 100 µl buffer AE.

3.3 PCR and cleanup

16S Barcoding Kit 1-24 (SQK-16S024) (Oxford Nanopore Technologies) was used to amplify the 16S gene. The samples were prepared according to SQK-16S024 protocol, version 16S_9086_v1_revT_14Aug2019. From this point on, all steps were followed according to this protocol. Post PCR, samples were loaded on a 1% agarose gel, set to 90V for 1h. Purity values were collected with a DS-11 spectrophotometer (DeNovix) and DNA concentration with Qubit before cleanup. Sample volume was then adjusted with nuclease free water to follow the previously mentioned protocol with required sample amount of 50 µl for magnetic bead cleanup. After cleanup, the barcoded DNA was stored at -20°C until all samples were prepared for sequencing.

3.4 Library preparation and sequencing

All barcoded PCR samples except *L. helsingborgensis* control culture, were diluted and mixed in a single tube with a final concentration of 5.50 ng/ µl with equal ratios of DNA from each sample. Library preparation was performed according to protocol by the addition of rapid sequencing adapters (Oxford Nanopore Technologies), the library preparation was then mixed with sequencing buffer, loading beads (both Oxford Nanopore Technologies), and nuclease free water, before it was loaded into the FLO-MIN106 SpotON flow cell in a MinION sequencing device. Output files were set to FAST5 format, minimum q-score=9, sequencing for 24 h, all other settings as standard. The sequencing was completed with MinKNOW v20.10.3, in MinKNOW core v4.1.2.

3.5 Basecalling and analysis

The FAST5 files generated in MinKNOW were converted into FASTQ files using Guppy software v6.1.2. The FASTQ files were then analyzed in EPI2ME desktop agent v3.4.2 with What's In My Pot? (WIMP) v2021.09.09, and Fastq 16S v2021.09.09. Minimum identity for

match was set to 95%, minimum qscore=9, BLAST E-value filter=0.01, minimum coverage 30% and max target sequences=3. Since the WIMP run misidentified 3/5 control *Lactobacillus* species, it was not used for further analysis. To evaluate the 16S sequence similarity between the *Lactobacillus* spp. identified by the Fastq 16S analysis, sequences were downloaded from NCBI. Multiple alignment of the sequences were then done with Clustal Omega.

3.6 In vitro inhibition testing

In order to isolate various *Lactobacillus* species for inhibition testing, one full intestine of *A. mellifera mellifera* and *A. mellifera ligustica* each were diluted in 1 mL of sterile saline solution containing 0.1% peptone, 0.9% NaCl and 0.1% tween 80. The samples were diluted to 1:1, 1:10, 1:100 and 1:1000 ratios and spread on MRS (ThermoFisher) 1.5% agar plates supplemented with 2% D-fructose (Sigma-Aldrich) and 0.1% L-cysteine (Thermo fisher scientific), which will be referred to as supplemented MRS culture. Samples were incubated at 37ºC for 72 hours in Anaerocult C mini bags (Merck). Selections were made based on colony morphology and isolated colonies were streaked on new plates containing previously mentioned media. Bacterial morphology was then examined in a microscope after Gram staining the cells. Isolates containing rod-shaped bacilli were catalase tested and transferred into 50mL falcon tubes containing supplemented MRS liquid medium, incubated in Anaerocult C mini bags in a shaking incubator at 120 rpm, 35ºC for 72 hours. After extracting 2 mL of culture from isolates for sequencing, the cultures were adjusted to pH 6.6 with NaOH and directly placed on ice. Next, OD_{600} measurements were recorded. The tubes were then centrifuged at 4100 rpm for 30 min at 18ºC before filter sterilization of separated supernatant through a 0.2 µm micropore nylon membrane syringe filter (Life Sciences). The filtered culture supernatants were then adjusted to a concentration equivalent of OD_{600}=1.0 based on values taken before filtering. The supernatants were then treated with 1000 U/mL of catalase (Sigma-Aldrich) incubated for 2 h, and after stored in -20ºC until inhibition testing. In the agar plate experiment, KBHI medium was used. KBHI medium was BHI medium supplemented with 20.4 g/L potassium dihydrogen phosphate (KH_2PO_4) and 1.5% agar. All the plates were inoculated with 100 µl of the same 72 h *M. plutonius* culture measured to OD_{600}=0.7. 5.5 mm sterile filter discs containing 10 µl of each supernatant sample were placed on the inoculated KBHI plates. Each supernatant was set up in technical triplicates. Positive control was the supplemented MRS media used to culture the *Lactobacillus*, with the addition of 10 µg/ml tetracycline (Alfa Aesar). Negative control was supplemented MRS media. The plates were incubated in a GasPak System (BBL) in microaerophilic conditions by lighting a candle inside to burn out most of the oxygen. Plates were then incubated at 35 ºC for 72h. In the liquid culture experiment, *M. plutonius* cultures were set up in technical triplicates, using KBHI medium, and 12.5% (v/v) of the prepared filtered culture supernatant from each of the *Lactobacillus* cultures. 15 mL falcon tubes were used for the experiment, filled to 2/3 in total. Positive control tubes contained the same ratio of supplemented MRS medium with 10 µg/ml tetracycline (Alfa Aesar), and negative control was the addition of supplemented MRS broth. An 1:100 aliquot of an overnight culture of *M. plutonius* with OD_{600}=0.7 were added to each tube, lids were completely closed and the tubes incubated horizontally at 35 ºC at 120 rpm. OD_{600} measurements were taken at 3 h, 6 h, 9 h, and 24 h.

3.6.1 Data analysis in vitro assay

The bacterial growth data from the liquid broth experiment was fitted to the solution of a logistic growth curve with Microsoft Excel by adjusting the parameters r and K in the equation:

$$N(t) = \frac{N_0 \times K}{N_0 + (K - N_0) \times e^{-r \times t}}$$

Where N_0 is the OD_{600} value at t=0, r the growth rate and K is the maximum OD_{600} cell count.

The best match was achieved by minimizing errors between predicted reads and collected data according to the the following function, where differences between raw data values and predicted values were adjusted to fit the logistic regression curve:

$$f(t) = \sum_T \big(Y(t) - X(t)\big)^2$$

Where T are the time points in hours =[0, 3, 6, 9, 24]

The following statistical analyses tests were completed in IBM SPSS v28. The Shapiro-wilk test of normality tested on the growth rate factors (r values) generated in the logistic regression model showed the data for Isolation 16, and *B. mellifer* group was not normally distributed. Since the data from *A. apinorum* did show a difference in mean value OD_{600} of 2.07 at 24 h, compared to negative control, an independent two-sample t-test were instead conducted between the negative control, and *A. apinorum*. Homogeneity of variances was controlled prior to the t-test. Additionally, for the last measurement at 24 h, an independent two-sample t-test were run between negative control and *A. apinorum*.

4 Results

4.1 DNA extraction

The concentration and purity of intestinal samples extracted with BT and ZB kit are listed in Table 1. Cultured samples of controls, and isolated *Lactobacillus* spp. were extracted with ZB kit, see Table 2.

Table 1. Intestinal sample purities and DNA concentration for both extraction kits.

Subspecies	**Colony**	**Kit**	**DNA-concentration (ng/µl)**	**$A_{260/230}$**	**$A_{260/280}$**
Ligustica	AVIL	BT Qiagen	35.6	1.92	1.87
Ligustica	ÅFB	BT Qiagen	87.0	2.23	2.06
Mellifera	Skedfors	BT Qiagen	59.0	2.37	2.05
Mellifera	LTF	BT Qiagen	42.1	1.73	1.89
Ligustica	AVIL	ZB Zymobiomics	28.1	1.24	2.15
Ligustica	ÅFB	ZB Zymobiomics	25.5	1.93	1.88
Mellifera	Skedfors	ZB Zymobiomics	19.9	1.48	1.88
Mellifera	LTF	ZB Zymobiomics	59.0	1.80	1.84

Mean values for the genomic extraction group samples were $A_{260/230}$ of 2.1 for BT, and 1.6 for ZB kit. $A_{260/280}$ were 2.0 (BT), and 1.9 (ZB).

Table 2. ZB kit extractions of isolated *Lactobacillus* spp., and cultivated control species sourced from from CCUG. Cultivated in supplemented liquid MRS cultures.

Sample	**DNA-concentration (ng/µl)**	**$A_{260/230}$**	**$A_{260/280}$**
Isolation 6	12.5	0.94	2.01
Isolation 9	18.3	1.09	2.08
Isolation 10	11.6	0.97	2.98
Isolation 13	18.1	0.96	1.78
Isolation 15	16.6	1.22	2.55
Isolation 16	16.1	0.85	3.22
B. mellifer	27.8	1.18	1.72
A. apinorum	15.1	0.69	1.80
L. helsingborgensis	12.7	0.91	1.99
L. kimbladii	33.8	1.78	1.91
L. kullabergensis	13.8	0.85	2.17

L. melliventris	11.0	0.92	1.95

Gel figures collected after DNA extraction show a difference in extracted DNA integrity between DNeasy blood and tissue kit, and ZB kit. As can be seen in Figure 2A, the DNA extracted with the BT kit has a lower DNA quality, as smaller fragments are visible in the lower part of the gel in all sample lanes. DNA extracted with the ZB kit seen in Figure 2, gel B shows no visible traces of small DNA fragments at the bottom part of the gel, as only longer fragments were retained. Samples in Figure 2, gel C show long fragment sizes of DNA. Since bands from ladders are on difference levels, and distance between bands on the gel, refer to:

From down to up, 1 kb ladder (NEB): 0.5 kb, 1.0 kb, 1.5 kb, 2.0 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb.

From down to up, 100 bp ladder (NEB): 100 bp, 200 bp, 300 bp, 400bp, 500/517 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1200 bp, 1517 bp.

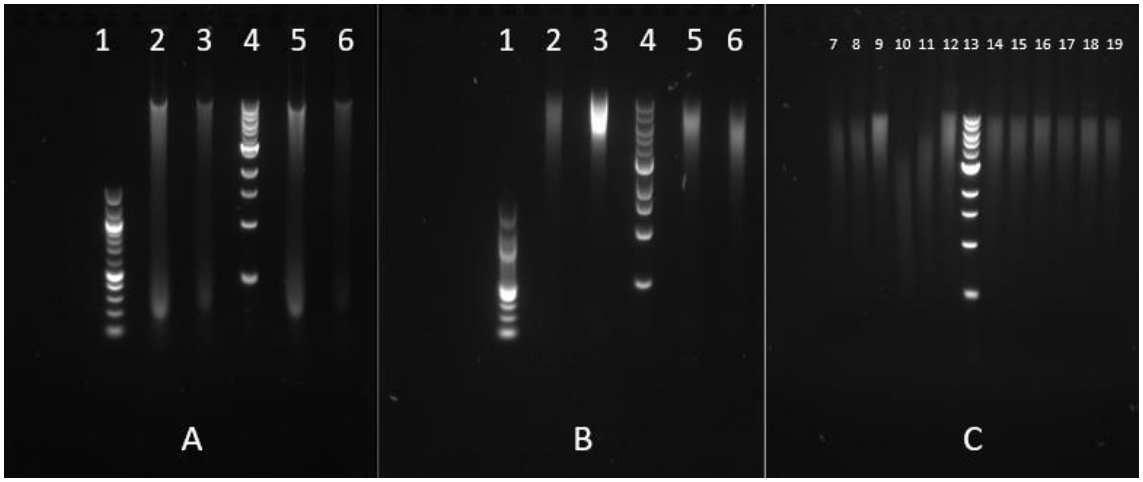

Figure 2. Extractions of *A. mellifera* microbiome DNA. Gel A shows intestinal sample DNA extracted with DNeasy blood and tissue kit. Gel B shows intestinal sample DNA extracted with ZB kit. Gel C shows cultured sample controls of *Lactobacillus* spp. and isolated samples of DNA, extracted with ZB kit. Gel A and B: Lane 1. 100bp DNA ladder(NEB); 2. *A. mellifera mellifera* Skedfors; 3. *A. mellifera mellifera* LTF; 4. 1kb DNA ladder (NEB); 5. *A. mellifera ligustica* ÅFB; 6. *A. mellifera ligustica* AVIL. Gel C: Lane 7. *L. melliventris;* 8. *L. kullabergensis*; 9. *L. kimbladii*; 10. *L. helsingborgensis*; 11. *A. apinorum*; 12. *B. mellifer;* 13. 1kb DNA ladder (NEB); 14. isolation 16; 15; 5. Isolation 15; 16. Isolation 13; 17. Isolation 10; 18. Isolation 9; 19. Isolation 6.

The 16S rRNA region was amplified on the PCR PTC-200 thermal cycler (MJ Research), and sample purity and concentrations were measured and can be seen in Table 3 for intestinal extracts, and Table 4 for isolated bacteria and *Lactobacillus* controls. Lower concentrations of amplified DNA was seen in samples extracted with the BT kit, compared to ZB samples. For the *L. helsingborgensis* control sample, it was omitted from the rest of the experiment and the values were not recorded.

Table 3. Sample concentration and purity post PCR with DNeasy blood and tissue kit BT and ZB kit. Bottom part of the table show comparitive values of the same samples post AMPure XP magnetic bead cleanup.

Subspecies	**Colony**	**Kit**	**DNA-concentraction (ng/µl)**	**$A_{260/230}$**	**$A_{260/280}$**
Ligustica	AVIL	BT Qiagen	8.6	0.88	1.54
Ligustica	ÅFB	BT Qiagen	3.6	0.86	1.54
Mellifera	Skedfors	BT Qiagen	6.1	0.89	1.54
Mellifera	LTF	BT Qiagen	6.5	0.87	1.55
Ligustica	AVIL	ZB Zymobiomics	7.1	0.89	1.52
Ligustica	ÅFB	ZB Zymobiomics	9.6	0.88	1.52
Mellifera	Skedfors	ZB Zymobiomics	18.4	0.89	1.52
Mellifera	LTF	ZB Zymobiomics	5.8	0.88	1.53
After Agencourt AMPure XP magnetic bead cleanup					
Ligustica	AVIL	BT Qiagen	18.0	2.27	1.72
Ligustica	ÅFB	BT Qiagen	9.6	1.79	1.82
Mellifera	Skedfors	BT Qiagen	17.3	2.18	1.91
Mellifera	LTF	BT Qiagen	16.2	2.33	1.88
Ligustica	AVIL	ZB Zymobiomics	25.2	1.95	1.93
Ligustica	ÅFB	ZB Zymobiomics	36.0	2.23	1.81
Mellifera	Skedfors	ZB Zymobiomics	68.0	2.35	1.92
Mellifera	LTF	ZB Zymobiomics	21.4	1.92	1.79

An independent-samples t-test comparing the extraction concentrations before magnetic bead cleanup between the kits resulted in the two groups having equal variance ($p=0.16$) according to Levene's Test for Equality of Variances. The mean DNA concentration of the BT kit (6.2 ± 2.1) and the ZB group (10.2 ± 5.7) was not statistically significantly different (95% CI, -3.4 to 11.4) $t_6=1.3$, $p=0.23$. Mean values of $A_{260/230}$ was 2.1 for both groups after magnetic bead cleanup, while $A_{260/280}$ was 1.83 for BT kit, and 1.86 for ZB kit samples.

Table 4. Sample purities and concentration for isolated colonies and *Lactobacillus* controls post PCR, before and after AMPure magnetic bead cleanup.

Sample	**CCUG number**	**DNA-concentraction (ng/µl)**	**$A_{260/230}$**	**$A_{260/280}$**
B. mellifer	63291T	14.7	Not measured	Not measured
A. apinorum	63287T	8.72	Not measured	Not measured
L. helsingborgensis	63635	1.27	Not measured	Not measured
L. kimbladii	63633T	26.2	Not measured	Not measured
L. kullabergensis	63632	9.42	Not measured	Not measured
L. melliventris	63629T	6.60	Not measured	Not measured
Isolation 6	-	38.2	Not measured	Not measured
Isolation 9	-	33.6	Not measured	Not measured
Isolation 10	-	28.4	Not measured	Not measured
Isolation 13	-	35.0	Not measured	Not measured
Isolation 15	-	27.0	Not measured	Not measured
Isolation 16	-	15.9	Not measured	Not measured
After AMPure XP magnetic bead cleanup				
B. mellifer	63291T	51.0	2.10	1.69
A. apinorum	63287T	27.6	2.95	1.81
L. helsingborgensis	63635	-	-	-
L. kimbladii	63633T	93.2	2.26	1.85
L. kullabergensis	63632	31.8	3.26	1.85
L. melliventris	63629T	18.9	3.68	1.90
Isolation 6	-	60.2	2.52	1.85
Isolation 9	-	58.0	2.49	1.82
Isolation 10	-	47.8	3.56	1.87
Isolation 13	-	55.6	3.15	1.88
Isolation 15	-	39.6	2.68	1.89
Isolation 16	-	54.6	2.62	1.89

Previous to cleaning the extractions with AMP magnetic beads, the PCR samples were loaded on a gel to determine if the fragment of interest was amplified. The 16S rRNA gene expected size is ~1550bp. In Figure 3, gel A, the BT extraction fragments in the sample wells are between the corresponding 1.5 kb and the 2.0 kb bands in the 1 kb DNA ladder (NEB). Figure 3, gel B, shows PCR samples extracted with the ZB kit. The samples are in level with the 2.0 kb band. The samples were cleaned up with AMPure XP beads and used for sequencing, except for *L. helsingborgensis* (Figure 3, gel C, lane 17). Since bands from ladders are on difference levels, and distance between bands on the gel, refer to:

From down to up, 1 kb ladder (NEB): 0.5 kb, 1.0 kb, 1.5 kb, 2.0 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb.

From down to up, 100 bp ladder (NEB): 100 bp, 200 bp, 300 bp, 400bp, 500/517 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1200 bp, 1517 bp.

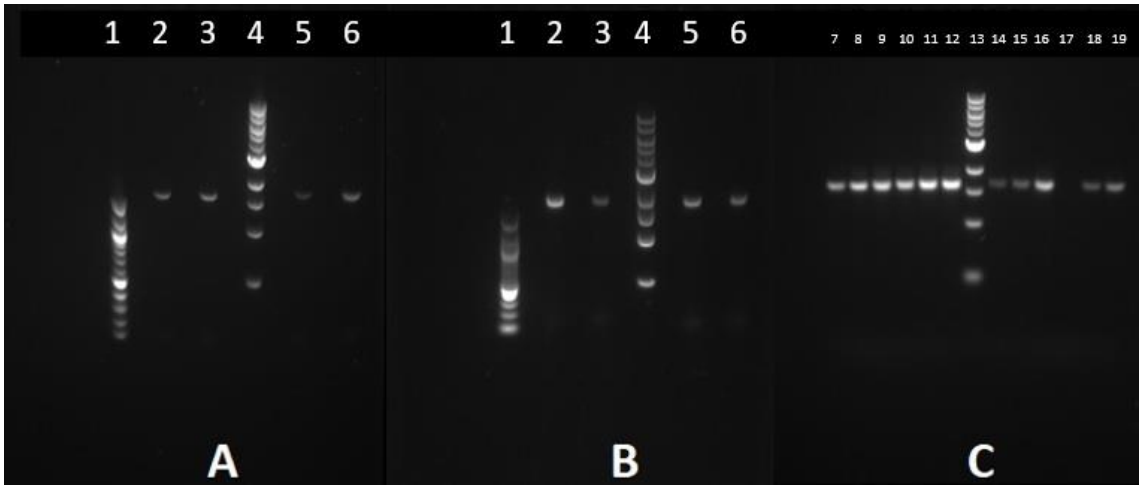

Figure 3. Sample extraction post PCR. Gel A contains intestinal samples extracted with DNeasy blood and tissue kit, Gel B intestinal samples extracted using ZB kit. Gel C shows *Lactobacillus* spp. controls and isolation samples extracted with ZB kit. Gel A and B: Lane 1. 100bp DNA ladder(NEB); 2. *A. mellifera mellifera* Skedfors; 3. *A. mellifera mellifera* LTF; 4. 1kb DNA ladder (NEB); 5. *A. mellifera ligustica* ÅFB; 6. *A. mellifera ligustica* AVIL. Gel C: Lane 7. *L. melliventris;* 8. *L. kullabergensis*; 9. *L. kimbladii*; 10. *L. helsingborgensis*; 11. *A. apinorum*; 12. *B. mellifer;* 13. 1kb DNA ladder (NEB); 14. isolation 16; 15; 5. Isolation 15; 16. Isolation 13; 17. Isolation 10; 18. Isolation 9; 19. Isolation 6.

4.2 Sequencing analysis

After loading the samples into the SpotON flow cell of type FLO-MIN106, the software found a total of 1037 nanopores active after the first MUX scan (Figure 4), whereas 440 nanopores were available for immediate sequencing. The total amount is 2048 nanopores divided into 512 pores. At the point of the last MUX scan after 23 h 13 m, there were 556 nanopores still active, whereas 316 nanopores were available for immediate sequencing.

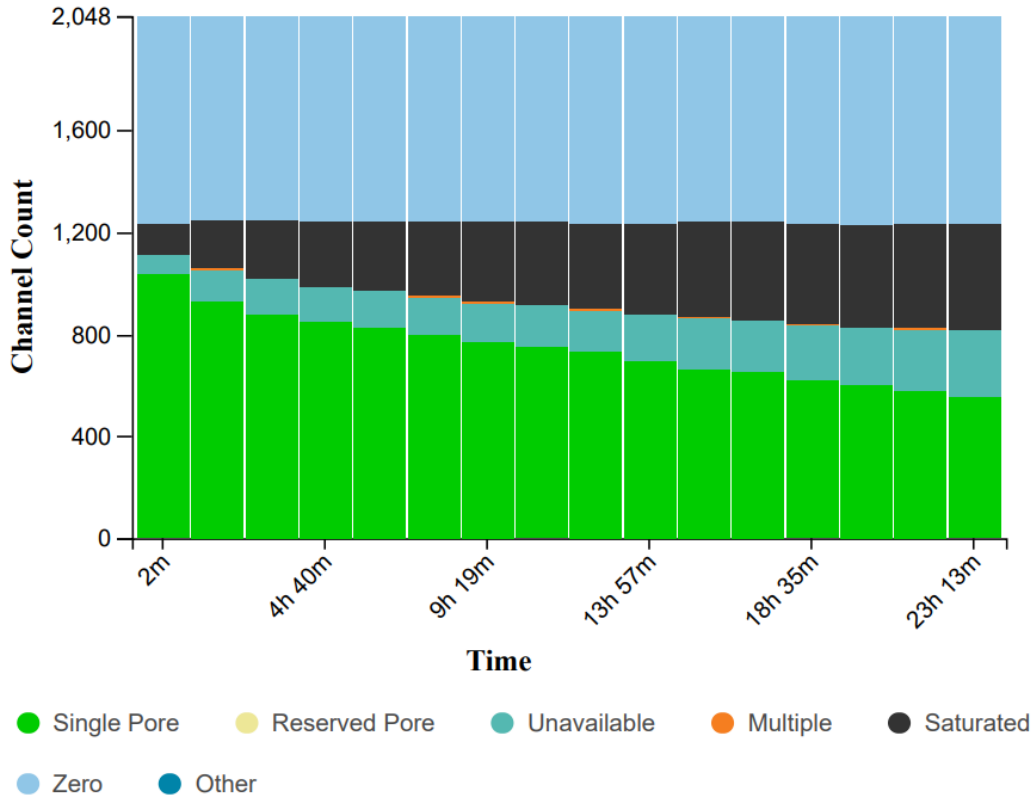

Figure 4. Active nanopore count during the 24 hour sequencing run with SpotON flow cell of type FLO-MIN106. The number of saturated and unavailable pores steadily increases as the sequencing run continues, while the number of active pores decreases.

The total amount of reads uploaded and registered in the EPI2ME Fastq 16S database analysis was 4.701.250 M. Start of the final analysis was: 2022-05-09 22:47:53, and finish: 2022-05-11 14:18:38. The analysis was manually stopped, since the final amount of 4.7 M reads did not increase after the final 24 h of analysis, despite the MinKNOW software reported registered total read count to 5.68 M. An overview of which sample represents which barcode and reads per barcode can be seen in Figure 5.

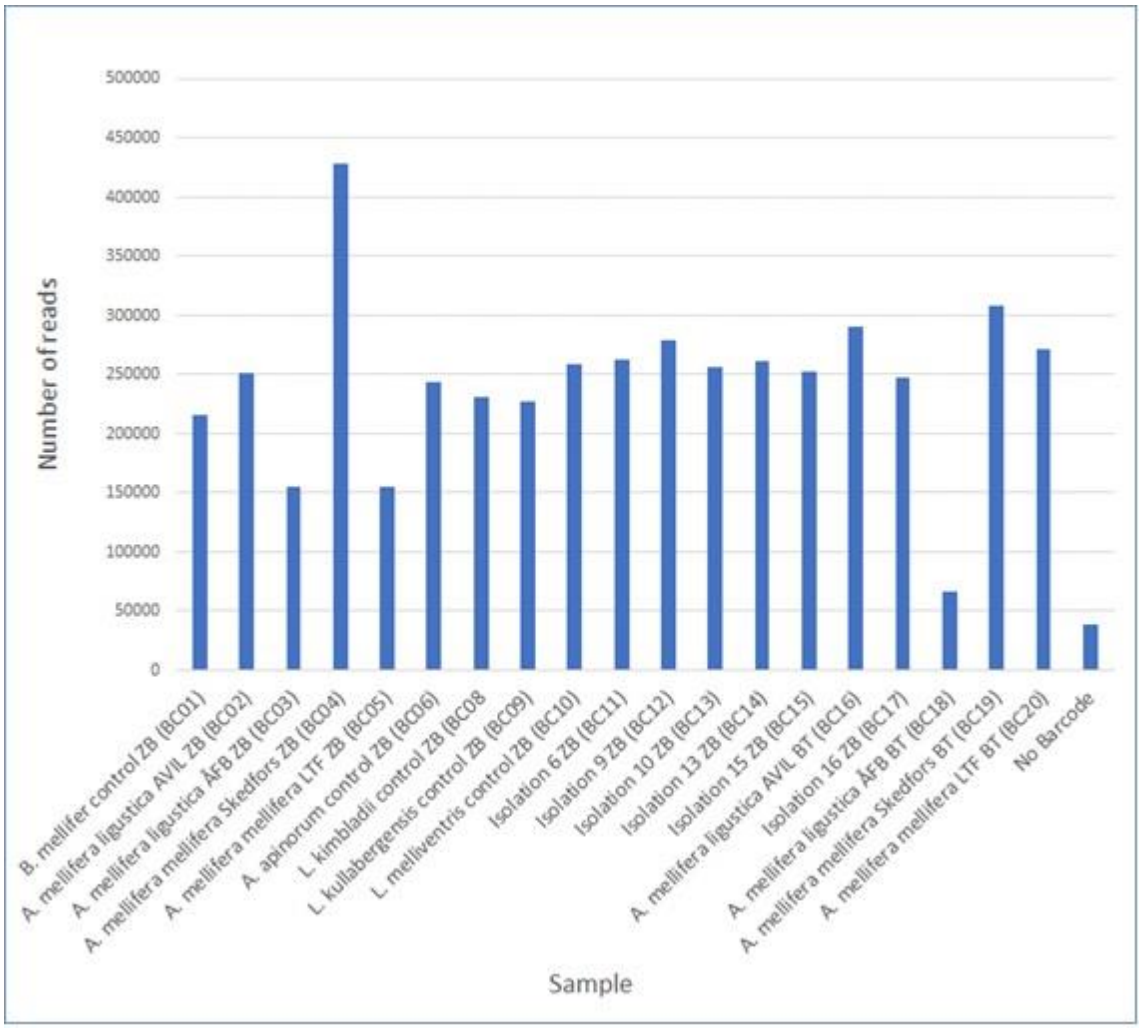

Figure 5. Bar chart of sample ratios for the sequencing run. The vertical axis show the number of reads per sample. Horizontal axis represents the barcodes attached to the samples. Sample identity is followed by kit abbreviations: ZB (Zymobiomics DNA miniprep kit), BT (DNeasy Blood and Tissue kit), and barcode number.

4.2.1 *Lactobacillus* controls, and isolated cultures

The number of matches per barcoded will be presented in this chapter. The percentages represents sequence matches to species, in percentage of matches per barcode, not sequence similarity percentage. Control species *B. mellifer* (BC01) was correctly identified in 99.7% of cases, by its previous name *L. mellifer*. *A. apinorum* control (BC06) reads matched to the correct identity in 55.2% of the reads, while 44.7% were labeled as *L. kunkeei*. *L. kimbladii* control (BC08) was categorized correctly for 98.1% of reads, while 1.1% of matches were categorized as *L. helsingborgensis*. *L. kullabergensis* control (BC09) had an 89.8% miscatecorization to *L. kimbladii*, while only 8.9% of reads were correctly identified as *L. kullabergensis*, and 1.1% reads matched with *L. helsingborgensis*. *L. melliventris* control (BC10) matched correctly in 99.6% of reads, with second species ratio at 0.1% match with *L. helsingborgensis*. Isolation 6 (BC11) was identified as *L. melliventris* (98.4%). Isolation 9 (BC12) had a 99.2% match to *L. kullabergensis*. Isolation 10 (BC13) was matched to *L. melliventris* (98.5%). Isolation 13 (BC14) had a 73.0% match to *L. kullabergensis*, a 25.6% match with *L. kimbladii* and a 1.3% match with *L. helsingborgensis*.

Isolation 15 (BC15) were categorized as *L. kullabergensis* (99.1%). Isolation 16 (BC17) also matched with *L. kullabergensis* (99.2%). Results of multiple alignment of 16S similarity between can be seen in Table 5. The 16S sequences has been gathered through NCBI database. To keep in mind when comparing the data, is that some sequences are categorized as partial. *L. mellis*; GenBank: JX099545.1, 1435 bp, *L. kimbladii*; GenBank: JX099549.1, 1437 bp, *L. helsingborgensis*; GenBank: JX099553.1, 1438 bp, *L. kullabergensis;* GenBank: JX099547.1, 1438 bp*, L. melliventris;* Genbank JX099551 1438 bp, *A. apinorum;* GenBank: JX099541.1, 1445 bp, *L. mellifer;* GenBank: JX099543.1, 1437 bp, *L. kunkeei;* GenBank: Y11374.1, 1517bp. The shortest sequence was 1435 bp, while the longest were 1517 bp, a difference of 82 bp. There were no further information from the NCBI database if fragments are missing and/or how long those missing fragments are.

Table 5. Percent identity matrix of the multiple alignment results of *L. helsingborgensis, L. melliventris, L kullabergensis, L. apis, L. mellis, L. kimbladii, L. kunkeei and L. mellifer*. Numbers depicted are 16S rRNA sequence similarity percentage.

Species	***L. apis***	***L. kunkeei***	***L. kimbladii***	***L. kullabergensis***	***L. helsingborgensis***	***L. melliventris***	***L. mellis***	***L. mellifer***
L. apis	100.0	98.55	86.57	84.59	84.31	84.59	88.19	87.57
L. kunkeei	98.55	100.0	87.24	84.95	84.61	85.33	88.34	88.00
L. kimbladii	86.57	87.24	100.0	99.30	98.61	98.46	89.30	90.54
L. kullabergensis	84.59	84.95	99.30	100.0	98.75	98.61	87.38	89.48
L. helsingborgensis	84.31	84.60	98.61	98.75	100.0	99.24	87.45	89.27
L. melliventris	84.59	85.33	98.46	98.61	99.24	100.0	87.38	89.27
L. mellis	88.19	88.34	89.30	87.38	87.45	87.38	100.0	92.82
L. mellifer	87.57	88.00	90.54	89.48	89.27	89.27	92.82	100.0

4.2.2 Intestinal samples

In total, sequencing results for the intestinal samples resulted in 1,063,244 (55%) classified, and 856,030 (45%) unclassified reads. Average accuracy was 97.0, average qscore 12.9, and average sequence length 1,580 bp. The total microbiome was composed of six genera: 54.4% *Lactobacillus* spp., 24.9% *Gilliamella* spp., 15.6% *Bartonella*spp., 3.1% *Snodgrassella* spp., 1.5% *Frischella* spp., 0.1% *Bombella* spp., and other genera 0.7%. The intestines were dominated by seven species of *Lactobacillus*: *L. helsingborgensis, L. melliventris, L. kullabergensis, L. apis (A. apinorum), L. mellis, L. kimbladii,* and *L. mellifer*. In "other" category depicted in Figure 6, and Figure 7, a few examples are total read counts: 817 *Lactobacillus bombicola*, 294 *Lactobacillus colini*, and 285 *Lactobacillus bombi* across all honeybee samples.

Extractions from subspecies *A. mellifera ligustica* originating from AVIL colony show large differences in *Lactobacillus* species distribution between extraction kits. Of all *Lactobacillus* present, 67.1% was *L. helsingborgensis*. In the comparative sample gathered with ZB kit, the ratio of *L. helsingborgensis* were 4.7%. The species to dominate the equivalent sample extracted with ZB kit was *L. kimbladii* (42.0%). Additionally, the other *A. mellifera ligustica* samples gathered from the ÅFB colony was dominated by *L. kimbladii* measuring 27.3% for BT extraction, and 22.2% with ZB. For complete overview of sample ratios for all *A. mellifera ligustica* samples, see Figure 6.

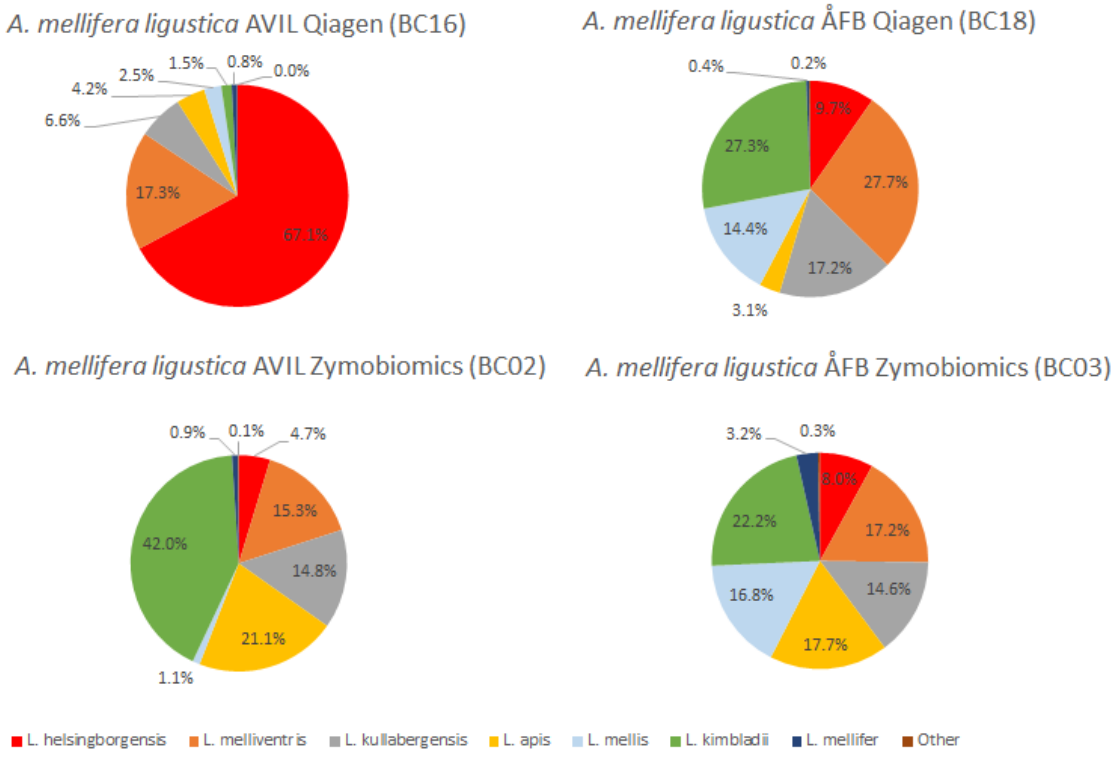

Figure 6. *Lactobacillus* species ratio data from *A. mellifera ligustica* extractions. The top two pie charts depict intestinal sample extracts from BT kit. Bottom row are samples extracted with ZB kit. Both pie charts to the left are extractions from the same AVIL colony. The pie charts to the right is from the same ÅFB colony.

Overview of *Lactobacillus* species in *A. mellifera mellifera* can be seen in Figure 7. Highest read count for *A. mellifera mellifera* samples from Skedfors colony were *L. kimbladii* at 25.3% (BT kit), and *L. mellis* measuring at 37.2% for the ZB sample. Ratios of LTF colony intestines were dominated by 49.4% *L. apis (A. apinorum)* in the BT extraction, while the ZB sample had the highest read count of 42.9% from *L. melliventris*.

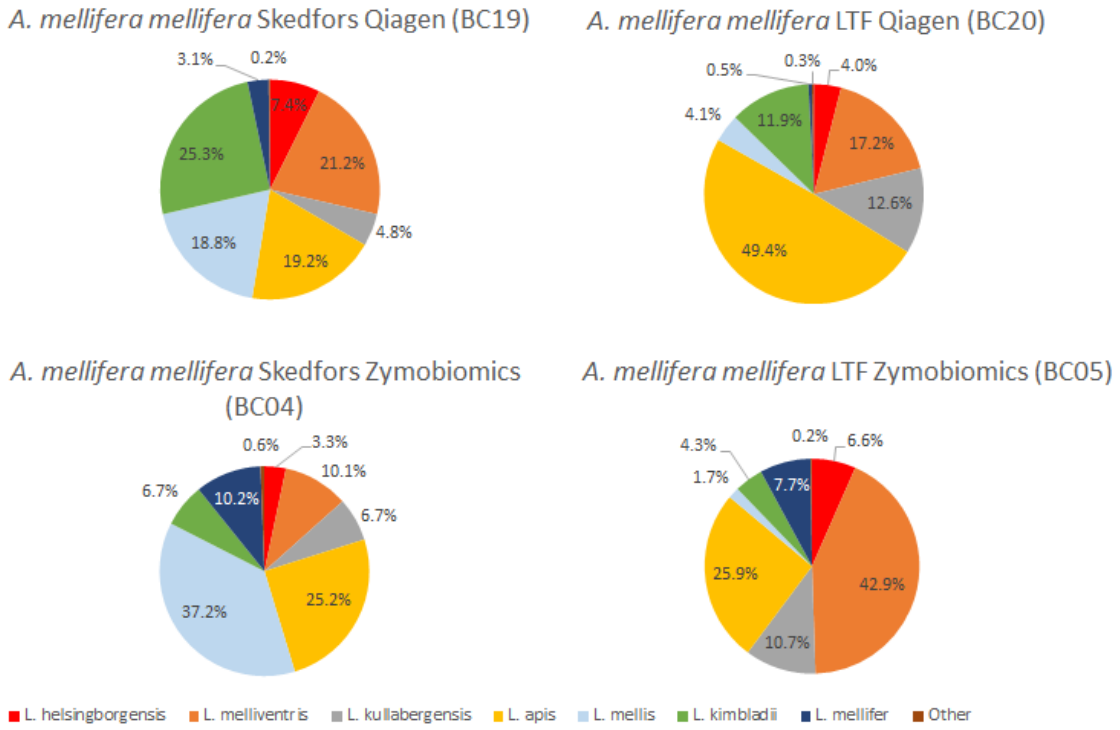

Figure 7. *Lactobacillus* species ratio data from *A. mellifera mellifera* extractions. The top two pie charts depict intestinal sample extracts with BT kit. Bottom row are samples extracted with ZB kit. Both pie charts to the left are extractions from the same Skedfors colony. The pie charts to the right is from the same LTF colony.

4.3 Inhibition testing

4.3.1 Agar plate experiment

At 72 h, the KBHI agar plates only showed signs of inhibition for the postitive control samples (10 µg/ml tetracycline). All other samples had healthy growth over the entire plates. No data analysis was therefore conducted based on these results.

4.3.2 Liquid broth growth experiment

Raw measurement data plotted on a scatter chart with connected lines can be seen in Figure 8. The logistic regression model resulted in a growth rate value (*r*) for each culture. The Shapiro-wilk test of normality resulted in p-values higher than cutoff value 0.05. P-values for the groups were higher than cutoff (0.05) in Levene's Test for Equality of Variances. An independent-samples t-test was run to determine if there were differences in mean growth rate (*r*-value) between the cell-free supernatant *A. apinorum* group, and negative control group. The growth rate of the the *A. apinorum* (0.64 ±0.03) and the negative control (0.61 ± 0.02) was not statistically significantly different (95% CI, -0.089 to 0.022), t4 = -1.7, p =0.17.

An independent-samples t-test was also run to determine if there were differences in mean OD_{600} values between the *A. apinorum* treatment and negative control group at 24 h. The mean OD_{600} of the *A. apinorum* group (3.93± 0.12) and the negative control group (6.00 ± 0.35) was statistically significantly different (95% CI, 1.48 to 2.65), t4 = 9.8, p<0.001. pH values were taken at the end of the experiment in negative control and *A. apinorum*, both recorded pH=5.2.

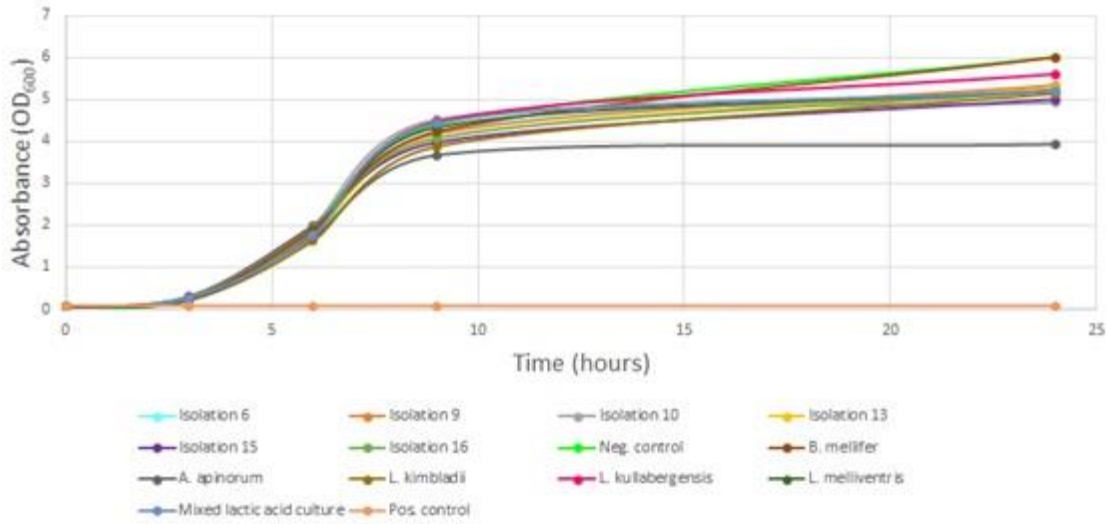

Figure 8. Raw data growth curve of *M. plutonius* with 12.5% (v/v) cell-free supernatant from *Lactobacillus* cultures. The data is generated from mean values of the sample triplicates. The light green line represents negative control as reference, positive control at the bottom showing no growth contained 12.5% (v/v) with 10 µg/mL tetracycline. The black line having an average OD_{600}=3.93 at 24 h, was *A. apinorum (L. apis)*. Negative control measured to OD_{600}=6.0 at 24 h.

5 Discussion

The objectives of this study was to design, plan, and test methods to be able to identify which species of the genus *Lactobacillus* was present in the intestines of *A. mellifera mellifera* and *A. mellifera ligustica.* The two subspecies of *A. mellifera* were sourced from two different colonies per subspecies, to investigate if there was a difference in microbiome composition between the subspecies, and difference colony populations. Inhibition testing of *Lactobacillus* isolates and controls were set up to investigate if the species had an inhibiting effect on *M. plutonius*. The results could help increase the knowledge about honeybee host-microbiota relationships.

5.1 DNA extractions

The DNA extraction kits evaluated for the study were DNeasy blood and tissue kit (BT kit), and Zymobiomics DNA miniprep kit (ZB kit). *Lactobacillus* spp. are Gram positive bacterial species with thick and hard to lyse cell walls (Hammes & Vogel, 1995). In order to extract nucleic acids from gram positive bacteria, there are several ways of breaking the cell wall in order to extract DNA. Chemical cell lysis reactants such as different lysozymes can be used, as well as physical disruption of the cell wall by bead beating (Chassy & Giuffrida, 1980). The concern is that the extraction method will cause fragmentation of the DNA, which could result in impaired PCR efficiency of the 16S gene, and further affect sequencing results (Liu et al., 2007; Yu & Morrison, 2004). Studies have reported increased DNA yields by applying bead beating protocols as an extra step in the DNA extraction protocols (de Boer et al., 2010; Fujimoto et al., 2004). However in a recent study, the bead-beating method showed slightly lower gDNA yield compared to chemical lysis to determine DNA efficiency in three gram-positive and two gram-negative species (Li et al., 2020). Both DNA extraction kits used in the study were applying a combination of the two methods, while only DNease blood and tissue kit implemented proteinase K treatment into the protocol. The results collected in this study showed that DNA integrity was higher in extractions from the ZB kit. Gel Figure 2 shows significant fragmentation of DNA with a high concentration of low length fragments migrating far down the gel in DNeasy blood and tissue extraction samples. However in Figure 2, gel B, the ZB samples show only detectable traces of DNA far up the gel, with high concentrations of DNA around the top, which fades rapidly further down the gel. There could be several reasons why the DNeasy blood and tissue kit samples show higher DNA fragmentation, one reason could be related to the Tissuelyzer more intense bead beating compared to the ZB kit. In a previous study, the Zymobiomics DNA miniprep kit was compared to the DNeasy blood and tissue kit samples collected from microbiota from poultry. Results did show a significantly higher ($p<0.05$) number of 16S copies from the ZB extractions (Abundo et al., 2021). Results of this study showed no significant difference ($p=0.230$) in mean concentration of 16S amplified fragements between the kits post PCR. This indicates the difference in DNA integrity seen in Figure 2, had no significant effect on PCR efficiency. It is possible the different extraction methods could introduce a bias towards Gram negative, or Gram positive species because of their difference in cell wall thickness. However, due to small sample groups with a high SD, its difficult to draw any conclusions based on that result. QC mean value results post gDNA extraction were within range of pure DNA ($A_{260/230}$ ~2.0-2.2) for the BT group at $A_{260/230}$=2.1, and 1.6 for ZB kit. A reasonable explanation for the low $A_{260/230}$ related to ZB kit is the presence of guanidinium thiocyanate,

a component present in the BT kit DNA Binding Buffer. According to Thermofisher, a low $A_{260/230}$ can be due to residual guanidine. $A_{260/280}$ were 2.0 (BT), and 1.9 (ZB). $A_{260/280}$ for pure DNA is around 1.8, and could vary due to pH, low DNA concentration, or phenol contamination. Slightly higher $A_{260/280}$ however is not indicative of an issue (Brian, 2015; Wilfinger et al., 1997). QC values for *Lactobacillus* controls and isolates extracted with ZB kit had a mean $A_{260/230}$ value of 1.03, which is lower than the intestinal mean for the ZB kit (1.6). Since the samples were centrifuged, and most but not all MRS culture were taken out, it is likely due to carbohydrate contamination. Presence of carbohydrates will lower the $A_{260/230}$ value. Post PCR and cleanup, the culture samples had a higher mean $A_{260/230}$ of 2.84. The cause of this is either the use of a blank with a different pH, or making a blank on a dirty pedestal (Wilfinger et al., 1997). Because of the aforementioned possible causes and the limited amount of sample, the samples were used in sequencing. It was therefore a compromise to not repeat the purification steps multiple times to achive "perfect" purity values.

5.2 Sequencing results

In the planning phase of this study, different primers were evaluated based on previous studies conducting similar research projects. It was found that previous studies were limited to shorter fragment sequences of the 16S region due to the sequencing methods used (Ellegaard & Engel, 2019; Kwon et al., 2004). The benefit of Oxford Nanopore Sequencing is the ability to sequence the whole 16S gene, and therefore not having to compromise which region/regions of the gene to be chosen for the analysis. The 27F and 1492R commercial Oxford Nanopore Sequencing primers included in the SQK-16S024 kit was used in the study. Although nanopore sequencing has a lower accuracy than Illumina MiSeq system, the error rate can be slightly mitigated by filtering out reads with lower Qscores in the analysis (Jain et al., 2018; McNaughton et al., 2019). Additionally, extractions of control species were sequenced with unique barcodes attached to evaluate the accuracy of the results. *L. helsingborgensis* control sample was omitted from the sequencing and growth experiments: The reason was that the cells did not generate any growth in the liquid broth, and as seen in Figure 3 (Gel C, lane 17), the 16S rRNA amplicon was not visible in the sample lane.

5.2.1 Control and isolated *Lactobacillus* samples

Starting with the correct matches for known coltrols, *L. mellifer* (*B. mellifer*) were correctly matched in 99.7% of cases, with the closest sequence similarity to *L. kunkeei* 98.55% (Table 5). *L. kimbladii* was categorized correctly for 98.1% of reads. *L. melliventris* matched correctly in 99.6% of reads, despite sharing a 99.24% identity with *L. kullabergensis*, 98.61% with *L. kullabergensis*, and 98.46% with *L. kimbladii* (Table 5). *L. apis (A. apinorum)* reads only matched to the correct identity in 55.2% of the reads, while 44.7% were labeled as *L. kunkeei*. *L. apis* has a 16S similarity of 98.55% with *L. kunkeei*, which is the species with the closest similarity. *L. kullabergensis* control had an 89.8% miscategorization to *L. kimbladii*, of which it shares 99.30% sequence similarity with. Only 8.9% of reads were correctly identified as *L. kullabergensis*, and 1.1% reads matched with *L. helsingborgensis,* of which it shares 98.75% of the sequence. The question is if these miscategorizations of control species is due to the NCBI database, the nanopore sequencing errors, or the

identification method used by CCUG to identify these species in stock. The NCBI database is however a well known standard database for 16S genotypic classification organisms, and uses more than 150 sources including *Catalog of Life* and the *Encyclopedia of Life*, and is being updated daily (Balvočiūtė & Huson, 2017). Adressing the CCUG stock information, it is not specified how the species strains were identified. For *L. helsingborgensis* and *L. apis*, only Api rapidID32strep is mentioned as an ID method. ID method information regarding *L. mellifer*, *L. kimbladii*, and *L. melliventris* were Api rapidID32strept, and 16S rRNA partial gene seq (Table A). The nanopore sequencing platform, previously reported to have an error rate of 6-15%, is also a likely explanation of the misidentifications (Jain et al., 2017; Loman et al., 2012). Moving on to the cultures of isolated *Lactobacillus* from the intesinal samples, Isolation 6 was identified as *L. melliventris* 98.4%, which is just slightly lower than the *L. melliventris* control (99.6%). Isolation 9 had a 99.2% match to *L. kullabergensis*, comparing to the control for the same species which was miscategorized as *L. kimbladii* in 89.8% of cases, which could mean the isolate is either of them, as there is no accurate reference. Isolation 10 was also matched to *L. melliventris* with a very similar match percentage as Isolation 6 (98.5%). Isolation 13 had a 73.0% match to *L. kullabergensis*, a 25.6% match with *L. kimbladii* and a 1.3% match with *L. helsingborgensis*. Comparing this result with the *L. kullabergensis* control, there are similarities, as the same three species are involved. If there was no control to compare with, the conclusion would be that it was a mixed culture, but it could be an isolated species, whether it is a *L. kullabergensis*, or a *L. kimbladii*. The results for Isolation 15 and 16 were categorized as *L. kullabergensis* for 99.1% and 99.2% of cases respectively, which causes some doubts on the results previosly discussed for the *L. kullabergensis* control. Since the nanopore sequencing managed to identify both isolates with a high percentage match, it indicates the control for that species could be a strain more similar to *L. kimbladii* according to NCBI database.

5.2.2 Intestinal samples

Results of the nanopore sequencing confirmed the presence of the *Lactobacillus* species *L. apis (A. apinorum)*, *L. kunkeei*, *L. kimbladii*, *L. helsingborgensis*, *L. melliventris*, *L. mellis*, and *L. kullabergensis*, found in *A. mellifera* in previous studies. In studies conducted in Sweden, the same seven *Lactobacillus* species has been found in *A. mellifera* intestines (Olofsson et al., 2014; Olofsson & Vásquez, 2008). *Lactobacillus* species: *L. bombicola, L. colini,* and *L. bombi,* which was grouped as category "other" in Figure 6 and 7, with a ratio ranging between 0.1% - 0.8% between samples. *L. bombicola* has previously been found in bumble bee guts of the species *Bombus pascuorum, Bombus lapidarius, Bombus hypnorum and Bombus terrestris* (Praet et al., 2015). It has a 97% 16S sequence similarity to *L. apis* (*A. apinorum*) and due to the low amount of matches (817 out of 1,063,244 total), it is likely the matches was because of sequencing errors. Additionally for *L. colini and L. bombi,* previously found in Northern Bobwhite (*Colinus virginianus*), and bumblebee *B. terrestris* respectively, a total of 294 and 285 matches are low to draw the conclusion that these species are part of the normal flora of the bees (Billiet et al., 2017; Zhang et al., 2017). Regarding the comparison of the different subspecies *A. mellifera ligistica,* and *A. mellifera mellifera* investigated in this study, there were no difference in the *Lactobacillus* species found in their intestines, regardless of colony location. This result has been confirmed in a previous study which investigated lactic acid bacterial flora between different subspecies of honeybee, where the low diversity of *Lactobacillus* was seen between all subspecies of the

study (Olofsson et al., 2011). Interestingly however, the ratios of these species were shown to vary a lot between samples in this study, as can be seen in Figure 6 and 7. The two intestines extracted from *A. mellifera ligustica* AVIL colony with BT kit contained 67.1% *L. helsingborgensis*. The other sample collected from the same subspecies and colony with ZB kit, had only 4.7% *L. helsingborgensis*. In the other samples from this study, the differences were not as large, but enough to detect microbiome ratio variances across colony, subspecies, and individual samples of the same colony population. This result indicates there could be large differences in individual workerbee microbiome within the same colony, in terms of the ratio of *Lactobacillus* species, which has been documented in previous research on the subject (Ahn et al., 2012; Moran et al., 2012) To confirm this, more studies with a higher sample size and the same extraction kit should be conducted, to remove any bias the kits might have caused.

5.3 In vitro inhibition of *M. plutonius*

5.3.1 Agar plate experiment

Isolated *Lactobacillus* colonies and known *Lactobacillus* spp. controls (Table A, appendix) were set up against the honeybee pathogen *M. plutonius* in two growth experiments. The purpose of the inhibition experiments were to investigate if some *Lactobacillus* species present in the intestines are able to protect the host honeybees by inhibiting the growth of *M. plutonius* with bacteriocins. It is known from previous studies that lactic acid bacteria inhibits growth of pathogenic bacterial species by lowering pH in the intestinal tract, as most bacteria grow optimally in a pH of 6.5-7.0 (Jin & Kirk, 2018; Sookkhee et al., 2001). Since the experiment was designed to examine bacteriocin activity, pH inhibition bias was removed by balancing the KBHI, and the filtered *Lactobacillus* supernatants to pH 6.6, respectively. Hydrogen peroxide produced by *Lactobacillus* is also known to have an inhibiting effect on many bacteria as it is very reactive and can form the toxic hydroxyl radical (^{1}OH) (Clifford & Repine, 1982; Hawes et al., 1996). The supernatants was therefore treated with catalase, an enzyme that breaks down hydrogen peroxide (Bonnichsen et al., 1947). The agar plate experiment resulted in no visual inhibition of the *M. plutonius* growth around the sterile fulter discs in any of the samples. However, for the positive control containing 10 μg/ml tetracycline, the discs had irregular inhibition zones of ~3-4 mm after 72 h. Comparing this result to previous studies where *M. plutonius* has been subjected to tetracycline, average inhibition zone size was reported in a 2014 study to be 37.3 mm for samples with the same tetracycline concentration, with 7 mm discs instead of 5.5 mm used in this experiment (Wu et al., 2014). The reason for reaching a lower inhibition zone is unclear, especially considering of the effectivity of the tetracycline in the liquid broth experiment.

5.3.2 Liquid broth experiment

In the first 6 hours of OD_{600} measurements, no inhibition of the *M. plutonius* cultures were seen in the data. It is therefore expected that the logistic regression modeling did not show statistical difference between *A. apinorum*, and negative control. It is perculiar that the growth curves are identical until t=9. A independent sample t-test comparing t=24 did however show significant difference in mean values between negative control and *A.*

apinorum. As no other studies were found comparing liquid broth cultures of *M. plutonius* to the point of this study, the comparisons will be related to inhibition experiments on agar plates. In a study from 2014, *L. apis* was isolated from *A. mellifera* intestines and the previous name *A. apis (*now *A.apinorum)* was suggested. *M. plutonius* and *P. larvae* was applied on MYPGP agar plates in triplicates, 0.2 ml culture of living *A. apis* was added to 8 mm diameter holes in the agar. The plates were incubated at 30 ºC for 24-48 h, and growth inhibition reached an average of 17 mm (Killer et al., 2014). Additional studies has been showing inhibition of both *P. larvae* and *M. plutonius* by applying living cultures of various *Lactobacillus* species in vitro (D Evans & Armstrong, 2005; Forsgren et al., 2010; Wu et al., 2014). The problem with in vitro testing with living cultures of both the pathogen, and the bacteria causing inhibition zones is that there is no definitive answer to what is causing the effect. As previously mentioned, inhibition of bacteria can be caused by pH reduction, nutrient competition, hydrogen peroxide, bacteriocins, or other unknown factors (Praet et al., 2018). The aim of this experiment was therefore to investigate bacteriocin activity specifically. The effect of antimicrobial bacteriocin activity originating from *Lactobacillus* spp. has been investigated for a long time, but not against honeybee pathogens, where the studies has often used living cultures for inhibition assays as previously mentioned (Benech et al., 2003; Prado-Acosta et al., 2010). As cell-free supernatant inhibition was not affected at all in the samples of this study until the 9 h measurement, a possible explanation could be that the bacteriocin expressed by *A. apinorum* has a lower optimum pH efficiency than pH 6.6, which was the start value of the culture. Since pH measurements would cause contamination, the pH was therefore not taken until after the experiment was completed, and recorded pH=5.2, for the cultures. The growth conditions of the *Lactobacillus* cultures in terms of pH, temperature, and media also affects the efficiency of bacteriocin production (Yang et al., 2018).

6 Conclusions

The project aim was to investigate which *Lactobacillus* species that were present in *A. mellifera mellifera* and *A. mellifera ligustica*, and if there was a difference in microbiome between hives. *L. apis (L. apinorum), L. mellis, L. mellifer, L. melliventris, L. kimbladii, L. helsingborgensis*, and *L. kullabergensis* was found in both subspecies across all colonies. The findings has been confirmed in previous studies on the subject (Martinson et al., 2012; Olofsson & Vásquez, 2008; Olofsson et al., 2011). It seems the difference in ratios of these seven species fluctuate drastically between individual worker bees, but larger sample size studies using the same extraction kits throughout the process needs to confirm the results, as the Dneasy blood and tissue kit used in this study caused heavy fragmentation of extracted gDNA. During the analysis of the sequencing results, doubts about the accuracy of the Nanopore sequencing, and the identification methods used by the source of *Lactobacillus* control species was raised. Control species *L. kullabergensis* only had correct match in 8.9% of cases, while Isolation 15 and 16 respectively were identified as L. kullabergensis in 99.1% and 99.2% of cases. If the error was due to the sequencing, accuracy would not be so close to 100% match for these isolates. However, *A. apinorum* control reads only matched to the correct identity in 55.2% of the reads, while 44.7% were labeled as *L. kunkeei.* The species share 98.55% of the 16S sequence, and the even split between the two could suggest errors caused the mismatch. The nanopore sequencing tool has however

proved to be an easy to use tool, which is useful in sequencing experiments where a general overview of organisms present in samples are investigated. In cases where sequence results need to have high accuracy, other methods such as Illumina sequencing are better suited. Interesting findings of growth inhibition against *M. plutonius,* likely caused by bacteriocin activity was found in the cell-free supernatant of *A. apinorum*. Since the growth inhibition had no effect on the early stages of bacterial growth, a suggestion for future research would be to test the bacteriocin in different growth medias, concentrations, pH values and temperatures to evaluate optimal conditions for the bacteriocin peptide(s).

7 Ethical considerations

The increase of antibiotic resistance has been an increasing concern for many years. Therefore, safety when handling these microbes are of high priority. *Lactobacillus* spp., are known from previous research of their probiotic effects, and further research is of interest in public health (Maragkoudakis et al., 2006). The *Lactobacillus* genus has been shown to inhibit growth of known pathogens in animals and humans, and careful consideration should be taken into account when conducting experiments on these effects towards harmful pathogens (D Evans & Armstrong, 2005). *M. plutonius* infects the larvae of honeybees, but is possible to clear infected individuals from a hive without needing to burn it, as per standard practice for *P. larvae,* which is another pathogen known to infect *A. mellifera*. However, *M. plutonius* could still escape the lab if studies are not done with caution, and infection risk needs to be avoided.

Several *A. mellifera* was put down to be able to conduct this experiment. For this project, it was not needed go through an ethical board, since insects and bacteria was investigated. In terms of moral and ethical implications, the study results are aimed towards increasing the understanding of the mutualistic relationships between the honeybees, and the microbiota in their intestines. The information could be used to lay the basis for further research, potentially leading to better honeybee health in the future.

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9 References

Abundo, M. E. C., Ngunjiri, J. M., Taylor, K. J. M., Ji, H., Ghorbani, A., Kc, M., . . . Lee, C.-W. (2021). Assessment of two DNA extraction kits for profiling poultry respiratory microbiota from multiple sample types. *Plos one*, *16*(1), e0241732. https://doi.org/10.1371/journal.pone.0241732

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. https://doi.org/10.1007/s12275-012-2188-0

Aiba, Y., Suzuki, N., Kabir, A. M. A., Takagi, A., & Koga, Y. (1998). Lactic acid-mediated suppression of Helicobacter pylori by the oral administration of Lactobacillus salivarius as a probiotic in a gnotobiotic murine model. *The American journal of gastroenterology*, *93*(11), 2097-2101. https://doi.org/10.1016/S0002-9270(98)00481-X

Allen-Wardell, G., Bernhardt, P., Bitner, R., Burquez, A., Buchmann, S., Cane, J., . . . Ingram, M. (1998). The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. *Conservation biology*, 8-17.

Allsopp, M. H., De Lange, W. J., & Veldtman, R. (2008). Valuing insect pollination services with cost of replacement. *PloS one*, *3*(9), e3128. https://doi.org/10.1371/journal.pone.0003128

Arredondo, D., Castelli, L., Porrini, M. P., Garrido, P. M., Eguaras, M. J., Zunino, P., & Antunez, K. (2018). Lactobacillus kunkeei strains decreased the infection by honey bee pathogens Paenibacillus larvae and Nosema ceranae. *Beneficial microbes*, *9*(2), 279-290. https://doi.org/10.3920/BM2017.0075

Balvočiūtė, M., & Huson, D. H. (2017). SILVA, RDP, Greengenes, NCBI and OTT—how do these taxonomies compare? *BMC genomics*, *18*(2), 1-8. https://doi.org/10.1186/s12864-017-3501-4

Benech, R. O., Kheadr, E. E., Lacroix, C., & Fliss, I. (2003). Impact of nisin producing culture and liposome-encapsulated nisin on ripening of Lactobacillus added-Cheddar cheese. *Journal of dairy science*, *86*(6), 1895-1909. https://doi.org/10.3168/jds.S0022-0302(03)73776-X

Bernet-Camard, M.-F., Liévin, V., Brassart, D., Neeser, J.-R., Servin, A. L., & Hudault, S. (1997). The human Lactobacillus acidophilus strain LA1 secretes a nonbacteriocin antibacterial substance (s) active in vitro and in vivo. *Applied and Environmental Microbiology*, *63*(7), 2747-2753. https://doi.org/10.1128/aem.63.7.2747-2753.1997

Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F., & Smagghe, G. (2017). Colony contact contributes to the diversity of gut bacteria in bumblebees (Bombus terrestris). *Insect science*, *24*(2), 270-277. https://doi.org/10.1111/1744-7917.12284

Bleidorn, C. (2016). Third generation sequencing: technology and its potential impact on evolutionary biodiversity research. *Systematics and biodiversity*, *14*(1), 1-8. https://doi.org/10.1080/14772000.2015.1099575

Bonnichsen, R. K., Chance, B., & Theorell, H. (1947). Catalase activity. *Acta chem. scand*, *1*, 685-709.

Brian, M. (2015). Assessment of Nucleic Acid Purity. In. Wilmington, MA, USA: Thermo Fisher Scientific.

Byrne, A., Supple, M. A., Volden, R., Laidre, K. L., Shapiro, B., & Vollmers, C. (2019). Depletion of hemoglobin transcripts and long-read sequencing improves the transcriptome annotation of the polar bear (Ursus maritimus). *Frontiers in genetics*, 643. https://doi.org/10.3389/fgene.2019.00643

Chapman, N. C., Sheng, J., Lim, J., Malfroy, S. F., Harpur, B. A., Zayed, A., . . . Remnant, E. J. (2019). Genetic origins of honey bees (Apis mellifera) on Kangaroo Island and Norfolk Island (Australia) and the Kingdom of Tonga. *Apidologie*, *50*(1), 28-39. https://doi.org/10.1007/s13592-018-0615-x

Chassy, B. M., & Giuffrida, A. (1980). Method for the lysis of Gram-positive, asporogenous bacteria with lysozyme. *Applied and environmental microbiology*, *39*(1), 153-158. https://doi.org/10.1128/aem.39.1.153-158.1980

Clifford, D. P., & Repine, J. E. (1982). Hydrogen peroxide mediated killing of bacteria. *Molecular and cellular biochemistry*, *49*(3), 143-149. https://doi.org/10.1007/BF00231175

Corby-Harris, V., Maes, P., & Anderson, K. E. (2014). The bacterial communities associated with honey bee (Apis mellifera) foragers. *PloS one*, *9*(4), e95056. https://doi.org/10.1371/journal.pone.0095056

Crane, E. (1991). Honey from honeybees and other insects. *Ethology Ecology & Evolution*, *3*(sup1), 100-105. https://doi.org/10.1080/03949370.1991.10721919

D Evans, J., & Armstrong, T.-N. (2005). Inhibition of the American foulbrood bacterium, Paenibacillus larvae larvae, by bacteria isolated from honey bees. *Journal of Apicultural Research*, *44*(4), 168-171. https://doi.org/10.1080/00218839.2005.11101173

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. https://doi.org/10.1016/j.mimet.2009.11.009

Eilers, E. J., Kremen, C., Smith Greenleaf, S., Garber, A. K., & Klein, A.-M. (2011). Contribution of pollinator-mediated crops to nutrients in the human food supply. *PLoS one*, *6*(6), e21363. https://doi.org/10.1371/journal.pone.0021363

Ellegaard, K. M., & Engel, P. (2019). Genomic diversity landscape of the honey bee gut microbiota. *Nature communications*, *10*(1), 1-13. https://doi.org/10.1038/s41467-019-08303-0

Eschenbach, D. A., Davick, P. R., Williams, B. L., Klebanoff, S. J., Young-Smith, K., Critchlow, C. M., & Holmes, K. K. (1989). Prevalence of hydrogen peroxide-producing Lactobacillus species in normal women and women with bacterial vaginosis. *Journal of clinical microbiology*, *27*(2), 251-256. https://doi.org/10.1128/jcm.27.2.251-256.1989

Forsgren, E., Olofsson, T. C., Váasquez, A., & Fries, I. (2010). Novel lactic acid bacteria inhibiting Paenibacillus larvae in honey bee larvae. *Apidologie*, *41*(1), 99-108. https://doi.org/10.1051/apido/2009065

Fujimoto, S., Nakagami, Y., & Kojima, F. (2004). Optimal bacterial DNA isolation method using bead-beating technique. *Memoirs Kyushu Univ Dep Of Health Scis Of Medical Sch*, *3*, 33-38.

Gisder, S., & Genersch, E. (2017). Viruses of commercialized insect pollinators. *Journal of Invertebrate Pathology*, *147*, 51-59. https://doi.org/10.1016/j.jip.2016.07.010

Hammes, W. P., & Vogel, R. F. (1995). The genus lactobacillus. In *The genera of lactic acid bacteria* (pp. 19-54). Springer. https://doi.org/10.1007/978-1-4615-5817-0_3

Hawes, S. E., Hillier, S. L., Benedetti, J., Stevens, C. E., Koutsky, L. A., Wølner-Hanssen, P., & Holmes, K. K. (1996). Hydrogen peroxide—producing lactobacilli and acquisition of vaginal infections. *Journal of Infectious Diseases*, *174*(5), 1058-1063. https://doi.org/10.1093/infdis/174.5.1058

Heydari, M., Miclotte, G., Van de Peer, Y., & Fostier, J. (2019). Illumina error correction near highly repetitive DNA regions improves de novo genome assembly. *BMC bioinformatics*, *20*(1), 1-13. https://doi.org/10.1186/s12859-019-2906-2

Jain, M., Koren, S., Miga, K. H., Quick, J., Rand, A. C., Sasani, T. A., . . . Fiddes, I. T. (2018). Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nature biotechnology*, *36*(4), 338-345.

Jain, M., Olsen, H. E., Paten, B., & Akeson, M. (2016). The Oxford Nanopore MinION:

delivery of nanopore sequencing to the genomics community. *Genome biology*, *17*(1), 1-11. https://doi.org/10.1186/s13059-016-1103-0

Jain, M., Tyson, J. R., Loose, M., Ip, C. L. C., Eccles, D. A., O'Grady, J., . . . Jansen, H. J. (2017). MinION Analysis and Reference Consortium: Phase 2 data release and analysis of R9. 0 chemistry. *F1000Research*, *6*. https://doi.org/10.12688/f1000research.11354.1

Jin, Q., & Kirk, M. F. (2018). pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science*, *6*, 21. https://doi.org/10.3389/fenvs.2018.00021

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., . . . Gerstein, M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature communications*, *10*(1), 1-11. https://doi.org/10.1038/s41467-019-13036-1

Kembel, S. W., Wu, M., Eisen, J. A., & Green, J. L. (2012). Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance. *PLoS computational biology*, *8*(10), e1002743. https://doi.org/10.1371/journal.pcbi.1002743

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*(Pt_1), 152-157. https://doi.org/10.1099/ijs.0.053033-0

Klein, A.-M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*, *274*(1608), 303-313. https://doi.org/10.1098/rspb.2006.3721

Kwon, H.-S., Yang, E.-H., Yeon, S.-W., Kang, B.-H., & Kim, T.-Y. (2004). Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS microbiology letters*, *239*(2), 267-275. https://doi.org/10.1016/j.femsle.2004.08.049

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews Microbiology*, *14*(6), 374-384. https://doi.org/10.1038/nrmicro.2016.43

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., . . . Knight, R. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology*, *31*(9), 814-821. https://doi.org/10.1038/nbt.2676

Lebrigand, K., Magnone, V., Barbry, P., & Waldmann, R. (2020). High throughput error corrected Nanopore single cell transcriptome sequencing. *Nature communications*, *11*(1), 1-8. https://doi.org/10.1038/s41467-020-17800-6

Li, X., Bosch-Tijhof, C. J., Wei, X., de Soet, J. J., Crielaard, W., Loveren, C. v., & Deng, D. M. (2020). Efficiency of chemical versus mechanical disruption methods of DNA extraction for the identification of oral Gram-positive and Gram-negative bacteria. *Journal of International Medical Research*, *48*(5), 0300060520925594. https://doi.org/10.1177/0300060520925594

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., . . . Law, M. (2012). Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology*, *2012*. https://doi.org/10.1155/2012/251364

Liu, Z., Lozupone, C., Hamady, M., Bushman, F. D., & Knight, R. (2007). Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic acids research*, *35*(18), e120. https://doi.org/10.1093/nar/gkm541

Loman, N. J., Misra, R. V., Dallman, T. J., Constantinidou, C., Gharbia, S. E., Wain, J., & Pallen, M. J. (2012). Performance comparison of benchtop high-throughput sequencing platforms. *Nature biotechnology*, *30*(5), 434-439. https://doi.org/10.1038/nbt.2198

Lu, H., Giordano, F., & Ning, Z. (2016). Oxford Nanopore MinION sequencing and genome assembly. *Genomics, proteomics & bioinformatics*, *14*(5), 265-279. https://doi.org/10.1016/j.gpb.2016.05.004

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E. (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. *International Dairy Journal*, *16*(3), 189-199. https://doi.org/10.1016/j.idairyj.2005.02.009

Martinson, V. G., Danforth, B. N., Minckley, R. L., Rueppell, O., Tingek, S., & Moran, N. A. (2011). A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*, *20*(3), 619-628. https://doi.org/10.1111/j.1365-294X.2010.04959.x

Martinson, V. G., Moy, J., & Moran, N. A. (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and environmental microbiology*, *78*(8), 2830-2840. https://doi.org/10.1128/AEM.07810-11

McNaughton, A. L., Roberts, H. E., Bonsall, D., de Cesare, M., Mokaya, J., Lumley, S. F., . . . de Lara, C. (2019). Illumina and Nanopore methods for whole genome sequencing of hepatitis B virus (HBV). *Scientific reports*, *9*(1), 1-14. https://doi.org/10.1038/s41598-019-43524-9

Menegon, M., Cantaloni, C., Rodriguez-Prieto, A., Centomo, C., Abdelfattah, A., Rossato, M., . . . Delledonne, M. (2017). On site DNA barcoding by nanopore sequencing. *PLoS One*, *12*(10), e0184741. https://doi.org/10.1371/journal.pone.0184741

Moran, N. A., Hansen, A. K., Powell, J. E., & Sabree, Z. L. (2012). Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PloS one*, *7*(4), e36393. https://doi.org/10.1371/journal.pone.0036393

Nicholls, S. M., Quick, J. C., Tang, S., & Loman, N. J. (2019). Ultra-deep, long-read nanopore sequencing of mock microbial community standards. *Gigascience*, *8*(5), giz043. https://doi.org/10.1093/gigascience/giz043

Olofsson, T. C., 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*(Pt 9), 3109. https://doi.org/10.1099/ijs.0.059600-0

Olofsson, T. C., & 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, T. C., Vásquez, A., Sammataro, D., & Macharia, J. (2011). A scientific note on the lactic acid bacterial flora within the honeybee subspecies Apis mellifera (Buckfast), A. m. scutellata, A. m. mellifera, and A. m. monticola. *Apidologie*, *42*(6), 696-699. https://doi.org/10.1007/s13592-011-0064-2

Pomerantz, A., Peñafiel, N., Arteaga, A., Bustamante, L., Pichardo, F., Coloma, L. A., . . . Prost, S. (2017). Real-time DNA barcoding in a remote rainforest using nanopore sequencing. *bioRxiv*, 189159. https://doi.org/10.1101/189159

Prado-Acosta, M., Ruzal, S. M., Allievi, M. C., Palomino, M. M., & Sanchez Rivas, C. (2010). Synergistic effects of the Lactobacillus acidophilus surface layer and nisin on bacterial growth. *Applied and Environmental Microbiology*, *76*(3), 974-977. https://doi.org/10.1128/AEM.01427-09

Praet, J., Meeus, I., Cnockaert, M., Houf, K., Smagghe, G., & Vandamme, P. (2015). Novel lactic acid bacteria isolated from the bumble bee gut: Convivina intestini gen. nov., sp. nov., Lactobacillus bombicola sp. nov., and Weissella bombi sp. nov. *Antonie van Leeuwenhoek*, *107*(5), 1337-1349. https://doi.org/10.1007/s10482-

015-0429-z
Praet, J., Parmentier, A., Schmid-Hempel, R., Meeus, I., Smagghe, G., & Vandamme, P. (2018). Large-scale cultivation of the bumblebee gut microbiota reveals an underestimated bacterial species diversity capable of pathogen inhibition. *Environmental Microbiology*, *20*(1), 214-227. https://doi.org/10.1111/1462-2920.13973
Prosperi, M. C. F., & Salemi, M. (2012). QuRe: software for viral quasispecies reconstruction from next-generation sequencing data. *Bioinformatics*, *28*(1), 132-133. https://doi.org/10.1093/bioinformatics/btr627
Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current opinion in insect science*, *26*, 97-104. https://doi.org/10.1016/j.cois.2018.02.012
Sookkhee, S., Chulasiri, M., & Prachyabrued, W. (2001). Lactic acid bacteria from healthy oral cavity of Thai volunteers: inhibition of oral pathogens. *Journal of applied microbiology*, *90*(2), 172-179. https://doi.org/10.1046/j.1365-2672.2001.01229.x
Tarpy, D. R., Mattila, H. R., & Newton, I. L. G. (2015). Development of the honey bee gut microbiome throughout the queen-rearing process. *Applied and Environmental Microbiology*, *81*(9), 3182-3191. https://doi.org/10.1128/AEM.00307-15
Taylor, M. A., Robertson, A. W., Biggs, P. J., Richards, K. K., Jones, D. F., & Parkar, S. G. (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. https://doi.org/10.1371/journal.pone.0225845
Vanbergen, A. J., & Initiative, t. I. P. (2013). Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment*, *11*(5), 251-259. https://doi.org/10.1890/120126
Wick, R. R., Judd, L. M., & Holt, K. E. (2019). Performance of neural network basecalling tools for Oxford Nanopore sequencing. *Genome biology*, *20*(1), 1-10. https://doi.org/10.1186/s13059-019-1727-y
Wilfinger, W. W., Mackey K Fau - Chomczynski, P., & Chomczynski, P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques*, *22*(3), 474–481. https://doi.org/https://doi.org/10.2144/97223st01
Winand, R., Bogaerts, B., Hoffman, S., Lefevre, L., Delvoye, M., Van Braekel, J., . . . Vanneste, K. (2019). Targeting the 16s rRNA gene for bacterial identification in complex mixed samples: Comparative evaluation of second (illumina) and third (oxford nanopore technologies) generation sequencing technologies. *International journal of molecular sciences*, *21*(1), 298. https://doi.org/10.3390/ijms21010298
Wu, M., Sugimura, Y., Iwata, K., Takaya, N., Takamatsu, D., Kobayashi, M., . . . Miller, T. (2014). Inhibitory effect of gut bacteria from the Japanese honey bee, Apis cerana japonica, against Melissococcus plutonius, the causal agent of European foulbrood disease. *Journal of Insect Science*, *14*(1). https://doi.org/10.1093/jis/14.1.129
Yang, E., Fan, L., Yan, J., Jiang, Y., Doucette, C., Fillmore, S., & Walker, B. (2018). Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *Amb Express*, *8*(1), 1-14. https://doi.org/10.1186/s13568-018-0536-0
Yoshiyama, M., Wu, M., Sugimura, Y., Takaya, N., Kimoto-Nira, H., & Suzuki, C. (2013). Inhibition of Paenibacillus larvae by lactic acid bacteria isolated from fermented materials. *Journal of invertebrate pathology*, *112*(1), 62-67. https://doi.org/10.1016/j.jip.2012.09.002
Yu, Z., & Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques*, *36*(5), 808-812.

https://doi.org/10.2144/04365ST04
Zhang, M. Z., Yang, M., Su, H., Rollins, D., & Zhang, S. (2017). Lactobacillus colini sp. nov., isolated from Northern Bobwhite (Colinus virginianus). *International Journal of Systematic and Evolutionary Microbiology*, *67*(2), 325-329. https://doi.org/10.1099/ijsem.0.001624

10 Appendix

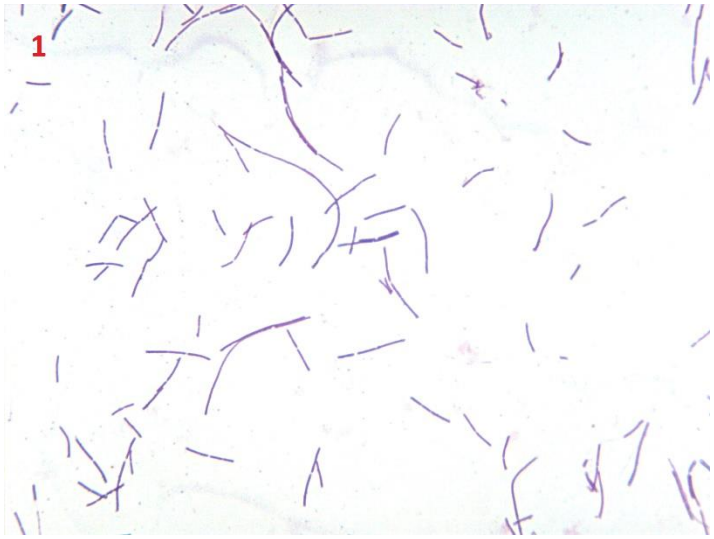

Figure A. Isolation 6, gram stained *lactobacillus* culture.

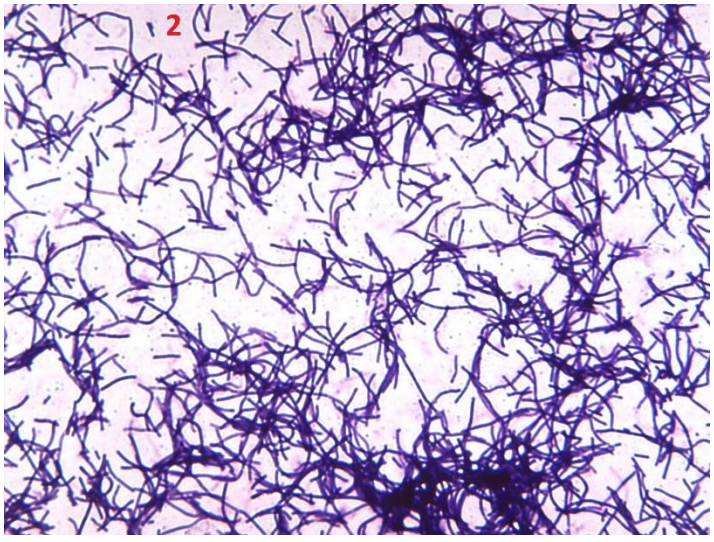

Figure B. Isolation 9, gram stained *lactobacillus* culture.

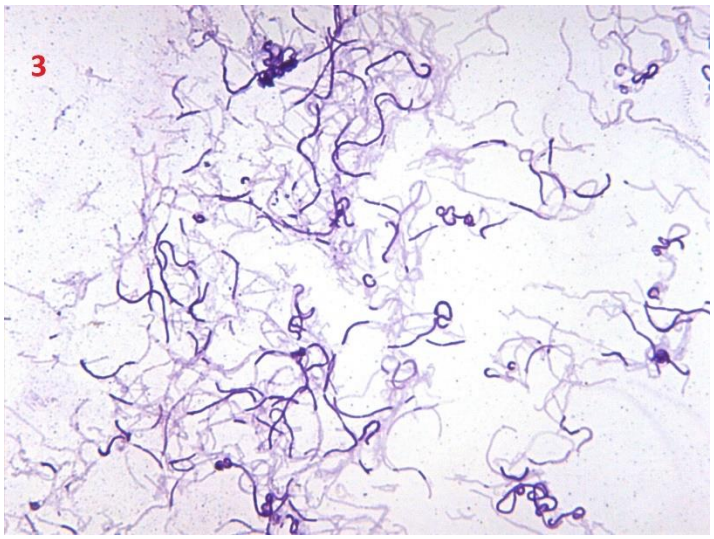

Figure C. Isolation 10, gram stained *lactobacillus* culture.

Figure D. Isolation 13, gram stained *lactobacillus* culture.

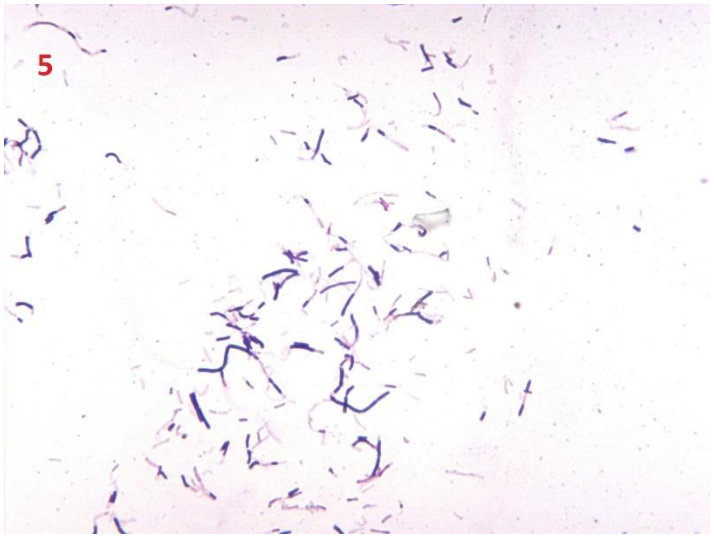

Figure E. Isolation 15, gram stained *lactobacillus* culture.

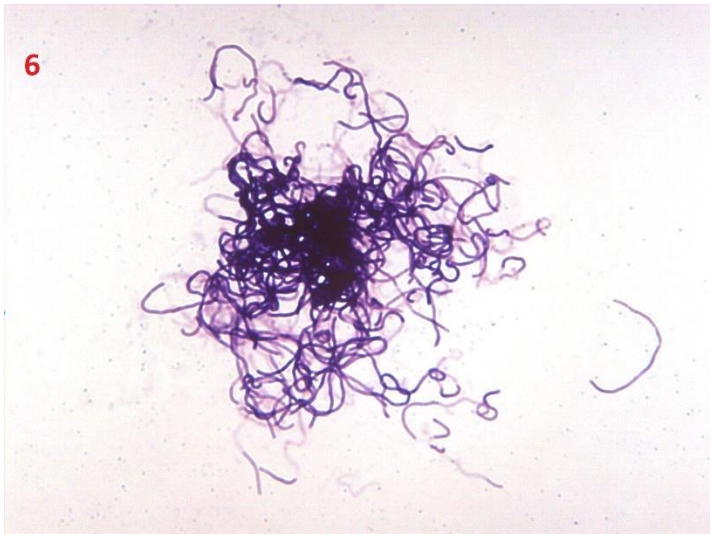

Figure F. Isolation 16, gram stained *lactobacillus* culture.

Figure G. Tools used to dissect the bees.

Table A. *Lactobacillus* control species and *M. plutonius* used in this experiment, collected from CCUG.se database.

Species	**ID nr**	**Previous name**
Bombilactobacillus mellifer	CCUG 63291T	*L. mellifera*
Apilactobacillus apinorum	CCUG 63287T	*L. apis*
Lactobacillus kimbladii	CCUG 63633T	*Lactobacillus* sp. Hma2N
Lactobacillus melliventris	CCUG 63629T	*L. melventris*
Lactobacillus kullabergensis	CCUG 63632	*L. olofsonii*
Lactobacillus helsingborgensis	CCUG 63635	*L. vasquezii*

Melissococcus plutonius	CCUG 62979	*Melissococcus pluton*