Degree project

UNIVERSITY OF SKÖVDE

# Seasonal shift in the intestinal microbiota of Honey bees, *Apis mellifera.*

Bachelor Degree Project in Bioscience

First Cycle 30 credits

Spring term 2023

Student: Jonathan Meijer

Supervisor: Erik Gustafsson

Examiner: John Baxter

## Abstract

Honey bees, *Apis mellifera*, are important pollinators across the world who provide the crucial function of maintaining the health and wellbeing of natural and agricultural ecosystems. However, in recent years, there has been a documented decline in honey bee populations worldwide. Research suggests that the intestinal microbiota of honey bees is crucial for their wellbeing and immune system as well as providing protection against harmful pathogens and affecting their growth and development. The aim of this study was to evaluate the seasonal shifts occurring within the microbiota of eight different honey bee communities and to compare the seasonal shift between autumn, winter and spring. This was done by Nanopore sequencing of the bacterial 16S rRNA gene. The results showed that there was no statistical difference between the examined species throughout this study. This is in line with current research suggesting that the intestinal microbiota of honey bees remains relatively stable. However, other studies have shown that there occur seasonal shifts in the genera within the intestines, which might suggest a larger shift occurring within the genera as opposed to the induvial species. In conclusion, no seasonal shift could be observed between the species examined throughout this study which suggests that the intestinal microbiota of honey bees remains stable throughout the year.

## List of abbrevations

| | |
|---|---|
| 16S rRNA | 16S ribosomal RNA |
| rRNA | Ribosomal RNA |
| RNA | Ribonucleic acid |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double-stranded deoxyribonucleic acid |
| PCR | Polymerase chain reaction |
| bp | Base pairs |

## Table of content

## Introduction

Honey bees, *Apis mellifera*, are important pollinators who provide the crucial function of maintaining the health and wellbeing of both natural and agricultural ecosystems. Honey bees contribute to the natural preservations of plant biodiversity, as well as contributing to the promotion of agricultural crop production. Honey bees are also important bioindicators of environmental pollution and are able to provide crucial information regarding how human activities affects the presence of environmental pollutants across ecosystems (Papa et al., 2022). It has been estimated that in North America alone, honey bees contribute to the pollination of more than 90 separate commercially grown crops (Status of Pollinators in North America, 2007). Moreover, honey bees provide an estimated economical contribution in the United States which reached over 11 billion US dollars in 2009 due to their role as agricultural crop pollinators (Calderone, 2012). Moreover, it has been estimated that honey bees and similar pollinators contribute around 190 billion US dollars annually to worldwide agricultural crop production (Paudel et al., 2015). In addition to their role as natural and agricultural pollinators, honey bees produce a wide range of natural products such as honey, beeswax, honey bee venom, propolis, pollen and royal jelly (Schmidt, 1997) which is estimated to serve as a source of additional income for over 600 000 beekeepers across the Europe Union alone (European commission, 2016). Moreover, it has been estimated that in Sweden, honey bee products contribute annually between 117 and 135 million SEK, and another 189 to 325 million SEK annually through their role as a pollinator in agricultural ecosystems (Massdöd av bin, 2010).

However, during the last couple of decades there has been a documented decline in honey bee communities (van Engelsdorp & Meixner, 2010; Goulson et al., 2015; Ellis et al., 2010). A few possible causes for this decline have been suggested to be due to either diseases or pathogens, an increased use of agricultural pesticides and antibiotics or due to a steady decline in available natural habitats for honey bees leading to a shortage of food (Goulson et al., 2015; Brettell & Martin, 2017; van Engelsdorp & Meixner, 2010; Ortiz-Alvarado et al., 2020). An example of this decline can be seen in the United States, where the annual losses of winter honey bee populations lie in the range of 30 % to 50 %, which has been suggested to be caused by poor nutrition, pesticides and/or bacterial, viral or parasitic infections (van Engelsdorp et al., 2017). A similar reported decline in honey bee communities has also been observed in Europe (Dainat et al., 2012; van Engelsdorp & Meixner, 2010). The results of this could be catastrophic, as for instance in Sweden, a 40 % decrease in the honey bee populations is estimated to generate losses in the range of 200 to 300 million SEK (Massdöd av bin, 2010). As natural pollinators are crucial to maintaining a future food security (Marshman et al., 2019), the need to be able to maintain healthy honey bee communities are of a high importance.

In recent years, there has been an increasing interest regarding how the intestinal microbiota of honey bees affects their health and immune system. There is increasing evidence which suggests that a compromised intestinal microbiota in honey bees negatively affects both their health and immune system (Anderson & Ricigliano, 2017; Raymann & Moran, 2018; Ye et al., 2021). It has been suggested that the intestinal microbiota plays a crucial role in honey bees' metabolism and immune responses, as well as providing protection against harmful pathogens and affects the growth and development of honey bees (Nowak et al., 2021; Kešnerová et al., 2017; Kwong & Moran, 2016). Therefore, gaining an insight in to the changes and function of the intestinal microbiota of honey bees might provide vital information which could mitigate the reported losses of honey bees' communities.

Furthermore, there is evidence showing how the increased use of agricultural antibiotics might disturb the intestinal microbiota of honey bees and increase the susceptibility for viral infection

(Deng et al., 2022; Powell et al., 2021). There is also evidence showing how exposure to insecticides and fungicides disturbs the intestinal microbiota of honey bees (Naggar et al., 2022).

The intestinal microbiota of honey bees is typically composed of a set of five different core genera, *Gilliamella*, *Snodgrassella*, *Lactobacillus Firm 4*, *Lactobacillus Firm 5* and *Bifidobacterium,* which together constitutes the majority of the whole intestinal microbiota community of honey bees (Kešnerová et al., 2020). The intestinal microbiota of honey bees is distributed throughout the digestive tract, but where the crop and midgut house a relative low abundance of bacteria, the majority of the microbiota is housed in the hindgut which is composed of the ileum and rectum (Kešnerová et al., 2017). The intestinal microbiota of honey bees is heritable within the community and is transmitted by social interactions amongst the bees themselves and/or through hive components. Initially, the newly-hatched larvae are lacking any internal microbiota, but eventually the larvae inherit their intestinal microbiota from the adult honey bees and/or hive components through feeding and secretion which is performed inside the colony (Kwong & Moran, 2016).

Even though the intestinal microbiota of honey bees is considered quite stable, it has been reported that certain genera might change and vary across honey bees throughout the seasons (Kešnerová et al., 2020). Other studies suggest that honey bees' microbiota changes throughout the foraging seasons, with intestinal microbiota being transmitted from the environment surrounding the colony (Ludvigsen et al., 2015). It might be reasonable to believe that there are to be some sort of changes in the intestinal microbiota of honey bees throughout the different seasons due to different physiological and lifestyle differences of bees active during the summer and winter, with a typical bee active during the summer tending to have a shorter life span, spanning a couple of weeks, while the bees active during the winter seasons life expectancy is in the range of months instead of weeks (Page & Peng, 2001). Other reasons might be that bees active during the summer are more likely to be exposed to pesticides than bees in hibernation during the winter (Krupke et al., 2012). Furthermore, bees in hibernation during the winter feed on stored food sources which might contain harmful pesticides and antibiotics (Krupke et al., 2012; Mullin et al., 2010). This might cause disturbances in the intestinal microbiota of honey bees, coupled with the fact that bees in hibernation during the winter tend not to defecate for longer periods of time (Bleau et al., 2020). However, very little is actually known about the changes occurring throughout the seasons in a community's intestinal microbiota.

The intestinal microbiota of honey bees can be analysed by Nanopore sequencing of the 16S rRNA gene which is commonly used for bacterial genera and species identification (Johnson et al., 2019). Nanopore sequencing works by constructing a biological nanopore into an artificial membrane, which is then subjected to a voltage. Before the sequencing can begin, a DNA molecule is prepared by attaching a leader adaptor and a motor protein to one stand of the DNA. During the sequencing, the motor protein that is attached to the DNA strand separates the dsDNA and passes the strands through the pore one base at a time. This causes a deviation in the current in the pore, and this change in the current can be correlated to which bases that are present in the pore at that time (Leggett & Clark, 2017). Moreover, the 16S rRNA gene is commonly used for taxonomical identification due to its occurrence in a large majority of bacterial genera, it being a highly conserved gene region with variable regions used to differentiate bacterial genera and species and also due to its size being large enough for bioinformatical purposes (Janda & Abbott, 2007; Santos et al., 2020).

## Research aim

The aim of this project is to examine whether the intestinal microbiota of honey bees, *Apis mellifera*, varies throughout the year. This will be done by examining the intestinal microbiota of

honey bees collected during different times of the year (autumn, winter and spring) and through sequencing of the 16S rRNA gene using Nanopore sequencing. Sequencing results will then be compared across the seasons to evaluate if any seasonal shifts occur. In doing so a better understanding of the seasonal shifts occurring within the intestinal microbiota of honey bees will be obtained.

Null hypothesis ($H_0$): There is no difference in the intestinal microbiota between the seasons. Alternative hypothesis ($H_1$): There is a difference in the intestinal microbiota between the seasons.

# Methods

## Dissection of the bees

The honey bees used throughout this study was collected from eight different hive communities located at the same site in Uddevalla Municipality in Sweden during the months September 2022, January 2023 and March 2023. One honey bee per group were used as a sample basis to evaluate the intestinal microbiota of each sample group. Only healthy-looking honey bees were used throughout the study to provide an accurate representation of the seasonal shifts occurring among the intestinal microbial species.

The tools used throughout the disinfection and dissections were submerged in a 2.8 % sodium hypochlorite solution and was left for at least 10 minutes. Before the dissection, the bees which had been euthanized by freezing as described in the ethical considerations section, were disinfected by transferring them to 50 mL falcon tubes containing 1 mL 1 % sodium hypochlorite solution per bee being disinfected and the tube was then gently rocked by hand for 2 minutes. After the disinfection the bees were washed three times in three separate 50 mL falcon tubes containing ultra-pure Milli-Q® water (Sigma-Aldrich). The bees were then transferred to sterile petri dishes.

The honey bees were then carefully dissected by first removing the legs, wings and head. A precise incision was made on the right side of the honey bees' lower abdomen next to the stinger, half-way up to the thorax. The same procedure was repeated on the left side of the abdomen. After the incisions were made, a small pointed tweezer was used to grasp the stinger and the intestines were carefully separated from the abdomen. The different intestinal samples were separated according to their sample group and placed in a ZR BashingBead™ Lysis Tubes (Zymo research) together with 750 µl Lysis Solution (Zymo research) and stored overnight at 4 °C and the extraction was performed the following day. The weight of the intestinal samples was recorded before the Lysis Solution (Zymo research) was added and the DNA extraction was performed.

## DNA extraction and amplification

The DNA was extracted from the intestinal samples to be used for identification of the existing intestinal microbiota through bacterial 16S rRNA gene sequencing.

The subsequent DNA extractions were performed using the Zymobiomics DNA miniprep kit (Zymo research) as described in the manufacturer instructions. The intestinal samples were lysed for 40 minutes in ZR BashingBead™ Lysis Tubes (Zymo research) using a Disruptor Genie (Scientific Industries). The extracted DNA was eluted in 50 µl ZymoBIOMICS™ DNase/RNase Free Water (Zymo research), instead of the suggested volume of 100 µl, to increase the final concentration of the samples. The concentrations and purity of the extracted DNA were then measured and recorded before the amplification of the bacterial 16S rRNA gene. The concentration of the extracted DNA was determined by Qubit™ 1X dsDNA High Sensitivity (HS) kit together with Qubit fluorometer 4 (Thermo Fisher Scientific) as per the manufacturer's instructions, while the purity was evaluated through DS-11 spectrophotometer (DeNovix).

The amplification of the 16S rRNA gene was performed using 16S Barcoding Kit 1-24 (SQK-16S024) (Oxford Nanopore Technologies) together with PCR PTC-200 thermal cycler (MJ Research). The amplification was performed as described in the SQK-16S024 protocol, version 16S_9086_v1_revT_14Aug2019, with the exception that the number of cycles were increased from 25 to 30 cycles. The amplifications were performed using the universal primers 27F and 1492R which were provided in the 16S Barcoding Kit 1-24 After the amplification of the 16S rRNA gene, the post-PCR products were verified on a 1 % w/v agarose gel to identify whether the correct fragment sizes had been obtained and if the amplification had been successful.

## DNA purification

The post-PCR samples were then purified using magnetic beads (Agencourt AMPure XP) together with a DynaMag™-2 Magnet (Thermo Fisher Scientific) as described in the 16S Barcoding Kit 1-24 The concentration of the purified post-PCR products was evaluated using Qubit™ 1X dsDNA High Sensitivity (HS) kit together with a Qubit fluorometer 4 (Thermo Fisher Scientific), while the purity was quantified using a DS-11 spectrophotometer (DeNovix).

## 16S rRNA sequencing

The 16S rRNA gene sequencing was performed using a MinION Mk1B (Oxford Nanopore Technologies) together with a Flongle Flow Cell (FLO-FLG001) (Oxford Nanopore Technologies). Before the purified and quantified post-PCR products were loaded onto the flow cell, they were pooled and diluted to a final concentration of approximately 12 ng/µl. The ready-to-sequence mix was then loaded onto the flow cell as described in the SQK-16S024 protocol, version 16S_9086_v1_revT_14Aug2019 with an input of between 3 and 20 fmoles. The recommended total volume of the ready-to-sequence library was also decreased from a volume of 11 µl to a volume of 5 µl. Roughly 4 ng from each diluted barcoded sample was added to the ready-to-sequence library mix and the volume adjusted with nuclease-free water up to a final volume of 5 µl. Each community was sequenced individually with roughly 4 ng from each seasonal group within the community being added. A mistake occurred with the second community sample group where the dilution was left out, and the final loading concentration was 120 ng/µl instead of the suggested 12 ng/µl.

The data acquisition and basecalling was performed using the software MinKNOW version 22.03.5 (Oxford Nanopore Technologies) during a four-hour sequencing with a quality score of 9. The generated data was then further analysed using EPI2ME desktop agent version 3.6.2 (Oxford Nanopore Technologies) together with the Workflow Fastq 16S 2023.04.21-1804452. The minimum and maximum length filter was set at 0, BLAST E-value filter was set at Default [e=0.01], minimum coverage percentage was set at 30, minimum identity percentage was set at 95 and the maximum target sequences was set at 3. Due to the small number of classified reads for community one and four, these were decided to be left out for future analyses.

## Statistical tests

The seasonal shifts among the top twelve species with the highest classifications were analysed. The groups were created based on whether the observed seasonal shift in the species among the communities increased or decreased between the autumn to winter groups, and then between the winter to spring groups. The statistical difference between the seasonal groups were then calculated by examining the increase or decrease of species across the evaluated seasons. Tables and charts displaying the number of classifications and the species used throughout the analysis were created using Microsoft Excel 2019 (Version 2304). To compare the groups, Chi-square tests were performed in Microsoft Excel 2019 and Fisher's exact test were performed using Social Science Statistics (2023). Both tests were performed with a significance level of 0.05 and a degree of freedom of 1.

# Results

## Dissection of the bees

The mean weight of the intestinal samples from each season can be seen in Figure 1. The weight of each separate sample can be seen in Appendix 1.

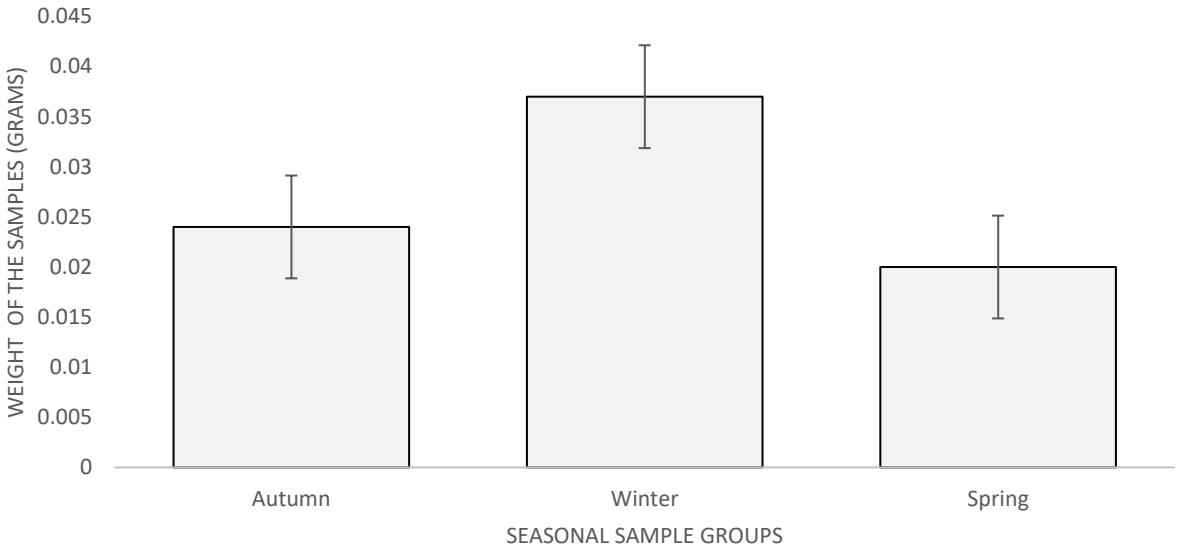


Figure 1. Mean weight of the bee intestinal samples (g) from the season's autumn, winter and spring together with the standard deviation.

The mean weight of the samples collected during autumn was 0.024 g with a standard deviation of 0.0087 g. The mean weight for the winter and spring samples was 0.037 g and 0.020 g respectively and a standard deviation of 0.012 g for the winter samples and 0.011 g for the spring samples. This demonstrates that the winter samples on average was heavier than both of the autumn and winter samples.

## DNA extraction and amplification

After the DNA extraction had been performed, the 16S rRNA genes were amplified and the products were verified using gel electrophoresis. The concentration and purity of the samples from the DNA extractions can be seen in Appendix 2, while the results from the amplification of the 16S rRNA gene can be seen in the gel electrophoresis displayed in Figure 2.

The lanes marked 1-3 were samples from community one, while the lanes 4-6 from community two, the lanes 7-9 from community three, lanes 10-12 from community four, lanes 13-15 from community five, lanes 16-18 from community six, lanes 19-21 from community seven and lane 22-24 from community eight. The lane marked 25 is from the second amplification of the winter sample from community eight. The lanes are in the order of spring sample, autumn sample and winter sample for all the communities, read from the left to the right.

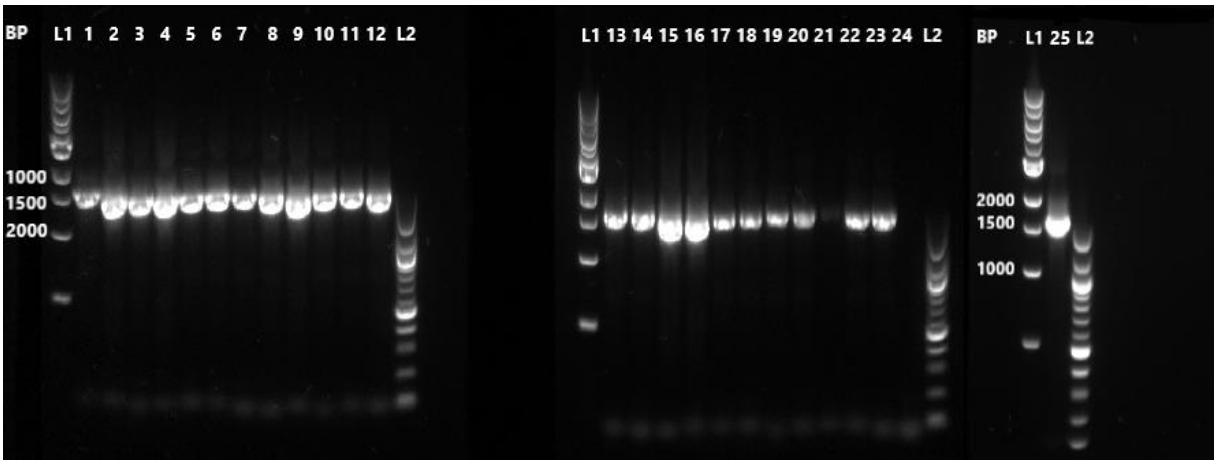


Figure 2. The results from the amplification of the 16S rRNA gene. Lanes marked 1-25 are from the communities 1 to 8 from left to right. The lanes marked L1 represents a 1.5 kb ladder, while the lanes marked L2 represents a 100bp ladder.

The bacterial 16S rRNA gene is expected to be around the size of 1500 bp (16S rRNA - Nucleotide - NCBI, n.d.) and the electrophoresis gels shows that the correct fragment sizes were obtained from the amplification.

## DNA purification

The concentration and purity of the amplified 16S rRNA gene after the purification can be seen in Appendix 2. The concentration of the samples after the purification ranged between 3.66 ng/µl to 63 ng/µl, while the purity of the samples ranged from 1.52 to 1.89.

## 16S rDNA sequencing

The classified and unclassified reads after the downstream analysis in EPI2ME desktop agent (Oxford Nanopore Technologies) of each pooled community can be seen in Table 1 together with their percentage. All the obtained classified reads were below 50 %, with the lowest number of classified reads being 686 and the highest number of classified reads being 67215. Moreover, the percentage of classified reads ranged from the lowest 2 % to the highest of 45 %.

Table 1 The amount of classified and unclassified reads after downstream analysis. Performed in EPI2ME together with Workflow Fastq 16S 2023.04.21-1804452. Each displayed community are pooled samples of the evaluated seasons (autumn, winter and spring) from the same community.

| Community | Classified reads | Unclassified reads | Classified reads (Percentage) | Unclassified reads (Percentage) |
|---|---|---|---|---|
| **Community 1** | 1203 | 22466 | 5 % | 95 % |
| **Community 2** | 60404 | 138159 | 30 % | 70 % |
| **Community 3** | 40390 | 82032 | 33 % | 67 % |
| **Community 4** | 686 | 35516 | 2 % | 98 % |
| **Community 5** | 57642 | 71358 | 45 % | 55 % |
| **Community 6** | 9069 | 20500 | 31 % | 69 % |
| **Community 7** | 28913 | 44893 | 39 % | 61 % |
| **Community 8** | 67215 | 84174 | 44 % | 56 % |

Tables containing all classified reads, excluding communities two and four, can be found in Appendix 3.

## Statistical tests

The bacterial species used throughout as an evaluation can be seen in Figure 3, where the sequencing results were pooled together according to season. All the separate communities and their bacterial species composition can be seen in Appendix 4. It can be seen from the species shown in Figure 3 that the communities' sampled during autumn were dominated by *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus apis* and *Bartonella apis*. For the communities sampled during the winter season *Gilliamella apicola*, *Bartonella apis*, *Lactobacillus mellis* and *Lactobacillus apis.* While the communities sampled during the spring season on large was dominated by *Lactobacillus melliventris*, *Bartonella apis*, *Lactobacillus apis* and *Gilliamella apicola*.

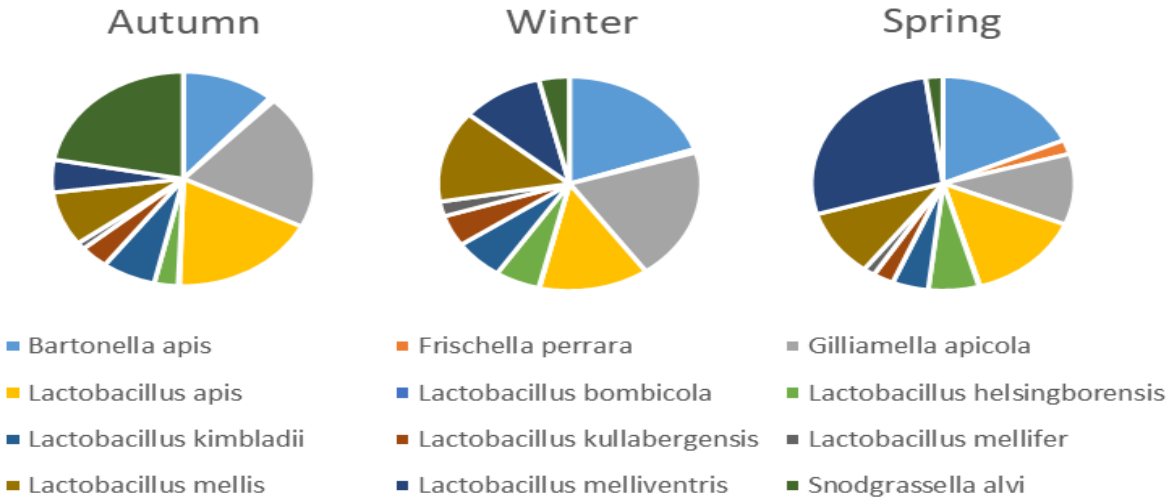


Figure 3. The seasonal shifts from all the examined communities combined in to single charts seperated according to their seasons. The top twelve aquierd species from the downstream-analysis performed in EPI2ME are represented in the charts.

While there seemed to occur a seasonal shift among the bacterial species when looking at the separate charts, the statistical analysis revealed that the difference was not statistically significant when analysed using Chi-square tests and Fisher's exact test, as can be seen in Table 2 and 3. The Chi-square tests displayed in Table 2 shows that only *Lactobacillus melliventris* was able to prove any statistical difference.

Table 2 The results from the Chi-square tests together with the $X^2$ and p-value from the statistical tests. All the evaluated species had a degree of freedom of 1 and significance level of 0.05.

| Community | $X^2$ | P-value |
|---|---|---|
| ***Lactobacillus melliventris*** | 5.33 | 0.021 |
| ***Bartonella apis*** | 3.09 | 0.079 |
| ***Lactobacillus apis*** | 0.56 | 0.34 |
| ***Lactobacillus mellis*** | 3.09 | 0.079 |
| ***Gilliamella apicola*** | 1.33 | 0.25 |
| ***Lactobacillus helsingborensis*** | 0.34 | 0.56 |
| ***Lactobacillus kimbladii*** | 1.33 | 0.25 |
| ***Lactobacillus kullabergensis*** | 1.33 | 0.25 |
| ***Frischella perrara*** | 1.33 | 0.25 |
| ***Snodgrassella alvi*** | 1.33 | 0.25 |
| ***Lactobacillus mellifer*** | 0 | 1 |
| ***Lactobacillus bombicola*** | 0.44 | 0.50 |

While the results from the Fisher's exact test seen in Table 3, shows that none of the species tested were able to prove a statistical difference across the seasons that were examined.

Table 3. The results from the Fisher's exact test together with the p-values from the statistical tests. All the evaluated species had a degree of freedom of 1 and a significance level of 0.05.

| Community | P-value |
|---|---|
| ***Lactobacillus melliventris*** | 0.08 |
| ***Bartonella apis*** | 0.24 |
| ***Lactobacillus apis*** | 1 |
| ***Lactobacillus mellis*** | 0.24 |
| ***Gilliamella apicola*** | 0.57 |
| ***Lactobacillus helsingborensis*** | 1 |
| ***Lactobacillus kimbladii*** | 0.57 |
| ***Lactobacillus kullabergensis*** | 0.57 |
| ***Frischella perrara*** | 0.57 |
| ***Snodgrassella alvi*** | 0.57 |
| ***Lactobacillus mellifer*** | 1 |
| ***Lactobacillus bombicola*** | 1 |

## Discussion

Previous studies regarding the intestinal microbiota of honey bees have reported that certain genera might change and vary depending on season (Kešnerová et al., 2020), with other studies suggesting that the intestinal microbiota changes across the foraging season and is transmitted from the environment surrounding the colony (Ludvigsen et al., 2015). Moreover, other studies suggest that the intestinal microbiota of honey bees remains relatively stable (Kwong et al., 2017). It might be reasonable to imagine that the intestinal microbiota might vary across the seasons due to different external factors. For instance, in their natural state honey bees in winter hibernation tend to live longer than bees active during the autumn and spring seasons (Page & Peng, 2001). Also, bees in winter hibernation do not defecate for longer periods of time (Bleau et al., 2020) which is likely to increase both the abundance of living and deceased bacteria within the intestinal tract. Furthermore, there are also studies which has shown that both pesticides and antibiotics might be stored in the food resources that bees feed upon during their hibernation (Krupke et al., 2012; Mullin et al., 2010). This might cause some disturbances in the microbiota and increase the risk for viral infections (Deng et al., 2022; Powell et al., 2021), which might in turn further influence their microbiota. Another reason that the intestinal microbiota might differ is that honey bees active during the foraging seasons are more likely to be exposed to pesticides (Krupke et al., 2012) than bees in hibernation during winter. However, very little is known about the seasonal shifts occurring within the intestinal microbiota of honey bees. Also, the large majority of the studies that has been conducted have focused on the changes occurring among the genera inhabiting the intestinal tract (Kešnerová et al., 2020; Ludvigsen et al., 2015; Li et al., 2022), and very little is known regarding the seasonal changes occurring between the different species inhabiting the intestines (Almeida et al., 2022; Hotchkiss et al., 2022) Therefore, the aim of this study was to examine the changes occurring within the intestinal microbiota of honey bees with the focus laying on individual species inhabiting the honey bee intestinal tract.

Interestingly, while measuring the weight of the intestinal tract, it was noted that the intestines dissected from the bees that were collected during the winter seasons had a higher weight average than their respective counterparts from the other sampled seasons as can be seen in Figure 1. However, this is to be expected since, as previously mentioned, honey bees in winter hibernation do not defecate for the duration of their hibernation. This leads to an increase in the intestinal content due to a build-up of both faeces and dead or alive bacteria. There are some indications that this might affect the subsequent 16S rRNA sequencing (Li et al., 2022). Whether this affected the final results remains a topic for further research. However, this should be regarded as a natural part of the intestinal microbiota of honey bees during their winter hibernation, so there

are valid reasons for including these samples when examining the seasonal shifts occurring within the microbiota.

Another interesting find was that during the dissections, it was observed that some of the communities that were examined displayed an unusual colour discolouration that was not observed during the other dissections. Communities five, six and seven displayed a black discolouration of the intestines and were highly fragile and tended to rupture. It is known that certain diseases affecting honey bees also affect their intestines, such as infections by *Nosema ceranae* which makes the intestines very fragile and causes the intestines to display a white discolouration (Galajda et al., 2021). This does not however seem to be the case due to the difference in the discolouration. However, it is also possible that the observed discoloration might be due to prolonged exposure to the sodium hypochlorite used during the disinfection and a subsequent necrosis of the intestines occurring due to prolonged exposure to the sodium hypochlorite (Slaughter et al., 2019).

Moreover, during the amplification of the 16S rRNA gene, the number of amplification cycles were increased from the suggested 25 cycles to 30 cycles. This decision was taken to increase the abundance of amplified material. While the amplification proved successful, as can be seen in Figure 2, it has been suggested that PCR amplification bias tends to increase with the number of cycles of amplification, with the increase being more prominent after 30 cycles (Sze & Schloss, 2019). Also, during the subsequent purification of the amplified material, it was noted that while the concentration was sufficient for the following 16S rRNA sequencing, the purity however tended to be a bit lower than what is recommended. Possible reasons for this might be due ethanol contamination that was left in the samples from the purification procedure, or possibly from contamination in the Tris-HCl buffer that was used to resuspend the purified DNA. Both of which are known to affect purity measurements (Koetsier & Cantor, 2019). However, a surprising find was that, as described in the SQK-16S024 protocol, version 16S_9086_v1_revT_14Aug2019, loading more than 20 fmol onto the flow cell could have detrimental effect on throughput. This was however not observed in the samples that was loaded in a volume of 120 fmol with the only noticeable difference being the increased number of reads which can be seen in Community 2, Table 1.

Furthermore, there are some issues regarding Nanopore sequencing and using the 16S gene region for species identification. It has been reported earlier that sequencing of the 16S gene have a low phylogenetic power at the species level and a rather poor discriminatory power for some genera. Meaning that due to certain genera sharing a high similarity of the 16S gene region there is an increased risk of faulty species classifications. An example of this is the genus *Bacillus,* where *Bacillus globisporus* and *Bacillus psychrophilus* shares a > 99.5 % similarity in the 16S gene region (Janda & Abbott, 2007). Moreover, there are limitations regarding the use of Nanopore sequencing, more precisely high error rates and also the need for a relatively high requirement of large amounts of nucleic acid materials that is needed during the sequencing (Wang et al., 2021). The aforementioned issues surrounding Nanopore sequencing and using the 16S gene for species identification might naturally have led to more wrongfully classified reads being included throughout this study. Also, the standard identity threshold used for microbial 16S rRNA sequencing analyses has been long been at 97 % (Edgar, 2018), while this study made the decision to set the identity threshold at 95 %. This did not prove to be any major cause of concern for the subsequent statistical analyses, but is still worth mentioning at is could have affected the number of classified reads, with more wrongfully classified reads having been included.

Regarding the seasonal shifts in the intestinal microbiota of honey bees, earlier research done by Kešnerová et al. (2020) has shown that there are statistical differences in the seasonal shift between genera as opposed to this study which showed little to no statistical difference among

the species as can be seen in Table 2 and 3. Which might indicate a larger seasonal shift occurring among the different genera as opposed to the individual species within the intestinal microbiota. Kešnerová et al. (2020) reported large seasonal shift occurring among genera *Lactobacillus firm-5* and *Bartonella* which can be seen in the findings from this study to be amongst the species generating the lowest p-value and therefore showing a slightly greater statical difference.

Moreover, during the Chi-square test the species *Lactobacillus melliventris* was able to prove a statistical difference in seasonal shift. Hotchkiss et al. (2022) have previously reported in their research that *Lactobacillus melliventris* is subjected to significant abundance shifts when exposed to pesticides, with the bacterial abundance declining after pesticide exposure. This might be a plausible reason as to the observed seasonal shift, with as previously mentioned, honey bees tend to be exposed to a higher level of pesticides during the foraging season (Krupke et al., 2012) compared to the other seasons. Furthermore, Almeida et al. (2022) reported that the abundance of *Lactobacillus melliventris*, as well as *Lactobacillus apis*, *Lactobacillus helsingborgensis*, *Lactobacillus kimbladii* and *Lactobacillus kullabergensis* has been observed to increase during the honey production season, which is in the spring and summer seasons. Castelli et al. (2021) suggests that the increased bacterial abundance of species within the genus *Lactobacillus* during the honey production season might be due to their importance in utilizing carbohydrates.

Another interesting find is that, as previously mentioned by Ludvigsen et al. (2015), the abundance of *Gilliamella apicola* seems to affect the abundance of *Snodgrassella alvi*, with samples indicating a high presence of *Gilliamella apicola* indicating a lower presence of *Snodgrassella alvi*. This is something can also be seen in the samples from this study. Interestingly enough, there was an increase in the number of *Snodgrassella alvi* classifications in a large portion of the bee samples collected during spring compared to the other seasonal samples. One possible reason for this might be that *Snodgrassella alvi*, as opposed to the majority of the bacterial species present in honey bee intestines, does not possess a glycolysis pathway and generates energy through gluconeogenesis instead. *Snodgrassella alvi* can there for not metabolizes sugars themselves, and might profits from acids accumulated in the intestines. *Snodgrassella alvi* could profit from the acidic environment produced by an increased abundance of acids produced by other bacterial species inhabiting the intestines and an increased abundance of *Gilliamella apicola* seen in a large portion of the spring samples (Quinn et al., 2023).

Moreover, Li et al. (2022) showed that the genus *Bartonella* in their research was the most dominant bacterial genera during the winter season. While there was some indication to this in the samples from this study, with communities three and five showing an increase in the number of *Bartonella apis* species, the pattern did not seem to be the same in the other evaluated communities. However, *Bartonella apis* was one of the most dominating species in the majority of the communities.

The finding in this study seems to generally be in line with earlier research indicating that the intestinal microbiota of honey bees is relatively stable as reported by Kwong et al. (2017), with all the core bacterial genera being present throughout the majority of the examined samples as can be seen in Figure 3. Curiously enough, there was a low abundance of species from the genera *Bifidobacterium* present in the samples, which can be seen in Appendix 3, and *Bifidobacterium* is reported to be one of the five core genera. However, this might not mean that there were no *Bifidobacterium* species present in the samples, and might instead mean that either method used to lyse the bacterial cells does not work for *Bifidobacterium* species, the primers used during the amplification did not work for this genus or that the Nanopore was unable to sequence this particular genus.

The research gathered from this study could be beneficial for further research since it demonstrates that the species within the intestines of honey bees remains relatively stable. As it has been demonstrated earlier that the intestinal microbiota of honey bees has a significant impact on their health and immune system (Anderson & Ricigliano, 2017; Raymann & Moran, 2018; Ye et al., 2021) the findings from this study are important as it can be used to provide further knowledge regarding the shifts occurring away from normal stable intestinal microbiota of honey bees.

## Suggestions for Further Research

It would be interesting to further evaluate the seasonal shifts in the species within the intestinal microbiota of honey bees with more focus on regional or geographical differences within the seasonal shifts. Also, more longitude studies of the shifts occurring between and within the species might also provide further valuable insights in to the intestinal microbiota of honey bees.

## Conclusion

The conclusion from this study showed that there while there occurred seasonal shifts among the species in the intestinal microbiota of honey bees, no large enough statistical difference could be proven to exist. This further proves that the species within the intestinal microbiota of honey bees remains relatively stable even when accounting for seasonal shifts, and this proves useful as it further adds to the knowledge regarding the intestinal microbiota of honey bees.

## Ethical considerations

There are always ethical considerations to be made when working with animals. It has been shown that honey bees experience pain and that they exhibit negative emotional states, and could be regarded as displaying emotions (Bateson et al., 2011). Therefore, it is essential to take the honey bees wellbeing into as high considerations as possible when handling them. The honey bees used throughout this project was frozen to death by being placed inside a freezer, which generated a relatively quick and painless death as a means to limit the unnecessary suffering for the honey bees used throughout this study. Moreover, the results gathered from this study could provide valuable insight in to the intestinal microbiota of honey bees and further the understanding of the interactions between honey bees health and microbiota.

## References


Almeida, E. a. C., Ribiere, C., Frei, W., Kenny, D., Coffey, M., & O'Toole, P. W. (2022). Geographical and Seasonal Analysis of the Honeybee Microbiome. Microbial Ecology, 85(2), 765–778. https://doi.org/10.1007/s00248-022-01986-x

16s rRNA - Nucleotide - NCBI. (n.d.). National Library of Medicine. Retrieved June 13, 2023, from https://www.ncbi.nlm.nih.gov/nuccore/?term=16s+rRNA

Anderson, K. E., & Ricigliano, V. A. (2017). Honey bee gut dysbiosis: a novel context of disease ecology. Elsevier, 22, 125–132. https://doi.org/10.1016/j.cois.2017.05.020

Bateson, M., Desire, S., Gartside, S. E., & Wright, G. A. (2011). Agitated Honeybees Exhibit Pessimistic Cognitive Biases. Current Biology, 21(12), 1070–1073. https://doi.org/10.1016/j.cub.2011.05.017

Bleau, N., Bouslama, S., Giovenazzo, P., & Derome, N. (2020). Dynamics of the Honeybee (Apis mellifera) Gut Microbiota Throughout the Overwintering Period in Canada. Microorganisms, 8(8), 1146. https://doi.org/10.3390/microorganisms8081146

Brettell, L. E., & Martin, S. F. (2017). Oldest Varroa tolerant honey bee population provides insight into the origins of the global decline of honey bees. Scientific Reports, 7(1). https://doi.org/10.1038/srep45953

Calderone, N. W. (2012). Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. PLoS ONE, 7(5), e37235. https://doi.org/10.1371/journal.pone.0037235

Dainat, B., van Engelsdorp, D., & Neumann, P. J. (2012). Colony collapse disorder in Europe. Environmental Microbiology Reports, 4(1), 123–125. https://doi.org/10.1111/j.1758-2229.2011.00312.x

Deng, Y., Yang, S., Zhao, H., Luo, J., Yang, W., & Hou, C. (2022). Antibiotics-induced changes in intestinal bacteria result in the sensitivity of honey bee to virus. Environmental Pollution, 314, 120278. https://doi.org/10.1016/j.envpol.2022.120278

EC (European Commission). (2016). Report from the commission to the European Parliament and the council. On the implementation of the measures concerning the apiculture sector of regulation (EU) no 1308/2013 of the European Parliament and of the council establishing a common organisation.

Edgar, R. C. (2018). Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics, 34(14), 2371–2375. https://doi.org/10.1093/bioinformatics/bty113

Ellis, J. D., Evans, J. D., & Pettis, J. (2010). Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. Journal of Apicultural Research, 49(1), 134–136. https://doi.org/10.3896/ibra.1.49.1.30

Galajda, R., Valenčáková, A., Sučik, M., & Kandráčová, P. (2021). Nosema Disease of European Honey Bees. Journal of Fungi, 7(9), 714. https://doi.org/10.3390/jof7090714

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science, 347(6229). https://doi.org/10.1126/science.1255957

Hotchkiss, M. Z., Poulain, A. J., & Forrest, J. R. K. (2022). Pesticide-induced disturbances of bee gut microbiotas. Fems Microbiology Reviews, 46(2). https://doi.org/10.1093/femsre/fuab056

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal of Clinical Microbiology, 45(9), 2761–2764. https://doi.org/10.1128/jcm.01228-07

Johnson, J. S., Spakowicz, D., Hong, B., Petersen, L. H., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature Communications, 10(1). https://doi.org/10.1038/s41467-019-13036-1

Kešnerová, L., Emery, O., Troilo, M., Liberti, J., Erkosar, B., & Engel, P. (2020). Gut microbiota structure differs between honeybees in winter and summer. The ISME Journal, 14(3), 801–814. https://doi.org/10.1038/s41396-019-0568-8

Kešnerová, L., Mars, R. a. T., Ellegaard, K. M., Troilo, M., Sauer, U., & Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. PLOS Biology, 15(12), e2003467. https://doi.org/10.1371/journal.pbio.2003467

Koetsier, G., & Cantor, E. (2019). A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers. New England Biolabs Inc, 1-8.

Krupke, C. H., Hunt, G. J., Eitzer, B. D., Andino, G. K., & Given, K. (2012). Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. PLOS ONE, 7(1), e29268. https://doi.org/10.1371/journal.pone.0029268

Kwong, W. K., Medina, L. A., Koch, H., Sing, K., Soh, E. S., Ascher, J. S., Jaffé, R., & Moran, N. A. (2017). Dynamic microbiome evolution in social bees. Science Advances, 3(3). https://doi.org/10.1126/sciadv.1600513

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

Leggett, R. W., & Clark, M. M. (2017). A world of opportunities with nanopore sequencing. Journal of Experimental Botany, 68(20), 5419–5429. https://doi.org/10.1093/jxb/erx289

Li, C., Tang, M., Li, X., & Zhou, X. (2022). Community Dynamics in Structure and Function of Honey Bee Gut Bacteria in Response to Winter Dietary Shift. MBio, 13(5). https://doi.org/10.1128/mbio.01131-22

Ludvigsen, J., Rangberg, A., Avershina, E., Sekelja, M., Kreibich, C. D., Amdam, G. V., & Rudi, K. (2015). Shifts in the Midgut/Pyloric Microbiota Composition within a Honey Bee Apiary throughout a Season. Microbes and Environments, 30(3), 235–244. https://doi.org/10.1264/jsme2.me15019

Marshman, J., Blay-Palmer, A., & Landman, K. (2019). Anthropocene Crisis: Climate Change, Pollinators, and Food Security. Environments, 6(2), 22. https://doi.org/10.3390/environments6020022

Massdöd av bin: – samhällsekonomiska konsekvenser och möjliga åtgärder. (2010). Jordbruksverket. Retrieved January 15, 2023, from https://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra09_24.pdf

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S. A., Simonds, R., vanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. PLOS ONE, 5(3), e9754. https://doi.org/10.1371/journal.pone.0009754

Naggar, Y. A., Singavarapu, B., Paxton, R. J., & Wubet, T. (2022). Bees under interactive stressors: the novel insecticides flupyradifurone and sulfoxaflor along with the fungicide azoxystrobin disrupt the gut microbiota of honey bees and increase opportunistic bacterial pathogens. Science of the Total Environment, 849, 157941. https://doi.org/10.1016/j.scitotenv.2022.157941

Nowak, A., Szczuka, D., Górczyńska, A., Motyl, I., & Kregiel, D. (2021). Characterization of Apis mellifera Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection—A Review. Cells, 10(3), 701. https://doi.org/10.3390/cells10030701

Ortiz-Alvarado, Y., Clark, D. A., Vega-Melendez, C. J., Flores-Cruz, Z., Dominguez-Bello, M. G., & Giray, T. (2020). Antibiotics in hives and their effects on honey bee physiology and behavioral development. Biology Open. https://doi.org/10.1242/bio.053884

Page, R. E., & Peng, C. Y. (2001). Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. Experimental Gerontology, 36(4–6), 695–711. https://doi.org/10.1016/s0531-5565(00)00236-9

Papa, G., Maier, R., Durazzo, A., Lucarini, M., Karabagias, I. K., Plutino, M., Bianchetto, E., Aromolo, R., Pignatti, G., Ambrogio, A., Pellecchia, M., & Negri, I. (2022). The Honey Bee Apis mellifera: An Insect at the Interface between Human and Ecosystem Health. Biology, 11(2), 233. https://doi.org/10.3390/biology11020233

Paudel, Y. P., Mackereth, R. W., Hanley, R. S., & Qin, W. (2015). Honey Bees (Apis mellifera L.) and Pollination Issues: Current status, impacts and potential drivers of decline. Journal of Agricultural Science, 7(6). https://doi.org/10.5539/jas.v7n6p93

Powell, J. E., Carver, Z., Leonard, S. P., & Moran, N. A. (2021). Field-Realistic Tylosin Exposure Impacts Honey Bee Microbiota and Pathogen Susceptibility, Which Is Ameliorated by Native Gut Probiotics. Microbiology Spectrum, 9(1). https://doi.org/10.1128/spectrum.00103-21

Quinn, A. J., Chazli, Y. E., Escrig, S., Daraspe, J., Neuschwander, N., McNally, A., Genoud, C., Meibom, A., & Engel, P. (2023). Foraging on host synthesized metabolites enables the bacterial symbiontSnodgrassella alvito colonize the honey bee gut. bioRxiv (Cold Spring Harbor Laboratory). https://doi.org/10.1101/2023.01.23.524906

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

Santos, A., Van Aerle, R., Barrientos, L., & Martinez-Urtaza, J. (2020). Computational methods for 16S metabarcoding studies using Nanopore sequencing data. Computational and Structural Biotechnology Journal, 18, 296–305. https://doi.org/10.1016/j.csbj.2020.01.005

Schmidt, J. O. (1997). Bee Products. In Springer eBooks (pp. 15–26). https://doi.org/10.1007/978-1-4757-9371-0_2

Slaughter, R. J., Watts, M., Vale, J. A., Grieve, J. R., & Schep, L. J. (2019). The clinical toxicology of sodium hypochlorite. Clinical Toxicology, 57(5), 303–311. https://doi.org/10.1080/15563650.2018.1543889

Status of Pollinators in North America. (2007). In National Academies Press eBooks. https://doi.org/10.17226/11761

Sze, M. A., & Schloss, P. D. (2019). The Impact of DNA Polymerase and Number of Rounds of Amplification in PCR on 16S rRNA Gene Sequence Data. mSphere, 4(3). https://doi.org/10.1128/msphere.00163-19

Van Engelsdorp, D., & Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology, 103, S80–S95. https://doi.org/10.1016/j.jip.2009.06.011

Van Engelsdorp, D., Traynor, K. S., Andree, M., Lichtenberg, E. M., Chen, Y., Saegerman, C., & Cox-Foster, D. (2017). Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology. PLOS ONE, 12(7), e0179535. https://doi.org/10.1371/journal.pone.0179535

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., & López-Baucells, A. (2021). Nanopore sequencing technology, bioinformatics and applications. Nature Biotechnology, 39(11), 1348–1365. https://doi.org/10.1038/s41587-021-01108-x

Ye, M., Fan, S., Li, X., Tarequl, I. M., Yan, C., Wei, W., Yang, S., & Zhou, B. (2021). Microbiota dysbiosis in honeybee ( Apis mellifera L . ) larvae infected with brood diseases and foraging bees exposed to agrochemicals. Royal Society Open Science, 8(1), 201805. https://doi.org/10.1098/rsos.201805

## Appendix 1. Wet weight of the dissected intestinal tract

Table 1. Weight of the intestinal samples from the different colonies and sample groups before DNA extraction.

| Colony | Sample group | Weight (Grams) |
|---|---|---|
| **Community 1** | **Autumn** | 0.0230 g |
| **Community 1** | **Winter** | 0.0498 g |
| **Community 1** | **Spring** | 0.0245 g |
| **Community 2** | **Autumn** | 0.0213 g |
| **Community 2** | **Winter** | 0.0270 g |
| **Community 2** | **Spring** | 0.0126 g |
| **Community 3** | **Autumn** | 0.0147 g |
| **Community 3** | **Winter** | 0.0552 g |
| **Community 3** | **Spring** | 0.0320 g |
| **Community 4** | **Autumn** | 0.0393 g |
| **Community 4** | **Winter** | 0.0498 g |
| **Community 4** | **Spring** | 0.0091 g |
| **Community 5** | **Autumn** | 0.0204 g |
| **Community 5** | **Winter** | 0.0246 g |
| **Community 5** | **Spring** | 0.0082 g |
| **Community 6** | **Autumn** | 0.0332 g |
| **Community 6** | **Winter** | 0.0321 g |
| **Community 6** | **Spring** | 0.0382 g |
| **Community 7** | **Autumn** | 0.0174 g |
| **Community 7** | **Winter** | 0.0252 g |
| **Community 7** | **Spring** | 0.0223 g |
| **Community 8** | **Autumn** | 0.0212 g |
| **Community 8** | **Winter** | 0.0362 g |
| **Community 8** | **Spring** | 0.0124 g |

## Appendix 2. Concentration and purity of the samples

Table 1. DNA concentration of the intestinal samples after DNA extraction.

| Community | Sample group | Concentration (ng/µl) | A260 | 260/230 | 260/280 |
|---|---|---|---|---|---|
| **Community 1** | **Autumn** | 22.6 ng/µl | 0.5946 | 1.10 | 1.92 |
| **Community 1** | **Winter** | 85.4 ng/µl | 1.9702 | 1.48 | 1.86 |
| **Community 1** | **Spring** | 21.2 ng/µl | 0.5402 | 1.07 | 2.06 |
| **Community 2** | **Autumn** | 32.4 ng/µl | 0.7574 | 1.35 | 1.89 |
| **Community 2** | **Winter** | 27.2 ng/µl | 0.6313 | 0.52 | 1.87 |
| **Community 2** | **Spring** | 21.6 ng/µl | 0.4971 | 1.39 | 1.85 |
| **Community 3** | **Autumn** | 30.4 ng/µl | 0.6830 | 1.40 | 1.88 |
| **Community 3** | **Winter** | 112.0 ng/µl | 2.2838 | 1.80 | 1.93 |
| **Community 3** | **Spring** | 47.4 ng/µl | 1.1543 | 0.66 | 1.85 |
| **Community 4** | **Autumn** | 37.6 ng/µl | 0.8626 | 1.00 | 1.85 |
| **Community 4** | **Winter** | 97.8 ng/µl | 1.9894 | 1.79 | 1.93 |
| **Community 4** | **Spring** | 11.7 ng/µl | 0.3322 | 0.70 | 1.82 |
| **Community 5** | **Autumn** | 30.8 ng/µl | 0.8303 | 1.16 | 1.87 |
| **Community 5** | **Winter** | 49.0 ng/µl | 1.2335 | 1.52 | 1.90 |
| **Community 5** | **Spring** | 16.6 ng/µl | 0.5239 | 0.98 | 1.94 |
| **Community 6** | **Autumn** | 36.2 ng/µl | 0.8626 | 1.16 | 1.92 |
| **Community 6** | **Winter** | 39.4 ng/µl | 1.0269 | 1.10 | 1.91 |
| **Community 6** | **Spring** | 38.0 ng/µl | 1.3206 | 0.64 | 1.49 |
| **Community 7** | **Autumn** | 25.8 ng/µl | 07406 | 1.27 | 1.99 |
| **Community 7** | **Winter** | 23.6 ng/µl | 0.5952 | 1.21 | 2.02 |
| **Community 7** | **Spring** | 42.2 ng/µl | 1.1658 | 1.33 | 1.92 |
| **Community 8** | **Autumn** | 45.6 ng/µl | 1.2014 | 1.03 | 1.92 |
| **Community 8** | **Winter** | 33.4 ng/µl | 0.9547 | 1.33 | 1.96 |
| **Community 8** | **Spring** | 23.4 ng/µl | 0.6988 | 1.20 | 1.98 |

Table 2. DNA concentration of the samples after DNA purification.

| Community | Sample group | Concentration (ng/µl) | A260 | 260/230 | 260/280 |
|---|---|---|---|---|---|
| **Community 1** | **Autumn** | 36.0 ng/µl | 0.2544 | 1.47 | 1.84 |
| **Community 1** | **Winter** | 43.0 ng/µl | 1.3055 | 1.88 | 1.86 |
| **Community 1** | **Spring** | 63.0 ng/µl | 1.1589 | 1.64 | 1.83 |
| **Community 2** | **Autumn** | 51.2 ng/µl | 1.4045 | 2.10 | 1.89 |
| **Community 2** | **Winter** | 44.2 ng/µl | 1.4821 | 1.60 | 1.75 |
| **Community 2** | **Spring** | 49.8 ng/µl | 1.4701 | 1.88 | 1.88 |
| **Community 3** | **Autumn** | 47.4 ng/µl | 1.4552 | 1.74 | 1.81 |
| **Community 3** | **Winter** | 60.0 ng/µl | 1.6210 | 1.92 | 1.81 |
| **Community 3** | **Spring** | 35.8 ng/µl | 0.9606 | 3.53 | 1.77 |
| **Community 4** | **Autumn** | 51.0 ng/µl | 1.1359 | 3.22 | 1.78 |
| **Community 4** | **Winter** | 44.6 ng/µl | 1.1885 | 1.93 | 1.77 |
| **Community 4** | **Spring** | 48.6 ng/µl | 1.3040 | 1.65 | 1.79 |
| **Community 5** | **Autumn** | 39.8 ng/µl | 1.0740 | 3.49 | 1.85 |
| **Community 5** | **Winter** | 53.0 ng/µl | 1.1753 | 3.63 | 1.87 |
| **Community 5** | **Spring** | 38.8 ng/µl | 0.9297 | 3.84 | 1.84 |
| **Community 6** | **Autumn** | 33.8 ng/µl | 1.0343 | 2.91 | 1.82 |
| **Community 6** | **Winter** | 25.8 ng/µl | 0.7900 | 3.32 | 1.77 |
| **Community 6** | **Spring** | 50.6 ng/µl | 1.1270 | 3.30 | 1.87 |
| **Community 7** | **Autumn** | 22.0 ng/µl | 0.6363 | 2.03 | 1.85 |
| **Community 7** | **Winter** | 3.66 ng/µl | 0.2341 | 0.43 | 1.52 |
| **Community 7** | **Spring** | 18.1 ng/µl | 0.3917 | 1.00 | 1.83 |
| **Community 8** | **Autumn** | 34.4 ng/µl | 0.8408 | 1.44 | 1.77 |
| **Community 8** | **Winter** | 29.0 ng/µl | 0.9128 | 1.43 | 1.74 |
| **Community 8** | **Spring** | 26.0 ng/µl | 0.7355 | 1.38 | 1.76 |

## Appendix 3. Tables showing the number of classified reads

Table 1. All classified reads from community 2 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Bacillus alkalitolerans*** | 0 | 0 | 1 |
| ***Bacillus iocasae*** | 2 | 0 | 0 |
| ***Bartonella apis*** | 0 | 4 | 2 |
| ***Bifidobacterium coryneforme*** | 1 | 0 | 0 |
| ***Bifidobacterium indicum*** | 1 | 2 | 1 |
| ***Bombella apis*** | 0 | 1 | 0 |
| ***Clostridium estertheticum*** | 0 | 1 | 0 |
| ***Commensalibacter intestine*** | 2 | 1 | 8 |
| ***Cutibacterium acnes*** | 0 | 1 | 0 |
| ***Ewingella americana*** | 1 | 0 | 0 |
| ***Frischella perrara*** | 1 | 8 | 368 |
| ***Gilliamella apicola*** | 2844 | 1213 | 449 |
| ***Gilliamella bombi*** | 1 | 0 | 1 |
| ***Gilliamella intestini*** | 2 | 4 | 3 |
| ***Gracilibacillus timonensis*** | 1 | 0 | 0 |
| ***Hafnia alvei*** | 4 | 0 | 0 |
| ***Herpetosiphon gulosus*** | 1 | 0 | 0 |
| ***Komagataeibacter europaeus*** | 1 | 0 | 0 |
| ***Lactobacillus bombicola*** | 31 | 11 | 40 |
| ***Lactobacillus amylovorus*** | 0 | 1 | 1 |
| ***Lactobacillus apis*** | 2911 | 597 | 1397 |
| ***Lactobacillus bombi*** | 21 | 13 | 5 |
| ***Lactobacillus colini*** | 3 | 7 | 3 |
| ***Lactobacillus delbrueckii*** | 5 | 2 | 1 |
| ***Lactobacillus helsingborensis*** | 2301 | 1448 | 1061 |
| ***Lactobacillus kimbladii*** | 349 | 1104 | 471 |
| ***Lactobacillus kullabergensis*** | 2156 | 694 | 1862 |
| ***Lactobacillus mellifer*** | 184 | 333 | 168 |
| ***Lactobacillus mellis*** | 4920 | 5456 | 447 |
| ***Lactobacillus melliventris*** | 3233 | 6403 | 1201 |
| ***Paenibacillus aceris*** | 0 | 1 | 0 |
| ***Paenibacillus glacialis*** | 1 | 0 | 0 |
| ***Pseudomonas libanensis*** | 0 | 1 | 0 |
| ***Roseomonas rosea*** | 1 | 0 | 0 |
| ***Savagea faecisuis*** | 1 | 0 | 0 |
| ***Serratia liquefaciens*** | 2 | 0 | 0 |
| ***Snodgrassella alvi*** | 614 | 257 | 13205 |
| ***Tatumella ptyseos*** | 7 | 0 | 0 |
| ***Tatumella punctata*** | 6 | 0 | 0 |
| ***Tatumella terrea*** | 53 | 0 | 0 |

Table 2. All classified reads from community 3 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Apibacter mensalis*** | 2 | 0 | 0 |
| ***Bacillus alkalitolerans*** | 0 | 0 | 1 |
| ***Bartonella apis*** | 648 | 7183 | 43 |
| ***Bartonella quintana*** | 0 | 1 | 0 |
| ***Bartonella rattimassiliensis*** | 1 | 0 | 0 |
| ***Bartonella tribocorum*** | 1 | 0 | 0 |
| ***Bifidobacterium indicum*** | 0 | 1 | 0 |
| ***Commensalibacter intestini*** | 1 | 0 | 0 |
| ***Euryhalocaulis caribicus*** | 0 | 1 | 0 |
| ***Ewingella americana*** | 0 | 2 | 1 |
| ***Frischella perrara*** | 232 | 1320 | 70 |
| ***Gilliamella apicola*** | 7630 | 612 | 6355 |
| ***Gilliamella bombi*** | 1 | 0 | 1 |
| ***Gilliamella bombicola*** | 1 | 0 | 0 |
| ***Gilliamella intestini*** | 5 | 0 | 2 |
| ***Gilliamella mensalis*** | 1 | 0 | 1 |
| ***Klebsiella pneumoniae*** | 0 | 1 | 0 |
| ***Lactobacillus amylovorus*** | 0 | 0 | 2 |
| ***Lactobacillus apis*** | 671 | 1182 | 1132 |
| ***Lactobacillus bombi*** | 3 | 1 | 3 |
| ***Lactobacillus bombicola*** | 12 | 13 | 13 |
| ***Lactobacillus colini*** | 1 | 2 | 0 |
| ***Lactobacillus delbrueckii*** | 1 | 2 | 0 |
| ***Lactobacillus helsingborgensis*** | 350 | 318 | 72 |
| ***Lactobacillus kimbladii*** | 197 | 174 | 2193 |
| ***Lactobacillus kullabergensis*** | 186 | 78 | 381 |
| ***Lactobacillus mellifer*** | 128 | 85 | 47 |
| ***Lactobacillus mellis*** | 1002 | 290 | 509 |
| ***Lactobacillus melliventris*** | 1183 | 1130 | 1304 |
| ***Monoglobus pectinilyticus*** | 1 | 0 | 0 |
| ***Paenibacillus motobuensis*** | 0 | 1 | 0 |
| ***Snodgrassella alvi*** | 598 | 31 | 1549 |
| ***Streptococcus oralis*** | 1 | 0 | 0 |
| ***Urmitella timonensis*** | 0 | 1 | 0 |

Table 3. All classified reads from community 5 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Bartonella ancashensis*** | 0 | 0 | 1 |
| ***Bartonella apis*** | 1858 | 4552 | 107 |
| ***Bartonella clarridgeiae*** | 0 | 0 | 1 |
| ***Bartonella elizabethae*** | 0 | 0 | 1 |
| ***Bartonella rattimassiliensis*** | 0 | 1 | 0 |
| ***Bartonella tribocorum*** | 1 | 0 | 0 |
| ***Bartonella vinsonii*** | 0 | 1 | 0 |
| ***Bifidobacterium coryneforme*** | 0 | 1 | 0 |
| ***Chromobacterium amazonense*** | 0 | 0 | 1 |
| ***Commensalibacter intestini*** | 0 | 0 | 1 |
| ***Frischella perrara*** | 2 | 484 | 108 |
| ***Gilliamella apicola*** | 2467 | 1320 | 3871 |
| ***Gilliamella bombicola*** | 0 | 0 | 1 |
| ***Gilliamella intestini*** | 1 | 0 | 2 |
| ***Gilliamella mensalis*** | 1 | 0 | 0 |
| ***Lactobacillus amylovorus*** | 1 | 0 | 0 |
| ***Lactobacillus apis*** | 3418 | 2423 | 8924 |
| ***Lactobacillus bombi*** | 5 | 8 | 4 |
| ***Lactobacillus bombicola*** | 22 | 22 | 74 |
| ***Lactobacillus colini*** | 8 | 4 | 0 |
| ***Lactobacillus delbrueckii*** | 0 | 1 | 0 |
| ***Lactobacillus helsingborensis*** | 1251 | 579 | 318 |
| ***Lactobacillus kimbladii*** | 2383 | 1844 | 677 |
| ***Lactobacillus kullabergensis*** | 939 | 214 | 329 |
| ***Lactobacillus mellifer*** | 714 | 428 | 234 |
| ***Lactobacillus mellis*** | 2773 | 1692 | 2625 |
| ***Lactobacillus melliventris*** | 1779 | 2851 | 652 |
| ***Snodgrassella alvi*** | 415 | 181 | 4252 |

Table 4. All classified reads from community 6 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Bartonella apis*** | 1333 | 313 | 32 |
| ***Bartonella quintana*** | 2 | 0 | 1 |
| ***Citrobacter braakii*** | 2 | 0 | 0 |
| ***Citrobacter freundii*** | 1 | 0 | 0 |
| ***Citrobacter murliniae*** | 10 | 0 | 0 |
| ***Clostridium sporogenes*** | 0 | 1 | 0 |
| ***Commensalibacter intestini*** | 0 | 0 | 1 |
| ***Frischella perrara*** | 25 | 3 | 1 |
| ***Gilliamella apicola*** | 157 | 709 | 1101 |
| ***Gilliamella bombi*** | 0 | 1 | 0 |
| ***Gilliamella bombicola*** | 0 | 1 | 0 |
| ***Klebsiella grimontii*** | 1 | 0 | 0 |
| ***Klebsiella oxytoca*** | 1 | 1 | 0 |
| ***Lactobacillus apis*** | 513 | 504 | 369 |
| ***Lactobacillus bombi*** | 0 | 1 | 1 |
| ***Lactobacillus bombicola*** | 2 | 6 | 3 |
| ***Lactobacillus colini*** | 0 | 3 | 0 |
| ***Lactobacillus helsingborgensis*** | 36 | 58 | 61 |
| ***Lactobacillus kimbladii*** | 20 | 31 | 663 |
| ***Lactobacillus kullabergensis*** | 16 | 75 | 10 |
| ***Lactobacillus mellifer*** | 58 | 33 | 30 |
| ***Lactobacillus mellis*** | 33 | 43 | 472 |
| ***Lactobacillus melliventris*** | 109 | 1318 | 112 |
| ***Paracoccus marinus*** | 0 | 1 | 0 |
| ***Snodgrassella alvi*** | 232 | 235 | 191 |

Table 5. All classified reads from community 7 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Bacillus iocasae*** | 0 | 1 | 0 |
| ***Bartonella ancashensis*** | 0 | 1 | 0 |
| ***Bartonella apis*** | 1407 | 2357 | 2339 |
| ***Bartonella elizabethae*** | 2 | 0 | 0 |
| ***Bartonella rattimassiliensis*** | 0 | 0 | 1 |
| ***Bartonella rattimassiliensis*** | 0 | 1 | 0 |
| ***Bartonella vinsonii*** | 7 | 0 | 0 |
| ***Bifidobacterium indicum*** | 1 | 0 | 0 |
| ***Breoghania corrubedonensis*** | 0 | 0 | 1 |
| ***Ewingella americana*** | 19 | 0 | 0 |
| ***Frischella perrara*** | 13 | 86 | 0 |
| ***Gilliamella apicola*** | 1181 | 1068 | 0 |
| ***Gilliamella bombicola*** | 1 | 0 | 0 |
| ***Gilliamella intestini*** | 2 | 2 | 0 |
| ***Komagataeibacter europaeus*** | 1 | 0 | 0 |
| ***Lactobacillus amylovorus*** | 0 | 1 | 0 |
| ***Lactobacillus apis*** | 999 | 2521 | 2000 |
| ***Lactobacillus bombi*** | 4 | 2 | 11 |
| ***Lactobacillus bombicola*** | 11 | 29 | 31 |
| ***Lactobacillus colini*** | 1 | 1 | 1 |
| ***Lactobacillus delbrueckii*** | 0 | 3 | 1 |
| ***Lactobacillus helsingborgensis*** | 317 | 476 | 761 |
| ***Lactobacillus kimbladii*** | 1723 | 398 | 741 |
| ***Lactobacillus kullabergensis*** | 496 | 297 | 395 |
| ***Lactobacillus mellifer*** | 274 | 262 | 161 |
| ***Lactobacillus mellis*** | 1117 | 750 | 2183 |
| ***Lactobacillus melliventris*** | 307 | 1418 | 923 |
| ***Snodgrassella alvi*** | 556 | 746 | 0 |

Table 6. All classified reads from community 8 separated by season.

| Species name | Autumn abundance | Winter abundance | Spring abundance |
|---|---|---|---|
| ***Apibacter mensalis*** | 1 | 0 | 0 |
| ***Bacillus iocasae*** | 1 | 0 | 0 |
| ***Bartonella ancashensis*** | 0 | 0 | 1 |
| ***Bartonella apis*** | 9828 | 1592 | 7809 |
| ***Bartonella henselae*** | 0 | 0 | 1 |
| ***Bartonella jaculi*** | 1 | 0 | 0 |
| ***Bartonella quintana*** | 0 | 0 | 1 |
| ***Bartonella rattimassiliensis*** | 4 | 1 | 4 |
| ***Bartonella tribocorum*** | 2 | 0 | 3 |
| ***Bartonella vinsonii*** | 1 | 0 | 2 |
| ***Bifidobacterium coryneforme*** | 0 | 2 | 0 |
| ***Bifidobacterium indicum*** | 1 | 3 | 0 |
| ***Bombella apis*** | 0 | 5 | 0 |
| ***Commensalibacter intestini*** | 1 | 0 | 3 |
| ***Enterobacter hormaechei*** | 1 | 0 | 0 |
| ***Frischella perrara*** | 147 | 0 | 39 |
| ***Gilliamella apicola*** | 2108 | 3892 | 5634 |
| ***Gilliamella bombi*** | 0 | 1 | 0 |
| ***Gilliamella bombicola*** | 2 | 0 | 0 |
| ***Gilliamella intestini*** | 0 | 2 | 5 |
| ***Gilliamella mensalis*** | 1 | 0 | 0 |
| ***Gluconobacter morbifer*** | 0 | 0 | 1 |
| ***Lactobacillus amylovorus*** | 0 | 2 | 0 |
| ***Lactobacillus apis*** | 2178 | 4875 | 2318 |
| ***Lactobacillus bombi*** | 2 | 5 | 3 |
| ***Lactobacillus bombicola*** | 22 | 39 | 22 |
| ***Lactobacillus colini*** | 0 | 8 | 3 |
| ***Lactobacillus delbrueckii*** | 0 | 2 | 2 |
| ***Lactobacillus helsingborgensis*** | 261 | 2582 | 334 |
| ***Lactobacillus kefiranofaciens*** | 0 | 1 | 0 |
| ***Lactobacillus kimbladii*** | 385 | 346 | 600 |
| ***Lactobacillus kullabergensis*** | 100 | 829 | 222 |
| ***Lactobacillus mellifer*** | 448 | 91 | 277 |
| ***Lactobacillus mellis*** | 1633 | 728 | 782 |
| ***Lactobacillus melliventris*** | 1628 | 9952 | 686 |
| ***Monoglobus pectinilyticus*** | 1 | 0 | 0 |
| ***Snodgrassella alvi*** | 477 | 211 | 737 |
| ***Solibacillus isronensis*** | 1 | 0 | 0 |
| ***Staphylococcus edaphicus*** | 0 | 1 | 0 |

## Appendix 4. Charts showing species seasonal shift.

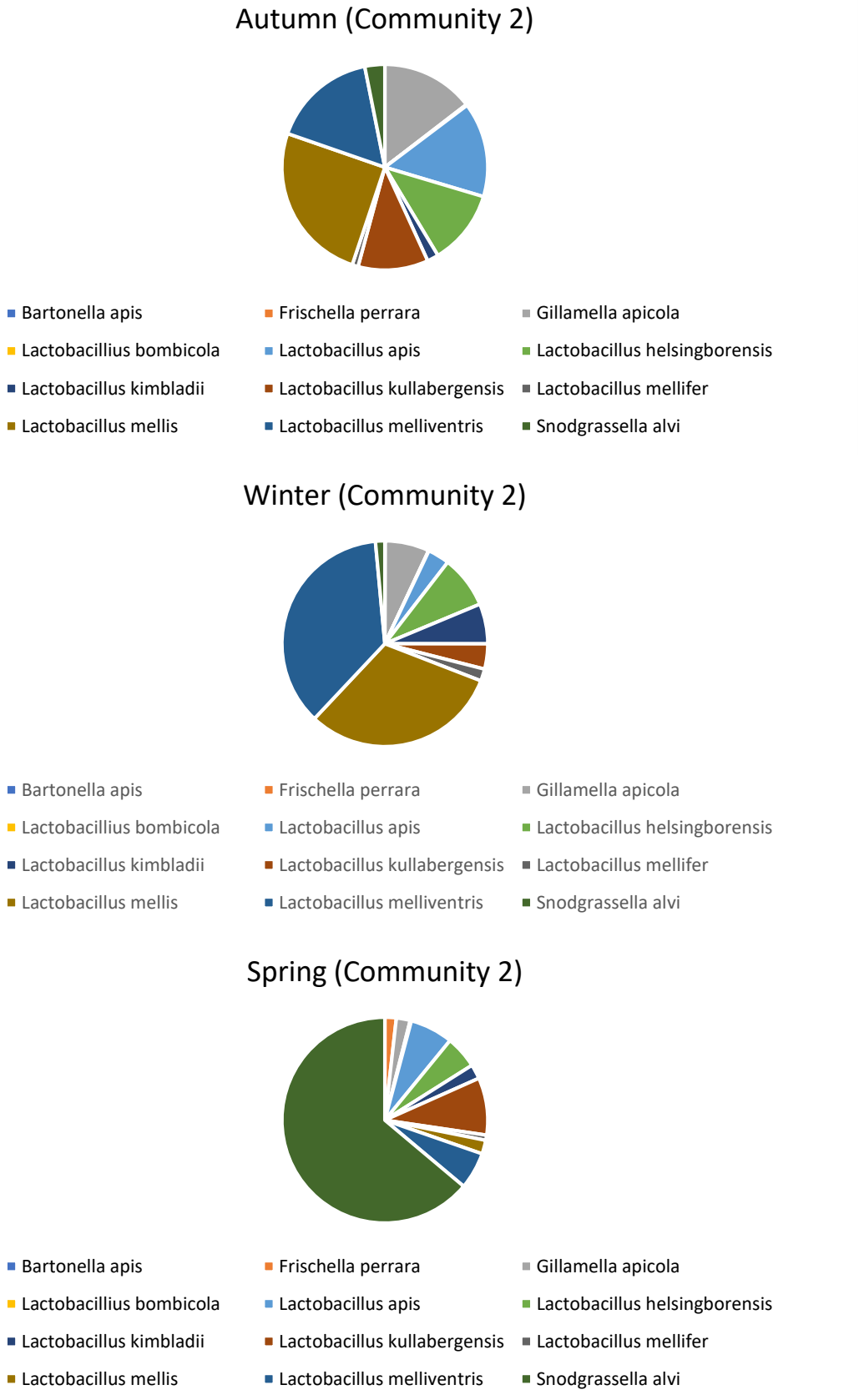


Figure 1. The seasonal shifts between the twelve examined species in community 2.

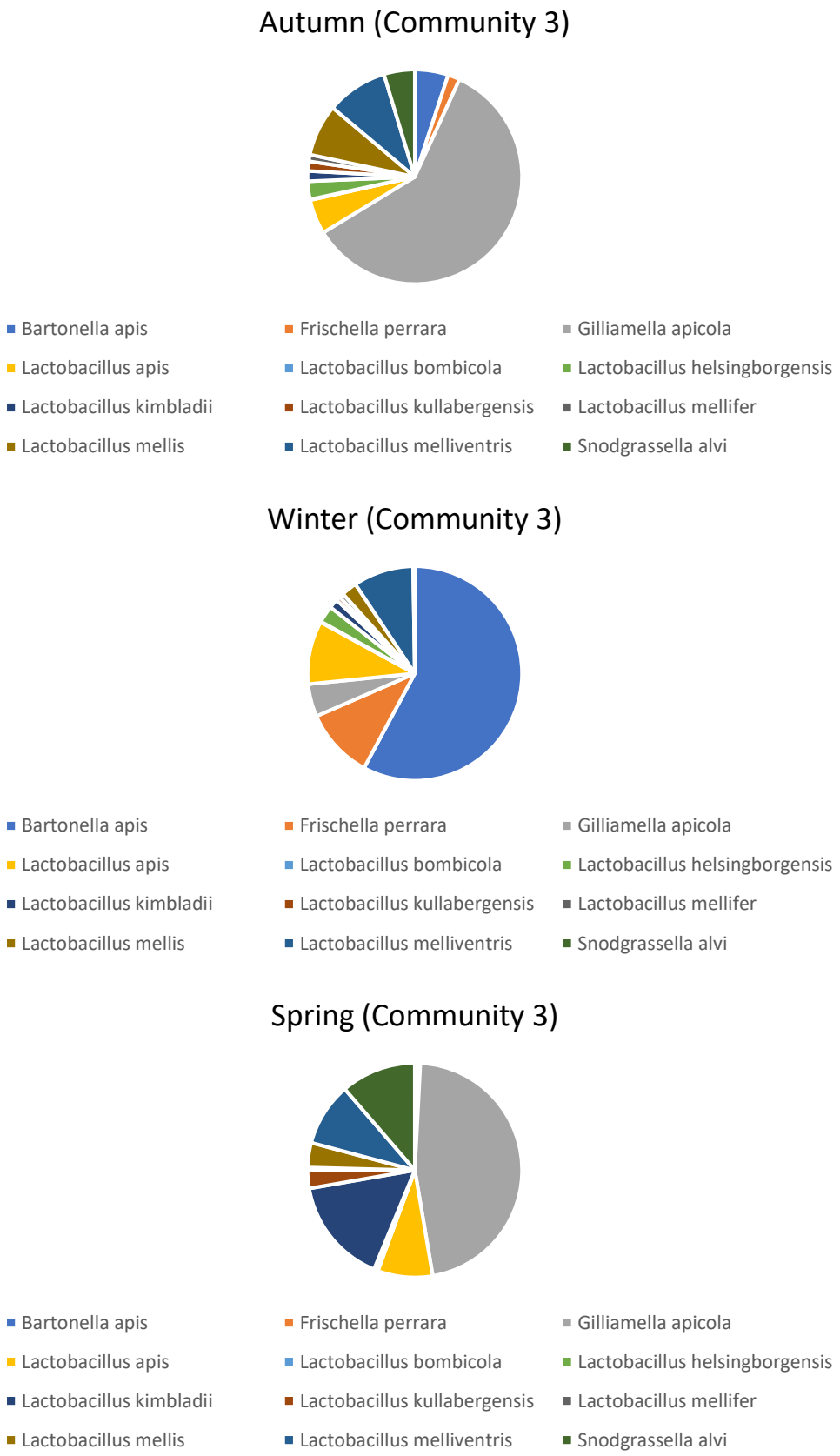


Figure 2. The seasonal shifts between the twelve examined species in community 3.


Figure 3. The seasonal shifts between the twelve examined species in community 5.

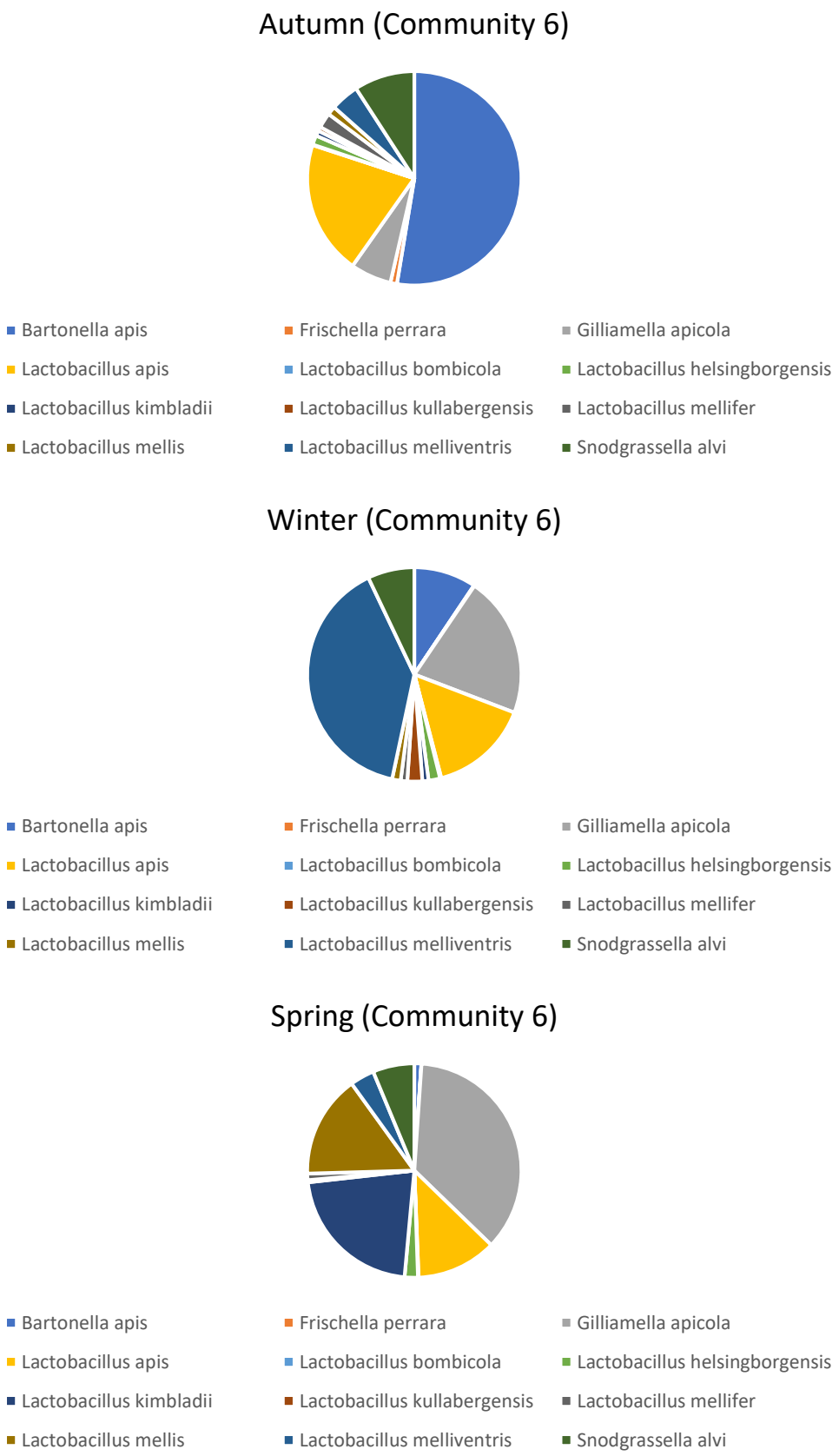


Figure 4. The seasonal shifts between the twelve examined species in community 6.


Figure 5. The seasonal shifts between the twelve examined species in community 7.

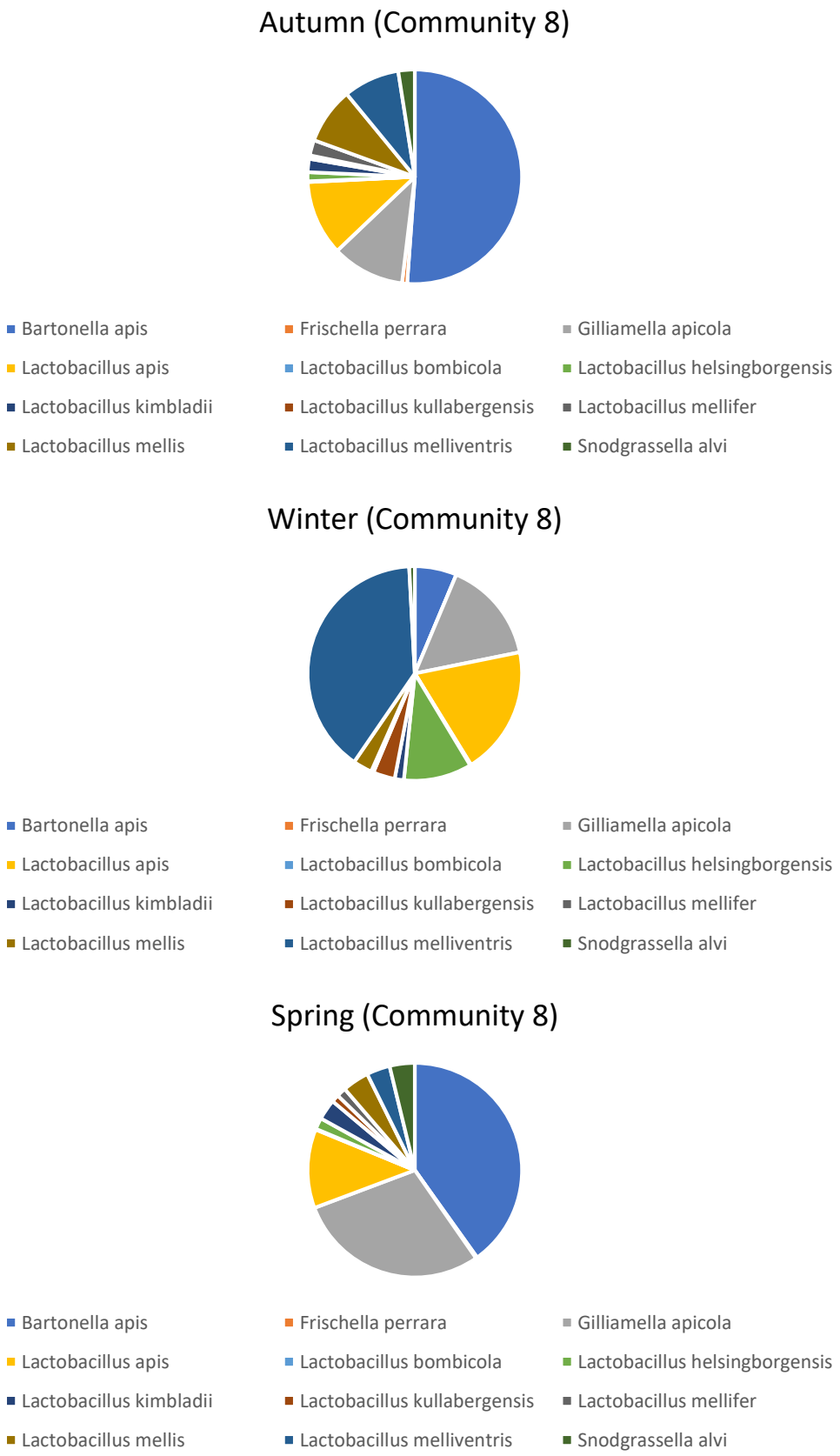


Figure 6. The seasonal shifts between the twelve examined species in community 8.