Bachelor Thesis

Phylogenetic and seasonal patterns in the microbiome of mosquito vectors

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Annotation

The aim of this thesis was to identify phylogenetic and seasonal patterns in mosquitoes’ microbiome. Thereby, the season around summer was targeted and the species *Culex pipiens* with the two forms *pipiens* and *molestus* as well as their hybrid form were of interest. For the microbiome analysis, the 16s rRNA gene was targeted in individual samples.

Declaration

I hereby declare that I have worked on my bachelor’s thesis independently and used only the sources listed in the bibliography.

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Content of the thesis

1 Abstract

Mosquitoes have a worldwide distribution and a high diversity with over 3000 species known. They are seen as a global health burden since they have the ability to transmit pathogens causing severe diseases such as the West Nile Virus. The main vector of this virus is the *Culex pipiens* species, which consists of two forms: *Culex pipiens pipiens* and *Culex pipiens molestus,* which can also hybridize. Even though morphologically these two forms are indistinguishable, they have a wide range of differences in their lifestyle that also affect their epidemiological relevance. For this reason, it is important to differentiate them, and the CQ11 molecular marker is a useful tool in this sense. Their microbiota could be also distinctive. The insight into the microbiome of mosquitoes like *Culex pipiens* is of interest because the gut microbiota has shown to have an effect on vector transmission as well as on other vector physiological factors. To analyze the microbiota of samples collected along several months (April-October) in Spain and Slovakia, the V4-V5 region of the bacterial 16s SSU rRNA was targeted. Prior to that, polymerase chain reaction (PCR) as well as agarose gel electrophoresis were used to identify the *Culex pipiens* species and forms. The microbiota composition of the samples was tested for seasonal as well as for form differences/similarities. In both cases no significant differences were found. Future studies may confirm that *Culex pipiens* host a common microbiome or find differences between the two forms using a broader sample range that overcomes the inter-individual differences. Seasonal effects should still be studied in more detail, especially combined with epidemiological data for the vectored diseases at the individual level.

2 Aims of the thesis

1. To assess the microbiome assemblage in natural populations of Culex pipiens mosquitoes in relation with their phylogenetic background (i.e. subspecies)
2. To identify the patterns of bacterial assemblage of individual *Culex pipiens* through the sampling season
3. To discuss the potential implications of the revealed microbiome phylogenetic and seasonal patterns in *Culex pipiens* vector's epidemiology

3 Introduction

There are more than 3000 species of mosquitoes (Insecta: Diptera: Culicidae), distributed between two subfamilies, called *Anophelinae* and *Culicinae*. Although they have worldwide distribution, India is the biogeographic zone with the largest diversity of species and the highest occurrence of mosquitoes (Jayakrishnan et al., 2018). Since mosquitoes can be vectors of severe diseases, they play a significant role in human's life (Tham et al., 2018) and, thus, research in this topic is abundant (for example: (Muturi et al., 2019; Saab et al., 2020; Sepulveda and Moeller, 2020)).

Mosquitoes have a complex life cycle, that is made up of four main metamorphosis stages: starting from an egg, they transform into larvae, then to a pupa, and finally emerge as an adult mosquito, the only terrestrial stage (Judd, 1998; Minard et al., 2013). In the larval stages, the mosquito mainly ingests plankton and bacteria, so a solid microbiota can already be established at the beginning of their life cycle (Minard et al., 2013).

3.1 Mosquitoes and their relevance as disease vectors

Mosquitoes are important disease vectors worldwide (Ciota and Kramer, 2013; Franz et al., 2015; Song et al., 2017). They transmit diverse pathogens, and the lack of vaccines and the resistance of these vectors against insecticides cause that the diseases they transmit keep spreading at an enormous speed, thus, more research is needed in the field of vector-borne diseases to ensure safety and health (Acharya and Bai, 2016; Petersen et al., 2013; Sallam et al., 2016; Song et al., 2017).

The vectorial capacity is a measure of the ability to transmit pathogens (Farajollahi et al., 2011; Schulz and Becker, 2018). This vector competence can be influenced by diverse factors such as genetics, environment or even the diverse microbiota compositions (Novakova et al., 2017). The microenvironment changes often and radically along the mosquito life cycle, as does the feeding behaviour of the different stages, and therefore, an intensive study on the gut interaction with the vector capacity might help when trying to control diseases which are caused by these vectors (Jayakrishnan et al., 2018).

3.1.1 West Nile Virus (WNV)

One of the mosquito-vectored diseases, the West Nile fever, is caused by a virus from the family Flaviviridae (West Nile virus, WNV) and is especially transmitted by mosquitoes to birds; mammals only play a role as dead-end hosts (Lustig et al., 2018; Patsoula et al., 2016). Most likely the origin of WNV is Africa, and birds spread it to other countries, for example Europe, through migration (Lustig et al., 2018). There are two lineages of WNV: one present in Europe, America, Middle East, Africa and India, and the other found in Madagascar as well as in sub-Saharan Africa (Patsoula et al., 2016). However, other sources claim the existence of more lineages (Marka et al., 2013).

West Nile fever is one of the most spread diseases worldwide (Ciota and Kramer, 2013). Outbreaks and virus spread are mainly influenced by factors called extrinsic and intrinsic. Extrinsic factors cover environmental factors such as rain, temperature or season, whilst intrinsic factors describe mosquito vectors' nutrition, longevity, as well as their competence and sensibility to virus infection, and the vectorial capacity (Ciota and Kramer, 2013). The diseases' clinical symptoms appear after an incubation time of 2-14 days, and most people suffer from sudden symptoms like headache, vomiting, low-grade fever and other mild ailment (Petersen et al., 2013). Some individuals also show illnesses that last up to a month and even neuroinvasive diseases, but the majority of infected humans fully recover (Petersen et al., 2013).

Culex pipiens (*Cx. pipiens*) is recognized as the main vector of the virus, even though the transmission to humans can also occur over blood transfusions and some organ transplants (Farajollahi et al., 2011; Petersen et al., 2013; Schulz and Becker, 2018).

3.2 *Culex pipiens* complex

As mentioned above, *Cx. pipiens* serves as major vector of WNV among other diseases. *Cx. pipiens* is not a single species but comprises what is called a species complex, which is a group of species so closely related through evolution that they cannot be easily distinguished morphologically (Farajollahi et al., 2011). The only difference among species of the *Cx. pipiens* complex is given in male genitalia, and females appear morphologically indistinguishable (Shaikevich et al., 2016). Molecular tools have shown to be a helpful alternative to differentiate between species and their given forms and hybrids (Shaikevich et al., 2016).

The *Cx. pipiens* complex includes six species: *Cx. quinquefasciatus* Say*, Cx. australicus* Dobrotworsky & Drummond*, Cx. pipiens pallens* Coquillet*, Cx. globocoxitus* Dobrotworsky*,* and

the two forms of *Cx. pipiens* Linnaeus, namely *Cx. pipiens form pipiens* and *Cx. pipiens form molestus* (Beji et al., 2017). The different species can be partly distinguished according to the areas they inhabit, which are specific for each of them. *Cx. quinquefasciatus* together with *Cx. pipiens* are known as the classical house mosquitoes and they are the most common species in inhabited areas and are therefore the most popular in association with humanity (Farajollahi et al., 2011). *Cx. quinquefasciatus* is mainly found in regions that are tropical or sub-tropical in Asia, America, Africa and Australia (Farajollahi et al., 2011). Within *Cx. pipiens*, *Cx. pipiens f. pipiens* was initially distributed between the areas of Northern Europe and Southern Africa, but later was also found in Northern as well as Southern America (Farajollahi et al., 2011). In contrast, the *Cx. pipiens f. molestus* can also be found in Japan, Australia and South Korea (Farajollahi et al., 2011). Both forms are found in Europe and also hybridization events are noted in European countries (Martínez-de la Puente et al., 2016).

3.2.1 *Culex pipiens* phylogenetic diversity

As already mentioned above, *Cx. pipiens* consists of two distinct forms: *molestus* and *pipiens*. These two forms differ in a variety of behavioral factors and often are the complete opposite of each other. To achieve egg development, the *pipiens* form needs a blood meal in advance, which is known as anautogeny. In contrast, for the *molestus* form no blood meal is required for the first batch of eggs (autogeny) (Osório et al., 2014). Regarding the nutrition of the two forms, *Cx. pipiens f. pipiens* mosquitoes are ornitophylic, which means that they seek for birds as their preferred host. On the other hand, *Cx. pipiens f. molestus* are mamophylic, so they preferably suck on mammals (Shaikevich et al., 2016). Hybrids of these two forms tend to live an opportunistic lifestyle, feeding on mammals and birds equally. These hybrid forms are of a significant importance in epidemiology, since they are often infected by feeding on birds, and then transmit pathogens further feeding on humans. Those hybrids are, thus, seen as the link between *Cx. pipiens* and WNV infection in humans (Farajollahi et al., 2011; Osório et al., 2014). Another differential characteristic is the winter behaviour: the *pipiens* form has a diapause (a term derived from the greek verb *diapauein*, which describes a certain inactivation of action; it is a certain state in which the mosquito is metabolically inactive, and this process is arbitrated by certain neurohormones (Diniz et al., 2017)), while the *molestus* form is active during the whole year (Osório et al., 2014). Aboveground habitats are mainly colonized by *Cx. pipiens f. pipiens*, while *Cx. pipiens f. molestus* prefer colder environments and live therefore in underground niches (Shaikevich et al., 2016). The form *molestus* could have developed this underground lifestyle as

an adaptation from the *pipiens* form to certain environments or localities, or they could alternatively derive from a southern *molestus* tribe that was already adapted to underground settings (Osório et al., 2014). Due to all these differences, there is some controversy if to consider *molestus* and *pipiens* as the same genetic entity (Osório et al., 2014). Another possibility that remains to be explored is if these differences in lifestyle could also be coupled with differences in their microbiome.

3.3 Microbiome in mosquitoes

Before the latest advances in molecular techniques gave the opportunity to take a closer look at the processes between a host eukaryotic system and the associated microorganisms, it was thought that most symbionts had rather a pathogenic than a beneficial effect (Bahrndorff et al., 2016). Thanks to new techniques like Next Generation Sequencing (NGS), it is now known that these microbes have a crucial effect on the host system in many ways, where processes like survival, the maintenance of the immune system, as well as social interactions rely to some extent on symbiotic microbes (Bahrndorff et al., 2016).
Microbiota (also referred to as microbiome in genetic terms) is the term used to define those microorganisms that live either within an organism or upon it (Thongsripong et al., 2018).
The bacteria of the microbiota have crucial roles in mosquitoes such as defense against pathogens (Bahrndorff et al., 2016), nutrition (Muturi et al., 2019) or reproduction (Hegde et al., 2018). Even if the mosquitoes' microbiota represents less diversity than that of other insect species such as the green bottle fly, it is highly variable (Bahrndorff et al., 2016; Hegde et al., 2015), and comparable to the microbial richness found in other blood feeding insects such as triatomines (Waltmann et al., 2019). The most abundant bacterial phyla in mosquito microbiota include Proteobacteria, Acinetobacter, Firmicutes and Bacteroidetes (Hegde et al., 2018; Jayakrishnan et al., 2018), including both aerobic and facultative anaerobic bacteria (Hegde et al., 2015). It is also important to highlight that, even though most studies mainly focus on bacteria in the microbiome, also fungi, viruses and protozoans are equally responsible for the richness of species in the mosquitoes' microbiota (Guégan et al., 2018; Hegde et al., 2015).

Adult mosquitoes obtain certain members of their microbiome very early in their development through vertical transmission, so these microorganisms evolve from the beginning till the end in the mosquito. Another part of the microbiome, however, is gained through the mosquito's nutrition, either along the development from the aquatic environment or as adults through blood-

meals and sugar-meals (Guégan et al., 2018). Particularly in the larval stages, the mosquitoes' microbiota adapts to the water environment surrounding the larvae, which results in similar microbiomes in both (Hegde et al., 2018). In the adult stage, since only females require a blood meal to complete the reproductive cycle, the differentiation between male and female mosquitoes in bacterial diversity is also crucial. Males tend to be colonized by bacteria of the phylum Firmicutes in their midgut, while the female's midgut is dominated by Gammaproteobacteria (Minard et al., 2013).

Space, shape and properties of the midgut build up a pleasant environment for bacteria, but also the contact to the outer environment makes the gut more favourable for microorganism colonization (Minard et al., 2013). Since bacteria are often located in the gut, in close vicinity to arboviruses and other pathogens acquired through feeding, this microbiota is especially important in determining the capability of a host to be infected by pathogens or to transmit them (Novakova et al., 2017). In fact, when a mosquito consumes a blood meal that is infectious, it is not automatically a vector. The virus first has to follow a certain path through the mosquitoes' body that may be interrupted by different barriers (Jupatanakul et al., 2014). The first barrier is in the midgut, where it has to replicate in the epithelium (infection barrier), then followed by the midgut escape barrier that would prevent the virus to follow the path through the rest of the body and to have the ability to infect more tissues. The last barrier occurs in the salivary glands, where the transmission to the vertebrate host would occur through the next blood meal (Jupatanakul et al., 2014). Only if all these barriers can be surmounted, the transmission of the disease is possible and the female mosquito is able to transmit the pathogen, therefore being infectious its whole lifetime (Jupatanakul et al., 2014; Schulz and Becker, 2018). The microbiota may play a role in the functioning of some of those barriers and, thus, prevent infection and disease transmission for example through the activation of immune signaling pathways (Jupatanakul et al., 2014).

3.3.1 Relationship between the microbiome dynamics and transmission of pathogens

It is of great importance to neither view the host system nor the microbiota as a separated unit, instead they should be considered together as a combined entity. For this reason, the term holobiont was created as an overall concept for hosts and their microbiota, as well as the resulting interactions. An arthropod vector within the theory of the holobiont is seen as a complex organism that is formed through interactions between the host and its microbiota (Guégan et al., 2018).

Microbiota and immune system interact, and for example microbes can have defensive functions such as excluding pathogens (Morella and Koskella, 2017). The system is controlled by signaling cascades that act upon the detection of a pathogens (Dennison et al., 2014). Different defensive mechanisms contribute to fight against pathogens, and for example in mammals the pathogens can be recognized by the skin microbiota and be excluded through antimicrobial production (Morella and Koskella, 2017). In insects, the microbiota production of antimicrobial peptides to fight pathogens results from the activation of certain immune pathways, such as the Toll pathway. Different pathways react to different viruses, bacteria, fungi or parasites forming the insect's specific immune responses (Dennison et al., 2014). Since the microbiome has an important role in immunity, it is also determining the susceptibility of vectors to be infected and their ability to transmit pathogens (Novakova et al., 2017).

3.3.2 Seasonal variation in the microbiome

Season has a huge impact on natural systems and also temperature shifts affect organisms lifestyle and fitness, with increasing temperatures decreasing animal's fitness (Altizer et al., 2006; Sepulveda and Moeller, 2020). A significant impact on the seasonality is caused by global events such as the global warming, which in turn has an effect on parasite dissemination (Altizer et al., 2006). This effect of variation in temperatures can also lead to an effect on gut microbiota of animals, influencing relative bacterial abundances (Sepulveda and Moeller, 2020). Higher temperatures are associated, for example in wood lice or fruit flies, with a relative increase in Proteobacteria in the microbiota (Sepulveda and Moeller, 2020).

As for mosquitoes, warmer climates favor more intensive feeding and faster development to maturity (Altizer et al., 2006). In contrast, if the climate is below ideal temperatures for mosquito development, they fail to develop and get rather inactive (Altizer et al., 2006). Also for parasites, cold temperatures are not advantageous because they stay immature or pass away before getting transmitted by mosquitoes (Altizer et al., 2006). In addition, increased seasonal rainfall usually happens in warmer regions, so the aquatic environment for the development of the larval stages is extended, and profusion of mosquitoes occurs as a result (Altizer et al., 2006), although rainfall can also cause damage in breeding sites due to overflows that result in larvae death (Benedum et al., 2018). In general, with warmer climate, more infected mosquitoes are present and therefore the risk of infections increases (Altizer et al., 2006).

Constant changes in the environment can also cause a huge impact on microorganisms and, thus, indirectly affect host's life cycle, since the microbiota is central to development and adaptation (Guégan et al., 2018). For example during the dry season in Africa, the mosquito microbiota showed more diversity than during the rainy one, but also the locality (meaning rural or urban regions) plays a role in the microbiota diversity found, as for the urban localities a higher diversity in the microbiota at the family level was found (Akorli et al., 2016). Seasonality, and more specifically temperature, also showed to have an impact on the abundance of *Wolbachia* and four other bacterial groups, namely *Acetobacteraceae, Bacteroidetes, Enterobacteriaceae* and *Asaia*, in adult mosquitoes (Novakova et al., 2017). In certain *Culex* larvae, the composition of bacteria differed among seasons, where *Firmicutes* were more abundant in winter compared to the period between end of summer and beginning of autumn, where *Cyanobacteria* and *Proteobacteria* dominated in the microbiota (Duguma et al., 2017). In spite of the fact that environmental associations with microbiota variations have been repeatedly found, in particular the seasonal patterns and the factors determining them are not yet well understood.

3.3.3 Phylogenetic patterns in the mosquito microbiome

Mosquito microbiomes can heavily differ among different species as well as within the same species (Hegde et al., 2018). Hegde et al. (2018) performed an interesting study, in which three mosquito species were compared and their microbiome was analyzed. Between *Cx. quinquefasciatus, Aedes aegypti* and *Aedes albopictus* different bacteria were observed. *Wolbachia* was shown as the most prevalent endosymbiont in *Cx. quinquefasciatus* as well as in *Aedes albopictus*, however, *Aedes aegypti* did not show any significant abundance of *Wolbachia*. Instead, Pseudomonas, *Zymobacter*, *Enterobacter* and *Tatumella* were observed in their microbiome. Besides *Wolbachia*, *Asaia*, *Halomonas* as well as *Shewanella* could be detected in *Cx. quinquefasciatus,* and *Zymobacter*, *Pseudomonas* and *Halomonas* in *Aedes albopictus* (Hegde et al., 2018). Hegde et al. (2018) and Novakova et al. (2017) found that *Proteobacteria,* like *Wolbachia*, have a dominating role in shaping some mosquitoes' microbiomes. The study of Novakova et al. (2017) showed that in the 11 mosquito species sampled in Canada, four bacterial strains including *Wolbachia, Serratia, Pseudomonas* as well as *Asaia* were the most prevalent. *Wolbachia* was abundant in higher numbers in three species of the genera *Culex* and *Coquilletidia*. However, when excluding *Wolbachia,* differences between species tend to be not significant (Novakova et al., 2017). In other genera, namely *Aedes, Anopheles* and *Ochleratus, Pseudomonas* show high numbers (Novakova et al., 2017).

This and other studies, however, did not test or differentiate the morphologically indistinguishable species or forms within the *Cx. pipiens* complex. The lower level phylogenetic patterns in the mosquito microbiota remain, thus, unexplored.

3.3.4 Microbiome in *Culex pipiens*

Culex microbiome studies from Muturi et al. (2016) showed Proteobacteria as the dominant phylum, followed by Firmicutes. Also, the families of *Rickettsiaceae, Enterobacteriaceae* and *Sphingomonadaceae* were found to be abundant in *Cx. pipiens* species (Muturi et al., 2016).
To be more precise, *Wolbachia* is the best indicator bacteria for some *Culex* species, including *Cx. pipiens. Wolbachia* is an important bacterial endosymbiont that contributes to the host's fitness and the ability to provide nutrients (Guégan et al., 2018). This bacterium can also limit the ability to carry certain parasites or pathogens for mosquitoes, causing lower infection rates as well as reduced virus transmission (Novakova et al., 2017). Wo*lbachia* is becoming of great interest to control mosquito-vectored diseases. Research concerning symbionts like *Wolbachia*, their introduction in mosquito populations, and their genetic manipulation can minder the spread of pathogens, shorten the life of the mosquito, or reduce the blood meal rate of the vectors (Muturi et al., 2016; Novakova et al., 2017).
In spite of the microbiota's relevance in the vectorial capacity of mosquitoes, particularly in *Cx. pipiens* which is the main vector of diseases like WNV in Europe, little is known about the specific environmental and genetic factors that determine the microbiota's patterns in these vectors. Determining the seasonal shifts and subspecific composition of the microbiota of *Cx. pipiens* will contribute to a better understanding of the mosquito holobiont and the epidemiology of the vectored diseases.

4 Materials and Methods

4.1 Mosquito Sampling

Female mosquito samples from two different countries, Slovakia and Spain, were collected in 2018. A total of 166 samples were collected, however only 113 of them were used for the microbiome analysis. From these 113, 76 samples were Slovakian; 66 were collected in Komarno, one in Podrecany, three in Bratislava and another six in Kosice. They were trapped with BG-Mosquitaire in all localities except for Kosice, where the BG-Sentinel trapping method was used. Samples were collected in summer months from June to August in roughly one to five-day intervals. As for Spain, a total of 37 samples were collected, where four originated from Palacio de Donana, 12 from Calatilla, 11 from Celestino Mutis and four from Los Alamos. In general, they were trapped with CDC traps, but for few of them also BG traps were used. Samples were collected from April to October at least once a month, and the most four times a month (in May).

Upon collection, the female mosquitoes were morphologically identified and selected for *Cx. pipiens* complex, and then preserved in All Protect solution (Qiagen) until processed in the laboratory. The exact sampling dates as well as the number of mosquitoes collected each day are summarized in Tables 1 and 2.

Table 1- Sampling dates in Slovakia in the Months June, July and August (2018)

Month	Day	Number of mosquitoes collected
June	13	5
	17	1
	18	1
	19	7
	21	2
July	5	3
	9	1
	10	11
	12	1
	13	1
	14	7
	15	4
	16	1
	17	6
	20	5
	30	2
August	5	5
	8	2
	11	5

	12	1
	13	1
	15	2
	18	1
	23	1

Table 2- Sampling dates in Spain in the months April-October (2018)

Month	Day	Number of mosquitoes collected
April	19	6
May	3	2
	24	2
	25	5
	26	3
June	24	4
	28	2
July	21	5
August	-	-
September	20	4
October	10	4

4.2 Mosquito Preparation

Mosquitoes were prepared for DNA/RNA extraction by removing wings and legs under sterile conditions, in order to reduce host DNA contribution to the final extraction yields. A three-step washing process followed: first rinse in sterile PBS buffer to remove the rest of the preservation solution; then a short rinse into absolute ethanol to remove any unwanted surface contaminants, and a final sterile PBS rinse step to remove ethanol residues. Then mosquitoes were immediately transferred to 350 μl of RLT buffer (Qiagen) and smashed until a proper homogenization occurred and no major rests of the mosquito's body could be seen. Following the homogenization, DNA/RNA extraction was performed using the Allprep 96 DNA/RNA Kit (Qiagen), thoroughly following the provided protocol. The main steps include the separation of the DNA and RNA fractions, the addition of several washing buffers followed by centrifugation and a final elution of the DNA or RNA that was left in the columns using ultrapure, RNAse-free water. DNA was stored at -20°C and RNA at -80°C until PCR preparation was carried out.

The extracted RNA was sent to a collaborator to check for WNV infection in our mosquito samples. All occurred to be negative and we continued our analyses with all of them.

4.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was carried out in order to amplify particular regions of the genomic DNA. In order to do so, a PCR mix was prepared as stated in Table 3. Two different markers were used to obtain different information: One to test for different *Culex* species (ACE gene) (Smith and Fonseca, 2004) and the other to identify different forms of *Cx. pipiens* (CQ11 microsatellite) (Bahnck and Fonseca, 2006). A negative control for each PCR reaction was prepared containing all the reagents but no DNA template.

Table 3- Markers ACE and CQ11: PCR mixture compositions and Primer Sequences

Marker	ACE	CQ11
PCR mixture	1 µl DNA sample 1 µl ACEpip 1µl ACEtorr 2 µl B1246s 5 µl ultrapure water 10 µl Master Mix	1 µl DNA sample 1 µl molCQ11R 1.5 µl pipCQ11R 1.5 µl CQ11F 2.5 µl ultrapure water 10 µl Master Mix
Primer Sequences	ACEpip: 5‘-GGAAACAACGACGTATGTACT-3‘ ACEtorr: 5‘-TGCCTGTGCTACCAGTGATGTT-3‘ B1246s: 5‘-TGGAGCCTCCTCTTCACGG-3‘	molCQ11R: 5‘-CCCTCCAGTAAGGTATCAAC-3‘ pipCQ11R: 5‘-CATGTTGAGCTTCGGTGAA-3‘ CQ11F2: 5‘-GATCCTAGCAAGCGAGAAC-3‘

The first primer pair was specific to identify the different species in the *Cx. pipiens* complex using the acetylcholinesterase (ACE) gene and the PCR cycles were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds; and a final step at 72°C for 5 minutes (Smith and Fonseca, 2004).

The second PCR with CQ11 microsatellite primer pairs aimed to identify whether the *Cx. pipiens* identified in the previous PCR were form *pipiens* or form *molestus.* The PCR was started with 94°C for 5 minutes, then 40 cycles with 94°C for 30 seconds, 54°C for 30 seconds and 72°C for

40 seconds followed; and a final extension step at 72°C for 5 minutes (Bahnck and Fonseca, 2006). The results of this PCR were used for the phylogenetic analysis of the thesis.

4.4 Gel preparation and interpretation

Following the PCR reactions, the samples were prepared for electrophoresis in 2% agarose gels, so all the bands could separate properly for all the markers. Before loading the gel, 8 µl of each sample (PCR product) were mixed with 2 µl of loading buffer. To compare the size of the bands, 5 µl of 100 bp ladder were loaded at the beginning and the end of each lane in 96-well gels. The electrophoresis was run at 210 V for approximately 50-55 minutes. The gels were analyzed under UV light and then evaluated according to the respective primers either for the ACE PCR products where a band between 634-636 bp was observed for *Cx. pipiens* (Smith and Fonseca, 2004), or to further identify the *Culex pipiens f. pipiens* and *f. molestus,* with the CQ11 marker, where bands of 200 bp or 250 bp were expected, respectively, while the presence of the two bands indicated the hybrid form (Bahnck and Fonseca, 2006).

4.5 16S library preparation

For analyzing the microbiome of the samples, the EMP 16S Illumina Amplicon Protocol was used as a guideline (http://www.earthmicrobiome.org/protocols-and-standards/16s/; accessed [23.03.2020]). To do so, the V4-V5 region of the 16s rRNA gene was targeted by specific primers: 515F (Parada et al., 2016) and 926R (Parada et al., 2016; Quince et al., 2011) that were modified to add a double-barcoding strategy.

The forward primer consists of a 5' Illumina adapter, a Golay barcode, a forward primer pad as well as linker and the forward primer (515F) itself: AATGATACGGCGACCACCGAGATCTACACGCTXXXXXXXXXXXXTATGGTAATT GT GTGYCAGCMGCCGCGGTAA.

For the reverse primer, the 3' Illumina adapter, a five-bp barcode, a reverse primer pad and linker and the reverse primer (926R) are included: CAAGCAGAAGACGGCATACGAGAT XXXXX AGTCAGCCAG CC CCGYCAATTYMTTTRAGTTT

For the PCR mixture, 10.5 µl PCR-grade water, 12.5 µl Q5 High-Fidelity 2X Master Mix (NEB), 0.5 µl of each 10 µM forward and reverse primer and 1 µl sample DNA were mixed for a volume of 25 µl, and PCR was carried out with the following conditions described in Table 4.

Table 4- PCR program conditions for 16S libraries

Temperature [°C]	Time	Repeats
98°	3 min	1 x
94°	45 sec	35 x
50°	60 sec	35 x
72°	90 sec	35 x
72°	10 min	1 x
4°	hold	

Each sample was amplified to obtain a total final volume of 50 µl and then checked via an agarose gel (500 bp band expected). The PCR products were then purified using AMPure XP beads (Beckman Coulter) and their concentration was measured using a Synergy H1 spectrophotometer (BioTek) prior to equimolar pooling. The pooled samples were sent for sequencing in an Illumina MiSeq run, with two reads of 300 bp each using v2 chemistry.
The following custom sequencing primers were used:
Read 1: 3' AATGATACGGCGACCACCGAGATCTACACGCT 5'
Read 2: 3' AGTCAGCCAGCCCCGYCAATTYMTTTRAGTTT 5'
Index read: 3' AAACTYAAAKRAATTGRCGGGGCTGGCTGACT 5'
The resulting fastq files with the sequencing outcome were sent back to our laboratory for bioinformatic analysis.

4.6 Analysis Pipeline for Illumina Amplicon Sequencing (16S)

The processing of the fastq files from Illumina was carried out using the "Analysis Pipeline for Illumina amplicon sequencing (16S)" from Novakova's Laboratory, which consists of several steps that are needed to get a filtered and high quality OTU (Operational Taxonomic Unit) table.
The read 1 file contains the forward reads of the sequences together with the forward barcode, the read 2 file contains the reverse sequences, and the index file contains the reverse barcode sequences. The quality of the reads was checked in FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed [20.05.2020]). The samples were summarized and listed in a metadata file, where all barcode combinations were matched to the corresponding samples. Then the barcodes of read 1 were extracted using QIIME1, followed by merging of the forward and reverse barcodes in a single file (Caporaso et

al., 2010). This step results in an index file which contains the barcode combinations present in our sequences and matching the metadata barcodes.

The data was demultiplexed in USEARCH (Edgar, 2010) and the per sample outputs were merged (meaning that the two reads of each sample were joint) into a single fastq file using a minimum overlap of 70 bp and a merged size range of 400-450 bp.

As a next step, sequence filtering was carried out; first the primers were trimmed off and then the reads were filtered using a maximum expected error of 5 and trimmed to the same length. This step is important, because low quality reads, as well as reads that are too short or too long, introduce bias in the results.

An OTU table was generated using USEARCH global alignment at 97% identity of the sequences. Then taxonomical assignments were performed using the SILVA132 database, which is specific for bacteria.

Finally, the OTU table was filtered again using QUIIME1 to remove very low abundant OTUs, mitochondrial, chloroplast and Archaea sequences that are amplified as a result of primer unspecificity, and to get a normalized dataset by rarefaction. Rarefaction is needed so the number of reads in each sample are equal, and therefore the samples can be compared.

In order to get the most reliable results, contaminants found in the negative controls included in our sequencing library, as well as bacterial infections detected in some mosquitoes, were removed from the data. Two datasets, with and without the typical *Culex* endosymbiotic *Wolbachia,* were constructed to get information about how the presence of *Wolbachia* affects the response of the microbiome to the different factors tested in the subsequent analyses. In the dataset included *Wolbachia* 106 samples were included, in the one excluding *Wolbachia* 49 samples were counted.

To get a general overview of the OTU distribution in the different categories tested, Microsoft Excel was used to generate pie charts, bar graphs and to calculate percentages from them. The outcomes are presented in the following section.

4.7 Statistical analysis

R was used for statistical analysis. Alpha diversity tables were generated using the usearch alpha_div command, and included Dominance, Equitability, Richness and Shannon indexes. The influence of the different study variables in the microbiome's diversity was tested by means of the Kruskal Wallis rank sum test or the Mann Whitney test depending of the number of groups in each variable. Every test was performed for the different variables, both including or excluding the endosymbiont *Wolbachia* in the analysis. The study variables included the country of sampling (samples from Slovakia and Spain in June and July, the only common dates), form (*pipiens*, *molestus* or hybrid) and monthly ranges for each country separately. For Slovakia, 15-day ranges were also tested.

5 Results

5.1 Gel analysis

A total of 166 samples were prepared for gel analysis using PCR with the two primer pairs: ACE and CQ11. All samples were examined in 2% agarose gels.

From the 166 samples analyzed in total, 10 samples (6.02 %) showed no amplification with the ACE primer, and three samples (1.81 %) showed an unspecific band of 800 bp; they were all excluded from the analyses. One sample (0.60 %) showed a band of 500 bp which corresponds to *Culex torrentium* species, and it was also not used for the analyses. 152 samples (91.57 %), showed a band of 600 bp and were, thus, identified as *Culex pipiens* species. From those, 113 samples were used for the microbiome analysis. Figure 1 shows the bands of 600 bp, corresponding to *Cx. pipiens* as well as samples tested negative (in row 1: lane 1, lane 21; in row 2: 19, 21).

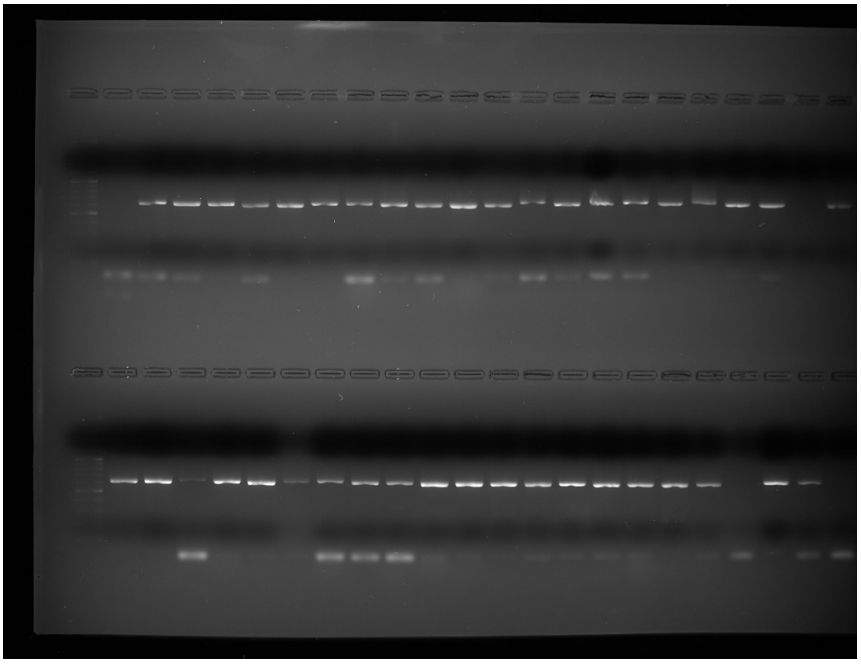

Figure 1- 2% agarose gel, ACE marker, 100 bp ladder

The samples were also amplified to examine the *Culex pipiens* form (*molestus* or *pipiens*) using the CQ11 marker. From the 113 samples used for the microbiome analyses, 15 samples (13.27 %) showed a band of 250 bp indicative of *molestus* form, while 88 samples (77.88 %) were identified as *pipiens* form with a band of 200 bp. The two different bands can be clearly seen in Figure 2, for example lane 1 is form *molestus* and lane 2 is form *pipiens*. 10 samples (8.85 %) gave bands of 200 and 250 bp simultaneously, which indicates the hybrid form *pipiens/molestus* (for example row 1, lane 19 in Figure 2).

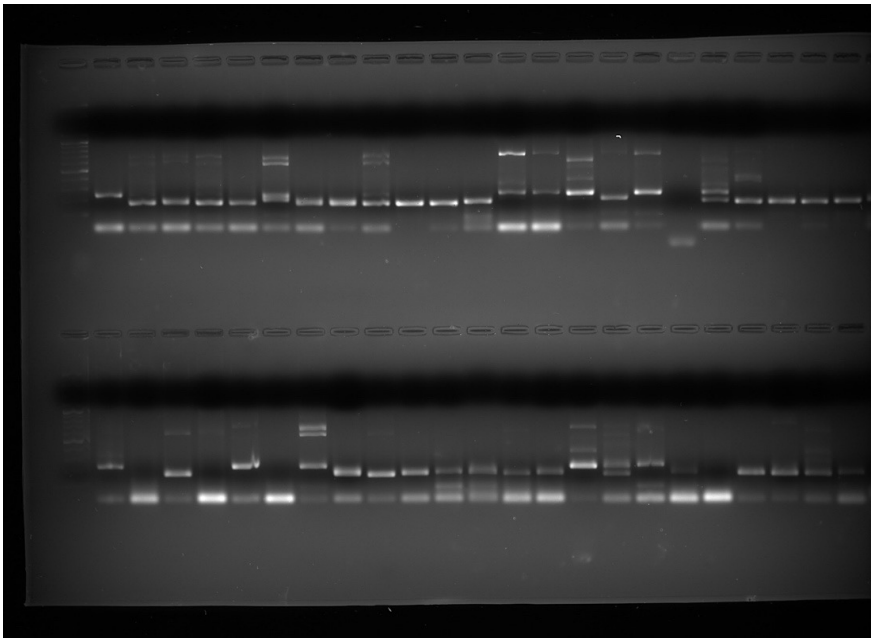

Figure 2- 2% agarose gel, CQ11 marker, 100 bp ladder

5.2 OTU distribution

5.2.1 General OTU distribution

A total of 67 OTUs were found, with *Wolbachia* being the most abundant bacteria (OTU 1), as expected for *Cx. pipiens*. Spanish samples had a total of 48 OTUs, while Slovakian samples had 61 OTUs. 40 OTUs were shared among the two countries.

Seven different bacterial phyla were found, with Proteobacteria as the most abundant one. However, there were also several OTUs from Firmicutes, Bacteroidetes and Actinobacteria. Deinococcus-Thermus, Verrucomicrobia and Planctomycetes were minoritary.

Wolbachia made up to 84% of the bacterial diversity found in our samples (see Figure 3). Therefore, another chart excluding *Wolbachia* was generated in order to get a more detailed information about the distribution of the rest of the OTUs, shown in Figure 4. Once OTU 1, *Wolbachia,* was excluded, also OTU 2, *Erwinia* (30%), OTU 19, *Asaia* (16%) and OTU 6, *Acinetobacter* (7%), showed a total abundance above 5% in the microbiome of our samples.

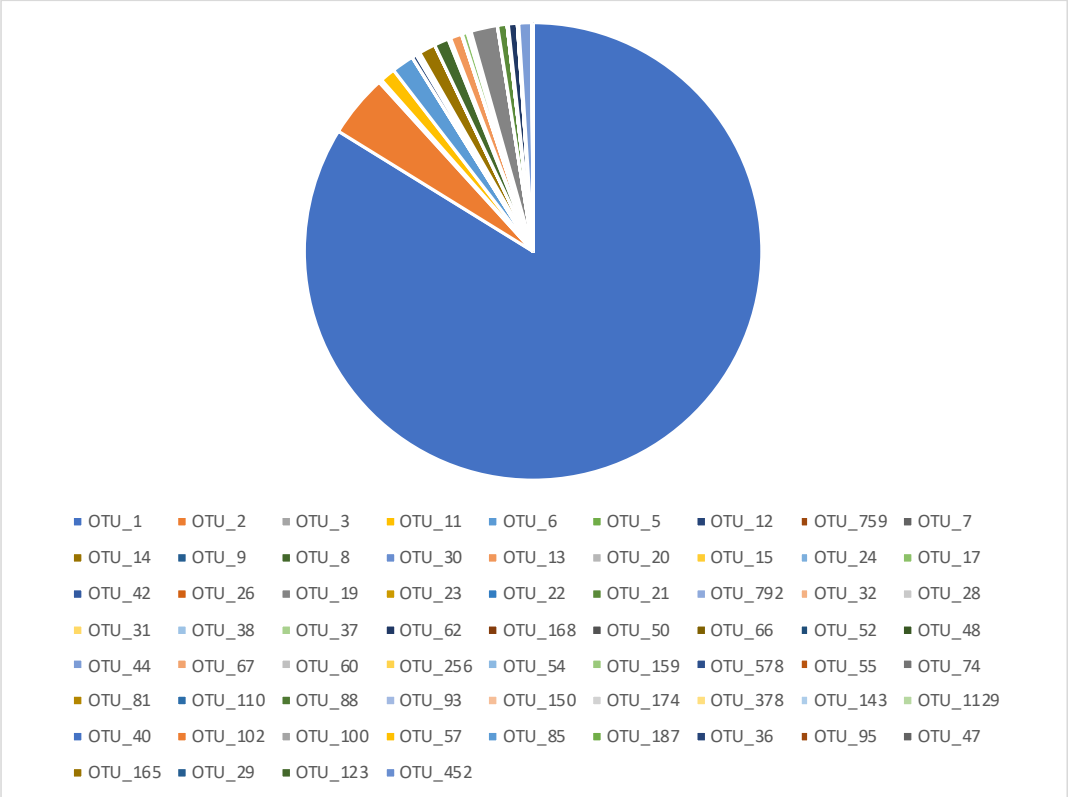

Figure 3- OTU distribution including all samples including *Wolbachia*

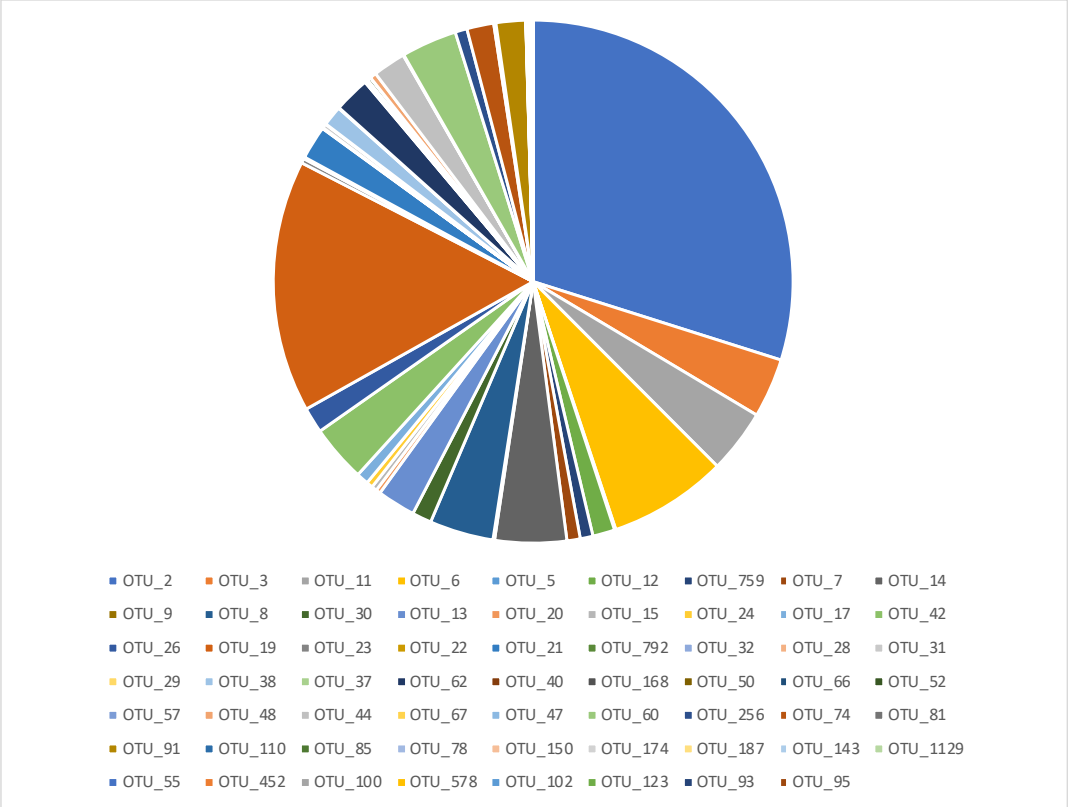

Figure 4- OTU distribution including all samples excluding *Wolbachia*

5.2.2 OTU comparison among individuals

The inter-individual variation of the microbiome profile of all samples was also evaluated. Figures 5 and 6 show these profiles (with and without *Wolbachia*, respectively) with all samples ordered by forms (first hybrids, then form *molestus* and then form *pipiens*), and within forms, by sampling date. In these figures it can be seen that sample individuality plays an important role in the microbiome profile. Figure 5 shows a high abundance of *Wolbachia* in the majority of the samples (93.46 %), however some (6.06%) also show really low abundance of this bacterium. Figure 6 shows the distribution of the rest of OTUs in the individuals, and a high variability can be seen. Due to the removal of *Wolbachia*, less samples passed the threshold of minimum number of sequences, and thus, there are less samples available in this dataset.

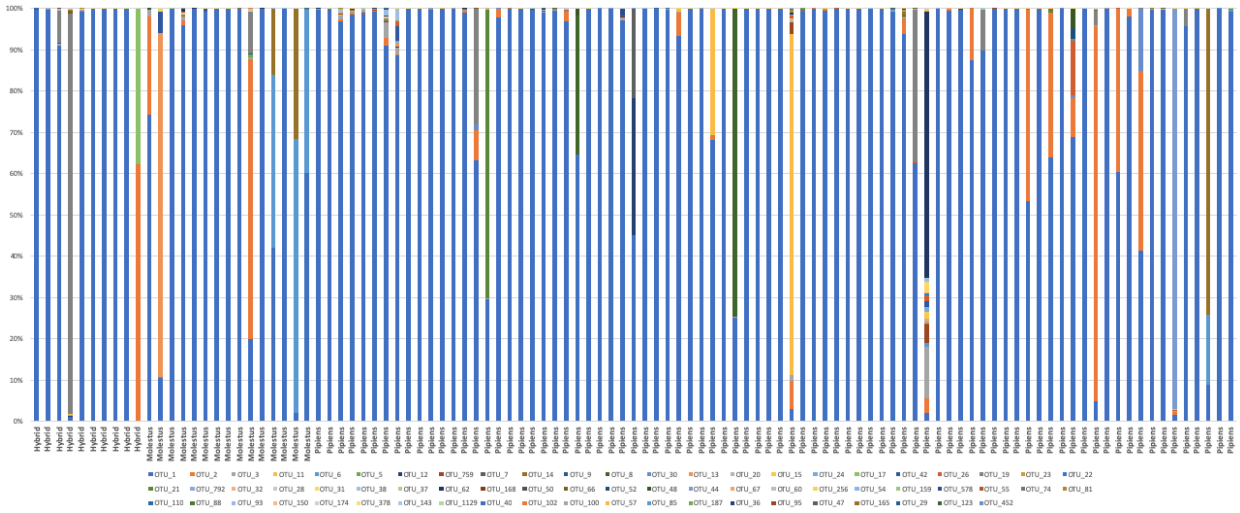

Figure 5- Microbiome profile of individual samples ordered according to different forms (hybrid, molestus, pipiens) including the endosymbiont *Wolbachia*

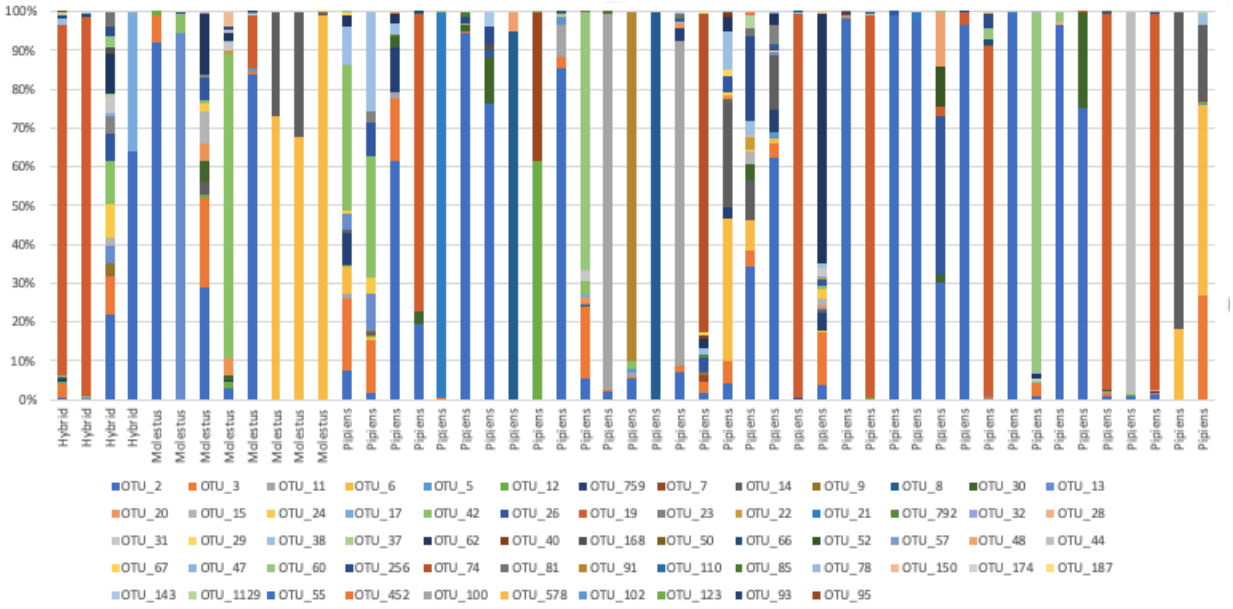

Figure 6- Microbiome profile of individual samples ordered after different forms (hybrid, molestus, pipiens) excluding the endosymbiont *Wolbachia*

5.2.3 OTU comparison between countries

The two countries, Slovakia and Spain, were analyzed separately and their specific OTU distribution was examined.

Figure 7 illustrates the abundance of *Wolbachia* in each of the two countries analyzed. The distribution between them was quite equal (Spain: 85%, Slovakia: 83%). The same chart excluding *Wolbachia* is shown in Figure 8 to illustrate the distribution of the rest of the bacteria in the microbiome. This figure shows the general differences between the two countries: OTU 2, *Erwinia*, was more abundant in Slovakia (35.19%) than in Spain (16.49%). Similar observations

were found for OTU 19, *Asaia*, which shows higher abundance in Slovakia (19.28%) compared to Spain (6.43%). OTU 6, *Acinetobacter*, had a high abundance in Spain (22.51%) and much less in Slovakia (1,33 %). Besides that, OTU 12, *Providencia* (Spain: 4.76%, Slovakia: 0.02%), OTU 8, *Thorsellia* (Spain: 0%, Slovakia: 5.57%), OTU 14, *Zymobacter* (Spain: 11.86%, Slovakia: 1,49 %), OTU 42, *Pseudomonas* (Spain: 10.99%, Slovakia: 0.55%), OTU 13, also *Pseudomonas* (Spain: 7.73%, Slovakia: 0.23%) and OTU 11, *Lonsdalea* (Spain: 0.09%, Slovakia: 5.49%), are differently abundant in the countries compared. However, this chart contains all samples analyzed together and, therefore inter-individual differences (for example affected by form or collection dates) are masked.

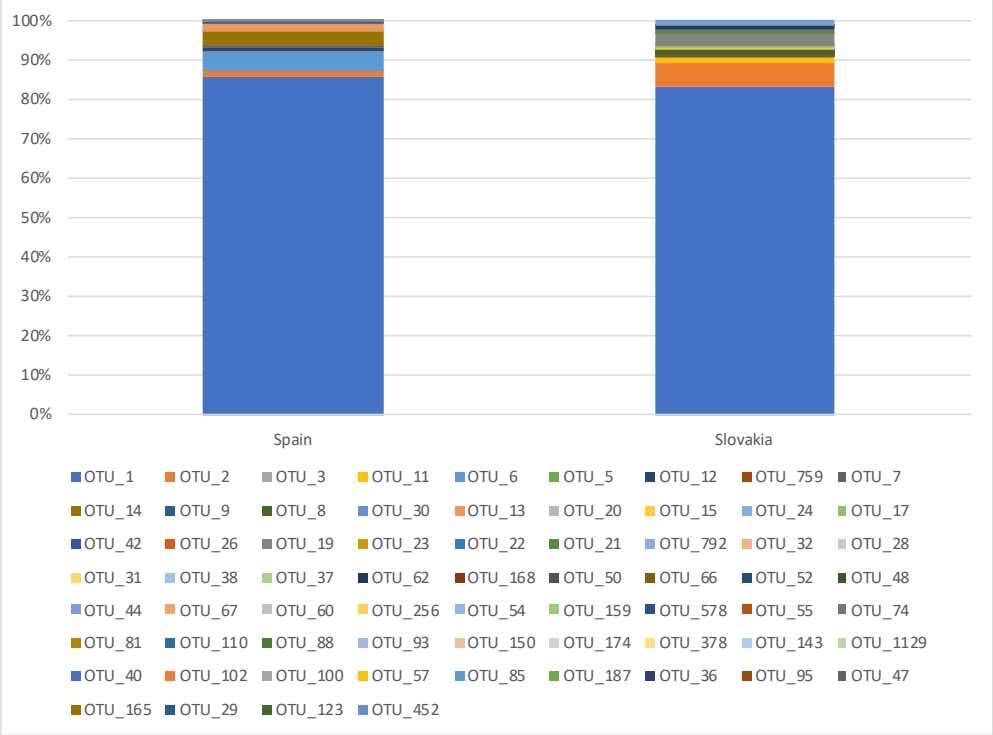

Figure 7- OTU distribution in the two countries, Spain and Slovakia, including *Wolbachia*

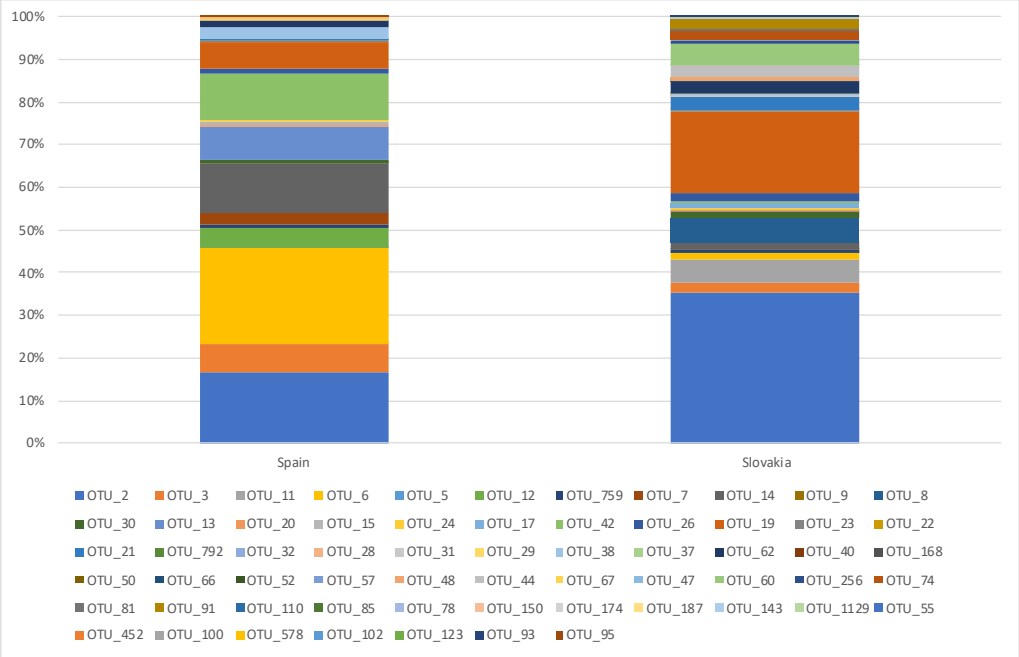

Figure 8- OTU distribution between the two countries, Spain and Slovakia, excluding *Wolbachia*

5.2.4 OTU comparison of different *Cx. pipiens* forms

The same analyses were performed to compare forms (i.e. *Cx. pipiens pipiens*, *Cx. pipiens molestus* and hybrid). The sample distribution can be seen in table 5. The great majority of the *molestus* mosquitoes was collected in Spain, and only one was collected in Slovakia. Figures 9 and 10 show the OTU distribution between the different *Cx. pipiens* forms, with and without *Wolbachia,* respectively. *Wolbachia* was abundant in all three forms (*pipiens:* 85.63%, *molestus:* 73.55%, hybrid: 79.07%) (see Figure 9). Considering all samples from both countries and all collection dates, when removing *Wolbachia*, OTU 2, *Erwinia*, showed a relatively high and quite equal abundance in both forms (*pipiens:* 31.59%, *molestus:* 25.91%) as well as their hybrids (21.60%). Differences were observed in OTU 19, *Asaia* (*pipiens:* 15.22%, m*olestus:* 1.71%, hybrid: 46.93%) , OTU 6, *Acinetobacter* (*pipiens:* 3.29%, *molestus:* 29.96%, hybrid: 0%), OTU 42, *Pseudomonas* (*pipiens:* 2.07%, *molestus:* 10.53%, Hybrid: 3.00%) and OTU 13, *Pseudomonas* (*pipiens:* 0.46%, *molestus:* 11.79%, hybrid: 1.23%).

Table 5- Number of samples used to test for different form (hybrid, *molestus*, *pipiens*) in both countries (Spain and Slovakia) of both datasets (including and excluding *Wolbachia*)

Dataset	Number of *pipiens*	Number of *molestus*	Number of hybrids
with *Wolbachia*	85	15	10
without *Wolbachia*	37	8	4

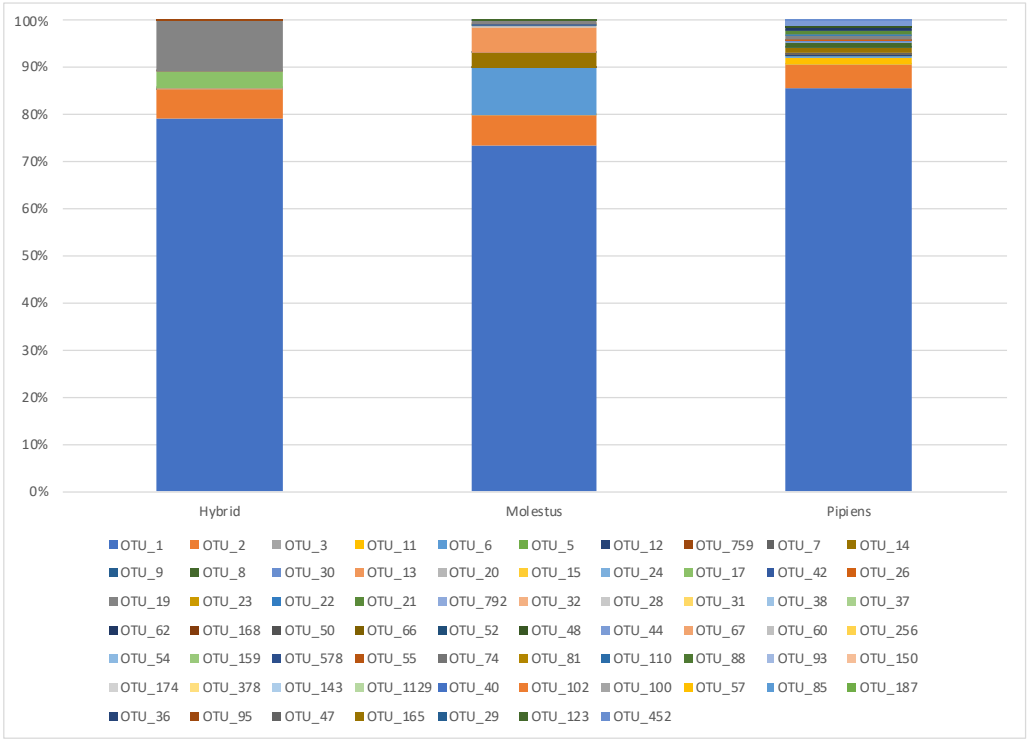

Figure 9- OTU distribution in different forms (hybrid, *molestus*, *pipiens*) including *Wolbachia*

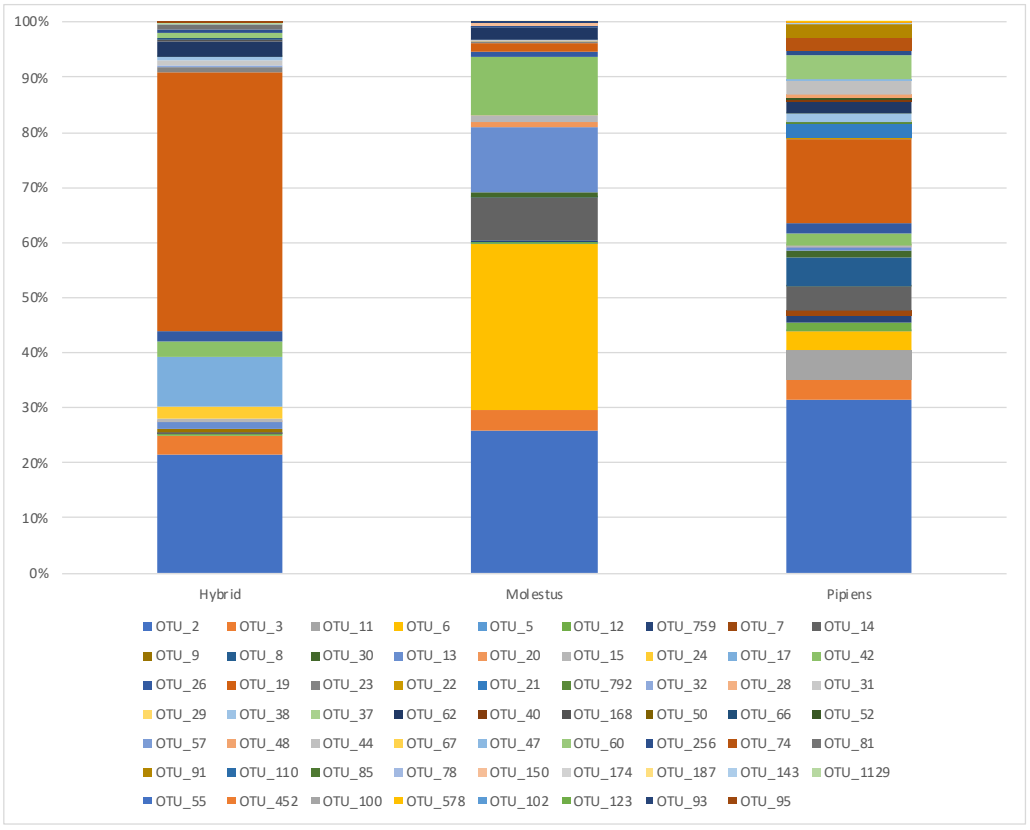

Figure 10- OTU distribution in different forms (hybrid, *molestus*, *pipiens*) excluding *Wolbachia*

5.2.5 OTU comparison of seasonal ranges in both countries

In order to check for seasonality, ranges were established for each country separately. In Spain, ranges of two months were made (Range 1: April & May, Range 2: June & July, Range 3: September & October), see Table 6, due to scattered and irregular collection events. The outcomes of this analysis are shown in Figures 11 and 12. Figure 11 shows the *Wolbachia* distribution in the three ranges: Range 1: 91.83%, Range 2: 90.87%, Range 3: 64.01%. Besides *Wolbachia*, also OTU 6, *Acinetobacter* (20.56%) and OTU 14, *Zymobacter* (15.29%) were abundant. Figure 12 shows the distribution of OTUs according to the seasonal ranges established, without *Wolbachia*. The greatest differences among the three ranges are seen in OTU 2, *Erwinia* (Range 1: 22.15%, Range 2: 32.67%, Range 3: 0%), OTU 3, *Staphylococcus* (Range 1: 10.37%, Range 2: 1.30%, Range 3: 5.36%), OTU 6, *Acinetobacter* (Range 1: 1.30%, Range 2: 0%; Range 3: 61.46%), OTU 12, *Providencia* (Range 1: 0.73%, Range 2: 20.53%, Range 3: 0.12%), OTU 7, *Orbus* (Range 1: 0%, Range 2: 12.73%, Range 3: 0.06%), OTU 13, *Pseudomonas* (Range 1: 18.03%, Range 2&3: 0%), OTU 42, *Pseudomonas* (Range 1: 25.53%, Range 2: 0.20%, Range 3: 0%) and OTU 19, *Asaia* (Range 1&3: 0%, Range 2: 30.00%).

Table 6- Number of samples used for the analysis in Spain 2-month Ranges (April-October) of both datasets (including and excluding Wolbachia)

Dataset	Range 1	Range 2	Range 3
with *Wolbachia*	17	11	8
without *Wolbachia*	6	3	5

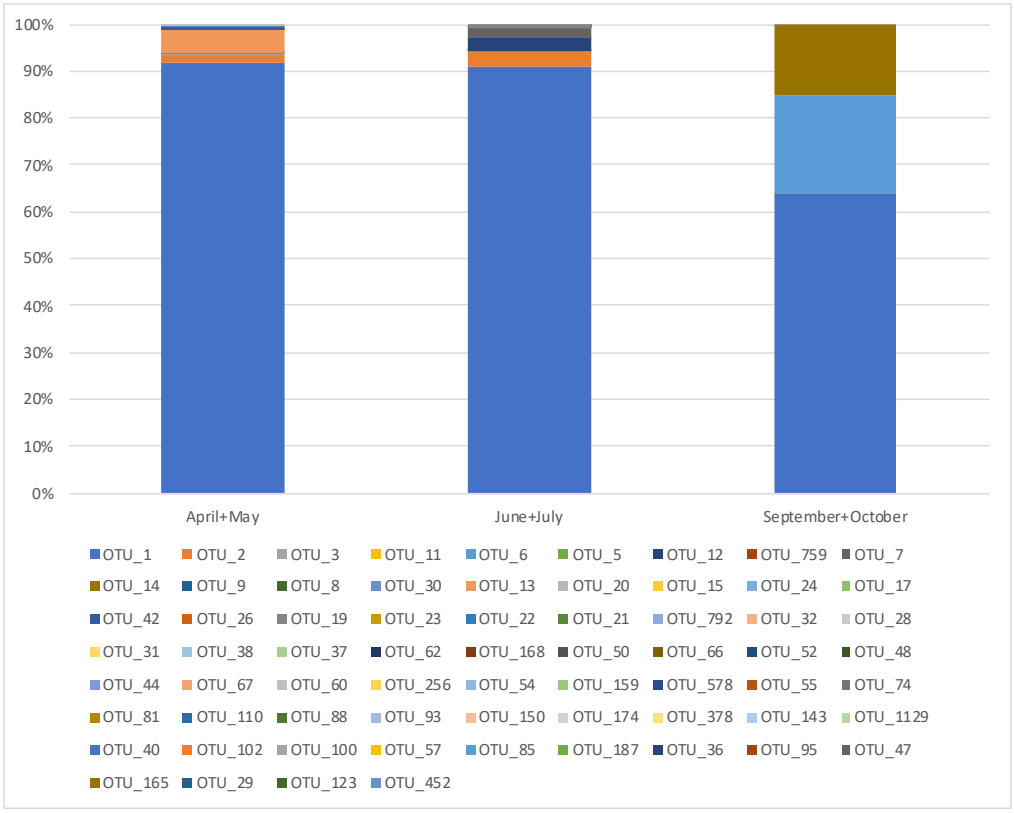

Figure 11- OTU comparison of Spanish 2-month ranges including *Wolbachia*

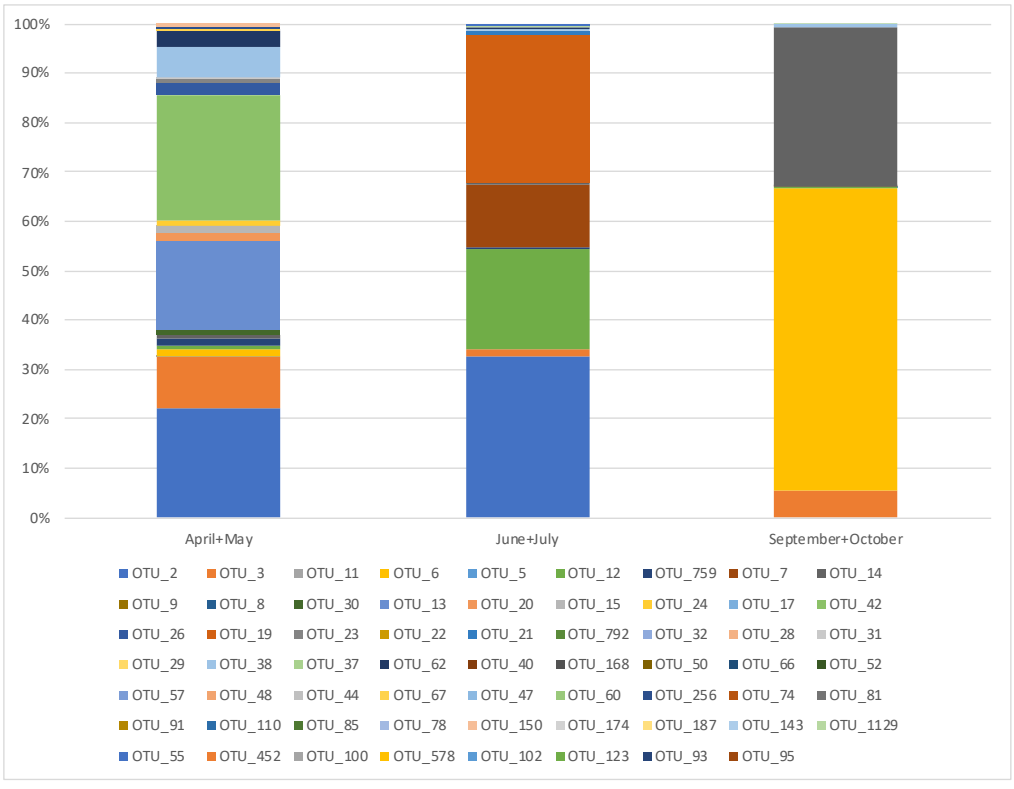

Figure 12- OTU comparison of Spanish 2-month ranges excluding *Wolbachia*

In Slovakia, June, July and August were compared by months (see Table 7). Figure 13 shows that *Wolbachia* is abundant in all three months, and decreases along the season (June: 90.92%, July: 85.13% and August: 68.08%). However, OTU 2, *Erwinia*, is also quite abundant in August with a percentage of 20.44%. Figure 14 shows the OTU distribution in the three months tested, without *Wolbachia*. Differences can be seen in OTU 2, *Erwinia* (June: 41.93%, July: 23.46%, August: 47.19%), OTU 11, *Lonsdalea* (June: 0.23%, July: 11.19%, August: 0.05%), OTU 8, *Thorsellia* (June: 15.80%, July: 5.88%, August: 0.01%), OTU 19, *Asaia* (June: 12.73%, July: 17.47%, August: 27.39%) and OTU 21, *Gluconobacter* (June: 16.58%, July&August: 0%).

Table 7- Number of samples used for the analysis in Slovakian monthly ranges (June, July and August) for both datasets (including and excluding Wolbachia)

Dataset	**June**	**July**	**August**
with *Wolbachia*	17	37	15
without *Wolbachia*	6	17	11

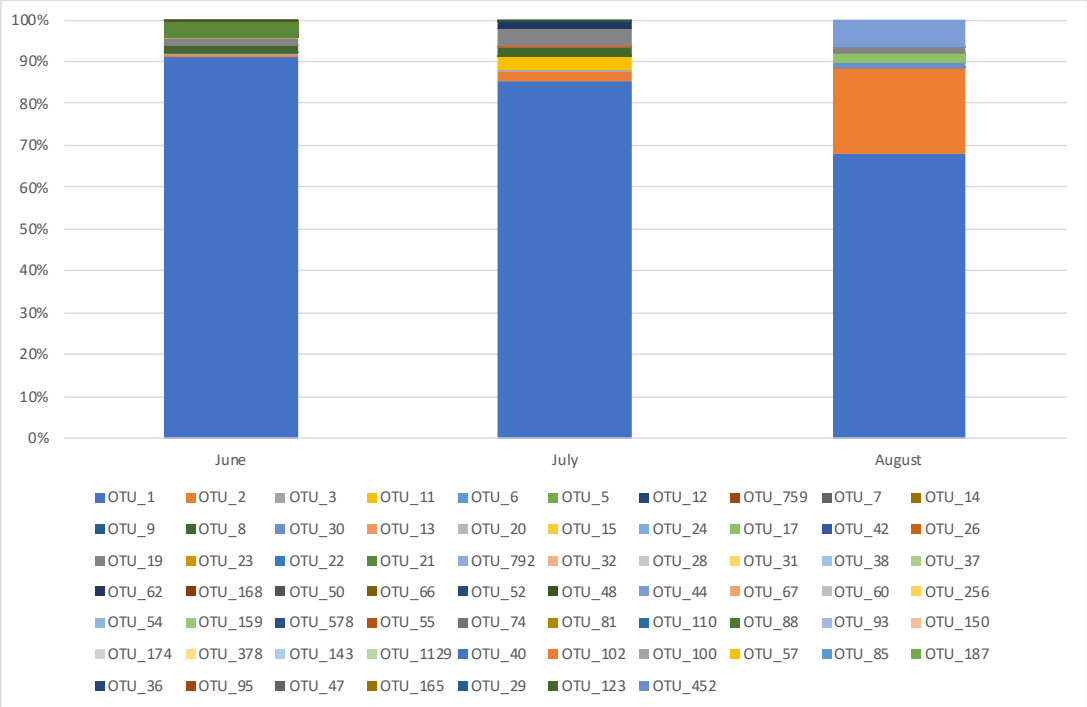

Figure 13- OTU distribution of Slovakian monthly ranges including *Wolbachia*

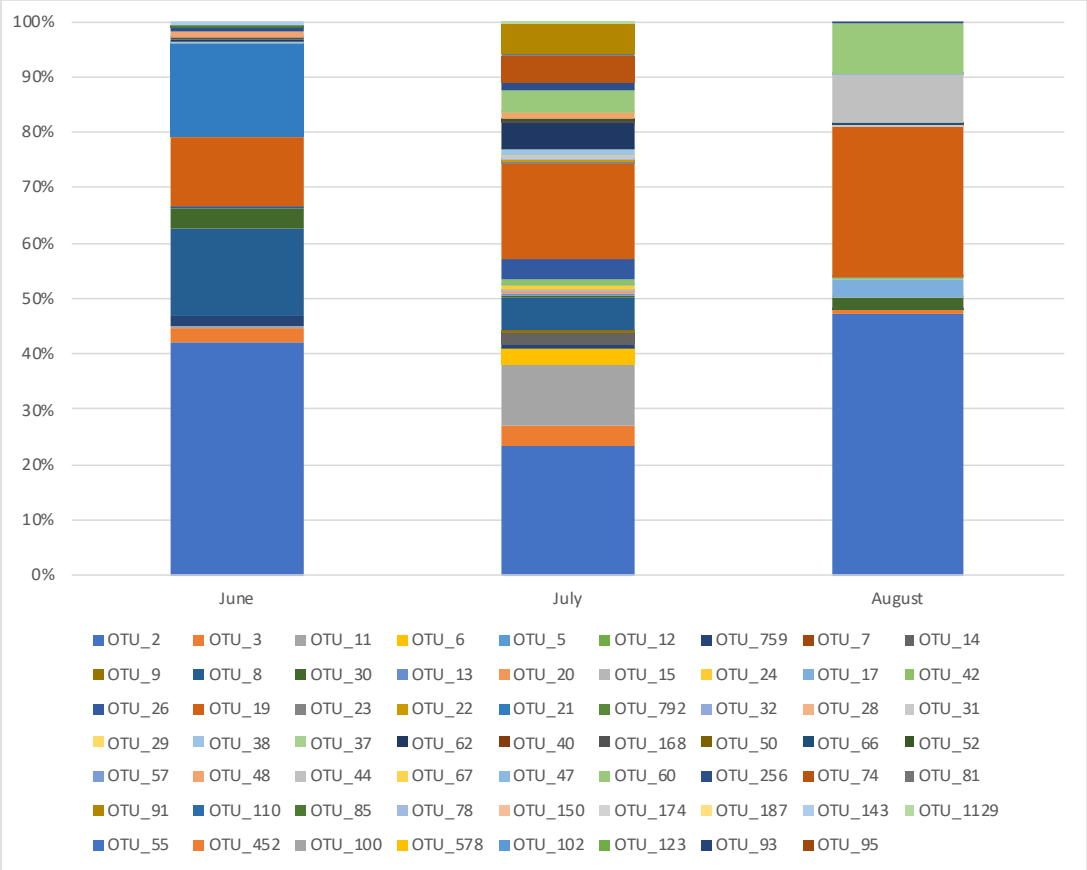

Figure 14- OTU comparison of Slovakian monthly ranges excluding *Wolbachia*

Besides the analysis of the three ranges for each month, also 15-day ranges were constructed and analyzed, to evaluate smaller scale effects of seasonality in our sampling. The ranges are shown in Table 8 below.

Table 8- Number of samples used for the analysis of 15-day ranges in Slovakian sample, to test for a smaller scale seasonality effect for both datasets (including and excluding *Wolbachia*)

Range	**Dates included**	**Number of samples including *Wolbachia***	**Number of samples excluding *Wolbachia***
1	June 13th – June 27th	17	6
2	June 28th – July 12th	14	7
3	July 13th – July 27th	21	9
4	July 28th – August 11th	12	9
5	August 12th – August 26th	5	3

Figure 15 shows that in all five ranges *Wolbachia* is abundant (Range 1: 90.92%, Range 2: 77.87%, Range 3: 90.04%, Range 4: 66.10% and Range 5: 79.33%). However, in Range 4 also OTU 2, *Erwinia,* is abundant (26.29%). Figure 16 shows that Range 5, which is entirely dominated by OTU 19, *Asaia* (64.57%) and OTU 44, *Cosenzaea* (32.83%). It has the least samples included, only three, so this may pose some bias in the observed diversity. In Range 1, OTU 2, *Erwinia* (41.93%), OTU 8, *Thorsellia* (15.80%) and OTU 21, *Gluconobacter* (16.58%) were the most abundant. In Range 2 OTU 11, *Lonsdalea* (27.14%), OTU 2, *Erwinia* (15.06%) and OTU 8, *Gluconobacter* (14.29%) were the most abundant ones. In Range 3 and 4 the dominant OTUs were OTU 2, *Erwinia* (29.24%; 60.71%) and OTU 19, *Asaia* (21.90%; 12.20%).

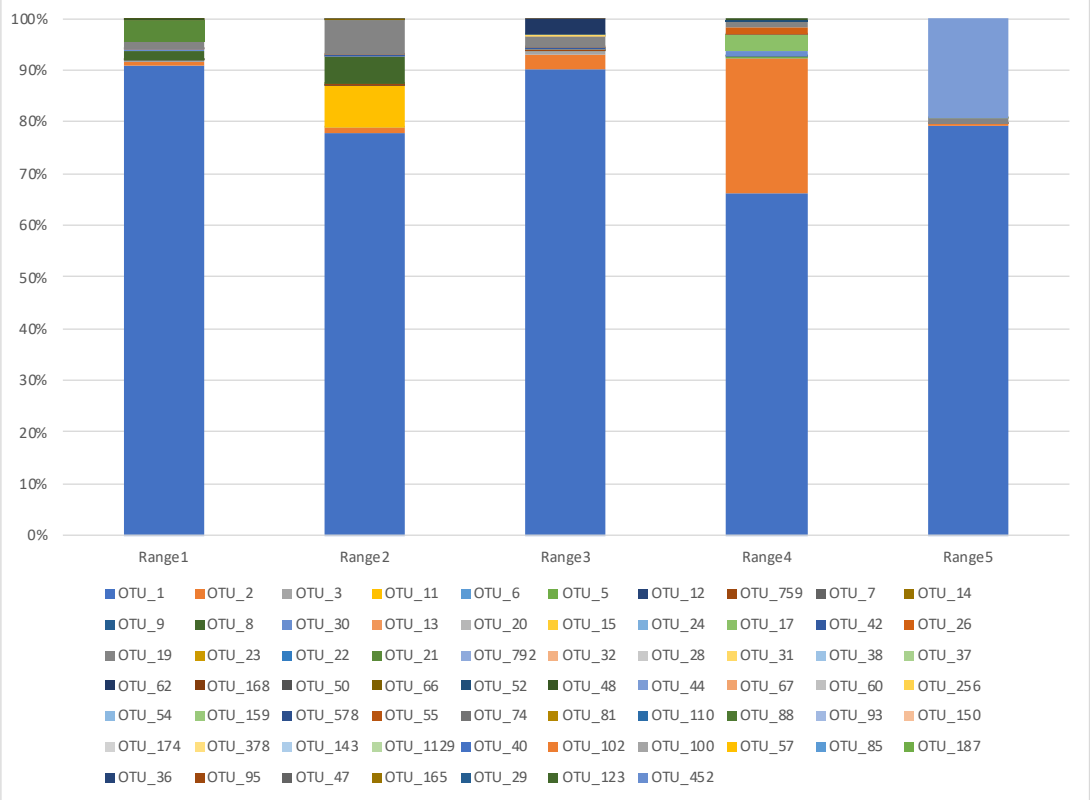

Figure 15- OTU distribution of Slovakian 15-day ranges including *Wolbachia*

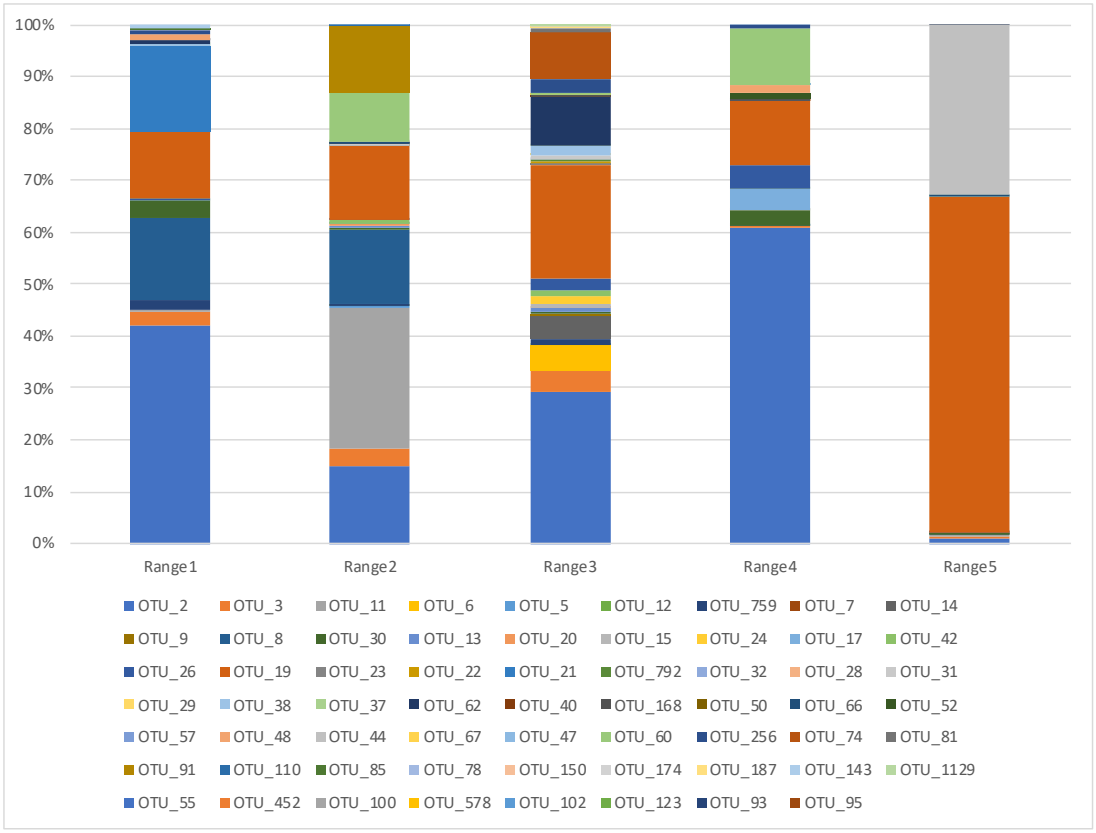

Figure 16- OTU distribution of Slovakian 15-day ranges excluding Wolbachia

5.3 Alpha-diversity

Although the analysis in Excel provides a basic overview of the OTU distribution, statistical analysis is necessary to get more precise results. R was used to test differences among country of origin, *Culex pipiens* forms and seasonality (2-month, month or 15-day ranges) in alpha diversity. For all the tested categories, means and standard deviations for the alpha diversity indexes were calculated and are summarized below in Tables 9 and 10.

Table 9- Alpha diversity means and standard deviations with data including *Wolbachia*

including Wolbachia			**Dominance**		**Equitability**		**Richness**		**Shannon index**	
Category	**Subcategory**	**N**	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
FormsSlovakia	Pipiens	62	0,1110	0,1856	0,1080	0,1728	6,7500	4,0119	0,3191	0,4952
	Molestus	1	-	-	-	-	-	-	-	-
	Hybrid	6	0,0927	0,1868	0,1012	0,1889	7,5000	4,9295	0,2314	0,3745
SeasonSlovakia	June	17	0,0965	0,1798	0,0992	0,1717	6,5882	3,1036	0,2539	0,4111
	July	37	0,1047	0,1782	0,0945	0,1504	7,1842	4,6141	0,3009	0,5055
	August	15	0,1612	0,2268	0,1643	0,2260	6,1333	3,6227	0,4039	0,5241
	Range1	17	0,0965	0,1798	0,0992	0,1717	6,5882	3,1036	0,2539	0,4111
	Range2	14	0,0951	0,1583	0,0886	0,1372	6,6429	4,6178	0,2678	0,4105
	Range3	21	0,0975	0,1801	0,0858	0,1466	7,5000	4,8378	0,2795	0,5196
	Range4	12	0,2305	0,2541	0,2264	0,2515	6,1667	3,8337	0,5845	0,6303
	Range5	5	0,0304	0,0378	0,0423	0,0484	6,6000	3,0496	0,1160	0,1280
Counries	Slovakia	54	0,1018	0,1787	0,0954	0,1571	6,8889	4,1421	0,2831	0,4788
(June and July)	Spain	11	0,1166	0,2253	0,0983	0,1857	5,1818	3,1247	0,2841	0,5268
	Both_compared	65	0,1043	0,1855	0,0959	0,1607	6,6000	4,0187	0,2832	0,4829
FormsSpain	Pipiens	19	0,1047	0,1958	0,1133	0,2036	6,7778	4,4133	0,2901	0,4725
	Molestus	14	0,1682	0,2284	0,1717	0,2386	6,1429	3,3249	0,4166	0,5246
	Hybrid	4	0,0431	0,0819	0,0387	0,0663	7,2500	3,9476	0,1382	0,2487
SeasonSpain	April&May	17	0,0668	0,1172	0,0705	0,1082	8,5294	4,2148	0,2245	0,3256
	June&July	11	0,1166	0,2253	0,0983	0,1857	5,1818	3,1247	0,2841	0,5268
	September&October	8	0,2491	0,2677	0,2897	0,3192	4,3750	1,6850	0,5832	0,6145
AllSamples	All	106	0,1155	0,1890	0,1152	0,1824	6,7273	4,0272	0,3130	0,4721

Table 10- Alpha diversity means and standard deviations with data excluding *Wolbachia*

excluding Wolbachia			**Dominance**		**Equitability**		**Richness**		**Shannon index**	
Category	**Subcategory**	**N**	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
FormsSlovakia	Pipiens	31	0,2502	0,2522	0,2761	0,2317	7,5806	4,2955	0,8383	0,8278
	Molestus	1	-	-	-	-	-	-	-	-
	Hybrid	3	0,4671	0,4249	0,6303	0,4889	9,3333	7,5056	1,5853	1,7827
SeasonSlovakia	June	6	0,2619	0,2232	0,3272	0,2045	5,1667	2,4833	0,8000	0,6664
	July	17	0,3410	0,3152	0,3400	0,2887	9,8889	4,8615	1,2084	1,1190
	August	11	0,1573	0,1515	0,2372	0,2711	5,8182	2,8920	0,4548	0,3143
	Range1	6	0,2619	0,2232	0,3272	0,2045	5,1667	2,4833	0,8000	0,6664
	Range2	7	0,1955	0,1815	0,2016	0,1613	8,4286	4,2762	0,6699	0,5696
	Range3	9	0,4059	0,3597	0,3981	0,3235	11,2000	5,2873	1,5022	1,3232
	Range4	9	0,2539	0,2283	0,3437	0,3161	5,4444	3,0867	0,7044	0,5898
	Range5	3	0,0518	0,0176	0,0812	0,0242	7,3333	0,5774	0,2343	0,0745
Counries	Slovakia	23	0,3098	0,2933	0,3271	0,2675	8,5652	4,8694	1,0670	1,0315
(June and July)	Spain	3	0,2370	0,2186	0,2426	0,1691	7,0000	4,3589	0,6407	0,4101
	Both_compared	26	0,3014	0,2830	0,3173	0,2569	8,3846	4,7588	1,0178	0,9844
FormsSpain	Pipiens	6	0,5105	0,2974	0,5408	0,2498	7,6667	4,7610	1,5168	1,0711
	Molestus	7	0,3298	0,2710	0,4828	0,3548	5,8571	4,9473	0,9984	0,9495
	Hybrid	1	-	-	-	-	-	-	-	-
SeasonSpain	April&May	6	0,5085	0,3378	0,5092	0,2768	10,0000	4,6904	1,7698	1,1802
	June&July	3	0,2370	0,2186	0,2426	0,1691	7,0000	4,3589	0,6407	0,4101
	September&October	5	0,3596	0,2295	0,6095	0,3414	3,6000	3,0496	0,8577	0,6063
AllSamples	All	49	0,3092	0,2780	0,3651	0,2848	7,6275	4,5693	1,0003	0,9455

The two countries, including only the samples collected in the same dates (June and July) were compared against each other. The following results were obtained for samples including *Wolbachia*: dominance (W=358.5, p-value= 0.2856), equitability (W= 354, p-value= 0.3228), richness (W= 384, p-value= 0.1271) and Shannon index (W= 358.5, p-value= 0.2858).

For samples excluding *Wolbachia* these results were found: dominance (W= 35, p-value= 1), equitability (W= 37, p-value= 0.88), richness (W= 43, p-value= 0.5193) and Shannon index (W= 39, p-value= 0.7481). All p-values exceed the threshold of 0.01 for significant differences.

Therefore, the samples from the two countries can be considered to have the same diversity according to the different metrics tested. These results were confirmed also using all the available samples (all collection dates), where again no significant differences were found.

Then, the Slovakian samples were tested for differences according to *Cx. pipiens* form, both including *Wolbachia*: dominance (X^2= 2.1549, p-value= 0.3405), equitability (X^2= 1.5036, p-value= 0.4715), richness (X^2= 2.6579, p-value= 0.2648) and Shannon index (X^2= 2.4433, p-value= 0.2947); and excluding *Wolbachia*: dominance (X^2= 1.1261, p-value= 0.5695), equitability (X^2= 1.7086, p-value= 0.4256), richness (X^2= 0.92471, p-value= 0.6298) and Shannon index (X^2= 0.64382, p-value= 0.7248). The outcome of this test was not significant for any of the alpha diversity indexes evaluated.
Testing for the different collection months in Slovakia lead to the following results when *Wolbachia* was included: dominance (X^2= 1.2059, p-value= 0.5472), equitability (X^2= 1.4179, p-value= 0.4922), richness (X^2= 0.80027, p-value= 0.6702) and Shannon index (X^2= 1.0071, p-value= 0.6044). Samples without *Wolbachia* provided these results: dominance (X^2= 1.4644, p-value= 0.4809), equitability (X^2= 0.88557, p-value= 0.6422), richness (X^2= 7.6582, p-value= 0.02173) and Shannon index (X^2= 2.0734, p-value= 0.33546). Therefore, there is no significant difference at 1% confidence level between the samples according to the month of sampling. The same test was also performed using 15-day ranges. The outcome with *Wolbachia* was: dominance (X^2= 2.4728, p-value= 0.6495), equitability (X^2= 2.6494, p-value= 0.6181), richness (X^2= 1.2064, p-value= 0.877) and Shannon index (X^2= 2.2513, p-value= 0.6897). And without *Wolbachia*: dominance (X^2= 3.4112, p-value= 0.4915), equitability (X^2= 3.7044, p-value= 0.4475), richness (X^2= 9.1395, p-value= 0.05771) and Shannon index (X^2= 2.8356, p-value= 0.5857). No significant differences were found using this smaller scale for the seasonal analysis.

Similar analyses were performed for the Spanish samples. Comparing the different *Cx. pipiens* forms lead to the following results when including *Wolbachia*: dominance (X^2= 1.1157, p-value= 0.5724), equitability (X^2= 1.124, p-value= 0.5701), richness (X^2= 0.21243, p-value= 0.8992) and Shannon index (X^2= 0.96544, p-value= 0.6171). And when excluding *Wolbachia*: dominance (X^2= 1.4735, p-value= 0.4787), equitability (X^2= 0.75646, p-value= 0.6851), richness (X^2= 1.1925, p-value= 0.5509) and Shannon index (X^2= 0.84218, p-value= 0.6563). There were no significant differences in alpha diversity among different forms in Spanish samples.
Testing for the 2-month ranges gave these results with *Wolbachia*: dominance (X^2= 2.3669, p-value= 0.3062), equitability (X^2= 2.9281, p-value= 0.2313), richness (X^2= 7.3043, p-value=

0.02594) and Shannon index (X^2= 2.4675, p-value= 0.2912). And the following without *Wolbachia*: dominance (X^2= 1.2571, p-value= 0.5334), equitability (X^2= 2.9281, p-value= 0.2313), richness (X^2= 7.3043, p-value= 0.02594) and Shannon index (X^2= 2.4675, p-value= 0.2912). No significant differences can be observed among the 2-month ranges established.

6 Discussion

Mosquitoes' gut microbiota is particularly important, because the different microorganisms can have an impact on vector susceptibility to virus infection (Novakova et al., 2017; Thongsripong et al., 2017). Therefore, it is necessary to gain more knowledge in this particular field and to understand different influences that can shape the mosquitoes' microbiota. The species *Cx. pipiens* is of special interest, because it is known to be the main vector of WNV in Europe (Vogels et al., 2017), and the interactions between the microbiome and virus transmission could help to control infection rates.

The main goal of this thesis was to examine seasonal as well as phylogenetic effects on the microbiome of mosquitoes of the *Cx. pipiens* species. For this reason, samples of two different countries, Spain and Slovakia, were collected in different months between April and October in 2018. Within the analyzed samples, *Cx. pipiens* form *pipiens* and *molestus*, as well as their hybrid forms, were found.

First the main OTU distribution, including taxonomy and abundance among samples, was examined. We found 67 OTUs in the mosquito microbiome. Proteobacteria was identified as the most abundant phylum (64.2% total abundance) in accordance with the studies of Zotzmann et al. (2017), with Alphaproteobacteria (37.2%) and Gammaproteobacteria (62.8%) as the only represented classes in our case. Muturi et al. (2016) found that these two are the dominant classes in *Cx. pipiens*. Moreover, the phyla Firmicutes, Bacteriodetes and Actinobacteria were identified as the next three major phyla. This outcome was also found for *Aedes albopictu*s by Yadav et al. (2016). Other minor phyla found in our samples were Planctomycetes (4.5%), Deinococcus-Thermus (1.5%) and Verrucomicrobia (1.5%). *Wolbachia*, a bacterium belonging to the phylum Proteobacteria and the class Alphaproteobacteria, was identified as the most abundant species in *Cx. pipiens* with a total relative abundance of 84%. This outcome was already discussed by Muturi et al. (2016), Thongsripong et al. (2018) and Novakova et al. (2017). Besides that, also *Asaia, Acinetobacter* and *Erwinia* were abundant in our samples. *Erwinia* and *Asaia* were mainly found to be abundant in Slovakia, while *Acinetobacter* showed higher abundance in Spanish

samples. *Acinetobacter* was also found to be prevalent in *Cx. quinquefasciatus* from India (Chandel et al., 2013), as well as in other mosquito species (Minard et al., 2013). *Asaia* can be found in nectars, so its occurrence could be a consequence of mosquito's nectar feeding (Minard et al., 2013). Osei-Poku et al. (2012) also found *Asaia* as an abundant species in *Aedes, Culex* and *Mansonia* species and stated also their possible importance in parasite interaction. Furthermore, *Asaia* can be transmitted maternally and therefore it is of increased importance in possible pathogen blockage (Zouache et al., 2011). *Erwinia* was the most abundant Gammaproteobacteria found in this study, in accordance with Thongsripong et al. (2018), who found *Erwinia* as one of the most abundant taxa in *Aedes aegypti*. It is also stated that *Erwinia* is a common bacterium in *Culex* species as well as in other genera, such as *Aedes* (Thongsripong et al., 2017).

The environmental influence in the microbiome is relevant for many species, as for *Aedes albopictus* and *Aedes aegypti*, where Zouache et al. (2011) identified an influence of capture sites on bacterial communities diversity. Moreover, Muturi et al. (2018) found an effect of host location on microbiome of different mosquito species. Muturi et al. (2019) characterized the alterations of microbiota of *Aedes aegypti* in response to blood-meal sources. These are just some examples that show the environmental influence in the microbiome composition. However, there is lack of available literature concerning the mosquito microbiome fluctuations among different months/seasons, in spite of the seasonality detected in epidemiological studies (Cairns et al., 2015; Olayemi et al., 2011; Reiner et al., 2015).

To analyze the phylogenetic effects on the microbiota's alpha diversity, we used the two forms of *Cx. pipiens, pipiens* and *molestus,* and their hybrids from Slovakia and Spain separately. Comparing these groups among each other, no significant differences were observed in any of the two countries. This lack of effect may be due to the close relationship between the groups compared, as we are considering a subspecific phylogenetic level. The outcome of this thesis indicates that the *Cx. pipiens* species harbors a typical microbiota not affected at lower phylogenetic levels. In fact, the most abundant bacteria found in this microbiota, namely the phyla Proteobacteria, Firmicutes and Actinobacteria (Jayakrishnan et al., 2018; Jupatanakul et al., 2014; Muturi et al., 2016), are also present in the two forms as well as their hybrid form. In the study of Duguma et al. (2017) it was found that there are differences between immature *Culex coronator* and *Culex nigripalpus* and they discussed the cause could be differences in certain genetic parameters (such as pH in the gut) or varying feeding behaviors. Again, Duguma et al. (2017), used early and late instar stages for their study, so their conclusions cannot be extrapolated to adults. However, the same pattern of species-specificity was found in a study including adults of 11 mosquito species (Novakova et al., 2017). In this thesis, we find those

differences do not occur at lower phylogenetic levels, in spite of the different feeding behaviors of the *Cx. pipiens* forms. This indicates that most likely the explanation for the possible inter-specific differences relies in some genetic mechanisms rather than in a direct effect of behavioral aspects. However, the high inter-individual variation among samples plays an important role in this thesis data. In Figures 5 and 6, it can be seen that there appears to be no clear pattern for the samples identified as the same form. Therefore, further sampling may be necessary to establish the microbiome patterns for phylogenetically close groups, if any. We also found that most of the samples collected are of the form *pipiens* rather than of the form *molestus*. Interestingly, the samples from Spain (mean latitude: 37.1N) did show a higher occurrence of samples identified as *molestus* than the ones from Slovakia (mean latitude: 48.3N), where only one sample was not form *pipiens.* This confirms and expands the results by Rudolf et al. (2013), who observed that *Cx. pipiens f. pipiens* was the most prevalent in Germany, and the occurrence of *Cx. pipiens f. molestus* increased in Southern regions.

To analyze different seasonal shifts and effects on the microbiome, *Cx. pipiens* mosquitoes were sampled from April to October, and different seasonal ranges were established to analyze the microbiome data. Only two months, June and July, were shared among the samples coming from Slovakia and Spain, and therefore we used only the data from these months to compare countries. No significant differences ($p > 0.01$) could be observed for the different alpha diversity indexes used, according to Mann-Whitney test. Therefore, we performed an analysis including all samples from both countries to increase sample size for the seasonality analysis. However, this test did not show significant differences, indicating a random distribution of microorganisms over time rather than a seasonal pattern. These results were confirmed within each country and over different time span ranges, and again the differences in microbiota alpha diversity were not significant. The study of Duguma et al. (2017), on the contrary, showed significant differences among sampling seasons in two different mosquito species, namely *Culex coronator* and *Culex nigripalpus.* There, highest bacterial diversity was found in a winter month (February), that also differed by composition from summer/autumn months. However, this study used immature (early or late instar) mosquito stages, which are exposed to completely different environmental conditions than adults. Indeed, Duguma et al. (2015) found that the microbial diversity of *Culex tarsalis* was less in adult species compared to their larvae, and microbiota differed significantly among the different life stages (from larvae to adults). This may also hold true for *Cx. pipiens*, and affect the way the microbiota reacts to the environment. In addition, our sampling only included months around summer, which could also potentially explain the different results we found. Novakova et al. (2017) showed a seasonal effect in a three-year period for *Culex*

pipiens/restuans and *Aedes vexans* complex from Canada that were sampled from June to September. We only used the data from a single year, but similar sampling dates; however, we used individuals instead of pooled mosquitoes (used in Novakova et al. (2017)), and the smaller sample size together with the inter-individual variation could determine the different results obtained in both studies. In fact, the results of this thesis show that the inter-individual variability in our samples is high, which can be interpreted from the Figures 5 and 6. Therefore, this high inter-individual variation may also mask the seasonal effects in our microbiome data, or on the contrary, reflect the actual absence of seasonal patterns when artificial pooling of the data is not used. Further studies should confirm one of these hypotheses.

7 Conclusion

The main aim of this thesis was to get an insight into seasonal as well as phylogenetic effects of *Cx. pipiens* microbiota. This species is the most extended in Europe, and the main vector of WNV. Concerning the phylogenetic effect on the microbiota, the existence of an inter-specific differentiation has been previously shown, although the mechanisms are not known. The use of the two forms of the same species, *Cx. pipiens,* as well as their hybrid form, allowed us to examine this from in a different perspective, including phylogenetically close individuals with quite different behaviours. The three groups studied did no show significant differences among them. This brings us to the conclusion that most likely the microbiota specificity relies on genetic mechanisms more than on behavioural differences. Future studies should focus on this direction to clarify the specific mechanisms driving the species-specific selection of the microbiota in mosquitoes. No seasonal effect could be observed in this study, which contrasts with previous studies. This could be due to the limited seasonal sampling (only around summer) and to the use of individuals, which present really variable microbiota composition. We suggest broadening the seasonal sampling to a longer period and also increasing the sampling numbers in the future, to further clarify if the seasonal effect does not really occur or if it is masked by methodological bias.

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9 Figure Appendix

10 Table Appendix