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Genetic structuration of tick Tunisian populations and the effect of plant essential oils on symbiotic bacteria diversity

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Introduction

Ticks are important vectors of several pathogens infecting humans and animals worldwide and are considered to be the first vectors of animal diseases and the second for human diseases after mosquitoes (Fuente et al., 2017; GRECH-ANGELINI et al. 2017). These arthropods do not only carry pathogens (viruses, bacteria, protozoa) but also diverse symbiotic and mutualistic microorganisms. (Greay *et al.*, 2018). Different studies have shown that tick's microbes are important contributors to the transmission of tick borne pathogens due to their roles in several biological processes implicated in the acquisition, maintenance, and transmission of pathogens, they also actively promote host phenotypic changes, and adaptation of ticks to new environments. Symbiotes also are implicated in the physiology of ticks, particularly, synthesis of some vitamins and tick's reproduction fitness (Pollet et al., 2020; Narasimhan and Fikrig, 2015). Accordingly, these symbionts play an important function by supplying ticks with essential nutrients such as vitamins (especially vitamin B) which are lacking in the ultra-specialized hematophagous tick diet. Indeed, several studies proved that *Francisella* like, *Coxiella* like, *Rickettsia* like have conserved all the genes coding for the pathway of vitamins B despite their highly reduced genome (Liu et al., 2016; Garcia Guizzo et al., 2017; Daniel et al., 2015). Duron et al. (2018) reported that experimental elimination of *Francisella* F-Om as an obligate nutritional mutualist of the African soft tick *Ornithodoros moubata* with antibiotic treatment resulted in physical abnormalities and impacted tick life-history traits, these effects were fully restored with a supplement of B vitamins. These results suggest that the *Francisella* F-Om symbiont has an essential nutritional role in the secretion of vitamin B. Furthermore, symbionts have an important role in tick's reproduction, Zhong et al suggested that after rifampin and tetracycline treatment of *A. Americanum* the tick reproductive fitness and larvae hatching was reduced, and interestingly it was related to the *Coxiella* like density.

Different tick species have been shown to harbor heritable symbiotic bacteria that are closely related to the *Coxiella*, *Rickettsia*, and *Francisella* genus. The endosymbiotic bacteria were identified in ticks from the genera *Haemaphysalis*, *Rhipicephalus*, *Amblyomma*, *Ornithodoros*, and *Argas*, being the most common maternally transmitted Coxiella like and Rickettsia like in ticks, however, other bacterial species, such as Midichloria and Francisella, apparently have successfully replaced the *Coxiella* like in other tick species (Zhong et al., 2007; Duron et al., 2018).

It is known that livestock farming plays a vital role in the agricultural economy of Tunisia and tick infestation causes serious problems particularly via the transmission of *Theileria annulata*, one of the most prevalent piroplasm affecting cattle in Tunisia (Darghouth et al., 1999). However, in Tunisia and more generally in North Africa, no previous work was, to our knowledge, carried out to explore the microbial community of the most important tick species and particularly those belonging to the *Hyalomma* genus. Beyond the generation of new knowledge, this research topic may lead to the identification of new control alternatives more friendly to the health triangle components, Environment Health, Human Health, and Animal Health. In this context, our study focuses on studying the diversity of tick microbiota in *Hyalomma* ticks infecting livestock in different bioclimatic and geographic regions to explore, firstly the microbial diversity and geographical dispersion in association to pathogen transmission, and to asses subsequently the potential antibacterial effects of some essential oils of aromatic plants on the tick reproductive performances and their symbionts. The hypothesis to be verified in this work considers that essential oils if affecting symbiotic bacteria could represent an alternative control option of tick populations, which has the potential to minimize the use of chemical acaricides, which are well known to have detrimental effects on the environment, human and animal health. To our knowledge, no study has been, to date, conducted using this approach which is relying on the use of sub-lethal concentrations of essential oils. For this, emphasis must be placed on the control of ticks through their microbiota using oil active biomolecules to reduce the transmission of pathogens.

Objectives

- Study by NGS the diversity of *Hyalomma* tick's microbiota from different species, regions, stages, sex, hosts and the genus of *Haemaphysalis* and *Rhipiciphalus* collected from cattle considering the dominant phylogenic characteristics of Tunisian *Hyalomma* ticks.
- Assess the effects of selected essential oils on the reproductive fitness of *Hyalomma* ticks and investigate their effects on the endosymbionts using real-time PCR~~.~~

Expected final deliverables

- New scientific knowledge on the major *Hyalomma* tick symbionts in Tunisia, their diversity and geographical distribution in relation to the species studied,

- More advanced experimental knowledge package on the effect of plan essential oils on tick reproduction and development and their potential of application as tick population control alternatives,
- New experimental knowledge package on the effects of plant essential oils on tick symbionts,
- Construction of an experimental basic prototype (active ingredient, active doses, mode(s) of application, target tick stages, costs, …) targeting the control of tick populations using plant essential oils, through the translation of the experimental knowledge packages into technological components.

Thesis project progress

During the second year of the thesis, we proceeded as follows:

1) Sample collection

we first carried out a massive ticks sampling during June, July, and August 2020. Ticks were collected from cattle in 6 different bioclimatic stages and 9 Tunisian governorates to explore the microbial diversity and investigate geographical dispersion and pathogen transmission as well as their endosymbionts in different regions in Tunisia (Figure 1).

Bioclimatic stages	**District**	**Sub-district**	**Farms visited (Numbers)**	**Surveyed cattle (Numbers)**	**Ticks Collected (Numbers)**
Upper semi-arid	Bizerte	Besbesia	3	68	79
Upper semi-arid	Ariana	Hessiènne	4	36	56
Subhumid	Bizerte	El Alia	1	7	20
Subhumid	Bizerte	Aousseja	1	16	33
Upper semi-arid	Seliana	El krib	5	40	45
Sub-humid	Bizerte	Ghezala	7	36	151
Humid to sub-humid	Jendouba	Fernena	7	56	32
Humid to sub-humid	Beja	Amdoun	4	25	27
Humid to sub-humid	Beja	Beja	3	10	7
Humid	Beja	Nefza	4	32	34
Upper Arid	Kairouan	El Ala	8	30	65
Upper Arid	Kasserine	Sebitla	7	35	53
Semi-arid	Zaghouan	Fahs	9	42	13
Lower semi-arid	Sousse	Kalaa Kbira	11	32	30
Humid	Jendouba	Tabarka	8	25	131

Figure 1. Sampling sites.

- Engorged nymphs were collected from cattle during October and November from El Hissiene site (Ariana, Tunisia).
- Engorged *Hyalomma scupence* female ticks were obtained from cattle in July 2020 from El Hissiene (Ariana, Tunisia) but the number of ticks was not sufficient to test the effects of essential oils on the reproductive fitness of *Hyalomma* ticks and investigate their effects on their endosymbionts using different doses that's why we are going to include *H. dromedarii* as a model due to the availability of this tick during the winter. We are planning in the summer 2021 to run additional assays on *Hyalomma scupense*.

Ticks were transported back to the laboratory of parasitology where they were identified according to the key of (Walker et al., 2004) prior to their storage at -80 until DNA extraction.

2) Ticks diagnosis

Tick's diagnosis was performed according to the morphological keys of Walker et al 2004; 2013. A total of 776 ticks were collected from cattle from 9 governorates of Tunisia, 68.7% were males and 31.0% were females. Due to the sampling design *Hyalomma scupence* was the main collected species, representing more than 40.0% of collected ticks (n = 776), followed by *Hyalomma marginatum* (17.80%) then *Hyalomma excavatum* (17.36%), Haemaphysalis punctata (18.81%), *Rhipiciphalus sp* (6.94%) and finally *Hyalomma impeltatum* (0.14%) (Figure 2).

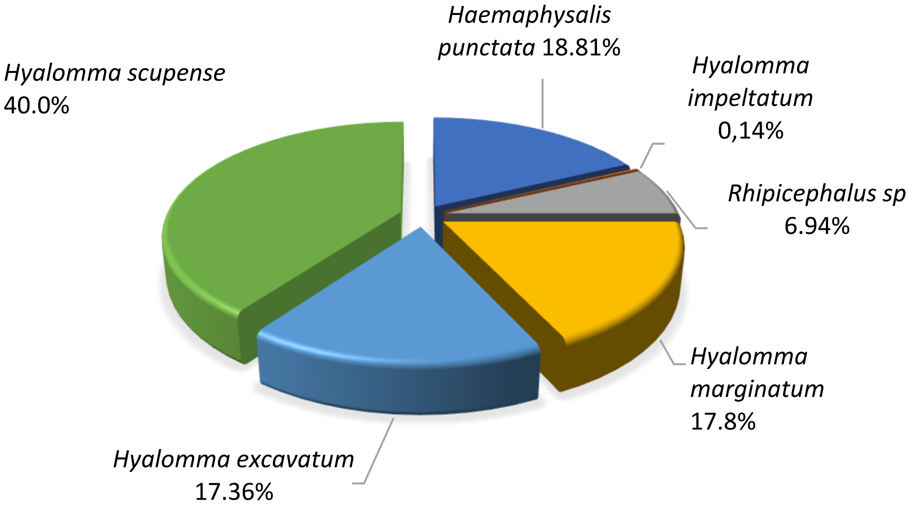

Figure 2. Tick genus/species identified in the present study.

3) Genomic DNA extraction and Next-generation sequencing (NGS)

DNA extraction from questing and gorged adult ticks, nymphs, and eggs was performed following last year's optimizations. DNA was extracted from individual adult ticks using the DNeasy Blood &Tissue Kit (QIAGEN, Valencia, CA), while DNA extraction from gorged nymphs and eggs was performed following optimization of the Wizard Genomics DNA kit (Promega, Madisson) protocol.

To verify the quality of our DNA and their concentration we used Nanodrop followed by the universal 16S rRNA polymerization chain reaction.

To the purpose of studying the microbial population of ticks, we prepared for next-generation sequencing a sample of 60 nymphs of *H. scupense* and 120 adult ticks distributed as follows, 30 *H. scupense* females, 30 *H. scupence* males, 30 *H. marginatum*, and 30 *H. excavatum* was used for this purpose. Besides, ticks' eggs were extracted in pools of 300 eggs using Promega kit. DNA individual samples were pooled together in equal concentrations for each species and stage using the following samples: the 30 adult ticks collected for *H. excavatum* and *H. marginatum*, 15 males and 15 females, and the 60 nymphs for *H. scupense*.

Now, we are waiting for the results of the microbial population which were analyzed using Illumina MiSeq sequencing of the V3-V4 variable region of the bacterial 16S rRNA gene.

4) Prevalence and Transmission of symbionts

-We started first with the PCR optimization of the housekeeping gene of *Hyalomma* ticks the ELF1A and then PCR of *Francsella* like, *Rickettsia* Like and Coxiella like. PCR assays were performed with specific primers (Table 1).

Table 1. Targeted genes, primers, and nucleotide sequences per tick species.

Species	Target gene	Primers	Nucleotide sequence(5'–3')	Refrence
Rickettsia-like endosymbiont	Citrate synthase gene (gltA)	gltA-F gltA-R	TCCTACATGCCGACCATGAG AAAGGGTTAGCTCCGGATGAG	Lan-Hua Li1 and al 2018
Coxiella-like endosymbiont	16S rRNA gene	L-CoxF L-CoxR	TGAGTGTTGACGTTACCCACAG GCATTTCACCGCTACACCG	Lan-Hua Li and al 2018
Francisella like endosymbiont	16S rRNA	Fran16S-F Fra16S-R	CAGGACTAGCTTATAGTTGCTG- CATCTGCGACAGCCTAAAAGC-	Jian-Nan L and al 2016
Housekeeping gene of *Hyalomma*	Elongation factor1-alpha	ELF1A- F ELF1A-R	-CGCAAGTCTGGCAAGTCTGA- -ATGCCACCAATCTTGTAGACG-	Nijhof et al. (2010)

To test whether the transmission of *Francisella* like, *Coxiella* like, and *Rickettsia* like has a transovarial transmission, samples of eggs of three different female *H. scupence* were screened. All the three pools of eggs were found infected with the three symbionts, also, eggs of *H. marginatum* and *H. excavatum* were positive with the three symbionts, which is consistent with vertical transmission of those endosymbionts in *Hyalomma* genus. For assessing the prevalence of symbionts in different tick stages, a total of 30 engorged nymphs were tested and the results show that 45% of them carry *Francisella* like bacteria, for questing ticks not all of them carry *Farncisella* like, however, the test of 20 engorged female showed that all the samples were positive with *Francisella* like, suggesting the reproductive role of those symbionts and their correlation with tick stage. Also, PCR results show that *Rickettsia* like is more abundant in *H. excavatum* than the other species of *Hyalomma*.

5) Detection of *Theileria annulata*

T. annulata infections were detected using cytochrome b gene-specific primers in PCR assay using specific primers cytoF (5'CAGGGCTTTAACCTACAAATTAAC3') and cytoR (5' CCCCTCCACTAAGCGTCTTTCGACAC 3') (Mhathbi et al., 2015).

The infection rate for engorged nymphs was 44%.

6) Essential Oils Extraction and Analyses

During May and June 2020, *Thymus capitatus* and *Mentha pulgeium* were collected at the flowering stage from their natural habitats from Takelsa (Nabeul, Tunisia), to test their essential oils on ticks' effects on reproduction and vitality at different doses and assess their effects on the symbionts. The extraction of essential oils was carried out by hydrodistillation of the aerial part of plants for three hours using a Clevenger-type hydrodistillation apparatus. During each extraction, 100 g of dried material was treated. The yield was calculated using the following equation:

$$RO = (M / B) \times 100$$

Where: RO: the yield of essential oils expressed in%; M: the mass of extracted essential oils in gram; B: initial plant biomass in gram (Olivia et al., 2014).

The essential oil yield (RO) of *Mentha pulegium* was 2.2 % while it was 1.2 % for *Thymus capitatus*. The Identification of compounds of EO was done using gas chromatography coupled with mass spectrometry (GC-MS). The predominant compound for *M. pulegium* was 62.98 % pulegone, However Chromatographic analysis of the essential oil of *T. capitatus* revealed that carvacrol (58.46%) and *P. cymene* 11.37 % were the main constituents (Figure 3 and 4).

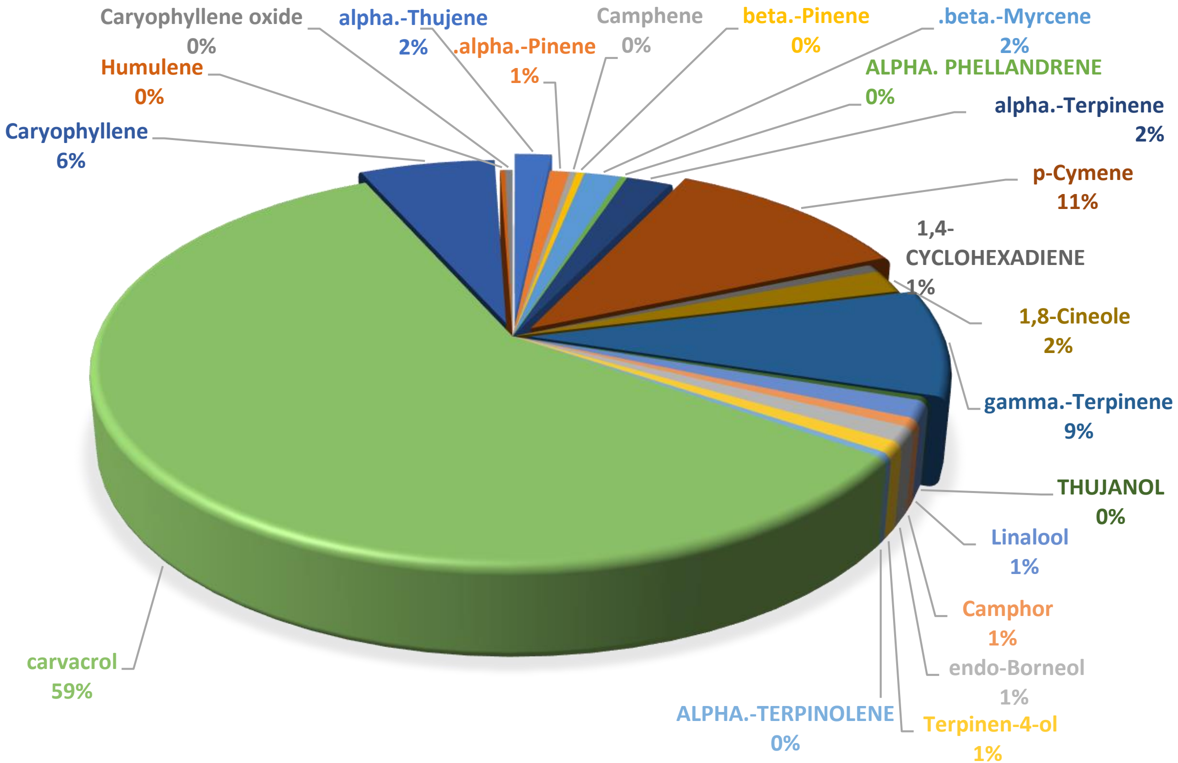

Figure 3. Identified compounds using GC-MS for *Thymus capitatus* extract.

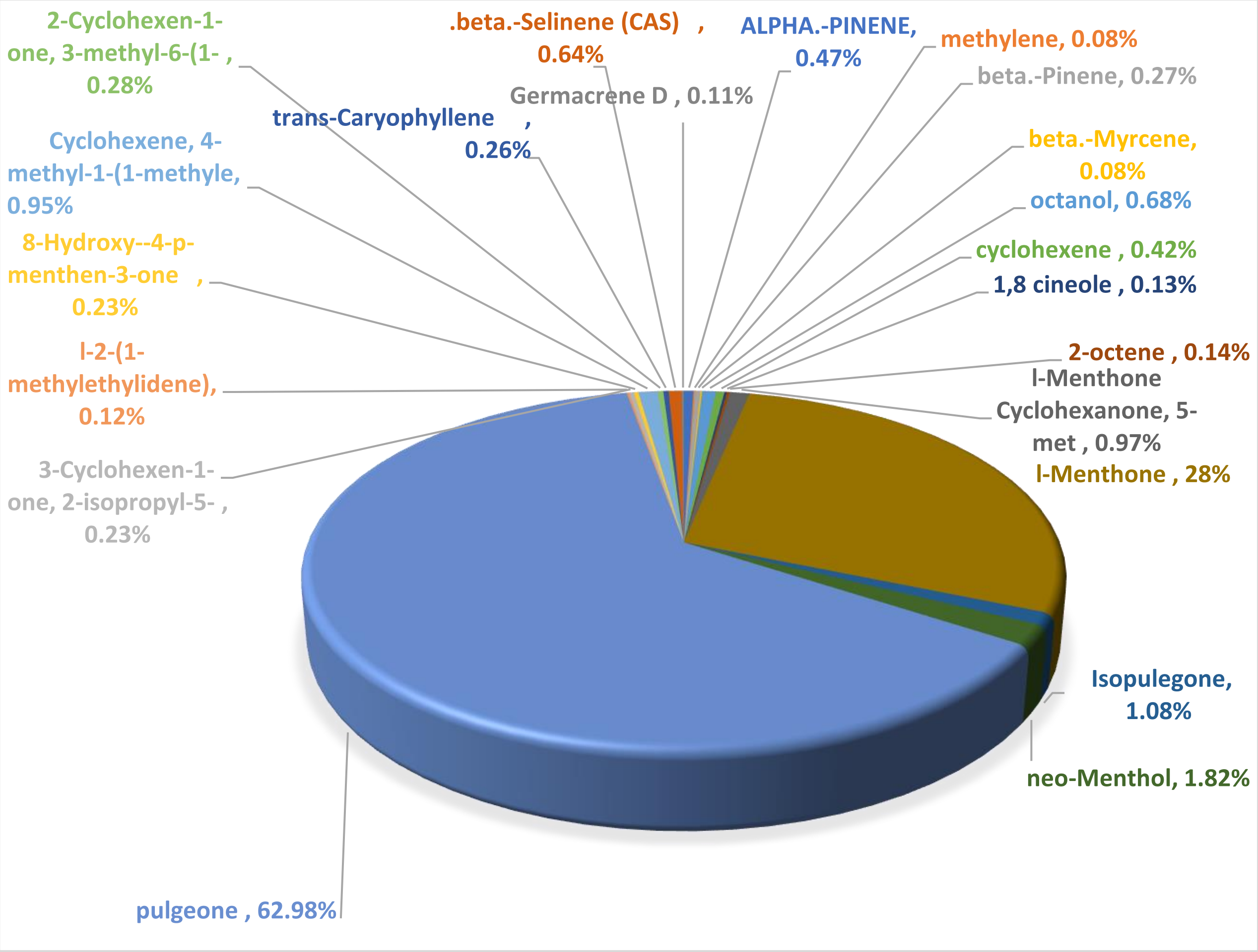

Figure 4. Identified compounds using GC-MS for *Mentha pulegium* extract.

7) Preliminary adult immersion test (AIT)

We prepared a series of essential oils concentrations diluted in Tween 80 at 2% used in Adult Immersion Tests (AIT) ranging from 3, 6, and 12μl/ml. engorged females were divided into groups composed of 6 ticks of homogeneous weight. Each group was immersed for 5min in a petri dish containing essential oil or, in a solution of tween 80 at 2% as a negative control and were then removed and dried with paper towels. Ticks were individually incubated at 27∘C and 90% relative humidity until the end of oviposition.

Reproductive efficiency and reproduction inhibition were calculated using the following equations:

$$OR = \frac{EW}{IFW}$$

$$OR\,(\%) = \frac{\mathrm{OR}\,(control) - \mathrm{OR}\,(treated)}{\mathrm{OR}\,(control)} \times 100$$

$$RI\,(\%) = \frac{RE\,(control) - RE\,(treated)}{RE\,(control)} \times 100$$

$$RE = \frac{EW}{IFW} \times \%E$$

where OR is the oviposition rate, IFW is the initial female weight, EW is the egg weight, E is egg eclosion, RE is the reproductive efficiency, and RI is reproduction inhibition (Djebir et al. 2019).

We have noticed from our preliminary test carried out of few ticks that the EO delayed significantly the oviposition and inhibited egg hatching. However, we should increase the number of ticks to test more EO with different concentration rates and study their impact on symbionts using real-time PCR.

We are going to repeat the test with a higher number of ticks, which, each bioassay will be repeated three times. After the oviposition, 300 randomly selected eggs from each female will be preserved in 70% ethanol for further DNA extraction and real-time PCR analysis. Another 300 selected eggs from each female will be put into a new container to estimate the hatching rate.

The rest of our work will therefore consist of continuing the study of the diversity of the tick microbiota (genus *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*), exploring the acaricidal activity of essential oils on their endosymbionts of *H. Dromedarii* to consider an alternative to control these pests and studying the phylogeny of *Hyalomma* ticks infecting livestock in different bioclimatic and geographic Tunisian regions.

Objectives	Activities
Study by NGS the diversity of *Hyalomma* ticks' microbiota from different species, regions, stages, sex, hosts and the genus of *Haemaphysalis* and *Rhipiciphalus*.	Tick sampling *H.dromedarii* + *H.impeltatum*
	Nymph sampling
	Ticks' culture
	DNA extraction
	NGS
	Bioinformatic analysis
Assess the effects of selected essential oils on the reproductive fitness	Engorged *H.scupence* sampling
	Engorged *H.dromedarii* sampling from Tataouine
	Ticks culture

of *Hyalomma* ticks and investigate their effects on their endosymbiotes using real time PCR.	Adult immersion test (AIT)
	Ticks' injection
	Study the reproductive effects (oviposition, hatching..)
	Viable larvae number
	DNA extraction from Eggs
	DNA extraction from larves
	PCR optimization and cloning
	Real time PCR for symbiotic bacteria of treated ticks with EO
	Statistical analysis
Study the phylogeny of Hyalomma ticks infecting livestock in different bioclimatic and geographic regions	rRNA 16S and rRNA 12S PCR
	Sequencing
	Bioinformatic analysis

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