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Sur le thème

Effect of withanolide-containing diet on gut microbial communities and AMP expression in two closely related Lepidoptera species : *Heliothis subflexa* and *Heliothis virescens*

Par

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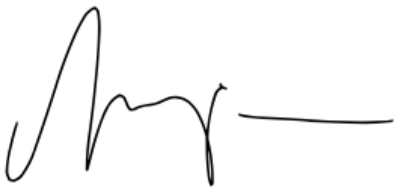

ABSTRACT

Every interaction between two organisms is a force driving the adaptation of one to the other and vice versa. This force of adaptation becomes more important as the number of interacting organisms and interaction levels increases. In this study we investigate the impact of withanolides, which are a group of plant secondary metabolites synthesized by *Physalis* plants, on the microbiota of two insect species in order to shed light on the mechanisms of insect-plant coevolution mediated by endosymbionts. We used the model system composed of the generalist lepidopteran moth *Heliothis virescens* and its close relative *Heliothis subflexa*, which is a specialist feeder of *Physalis* plants. We showed by means of bacterial 16S rRNA gene sequencing that *H*. *subflexa* has a more diversified gut microflora than *H. virescens*. Both gut bacterial communities were largely dominated by the genus *Enterococcus*. Withanolide supplementation to diet given to larvae had no significant effect on the diversity and richness of gut symbionts. In addition, we assessed the temporal expression levels of two antimicrobial peptides - i-type-lysozyme and attatcin-1 - under withanolide treatments on third instar larvae. We showed that the expression of attacin-1 was not affected by any of the treatments; however, i-type-lysozyme was upregulated after 24 hours.

Key words: microbial community, host-plant adaptation, withaferin A, withanolides, *Heliothis subflexa*, *Heliothis virescens*, Physalis, AMPs

RÉSUMÉ

Un des thèmes phares de la biologie de l'évolution est l'étude des interactions entre deux organismes et comment ces interactions deviennent des moteurs d'adaptation, ou plus précisément de coadaptation. Un exemple célèbre et largement étudié est celui des interactions entre les insectes et les plantes. Cette coévolution, souvent appelée « course aux armements », promeut plus souvent la spécialisation des insectes à un nombre réduit de quelques espèces de plantes phylogénétiquement proches qu'à l'expansion de la gamme d'hôte à un grand nombre de plantes (Ehrlich et Raven, 1964, Fox, 1981, Jaenike, 1990).
Les plantes ont développé des mécanismes de défense pour se protéger contre les herbivores au moyen, entre autres, de composés appelés «métabolites secondaires de plantes» (MSP). Les insectes quant à eux ont évolué des moyens pour détoxifier leur nourriture, devenir tolérant voire même séquestrer les toxines végétales pour servir leurs propres intérêts (Heckel, 2014). Outre la gestion des toxines de plantes, les insectes doivent également résister aux attaques d'un grand nombre de pathogènes (Lacey et al., 2001). Cependant, comme pour la plupart des animaux, les insectes ne sont pas seuls pour y faire face. Ils sont accompagnés de microorganismes qu'ils portent dans leur intestins, et qui leur procure une grande variété de bienfaits (Frago et coll., 2012, Janson et coll., 2008, McFall-Ngai et coll., 2013). Les études montrant l'importance des microbes intestinaux sur ce sujet se sont multipliées au cours de la dernière décennie. Les symbiotes intestinaux sont connus pour dégrader les matières végétales indigestes, détoxifier l'organisme des MSP, et protéger l'hôte contre les agents pathogènes. Ils sont ainsi considérés comme une potentielle force évolutive capable de provoquer des changements de gamme hôtes chez les insectes. Dans certains cas, le microbiote intestinal de l'insecte serait même impliqué dans le développement de résistances aux insecticides (Pietri et Liang, 2018).

Cette présente étude porte sur le système *Heliothis subflexa* et son espèce soeur *Heliothis virescens*. *H. subflexa* est un lépidoptère spécialiste des *Physalis* (Solanaceae) qui produisent des withanolides pour se défendre, alors que *H. virescens* peut se nourrir sur plus d'une dizaine de famille de plantes différentes excepté des *Physalis* (Brazzel, 1953; Cho et al., 2008). Les withanolides sont des lactones steroïdiennes avec un ergonstane pour structure centrale (Misico et al., 2011). Ces molécules sont reconnues comme étant des anti-appétants efficaces (Ascher et al., 1980), des antagonistes des ecdystéroïdes (Dinan et al., 1996), des immunosuppresseurs (Garcia et al., 2006) et pour présenter une activité antimicrobienne (Kurup, 1956). Ils sont répulsifs ou nocifs pour une grande quantité d'organismes. Une étude récente menée par Barthel *et al.* (2016) a montré dans des expériences d'alimentation que les withanolides bénéficiait à *H. subflexa.* Celui voyait en effet sa prise de poids relatif augmenter lorsque il en consommait. D'autre part, la consommation de withanolides avait un effet négatif sur *H. virescens* qui se traduisait par une perte de poids relatif en comparaison du contrôle. Les effets des withanolides portaient également sur l'immunité des deux insectes, l'activité phenoloxidase (PO) était augmentée chez *H. subflexa* alors qu'elle restait inchangée chez *H. virescens*. De plus l'apport de

withanolides à leurs régimes alimentaires provoqua chez *H. subflexa* une augmentation non significative de l'expression de la plupart des gènes de l'immunité alors que ces mêmes gènes étaient réprimés chez *H. virescens.* Ces résultats ont inspiré la présente étude qui s'organise autour de deux questions : les microorganismes intestinaux d'*H. subflexa* interviennent-ils dans son adaptation aux *Physalis* ? Il y a t il un changement de composition de la microflore intestinale de ce dernier qui puisse expliquer la capacité de cet insecte à se nourrir de *Physalis* ? Et deuxièmement, cette étude s'intéresse à l'aspect temporel de la réponse immunitaire de cet insecte aux withanolides. Cette réponse est-elle rapide ou bien s'inscrit elle dans la durée ? Et quelle en est l'ampleur en terme que quantification de la surexpression des gènes de l'immunité ?

Pour répondre à ces deux problématiques deux expériences de nutrition comprenant deux conditions de traitements ont été établies. Le premier traitement consistait en un extrait purifié de withanolide provenant de *Physalis peruviana* tandis que le second était du withaferin A, un withanolide disponible dans le commerce. Le traitement control était le méthanol, solvant dans lequel ces composés étaient dissouts. La première expérience consistait à appliquer les traitements de withanolides sur un régime alimentaire artificiel et de permettre à des larves de *H. subflexa* et *H. virescens* de s'en nourrir pour une durée de 48h. A l'issue de ce temps d'exposition les intestins des larves ont été disséqués et l'ADN génomique extrait. Les échantillons ont ensuite été envoyés à séquencer pour le gène bactérien ribosomal 16S. Les données brutes ont été traitées dans QIIME2 par le Dr. Shantanu Shukla qui a produit un tableau répertoriant le nombre de comptage obtenu par échantillon pour une séquence donnée. La seconde expérience consistait à exposer les larves à ces mêmes traitements sur des durées de une, six 12 et 24 heures, à l'issue desquelles l'ARN total a été extrait des larves entières, converti en ADN complémentaire et utilisé dans des réactions de PCR quantitatives en temps réel. Cela a permis de suivre au cours du temps l'expression relative de deux gènes de l'immunité : le peptide antimicrobien (AMP) attacine-1 et le lysozyme 1 de type i en réponse aux traitements.

Les résultats de l'analyse métagénomique des intestins a montré que *H. subflexa* a une microflore intestinale plus diversifiée que celle de *H. virescens*, comprenant 70 familles bactériennes différentes contre seulement cinq chez *H. virescens*. Cependant le nombre total de séquences quantifiées dans les échantillons de *H. subflexa* variaient grandement. Certain échantillons comprenant seulement 388 ou 423 séquences lorsque tous les échantillons de *H. virescens* en avaient plus de 22687. Les deux communautés bactériennes intestinales étaient largement dominées par le genre bactérien *Enterococcus*. Ce dernier prévalait à 99,89% chez *H. virescens* et à 84,57% chez son espèce soeur. Ces observations ne sont pas inhabituelles pour des populations d'insectes de laboratoires. L'ajout de withanolides au régime alimentaire administré aux larves n'a pas eu d'effet significatif sur la diversité et la richesse intraspécifique de leurs symbiotes intestinaux. Seul la proportion relative a été impactée chez *H. subflexa* où l'ordre des Bacillales composait seulement 0,27% de la communauté totale sur le contrôle mais représentaient 4% et 31% lorsque les larves étaient exposées à l'extrait de withanolide et au withaferin A respectivement. Ces résultats suggèrent que *H. subflexa* n'a pas de communauté bactérienne résidente mais plutôt

transitoire. Il a ensuite été montré que l'expression du gène de l'attacine-1 n'était pas affectée par les traitements alors le gène du lysozyme 1 de type i était surexprimé de manière significative après 24 heures chez les deux espèces comparé au temps zéro. Cependant, l'expression des deux gènes de l'immunité ne différait pas dans les conditions de traitement par rapport au contrôle, et ce pour tous les temps étudiés.

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TABLE OF CONTENTS

INTRODUCTION

One major field of evolutionary biology is the research on interactions between organisms and how these interactions become drivers of adaptation, or more precisely co-adaptation. A well-known system often chosen to tackle this topic is the interactions between insects and plants. This coevolution, often referred to as an “arms race”, prompts more specialization of insects feeding on a restricted number of closely related plant species than the expansion of their host-plant range (Ehrlich and Raven, 1964; Fox, 1981; Jaenike, 1990). Plants have evolved defense mechanisms to protect themselves against herbivory by the means of, among others, compounds called « plant secondary metabolites » (PSM). Insects in return have developed the competence to detoxify their diet, become tolerant to or even sequester the plant toxins to serve their own interest (Heckel, 2014). In addition to toxic PSMs, insects have to deal with a high numbers of pathogens (Lacey et al., 2001). However, like most animals they are not alone to deal with this challenging environment. They are accompanied by microorganisms, bearing them into their gut lumen, which provide them with a wide variety of benefits (Frago et al., 2012; Janson et al., 2008; McFall-Ngai et al., 2013). The number of studies showing the importance of gut microbes on that matter have increased over the past decade. Gut symbionts are known to process indigestible plant material, degrade PSMs, outcompete pathogens and are therefore seen as an evolutionary force able to drive shifts in insect host-plant range. In some cases the insect gut microbiota is even thought to be involved in the development of insecticide resistance (Pietri and Liang, 2018).

This study focuses on the model species *Heliothis subflexa*, which is a specialist insect feeding on one genus of the Solanaceae family: the *Physalis.* In chemical ecology there is a growing interest in this insect for its capacity to cope with withanolides while *Heliothis virescens*, a sister species which feeds 14 different plant families, can’t (Brazzel, 1953; Cho et al., 2008). *H. subflexa* is mostly found to be feeding on the fruits. These berries are protected by a membrane which forms an inflated calyx or « lantern » (Oppenheim and Gould, 2002; Sisterson and Gould, 1999). Inside this calyx, the larva is sheltered from most biotic and abiotic stresses (pathogens, drought, sun etc.). However, taking advantage of this natural shelter comes at the cost of having to cope with withanolides, the major defensive PSM of *Physalis* plants. Withanolides are steroidal lactones with an ergostan core structure (Misico et al., 2011). These molecules are recognized to be effective antifeedants (Ascher et al., 1980), ecdysteroid antagonists (Dinan et al., 1996), immunosuppressants (Garcia et al., 2006), and to display antimicrobial activity (Kurup, 1956). They are, repellent or harmful to a wide range of organisms. In this context, how does *H. subflexa* readily feed and thrive on *Physalis* plants? What benefits does *H. subflexa* earn from its host plant? In light of the antimicrobial and immunosuppressing effect of withanolides, Barthel *et al* (2016) studied this host-plant specialization, and contributed to the emerging field of nutritional ecology (Barthel et al. 2016). They examined the effect of withanolide extracts from *Physalis peruviana* supplemented to artificial diet on larval relative weight gains, survival rates and immune indicators in both *H. subflexa* and *H. virescens.* They found that *H. subflexa* was positively affected by withanolides and had an increase in relative weight gain, much higher

than when it fed on regular diet. *H. virescens* on the other hand was negatively affected by withanolides, which led to a decrease in weight gain. Secondly, withanolides significantly increased survival of *Bacillus thuringiensis* (*Bt)* infected *H. subflexa* larvae. Additionally, withanolides seem to increase the phenoloxidase activity (PO) and significantly upregulate the gene expression of almost all antimicrobial peptides (AMPs) identified in this species. On the contrary, *H. virescens* did not benefit from withanolides when challenged by *Bt*, to which it stayed susceptible. The majority of AMPs identified in this species were downregulated or unaffected by withanolide treatments and PO activity stayed unchanged.

These results and observations inspired us to explore in this present study two aspects of the ecological and physiological impact of withanolides in *H. subflexa* compared to *H. virescens*. First, there is the possibility that withanolides shape the gut microbial community in *H. subflexa* resulting in a microbial-driven beneficial effect that allows the insect to thrive on *Physalis* plants. Withanolides might be responsible for a shift in the gut microbial community of *H. subflexa* favoring bacteria involved in the activation, detoxification, and breakdown of withanolides and which could explain the diverging food strategy between the two species (specialist *vs* generalist). Secondly, knowing the timescale at which immune genes are up or downregulated by withanolides would allow us to evaluate how fast *H. subflexa* and *H. virescens* respond to withanolide after starting to ingest them. Does *H. subflexa* quickly respond to withanolides intake or is it a mechanism observed on the long-term? It would also tell us how strong this response is and how it evolves overtime. To tackle this second aspect, the choice of AMPs and immune-related genes was based on the data provided by the RNAseq analysis in the study of Barthel *et al.* (2016). Two genes were chosen so that both had significant fold changes in expression levels in *H. virescens*, while being unaffected in *H. subflexa*. The AMP attacin-1, which was significantly downregulated and the i-type lysozyme-1, an immune-related gene that was significantly upregulated made good candidates.

Barthel *et al.* used crude and purified extracts of withanolides to carry out their study. Here was added the evaluation of a pure and known withanolide compound, easily available on the market: withaferin A.

LITTERATURE REVIEW

Insects manage to thrive in a tremendously challenging environment. They are constantly confronted with diverse biotic and abiotic stresses: extreme thermal stresses, predation, pathogen and parasite infections as well as plant defense strategies. The access to a food source in quantity and quality is the cornerstone of physiological performance of insects (Schoonhoven et al. 2005; Vogel and Musser 2013). Most insects have to consume high quantities of plant material to meet the nutritional requirements needed for the completion of their costly processes of development (Ayres and MacLean 1987; Huffaker 1999; Lee et al. 2006). The consumption and digestion of plant material requires insect's ability to overcome the successive mechanical and chemical barriers raised by plants to avoid herbivory. Plants have developed many ways to protect themselves from herbivores including the use of allelochemicals. These plant secondary metabolites include toxins, repellents, feeding deterrents, volatile organic signals that may interfere with insects' behavior, growth, fitness and development. The ability to properly address the challenges posed by their host plants is of major importance for the larval fitness and survival throughout its life cycle.

Plant secondary metabolites (PSMs) are organic molecules that are usually not of primary importance for the plant. They are often considered as by-products of the synthesis of primary metabolites, essential for the plant development and growth (Herbert 1989). Although some PSMs play a physiological role such as tools for food transport and storage or osmoregulatory solutes concentrated to avoid nutrient deficiency or water losses (Gershenzon 1984; Charles, Joly, and Simon 1990) a large body of evidence shows that PSMs are involved in plant defenses against pathogens and herbivores (Ehrlich and Raven 1964; Bennett and Wallsgrove, n.d.; Theis and Lerdau 2003; Mao et al. 2007). The classification of PSMs encompasses terpenoids, alkaloids and phenolic compounds. To date, the literature states that about 50000 PSMs have been described but it is very likely that this is only a small fraction of the diversity present in the plant kingdom (De Luca and St Pierre 2000; Wink 2003). Altogether, the PSM signature of a plant is very complex and unique. It is believed that this unique set of chemicals throughout species was shaped by plants coevolution with insects and pathogens (Ehrlich and Raven 1964; Wink 1999). Every species evolved its own allelochemicals cocktail in a process of bilateral adaptation and counter-adaptation between plants and their natural enemies (Ehrlich and Raven 1964). This being said, strong similarities among plant taxons are observed (Wink 2003; Chen 2008; Heckel 2014). For instance, glucosinolates are widely produced among Brassicaceae (Heckel, 2014) whereas cyclic hydroxamic acids are almost exclusively produced in Gramineae (Niemeyer 1988). In Solanaceae secondary metabolites are as varied as propane, pyridine, ecdysteroids, steroid alkaloids, diterpenes and withanolides (Wink 2003; Griffin and Lin 2000; Baxter, Harborne, and Moss 1998).

However, while plants unfold strategies to deter herbivory, insects in return develop means to circumvent these defenses (Ehrlich and Raven 1964; Vogel and Musser 2013). The success of this response is at the base of larval fitness and survival of insect populations through time. The mechanisms underlying the ability of herbivorous insects to thrive on

plants are vast and finely tuned, although most of these aspects are still obscure. Insects are able to digest a large diversity of plant matter (Schoonhoven et al. 2005; Watanabe and Tokuda 2010) and detoxify the most toxic compounds present in their diets (Heckel 2014). Some highly specialized species twist plant defenses by sequestrating phytotoxins to serve their own purposes (Heckel 2014; Petschenka & A. Agrawal 2016) while others engaged in an intimate relationship with symbionts to help them accomplish particular biochemical transformations (Brauman et al. 1992; Warnecke et al. 2007) or elude plants defenses (Hansen and Moran 2014; Hammer and Bowers 2015). It has been known for long that microbes play an important role in insect biology, physiology and development (Sommer and Bäckhed 2013). Symbiosis is ubiquitous in nature: among plants (Koh and Hik 2007; Patriarca, Tatè, and Iaccarino 2002), animals (McFall-Ngai et al. 2013; Sullam et al. 2012) or fungi (Rouland-Lefèvre 2000; Smith, Jakobsen, and Smith 2000) and influence all aspects of host biology (Bjorndal 1997; Frank 1997; Ley et al. 2008; Wernegreen 2012; Kwong and Moran 2015).

This literature review will first cover the diversity and biological properties of withanolides, present in *Physalis* plants, before tackling the role of gut bacterial communities in insects. Finally, a closer look will be taken at the model species *Heliothis subflexa* and *Heliothis viresens* in the light of both previous topics.

Withanolides, plant secondary metabolites.

***Physalis*, withanolides and withaferin A.** *Physalis peruviana*, also known as the « Cape gooseberry », is an economically important and is the most cultivated species of the *Physalis* genus worldwide. It is the second most important fruit crop for export in Columbia (Salazar et al. 2008), which remains the world's largest exporter of *Physalis* right before South Africa (Muniz et al. 2014). *Physalis peruviana* is also grown in Brazil, Peru and Kenya (Fischer, Herrera, and Almanza 2011). As many other Solanaceae, the Cape gooseberry has been reported to accumulate withanolides (Bagchi et al. 1984; Neogi, Sahai, and Ray 1986; Oshima et al. 1989; Baumann and Meier 1993; Dinan et al. 1996; Misico et al. 2011). Withanolides represent a group of naturally occurring steroidal lactones with a C28 ergostane skeleton (Misico et al. 2011) harboring strong biological properties, which appear to be restricted to the Solanaceae (Baxter, Harborne, and Moss 1998; Wink 2003). Within Solanaceae only a certain number of tribes and genera belonging to the subfamily Solanoideae produce withanolides: *Withania, Acnistus, Physalis, Jaborosa, Datura, Lycium, Iochroma, Nicandra, Trechonaetes, and Witheringia* (Christen 1989; Glotter 1991; Misico et al. 2011). A handful of papers have extensively reviewed the structural aspects and classification of this group of compounds (Glotter 1991; Ray and Gupta 1994; Anjaneyulu, Rao, and Lequesne 1997; Veleiro, Oberti, and Burton 2005; Misico et al. 2011) as well as their biological proprieties (Budhijara, Krishan, and Sudhir 2000; Su et al. 2004). Four major plant genera contribute to the withanolide core structure: *Jaborosa* Juss., *Datura* L., and

Physalis L. from the North and South American continents, and *Withania* Pauq (Misico et al. 2011).

Since their first successful isolation in the mid-1960s (Misico et al. 2011), withanolides sparked interest from medical and pharmaceutical research for their numerous biological activities such as anti-inflammatory, immunosuppressants (Castro et al. 2008; Yu et al. 2010), cytotoxic (Lan et al. 2009), antibacterial (Silva et al. 2005) and antifungal activity (Misico et al. 2011). The number and variety of proprieties is immense: antitumor (cancer chemopreventive, slowing tumor growth), radiosensitizing, adaptogenic, immunomodulating, antioxidant and antistressor effect have been reported as well (Budhiraja and Sudhir 1987; Budhijara, Krishan, and Sudhir 2000). In this respect the best-known component is withaferin A, which has been investigated extensively (Yang et al. 2007; Mandal et al. 2008; Stan et al. 2008) (Figure 1). Withaferin A was long considered as the basic template for withanolides (Misico et al. 2011). It is particularly abundant in *Withania somnifera* Dun. (Gottlieb et al. 1987; Mohan et al. 2004) and *Acnistus arborescens* (Kupchan et al. 1969). Withaferin A was historically used in traditional Indian Ayurveda medicine (Grosourdy 1864; Dhalla, Sastry, and Malhotra, n.d.; Misra 1998). It was first isolated by Kurup in 1956 (Kurup 1956) and later extracted and characterized by Lavie and collaborators in 1965 (Laviee, Shvo, and Glotter 1965). The roots and the leaves contain high levels of the sought-after molecule and were traditionally used for the treatment of arthritis or disorder of the uterus and menstrual cycle (Mishra et al. 2000). Other examples of ancient use of withanolides from plants are known: extractions from *Datura metel* in traditional Chinese medicine (Misico et al. 2011), *D. brachyacantha* extracts used to relieve from stomach ache by the Raqaypampeños, a Bolivian ethnic group (Bravo et al. 2001), or *Physalis* species in Asia and the Americas (Misico et al. 2011). Recent studies focus first and foremost on the potential use of these steroids in cancer treatment (Thaiparambil et al. 2011; Dhanani et al. 2017; Khan et al. 2017; Achar et al. 2018).

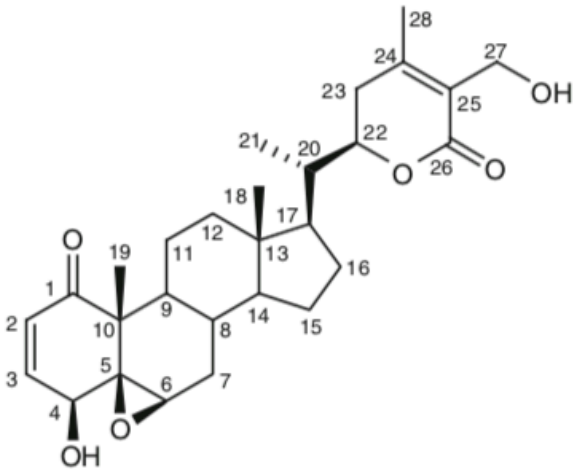

Figure 1 Structure of withaferin A (Misico et al., 2011)

Withanolides have detrimental effects on insects and microorganisms.

Growth inhibitor and feeding deterrent. The research on the feeding deterrence of solanaceous plants to insects started when Yamamoto and others noticed that a few solanaceous species were repellent to the larvae of the tobacco hornworm *Manduca sexta*, although this oligophagous insect is restricted to the Soleanaceae family (Nalbandov et al., 1964; Yamamoto and Fraenkel, 1960). Nalbandov and coworkers managed to extract the so-called « repellent factor » which they called « nicandrin » (nicandrenone or Nic-1, from the larger nicandrenoids group) from *Nicandra physalodes*. They revealed its deterrent proprieties, long before the description of its chemical structure (Begley et al., 1972b, 1972c). Later, Ascher and collaborators investigated the feeding deterrence of several other withanolides. It was first demonstrated that withanolide E and 4/β-hydroxywithanolide E are the main withanolides represented in *Physalis peruviana* (Ascher et al., 1980; Baumann and Meier, 1993). Withanolide E and its 2,3-dihydro-β-hydroxy derivative (III) were found to be the most potent antifeedants when compared to five other withanolides on the larvae of *Spodoptera littoralis* Bois (Ascher et al., 1980). Nic-1 (nicandrenone) had no significant deterrent effect on *S. littoralis* (Ascher et al., 1980), *or Pectinophora gossypiella* Saund, *Heliothis virescens F ,* and *Helicoverpa zea* Boddie (Elliger ; personnal communication to E. Glotter) although it was previously found to be a feeding deterrent for *Manduca sexta* and a toxic agent in house flies (Nalbandov et al., 1964). During the following years Ascher *et al.* also demonstrated the antifeedant activity of other withanolides on *Epilachna varivestis* Muls (Ascher et al., 1981, 1987), *S. littoralis* and *Tribolium castaneum* Herbst (Ascher et al., 1987). Feeding deterrence is closely intertwined with growth inhibition and the boundaries between them are often unclear. Hence, Elliger and collaborators showed that leaf extracts of *P. peruviana* had a strong inhibitory impact on growth of *H. zea* larvae (Elliger and Waiss, 1989). They expressed larval growth as a percentage of control values and introduced the term ED50 (Effective Dosage). It is defined as « the concentration of additive required to reduce larval growth to fifty percent of control weights » (Elliger and Waiss, 1989). Some saccharide esters obtained by fractionation[1] and closely related to withanolides had ED values as low as 5.4ppm such as the 11b-hydroxy diglucoside ester (Elliger et al., 1994; Waiss et al., 1993). However, none of the compounds tested were lethal to the insects indicating that the growth inhibitory effect was a consequence of feeding deterrency, provoking to the semi-starvation of the animal (Elliger et al., 1994).

In view of nicandrenone properties on insects, Mareggiani and collaborators (Mareggiani et al., 2000) investigated the properties of three salpichrolides extracted from *Salpichroa origanifolia.* Salpichrolide A, salpichrolide C, and salpichrolide G had clear detrimental effects on the common fly *M. domestica* larvae as the percentage of mortality rose with higher doses of compounds. Further on, these compounds were tested on the stored grain pest *Tribolium castaneum* (Mareggiani et al., 2002) and the Mediterranean fly *Ceratitis capitata* (Bado et al., 2004). In all three cases, the chemicals added to the diets

[1] **Fractionation** is a separation process in which a certain quantity of a mixture (gas, solid, liquid, enzymes, suspension, or isotope) is divided during a phase transition, into a number of smaller quantities (fractions) in which the composition varies according to a gradient. Fractions are collected based on differences in a specific property of the individual components. A common trait in fractionations is the need to find an optimum between the amount of fractions collected and the desired purity in each fraction. Fractionation makes it possible to isolate more than two components in a mixture in a single run. This property sets it apart from other separation techniques (Wikipedia).

caused significant developmental delays and lethal effects on larvae whereas adult exposure in *Ceratitis capitata* resulted in high mortality rates in all cases. The authors observed similar developmental delays when insects were fed with low nutrition diets and salpichrolides leading to the hypothesis that these compounds act as feeding deterrents. The identity of the functional group responsible for the biological activity is still unclear although it was shown several times that the level of oxidation of the ring in the side chain plays an important role (Bado et al., 2004; Mareggiani et al., 2000, 2002). From the analysis of modified analogues, it was shown that the hemiketal moiety in the side chain was crucial for exerting an antifeedant effect as salpichrolide B, obtained by synthesis, was the most active compound among all salpichrolides tested (Bado et al., 2004). According to Enriz *et al.* (2000) it appears also that the minimal structure requirements to produce a biological response in the insect is a furan ring in the side chain and a carbonyl αβ-unsaturated (or *spiro*-epoxide) lactone (Figure 2)(Enriz et al., 2000).

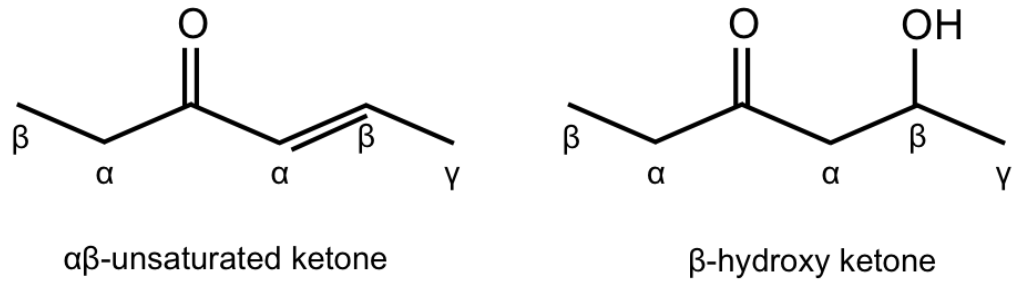

Figure 2 Structure of αβ-unsaturated ketone and β-hydroxy ketone moieties present in withanolides

Ecdysteroid activity. The ecdysteroid hormonal mechanisms are essential for development in insects. Withanolides have demonstrated antagonistic activities to the action of ecdysteroids such as 20-hydroxyecdysone (20E) in *Drosophila* BII cell lines (Clément et al., 1993) containing the ecdysteroid receptor - EcR (Dinan et al., 1996, 1999, 2001). Plants are known to contain phytoecdysteroids, secondary metabolites with agonistic or antagonistic effect to insect hormones, which function is presumably to disrupt the development and reproduction of insect herbivores. Dinan *et al.* (1996) argue that the antagonistic activity of withanolides is mediated by an oxygen-containing function at C-3 where a hydroxyl (-OH) confers greater activity than a methoxy group (-OCH3) and an αβ-unsaturated lactone in the side-chain ring (Dinan et al., 1996). However withanolides with a C-3 hydroxyl function are rare in nature and it is believed that upon herbivory, the alkaline environment of the insect gut yields 3-hydroxy compounds subsequently mimicking 20E. It is therefore thought that the majority of withanolides tested were biologically inactive *per se* when tested in BII cell lines but that some might be activated after ingestion by the herbivore (Savchenko et al., 2000). The authors further hypothesize that Solanaceae originally produced withanolides as insect ecdysteroid antagonists and that insect herbivores assimilated them as feeding deterrents.

Antimicrobial activity. The antimicrobial activity of withanolides was first reported in withaferin A, long before its structure was elucidated (Kurup, 1956). Since then many other withanolides have been screened for related properties and yet, data is scattered

throughout the literature with no consistent frame of work making it difficult to organize the results. The antibacterial properties of withaferin A are particularly effective against acid-fast bacilli and gram-positive microorganisms (Ben-Efraim and Yarden, 1962). Chatterjee and Chakraborti (1980) studied the antibacterial effect of withaferin A and five related withanolides compounds: all molecules were found to be only active against gram-positive bacteria and had low or no effect on gram-negative bacteria. They also reported that 27-deoxywithaferin A and 20-deoxywithanolide D were the most active among the compounds tested (Chatterjee and Chakraborti, 1980). Leaves and root extracts of the Argentinian shrub *Dunalia brachyacantha*, from which withaferin A was also reported (Bravo B. et al., 2001), has shown moderate antimalarial activity *in vivo* and strong activity *in vitro* (Muñoz et al., 2000). *D. brachyacantha* produces withanolide glycosides, named dunawithanines and are particularly active against *Trypanozoma cruzi*, *Leishmania* spp., *Bacillus subtilis,* and *Staphylococcus aureus* (Bravo B. et al., 2001). Withasteroids are often shown to display antibacterial activity against a few numbers of bacterial species, when completely harmless to others. For instance, while 4-deoxywithaperuvin showed a moderate activity against *B. cereus*, *B. subtilis* or *Streptomyces spp* it was inactive against *Micrococcus luteus*, *M. roseus* and *S. aureus* (Abou-Douh, 2002). As for withaferin A, it was tested against *B. subtilis* against which it exhibited strong activity but turned out to be moderate against *Escherichia coli*, *S. aureus* or completely harmless for *Pseudomonas aeruginosa* (Misico et al., 2011). An extensive number of studies investigating the properties of withanolides can be found in the literature (Arora et al., 2004b; Helvacı et al., 2010; Mwitari et al., 2013; Silva et al., 2005). More recently Barthel *et al.* (2016) tested the antibacterial activity of crude extracts from *P. peruviana* leaf homogenates (Barthel et al., 2016b). Crude extracts had strong effects on vegetative cells of *Bt*, *B. subtilis* and *Lactobacillus casei*. On the contrary, the homogenates were inactive against *E. coli*, *Serratia entomophila* and *Pseudomonas putida.* These results were not repeatable with crude homogenates from other solanaceous species such as potato, or brassicaceous plants like rapeseed. To test whether this activity could be related to the withanolides contained in *P. peruviana* leaves, Barthel *et al.* tested the inhibitory effect of purified withanolides extracted from the plant. However, the experiment was carried out on *Bt* spores only, thereby assessing the sole germination rate of spores and, however, not providing any data on vegetative cells.

Immunomodulating properties. As mentioned above, withanolides have been reported to possess immunosuppressive and immunostimulating proprieties (Budhijara et al., 2000). Application of withaferin A on Ehrlich ascites carcinoma (EAC) in mice prompted growth inhibition of the tumorous cells and resistance of the treated mice when exposed to a second challenge on EAC (Shohat and Joshua, 1971). The authors of the study observed a striking proliferation of macrophages around EAC supporting the action of withaferin A by immune activation. Withaferin A has also shown immune suppression activity when tested in rats against adjuvant arthritis (Budhijara et al., 2000). Bähr and Hänsel (1982) showed that the proliferation of murine spleen cell cultures was inhibited by lycium substance A, present in *Withania somnifera* and *Lycium chinense,* which hinted for the immunosuppressive activity of this withanolide (Bähr and Hänsel, 1982). Withaferin A and withanolide E

demonstrated immunosuppressive effects on human B and T lymphocytes and mice thymocytes. The two compounds, and especially withaferin A, which had a broader range, prevented the formation of EAC and E rosettes by normal human B and T lymphocytes (Shohat et al., 1978). There are many studies addressing the immunomodulating effects of withanolides on mammalian cells (Ghosal et al., 1989; Huang et al., 2009; Stan et al., 2008; Yu et al., 2010b) along with comprehensive reviews on the therapeutic potential of withanolide-producing plants (Maurya, 2017).

Physallins are a group of seco-steroids found in solanaceous plants (Garcia et al., 2006). Based on the many physiological aspects affected by physalins in mammalian cells (Soares et al., 2003, 2006; Vieira et al., 2005), Garcia *et al.* (2006) hypothesized that physalins might be able to modify immune reactions in *Rhodnius prolixus* (Garcia et al., 2006). They tested the effect of physalins on *R. prolixus* and showed that infected insects had a higher mortality rate than uninfected ones. Although PO activity and total hemocyte counts in the hemolymph were unaffected, physalin treatments lead to strongly blocked hemocyte micro-aggregation, reduction of nitric oxide production and an enhanced parasitemia in the hemolymph (Garcia et al., 2006). When *R. prolixus* larvae were fed with blood containing physalins and challenged with *Enterobacter cloacae* β-12 their mortality rates were three times higher than the controls. Insects showed also significant differences in lysozyme activity in the hemolymph and a reduced antibacterial activity in inhibition zone assays (Castro et al., 2008b). Hemocyte phagocytosis and micro aggregation are induced for instance by the platelet-activating factor (PAF) and regulated by PAF-acetyl hydrolase (PAF-AH) activities (Machado et al., 2006). In further studies Castro *et al.* showed that the activity of PAF-acetyl hydrolase was significantly enhanced under physalin B treatment and lead to a depression of PAF in hemolymph, thus a reduction in hemocyte phagocytosis and microaggregation (Castro et al., 2009).

A recent study from Barthel *et al.* (2016) demonstrated that the Heliothine species *H. subflexa* showed an increase of phenoloxidase (PO) activity when fed with leaf extracts of *P. peruviana* along with the upregulation of most identified immune genes in this species. Interestingly, the PO activity in its close relative *H. virescens* was unaffected when tested under the same conditions. Furthermore, the expression of the majority of immune genes and immune-related genes were found to be significantly downregulated in RNAseq analysis.

Insect gut bacterial communities

Most animals are colonized by microorganisms, with which they maintain close symbiotic relationships that fundamentally impact their own biology. Many studies have comprehensively reviewed the benefit animals get from association with microbes. They are known to impact the development, physiology and sex determination of the host. They also shape its immunity, enhance the metabolism as well as mediate ecological interactions (Frago et al., 2012; Janson et al., 2008; McFall-Ngai et al., 2013; Moran, 2002; Sudakaran et al., 2017; Voirol et al., 2018). Host-microbe interactions are so complex and widespread that the concept of a superorganism, or « holobiont », has been proposed (Bordenstein and

Theis, 2015; Gilbert et al., 2012; Russell et al., 2014; Vavre and Kremer, 2014; Zilber-Rosenberg and Rosenberg, 2008).

Bacteria benefit to the insect biology at various levels. The benefits provided by microbial symbionts to their insect hosts can be remarkably diverse (Dillon and Dillon, 2004; Dunbar et al., 2007; Moran, 2001; Moran and Telang, 1998; Wernegreen, 2005). Insect bearing endosymbionts rely on them for reproduction, digestion, social interaction through pheromone production, natural enemies protection or immunity and supply with essential nutrient (Gil et al., 2004; Wernegreen, 2002).

Insects living in extreme abiotic environments or in resource-limited niches see their fitness enhanced when inhabited by microbes. This was, among many others, shown in the pea aphid *Acyrthosiphon pisum* where clones had their fecundity and longevity increased under heat stress when infected with endosymbionts (Chen et al., 2000; Dunbar et al., 2007; Montllor et al., 2002).

In insects, symbionts can either be extracellular as it is the case with most gut endosymbionts, or intracellular. In the latter, microbes infest host in specialized cells called bacteriocytes and are well known to inhabit phloem or blood feeding insects, often suffering from a lack of essential nutrients (Douglas, 1998). The best-studied endosymbionts in insects are *Wolbachia* and *Spiroplasma* species (Ahmed et al., 2015; Jiggins et al., 2000; Narita et al., 2007), which colonize the host's reproductive tissues and manipulate its physiology to increase the chances of their own transmission success (Ahmed et al., 2015; Hiroki et al., 2002; Jiggins et al., 2000; Tagami and Miura, 2004). In *Wolbachia* the main effect observed on hosts is a shift in sex ratio in favor of females that can be achieved by male killing (Charlat et al., 2007; Dyson and Hurst, 2004). The alleged benefit for the symbiont is the possibility to end up with a higher number of infected female offspring and rapidly spread into the population (Jiggins et al., 2000). Microbes interfere also in insect communication. In the case of *Drosophila* or tephritid fruit flies, some non-obligatory symbiotic yeasts thriving on ripening fruits emit volatile organic compounds to drive the choice of females' oviposition sites (Christiaens et al., 2014; Davis et al., 2013; Piper et al., 2017b). In locust, the vast swarms that cause important crop losses are promoted by guaiacol, a key pheromone substance found in locust fecal pellets and actually produced by a bacterium living in the insect's gut (Dillon et al., 2000).

Resident gut bacteria are also involved in their host protection against pathogens (Dillon and Dillon, 2004; Flórez et al., 2015). Mutualistic microbes outcompete pathogens by establishing a so-called 'colonization resistance' in the gut preventing them to colonize and infect the host (Dillon and Dillon, 2004). It was seen in *Homona magnanima* larvae, which were susceptible to *Bacillus thuringiensis* (*Bt*) when reared aseptically, but less susceptible when not (Takatsuka and Kunimi, 2000). The strategies by which gut bacterial communities hinder pathogen development rely on bactericidal substances that selectively suppress external bacteria without affecting autochthonous ones (Dillon and Dillon, 2004). Shao *et al.* (2017) recently highlighted this phenomenon in *S. litoralis* in which the very abundant *Enterococcus mundtii* produces an antimicrobial compound active against pathogens like *Listria*, but not against resident bacteria (Shao et al., 2017). Mass action and competition for

resources in the gut, without affecting the host's needs, or stimulating the host's immune system are also strategies of choice for endosymbionts (Berg, 1996; Ferrari et al., 2004; Loker et al., 2004; Scarborough et al., 2005). In addition, insects can associate with microorganisms to defend themselves to a much larger scale. The gall maker *Asteromyia carbonifera* was reported to use a symbiotic fungus to render attacks by the parasitoid wasp *Torymus capite* inactive (Weis, 1982). Similar examples were studied in aphids by Oliver *et al.* (Oliver et al., 2003, 2005, 2006).

Gut microbes are involved in insect-host plant interactions. Plant-insect interactions are complex and mediated by a battery of molecules from each party that interfere with one another. Microorganisms may step in for their host's wellbeing. This had been suggested as associations were already observed in human pharmaceutical studies, in which gut bacterial composition turned out to be an important factor of drug efficiency or toxicity (Haiser et al., 2013; Nicholson et al., 2005). In addition, the major role bacteria play in rumens of vertebrate herbivores (Jones and Megarrity, 1986; Smith, 1992), had also lead to this hypothesis (Clark et al., 2010; Feldhaar, 2011; Felton and Tumlinson, 2008; Frago et al., 2012; Janson et al., 2008). Plants host an impressively high amount of microorganisms such as endophytic fungi. Some of these symbionts live asymptomatically within plant tissues and produce alkaloid-based antifeedant molecules that play a key role in the host defense against herbivores (Arnold and Lutzoni, 2007; Kogel et al., 2006; Koh and Hik, 2007). On the other hand, insects also carry beneficial microorganisms helping them to circumvent plant defenses. The Colorado potato beetle (*Leptinotarsa decemlineata*), a worldwide and longstanding major pest of potato plants, has been reported to host bacteria in its oral secretions that suppress defense mechanisms in tomato (*Solanum lycopersicum*) (Chung et al., 2013). The bacterial community of some specialized niche feeders has drawn high attention from a large scientific community for their crucial importance in host ecological traits. Here it has to be mentioned that aphids harboring large bacterial communities provide universally required nutrients, protect them from parasitism and helping them to overcome plant defenses (Chen and Purcell, 1997; Douglas, 2009; Frago et al., 2012).

- *Gut symbionts enhance nutrient acquisition in plant feeding insects.*

In terms of acquiring essential nutrients, gut symbionts have proven themselves very useful to their insect host. Some microbes can for instance are capable of biosynthesizing limiting metabolites and essential vitamins crucial for the host's growth and development (Dillon and Dillon, 2004; Douglas, 1998; Nakabachi and Ishikawa, 1999). Nitrogen is often the limiting component in insect diets due to the high carbon-to-nitrogen ratio of plant matter (Raven, 1983; Schoonhoven et al., 2005; Waldbauer and Friedman, 1991). One adaptation to this nitrogen-poor diet is to increase the intake of food (Mattson, 1980). This is particularly true for phloem feeders, which suck up an incredible amount of plant sap resulting in excretion of a sugary substance - called honeydew - in order to maintain osmosis (Ashford et al., 2000; Douglas, 2006). Another adaptation is the development of associations with microbes able to fix nitrogen and biosynthesize molecules with high nitrogen content that are easier for insects to metabolize (Nardi et al., 2002; Voirol et al., 2018). In termites for instance it has

been demonstrated that some bacteria fix dinitrogen into ammonia, which is then further assimilated into amino acids and vitamins by other gut symbionts (Brune, 2014; Fröhlich et al., 2007). Indiragandhi *et al.* (2008) showed that many bacteria isolated from the gut of *Plutella xylostella* were able to fix nitrogen *in vitro*, which might indicate that the same mechanisms might occur in other Lepidoptera as well (Indiragandhi et al., 2008).

Another key characteristic of gut symbionts is the ability to assist their host in the breakdown of recalcitrant plant compounds (Genta et al., 2006). The primary cell wall is a complex and heterogeneous meshwork of polysaccharides comprising cellulose, hemicellulose and pectins along with proteins (Cosgrove, 2005; Pauchet and Heckel, 2013). Research on the mechanisms of degradation of plant cell walls focused originally on bacteria and fungi degraders (Bateman and Millar, 1966). Plant cell wall degrading enzymes (PCWDEs) include cellulases, hemicellulases and pectinases (Danchin et al., 2010; Juge, 2006; Kikot et al., 2009). These enzymes were long thought to be absent in the animal kingdom until the first was discovered in termites (Watanabe et al., 1998). Xylophagous insects, such as termites or beetles in the Chrysomeloidea and Curculionoidea superfamilies (Calderón-Cortés et al., 2012) produce these enzymes for the uptake of carbohydrates from their diet. These genes were found to be encoded in crickets (Orthoptera), stick insects (Phasmatodea), cockroaches (Dictyoptera), aphids (homoptera), beetles (Coleoptera) and bees (Hymenoptera) (Engel et al., 2012; Watanabe and Tokuda, 2010). Surprisingly, evidence for PCWDEs in herbivorous insects from the order Lepidoptera is scarce (The International Silkworm Genome Consortium, 2008; Walters et al., 2012; You et al., 2013; Zhan et al., 2011). One of the rare examples known to date is a family of predicted cellulases-encoding genes in the hesperiid butterfly *Lerema accius* (Cong et al., 2015), which wasn't found in its close relative *Achalarus lyciades* (Shen et al., 2017). It is hence suggested that lepidopteran species lost these genes and may rely solely on symbionts for cellulose degradation (Voirol et al., 2018). Recently, a metagenomic study of *P. xylostella's* gut microbiome has shown that it contained thousands of genes from six families encoding for, among others, cellulases (Xia et al., 2017b).

- *Role in defeating plant defenses*.

Gut microorganisms can either be tolerant or degraders of plant allelochemicals. Both these behaviors may come from the extraordinary diversity of biosynthetic capabilities of microorganisms, bacteria and fungi included. Bacteria are known to thrive in the most extreme environments (Rothschild and Mancinelli, 2001). They are also famous for gaining energy in anaerobic conditions by oxidizing highly reduced molecules such as hydrogen sulfide to fix CO_2 and synthesize organic compounds (Grieshaber and Völkel, 1998). This particularity benefits in various ways to insects. Cyanogenic glucosides release cyanide upon herbivory by the action of a β-glucosidase (Vetter, 2000), which is highly toxic for respires (Solomonson, 1981). Because many bacteria are fermenters or do not rely on respiration to yield energy and fix carbon, they might be resistant to the toxic effect of cyanide. Moreover, some cases are known where a bacterial nitrogenase converts cyanide into ammonia (Li et al., 1982). Only microorganisms are able to completely mineralize aromatic compounds comprising phenolics, terpenes or alkaloids (Boll et al., 2014) and in some cases they will

readily use them as their sole source of carbon and energy (Laskin and Lechevalier, 1984). There are many examples of studies attempting to characterize the interactions between microbes and plant allelochemicals but in insects the conclusions are mostly lacking direct evidence. In the highly alkaline lepidopteran gut, phenolic compounds are degraded to quinones and release cytotoxic reactive oxygenic species (ROS). *Enterococcus* species for instance can produce ROS detoxifying enzymes such as the superoxide dismutase or catalases (Xia et al., 2017b). Mountain pine beetles (*Dendroctonus ponderosae)* bear bacteria with a genome enriched in genes coding for decarboxylases breaking down oxalate (Nikoh et al., 2011). In conifer-feeding bark beetles and weevils, gut symbionts were shown to be involved with the detoxification of terpenes (Adams et al., 2013; Berasategui et al., 2017; Boone et al., 2013). The gypsy moth larvae subsist on diets with high content of monoterpenes (Broderick et al., 2004; Powell and Raffa, 1999) and it has been suggested that this is likely due to the presence in the gut of *Rhodococcus,* which degrades these compounds in high alkaline environments (Vlugt-Bergmans and Werf, 2001). Another well understood means of plant defenses are proteinase inhibitors (PI) employed to hinder the digestive activity of insect gut enzymes (Zhu-Salzman and Zeng, 2015). In this, bacterial proteases may contribute to counterbalance the loss of PI-sensitive insect proteases (da Silva Fortunato et al., 2007). These characteristics suggest that co-occurring gut microorganism communities may be crucial to the insect in allowing it to feed on a chemically defended plant.

Gut microbial communities in Lepidopteran species. To date, little is known on the microbiome of insects feeding on foliage such as Lepidopteran larvae. However, this poor perception is more likely due to little evidence rather than an evidence of absence (Broderick et al., 2004; Genta et al., 2006; Mondy and Corio-Costet, 2000). There were various bacteria isolated from caterpillars intestinal tracts (Kim et al., 2013; Steinhaus, 1941), but the study of the gut composition at the community level appeared only in recent work (Hammer et al., 2014; Tang et al., 2012). This is largely due to the advent of molecular tools, high throughput sequencing and metagenomics (Shi Weibing et al., 2010). Most lepidopteran insects feed almost exclusively at larval stage (Hammer et al., 2017) on plant matter (Carter, 1984), and like in most insect groups their gut content is not sterile (Dillon and Dillon, 2004). In addition, toxic plant secondary metabolites were shown to be detoxified by gut microbes in Lepidoptera (Morrison et al., 2009). However, no strict symbiotic interaction has been found in this order so far and it is even suggested by some authors that Lepidoptera may lack a resident microbiome (Hammer et al., 2017). Yet, recent studies have shown that there is a high variability in gut-associated microbes among lepidopteran species (Hammer et al., 2017; Staudacher et al., 2017). This is thought to be due to the simple anatomy of the caterpillar's gut, merely consisting in a tube where food passes quickly and steadily through, normally within a few hours (Tang et al., 2012), making it a challenging environment for bacteria to thrive in. It is known from work on other holometabolous insects that the larval stage may harbor fewer or no microbial symbionts (Lauzon et al., 2009), similar or a completely different community as adults (Arias-Cordero et al., 2012; Wong et al., 2011).

In Lepidoptera, the gut communities are often dominated by the phyla Proteobacteria or Firmicutes along with Actinobacteria and Bacteroidetes also often occurring (Chen et al., 2018; Hammer et al., 2017; Voirol et al., 2018; Xia et al., 2017b). Voirol and collaborators (2018) screened the literature and made an exhaustive summary of the gut microbial composition of 30 lepidopteran species. The most represented families of bacteria were Enterobacteriaceae, Bacillaceae, Pseudomonadaceae, Staphylococcaceae and Enterococcaceae, present in more than 60% of all surveyed species. In the same study, genera such as *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Enterobacter*, and *Enterococcus* were the most common identified. The authors also noted that the lepidopteran gut microbiome was characterized by a high variability between species and among individuals of the same species. Gut bacterial communities also vary greatly throughout the life stages if the larvae (Tang et al., 2012). In many other studies the Enterobacteriales from Porteobacteria dominated gut communities (Hammer et al., 2014; Staudacher et al., 2017; Xia et al., 2017b) and are widely known as laboratory-associated order.

***Heliothis virescens* and *Heliothis subflexa*.** The Heliothinae are a Lepidoptera subfamily counting roughly 365 species of noctuid moths (Matthews, 1999). Major agricultural pests striking at a global scale are part of this group such as the cotton bollworm *Helicoverpa armigera,* the tobacco budworm *Heliothis virescens.* A comprehensive study has re-assessed the phylogeny and classification within heliothines based on a total of 71 known species and 16 outgroup species (Cho et al., 2008). The host plants for at least a quarter of the studied species are known along with the feeding preferences. Cho *et al.* 2008 established that *ca.* 70% of the latter were monophagous or oligophagous feeding on a unique or a few closely related plants (Matthews, 1999; Mitter et al., 1993). Heliothines are for 80% specialized feeders of the Asteraceae or related families such as Lamiaceae, Scrophulariaceae, Polemoniaceae or Solanaceae (Cho et al., 2008). *Heliothis subflexa* is restricted and occasionally a pest of Solanaceae plants (Mitter et al., 1993)**.** The study of Cho *et al.* (2008) is based on the sequence analysis of three genes: two nuclear genes, elongation factor-1-α (EF-1α) a dopa decarboxylase (DDC) and one barcoding region of the mitochondria cytochrome oxydase I (COI). All three genes supported the pairing of *Heliothis virescens* and *Heliothis subflexa,* which confirm the really close relation that exists between these two species. This is fairly important when considering that these two species have become a pair of preference to study the genetic ground of differences in host-plant selection (Sheck and Gould, 1996) or pheromone communication mechanisms (Choi et al., 2005; Groot et al., 2005a, 2005b, 2006; Sheck et al., 2006).

MATERIALS AND METHODS

Insects and rearing. *Heliothis subflexa* was collected near Gainesville, Florida and was provided by the USDA Insect Attractant, Behavior and Basic Biology Research Laboratory. From 1986 until 2006, and their transfer to Jena, the insects were reared at North Carolina State University (NCSU) on a corn-soy blend-based artificial diet (described in Barthel et al. 2016). *Heliothis virescens* was collected near Clayton in North Carolina and provided by NSCU where they had been reared on pinto bean based artificial diet (Barthel et al. 2016). In Jena, both species larvae were reared on a wheat germ and soy flour base (19g agar; 144g Dry Mix F9772 (Frontier Scientific) per 875ml of water) artificial diet until pupation. Adults were placed in plastic cups when they emerged and were fed with a 10% honey water solution (v/v). Adults were subsequently used for single pair mating to keep track of larvae lineage. Matings were done as follows: one female and one male were selected and placed in a paper cup covered with an egg cloth. The cups containing mating couples were then placed in a climate chamber (Snijder) at 26°C, 55 ±10% relative humidity and 16:8 hours (L:D). Egg cloths with eggs were collected every three to four days and placed on Petri dishes with artificial diet. When reaching ~ fourth instar, larvae were separated and transferred into single plastic cups in a climate chamber at 12°C, 55 ±10% relative humidity and 16:8 hours (L:D), to complete their life cycle.

Withanolide extract and withaferin A. Former lab members of the team prepared the withanolide extract used for the experiments. Leaves and fruits from *Physalis peruviana* grown in the greenhouse of the Max Planck Institute for Chemical Ecology in Jena, were frozen at -20°C before desiccation in a lyophilisator at -80°C for two days. A crude extract was obtained after three extractions with methanol (2l) from 5g of the dried preparation. This crude extract was then further processed to separate withanolides from flavonoids and other leaf components in order to obtain a purified extract of the steroid lactones. Withaferin A was purchased from BOC Sciences (#B0084-120734). The activity of both products was verified in *Bt* spores germination bioassays as described in Barthel *et al.* (2016) (Figure III, annexes p. 54, Marie Pauline Sell).

Feeding assays. To assess the effect of withanolides on the gut microbial community of *Heliothis subflexa* and *Heliothis virescens*, larvae were supplemented with a purified withanolide extract or withaferin A to 1ml of artificial diets in 24-well polystyrene plates (VWR International, Darmstadt, Germany). Withaferin A and the purified withanolide extract were dissolved in 40% methanol to a final concentration of 100µg.ml^{-1} (5mg withanolide extract or withaferin A; 4ml methanol; 6ml Milli-Q® water). This concentration was set according to previous data obtained by Barthel *et al.* (2016) and is relevant to naturally occurring concentrations in *Physalis peruviana* fruits (Baumann and Meier, 1993). After pipetting 200µl of treatment solution onto the diet, the plates were allowed to dry under a fume hood for 6h in order to let the solvent evaporate completely. In the control, larvae

were provided with diet supplemented with the solvent only (40% methanol). Late second-instar larvae were sorted and placed into single wells containing the supplemented diet. The 24-well plates were kept together in a clip box and placed in a climate chamber (Snijder) for 48h at 26°C ±10% relative humidity and 16:8 h (L:D). After less than 24h all larvae had molted to third instar. For both species 15 larvae were tested on each treatment (n=45 per species). After 48h, larvae were taken out of the climate chamber and placed on ice in 25ml Falcon tubes in order to immobilize them for gut dissection. Dissection was carried out under sterile conditions and larvae were surface sterilized to remove bacteria and frass from the outer cuticle. To do so, insects were placed successively 10 to 15s in 2ml Eppendorf tubes containing 1% (w/v) sodium dodecyl sulfate (SDS) solution, sterile water, 90% EtOH and sterile water (x2). Guts were dissected in sterile Petri dishes under microscope with dissecting tools sterilized beforehand with bleach and rinsed in sterile water. When a larval gut was flat and tended to sheer when squeezed by the tweezers, another larvae from the same treatment group was chosen and dissected. Dissected guts were placed at once in 2ml Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until DNA extraction. Additionally, three pieces of diet for every treatment condition was sampled and processed the same way the insect guts were.

To monitor the temporal expression profile of immune genes in response to a withanolide supplemented diet, third-instar larvae were placed into single wells containing 1ml of diet and 200µl of treatment (withanolide extract, withaferin A and 40% methanol as a control). The 24-well plates were kept in a climate chamber as described above and three larvae were subsequently collected at each following time points: t+1, t+6, t+12, and t+24 hours, where ‘ t ’ represents the beginning of the experiment. For each species 15 larvae were tested on each treatment. At time point t=0 three larvae which were not exposed to one of the treatments were collected and the 12 remaining larvae were placed on diet. None of the larvae molted during the exposure to treatments. When collected, single larvae were placed in a 2ml Eppendorf tube and snap frozen in liquid nitrogen. All tubes were then stored at -80°C until RNA extraction.

Genomic DNA extraction and purification. DNA was extracted according to the standard CTAB/chloroform method (see detailed protocol in annexes p. 57). Guts were previously grinded in 500µl TES Buffer [10mM tris(hydroxymethyl) aminomethane hydrochloride (TRIS-HCl) pH 8, 10mM ethylenediaminetetraacetic acid (EDTA), 0,2% SDS] and 4µl lysozyme from chicken egg white (100mg/ml) (Sigma-Aldrich). Two beads were added to the tubes and guts were processed in a tissue lyser for 5min at 30Hz after which they were incubated at 37°C for 30 min. An additional incubation step was performed by adding 2.5µl of proteinase K (20mg/ml) (Thermo Fisher Scientific) for 4h at 56°C. DNA was resuspended in 100µl of sterile water and cleaned using the DNeasy PowerClean Pro Cleanup Kit (Qiagen) following the manufacturer’s instructions. A blank extraction was performed in order to later rule out potential contaminants.

Standard PCR, gel extraction and sequencing. Standard PCRs were run on the genomic DNA extracted from the larval guts. These were performed using PfuTurbo polymerase and the 515f/806r primer pair targeting the bacterial 16S rRNA gene. A gel extraction and purification was performed in order to sequence two genomic unknown fragments. Fragments were cut out from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA). Purified DNA was further cloned into a pCR4-TOPO10 vector and transformed One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fischer Scientific). Transformed bacteria were plated on Petri dishes supplemented with ampicillin and displayed at 37°C overnight. For each of the two fragments, eight colonies were selected and incorporated into 5ml Falcon tubes of LB medium using toothpicks, after what they were placed at 37°C overnight. The plasmids containing the fragments of interest were recovered from bacterial cultures using the GeneJET Plasmid Miniprep Kit (Thermo Fischer Scientific) following the manufacturer's instructions. 140ng of purified plasmid DNA and the 515f/806r primer pair were used for the Sanger sequencing reactions. Henriette Ringys-Beckstein, laboratory technician, kindly performed the handling of the last following steps: sample preparation was performed on a Tecan Freedom EVO 150 robotic workstation (Tecan). The sequencing was carried out on a ABI 3730 xl automatic DNA sequencer (PE Applied Biosystems).

Plating of *H. subflexa* gut content. In order to check whether *H. subflexa* harbored bacteria, gut content of one individual were plated out on agar Petri dishes. The insect was surface sterilized as described earlier and dissected under the same sterile conditions. The freshly dissected gut was placed in an Eppendorf tube containing 500µl of previously filter sterilized phosphate-buffered saline (PBS). Then the gut was cautiously grinded with an Eppendorf-adapted pestle and two serial dilutions were made, 150µl from which was pipetted on agar Petri dishes. A Petri dish with plated PBS only served as control. The Petri dishes were placed at 37°C for 24h to allow bacterial colonies to grow (Figure I, annexes p. 52).

Real time quatitative PCR (qPCR). Total RNA was extracted from whole larvae for real-time quantitative PCR (RTqPCR). Each larva was crushed in a tube with a pestle and RNA was extracted with TRIzol® Reagent (Invitrogen Thermo Fischer Scientific). RNA extracts were treated with TURBO DNase (Invitrogen Thermo Fischer Scientific) for 30min at 37°C in order to remove genomic DNA and purified by RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quantity and purity of the RNA was assessed with a N60 NanoPhotometer® (Implen). When a RNA sample appeared suspicious in a qPCR reaction it was additionally analyzed with the RNA 6000 Nano LabChip kit on a 2100 Bioanalyser (Agilent Technologies). cDNA was subsequently synthesized using the VersoTM cDNA synthsis kit (Thermo Fischer Scientific). All RNA concentrations were equalized to 50ng.µl-1 (1000ng) before use of the kit for an input of 2.5ng.μl^{-1} of RNA equivalent in qPCR reactions. All RT-qPCR reactions were carried out using the SYBR® Green 2-Step QRT-PCR Kit (Thermo scientific) following the manufacturer's protocol and were performed in 96-well hard shell plates on a CFX Connect Real-Time System (Bio-Rad). The abundance of attacin-1 and i-type lysozyme transcripts were expressed as RNA molecules

per 1000 RNA molecules of the ribosomal protein S18 (RpS18) reference gene. Primers were designed with Primer3 (version 0.4.0) for RpS18 and attacin-1 in *H. subflexa* and for RpS18 and i-type lysozyme in *H. virescens*. i-type lysozyme in *H. subflexa* and attacin-1 in *H. virescens* were designed by hand (Table 1). Specificity of amplification of each pair of primers was assessed by dissociation curve analysis along with an efficiency assay using the CFX Manager software (version 3.1). To assess the efficiency, a standard curve was first established from Cq-values of qPCRs run on dilution series of cDNA. The efficiencies were calculated thanks to the integrated calculator of the CFX Manager software (version 3.1) and based on the slope of the standard curve. Controls included non-template control (NTC) and RNA template (NRT). Three technical replicates were carried out for each reaction.

Table 1 List of the primer pairs designed for the study of attacin-1 and i-type lysozyme expression over time by RT-qPCR.

Species	Oligoname	Sequence	Design	Efficiency
Heliothis virescens	RpS18_Vir_for RpS18_Vir_rev	GCGTGCTGGAGAATGTACTG (20) TATTGCCTGGGGTTGGACAT (20)	Primer3	1.78
	Att_Vir_for Att_Vir_rev	CAGCAATTACGCCAGACTGC (21) TTATCATTATGGAACAGGTTCGCG (24)	By hand	1.78
	Lyso_Vir_F Lyso_Vir_R	TTGAAGACTGCGCCAAAGAC (20) CGTGGTTGCAATCCTTTCCA (20)	Primer3	2,12
Heliothis subflexa	RpS18_Sub_for RpS18_Sub_rev	TATGCGTCACTACTGGGGTC (20) TCCTCTCCTGCCAGTAGTCT (20)	Primer3	1.75
	Att_Sub_F Att_Sub_R	GACAACCATGACGTCTCAGC (20) GAGTGCGAGCTACCCCTAAT (20)	Primer3	1.96
	Lyso_Sub_for Lyso_Sub_rev	CCGTTGCAATCCTTTCCG (18) TGATAACCCTGCCTGATGACG (21)	By hand	1.88

DNA sequencing. Genomic DNA extracted from the guts was pooled with equal input of DNA quantity from each sample to a final concentration of 22.5ng.µl^{-1}. An equivalent of 20µl from each pool was sent to MR DNA (Shallowater, TX, USA) for library preparation and sequencing of the bacterial 16s rRNA V4 variable region. Amplicons were prepared with the barcoded forward primer 515f (5' - GTGYCAGCMGCCGCGGTAA - 3') and reverse primer 806r (5' - GGACTACNVGGGTWTCTAAT - 3') (Caporaso et al., 2012). Libraries were pooled and the sequencing was performed on an Illumina MiSeq platform using the 2 x 300bp paired-end sequencing technology. Sequences were processed using MR DNA pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX, USA) and deposited on BASESPACE (basespace.illumina.com).

Data processing. Dr. Shantanu Shukla recovered the sequences from BASESPACE and processed the libraries in QIIME2 (2018.6) (Caporaso et al., 2010). The paired-end sequences were demultiplexed using the default cutoff of quality score=2 and trimmed at 249 and 250bp so that any shorter sequence was removed before further analysis. Unlike most previous methods that clustered sequences to OTUs, here sequences were compared to one another using the DADA2 sample inference method (version 1.9.1) (Callahan et al., 2017, 2018). Taxonomy of sequences were assigned using the Greengenes 16S rRNA gene

database (version 13_8) (DeSantis et al., 2006) using a Naïve Bayes classifier. When using universal bacterial primers like the 515f/806r primer pair amplification of non-bacterial RNA gene sequences might happen (Ghyselinck et al., 2013; Hanshew et al., 2013). Here, all non-bacterial sequences including insect sequences, chloroplast, mitochondria and any unassigned reads were discarded from the data set prior to downstream analysis. When extracting genomic DNA from each sample, an additional blank extraction was carried out and sequenced as well. During the analysis, any sequence which proportion was higher in the blank than any other sample was removed. The occurrences of bacterial sequences per sample were highly divergent between the two species with also a high heterogeneity within *Heliothis subflexa* samples. While none of the samples in *H. virescens* had less than 22687 counts, six samples in *H. subflexa* harbored less than 1000 counts. For this reason the samples were not rarefied for subsequent diversity analyzes.

Diversity analysis and observed richness. The outcome of the QIIME2 analysis consisted in an Excel sheet, which displayed for each sample the number of bacterial sequences. This data sheet was used to assess the diversity and richness in gut bacterial communities among species and in different treatment conditions. The α-diversity was calculated at family level for each insect species and each treatment based on the Shannon-Wiener diversity index that was calculated for all 30 samples.

Statistical analysis. Data was analyzed in R (version 3.5.1). The difference of diversity and richness between *H. subflexa* and *H. virescens* gut communities were statistically analyzed using a Wilcoxon rank sum test. Then, a Kruskal-Wallis test was performed to assess whether diversity and richness were affected by the treatments. The influence of a withanolide extract and withaferin A on gene expression levels over time was statistically analyzed as follows: first the influence of treatments and time on expression levels of a given gene in a given insect species was assessed in an ANOVA, when data was normally distributed. If not, gene expression, taken as the variable to explain, was transformed by a log function or computed to its square root and was later used as such in the ANOVA. Treatments and time points were used as explanatory variable with respectively three and five modalities. Secondly, multiple comparisons were performed using pairwise t-tests followed by a FDR correction when analyzing the effect of treatments or time points separately on the genes expressions levels.

RESULTS

Effects of a withanolide extract and withaferin A supplemented diets on *H. virescens* and *H. subflexa* gut microbiota.

This experimental design aimed at shedding light on the effect of a withanolide extract and withaferin A on the bacterial gut composition of two closely related Lepidoptera species. In order to assess the integrity of the bacterial DNA extracted from the guts, a handful of randomly selected samples were briefly analyzed via gel electrophoresis (Figure 3). The amplicon has an expected length of *ca*. 350bp depending on the bacterial species. The positive control consisted in an *E. coli* colony picked with a toothpick and dipped into 100µl of PBS, from which 1µl was used for the PCR reaction. The negative control was a non-template control. In *H. virescens* the three chosen samples resulted in large and bright bands of the expect length (300bp), whereas in *H. subflexa* the bands ranked at 500bp and were less bright. The gDNA extracted from the diet sample resulted in a band at 300bp as well, very similar to the bands in *H. virescens.* So as to identify the sequences amplified by the primers in both species, a piece of gel containing the corresponding band was cut out, purified with the and sequenced after cloning the amplicon into a pCR4-TOPO10 vector. The sequences obtained were identified using BLAST homology searches. Most cloned sequences originating from *H. virescens* matched fragment of the bacterial 16S rRNA gene of *Enterococcus* species. But interestingly, all sequences in the sample from *H. subflexa* were identified as a fragment of the S18 rRNA gene of the insect host and other close Lepidoptera species (*H. zea*, *H. armigera* at respectively 97% and 94% of similar identity). In order to assess if this outcome was due to a mispriming of the primer pair or an absence of bacterial DNA in the samples, two additional PCRs were carried out. First, a DNA quality control was performed by PCR followed by a gel electrophoresis on all 33 samples using two primer pairs targeting EiF4a and RpS18, two housekeeping genes conserved in *H. virescens* and *H. sublflexa* (Figure II, annexes p. 52). Secondly, a standard PCR targeting a larger fragment of the bacterial 16S rRNA gene was performed using the FD1/RP2 primer pair (Figure 3). Here again, all samples from *H. virescens* lead to clear and large bands at the expected length (1400bp) whereas no bands were detectable in the six chosen samples from *H. subflexa*. These preliminary results showed that third instar *H. subflexa* larvae might bear very low amounts of gut bacteria.

Variability of gut bacterial communities between the two species. The table of sequence counts obtained by the QIIME2 analysis from Dr. Shantanu Shukla allowed a quick confirmation of the trend observed with the standard PCRs. In *Heliothis virescens* the number of counts per sample ranged from 22687 to 33617 whereas in *Heliothis subflexa* six samples out of 15 had less than 1000 counts, some as low as 388. The samples in *H. subflexa* bore a rather high amount of host RPS18 gene, which confirmed the outcome of the sequencing performed in the lab.

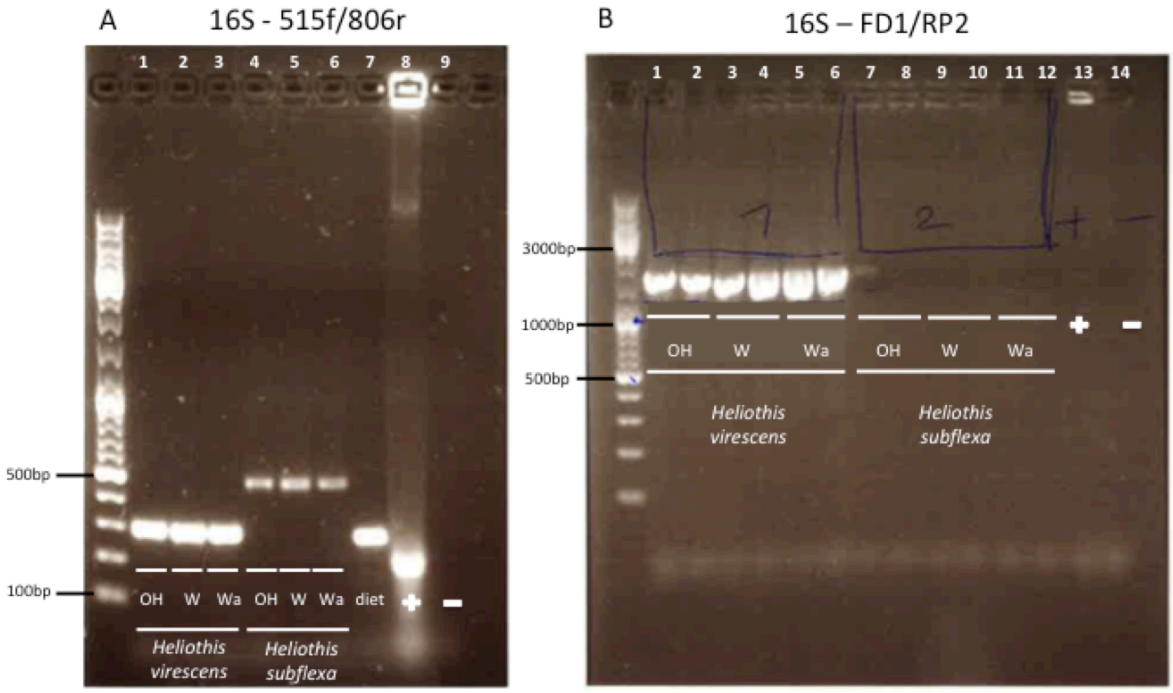

Figure 3 Gel eletrophoresis of bacterial 16S rRNA fragments from selected gut genomic DNA samples of *H. subflexa* and *H. virescens*. (A) Each fragment is targeted with the 515f/806r primer pair. From the left to the right: the first three lanes represent one randomly selected sample out of the five of each treatment conditions in *Heliothis virescens*, the three following lanes are from *H. subflexa,* lane n°7 was obtained using gDNA extracted from the feeding assay diet supplemented with withaferin A. (B) Fragments were targeted with the FD1/RP2 primer pair. The first six lanes are samples from H. virescens while the six following are from H. subflexa. For each species pool 1 and pool 2 of each treatment were chosen for quality check. Treatment conditions are abbreviated as follows: OH methanol, W: withanolide extract, Wa: withaferin A. The positive control, indicated by a ' + ', was a bacterial colony dipped into 100µl of PBS, from which 1µl was used as template DNA for the PCR reaction. The negative control, indicated by a ' - ' is a non-template control. The molecular-weight size marker has the form of a 100bp DNA ladder and is located in the leftmost lane.

The difference in richness of bacterial phylotype between the two species was striking. The bacterial community in *H. virescens* was composed of only two phyla: the Firmicutes, by far the most abundant, accounting for 99.96% of the total bacterial community, and the Proteobacteria accounting for 0.04% (Figure 4, A). In *H. subflexa*, a total of 11 Phyla were represented with only four of them accounting each for more than 0.01% of the total community. Among them, the Firmicutes were also the most dominant phyla, followed by the Proteobacteria (1.58%), the Actinobacteria (0.34%) and the Bacteroidetes (0.11%) (Figure 4, B). At the family level, Enterococcaceae, prevailing at 99.89% in *H. virescens* and 84.57% in *H. subflexa*, dominated the gut communities of both species (Figure 5). In both cases all sequences of Enterococcaceae bacteria belonged to the sole genera *Enterococcus* with two species identified: *Enterococcus casseliflavus* and *Enterococcus cecorum*. Interestingly, *E. casseliflavus* was only present in *H. virescens* and *E. cecorum* only in *H. subflexa* representing respectively 0.03% and 0.11% of *Enterococcus* found in their guts. The remaining 99.97% and 99.89% belonged to unidentified species. Observed richness in *H.*

subflexa was significantly higher than in its sister species ($P < 0.001$) with a total of 70 bacterial families co-occurring in the gut, whereas *H. virescens* counted only five. The α-diversity based on the Shannon-Wiener index (which was calculated for each sample) was hence significantly higher in *H. subflexa* than in *H. virescens* guts ($P < 0.005$).

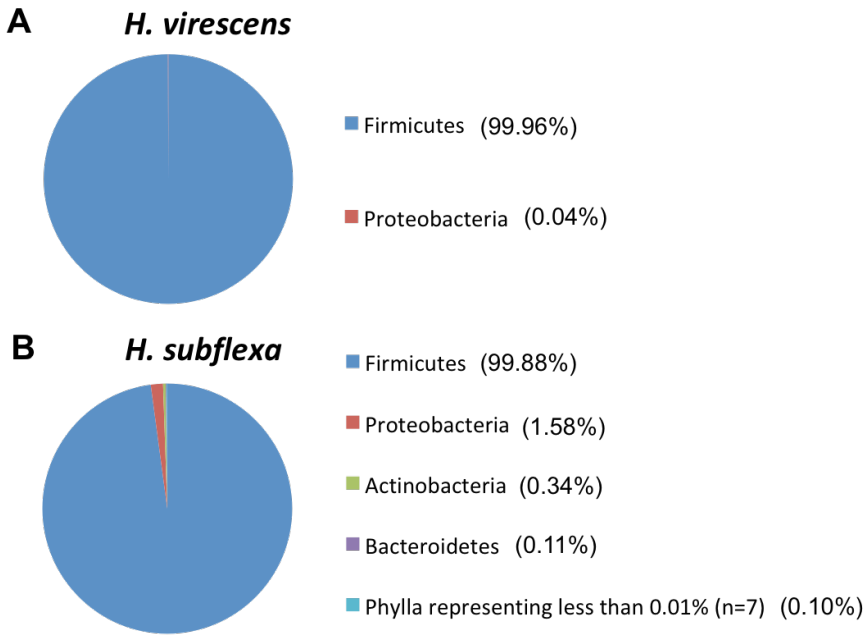

Figure 4 Gut bacterial community structures in *H. virescens* and *H. subflexa* at phylum level. The pie charts are constructed from bacterial 16S rRNA gene sequences, gathering the data in both species from all treatment conditions together. Phyla representing less than 0.01% of the total relative abundance were computed together. Relative abundance is shown as a percentage of total count per species. (A) Gut composition of *H. virescens* consisting of only two phyla. (B) Gut composition of *H. subflexa* shared by 11 phyla.

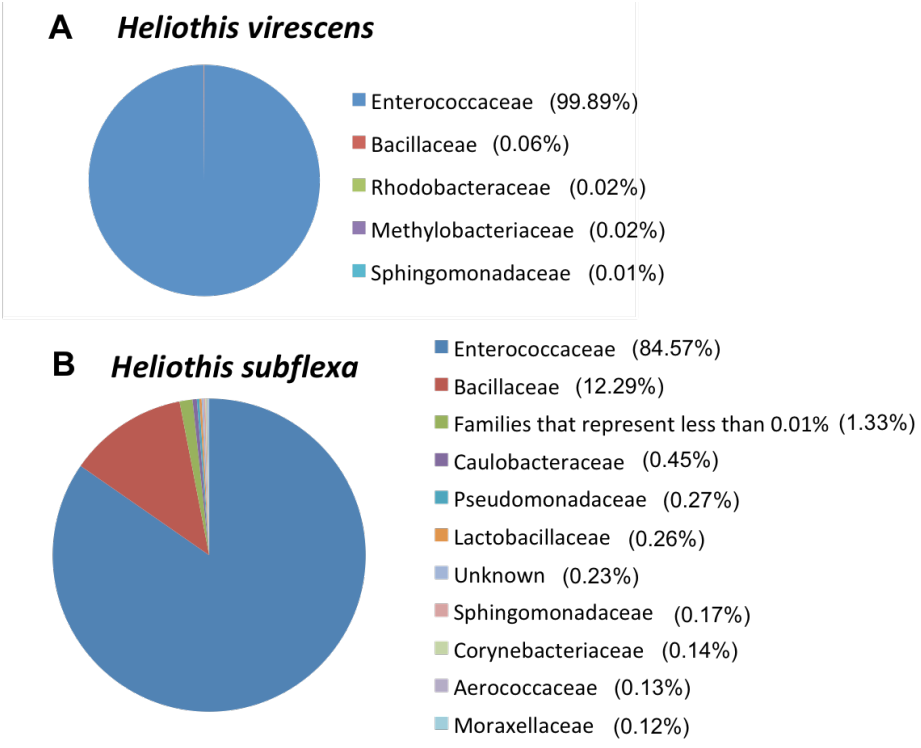

Figure 5 (former page) Gut bacterial community structures in *H. virescens* and *H. subflexa* at family level. For pie charts construction refer to Figure. (A) Gut composition of *H. virescens* consisting of only five families dominated at 99.89% by Enterococcaceae. (B) Gut composition of *H. subflexa* shared by 70 families and dominated by Enterococcaceae. 60 families represented less than 0.01% of the total gut community.

Impact of the withanolide extract and withaferin A on gut bacterial composition. The treatments had no significant influence on the richness and diversity of gut bacterial communities in both species. The Shannon-Wiener index was calculated at the family level for each gut and was used to estimate the gut diversity for a given treatment (n=5). In *H. subflexa* the diversity and richness varied greatly among samples compared to the gut diversity and richness of *H. virescens* (Figure 6). In *H. subflexa* the richness was not influenced by the withanolide extract or withaferin A treatments (P = 0.7326) and nor was the diversity (P = 0.9324). The same observations were made in *H. virescens* gut diversity (P = 0.09285) and richness (P = 0.3035). This being said, there is a slight variation in the relative proportion of bacteria in both species although it might be anecdotic in *H. virescens*. In *H. subflexa* however, bacteria from the order Bacillales, which accounted for roughly 0.27% in the control samples, increased in proportion to 4% in the withanolide extract samples and represented 31% of the whole gut community in the withaferin A samples (Figure 7). Simultaneously, the Lactobacilles encompassing genera such as *Enterococcus*, *Lactococcus* and *Streptococcus,* decreased from 98.5% of total bacterial community in the control samples to 67.5% in withaferin A samples (Figure 7).

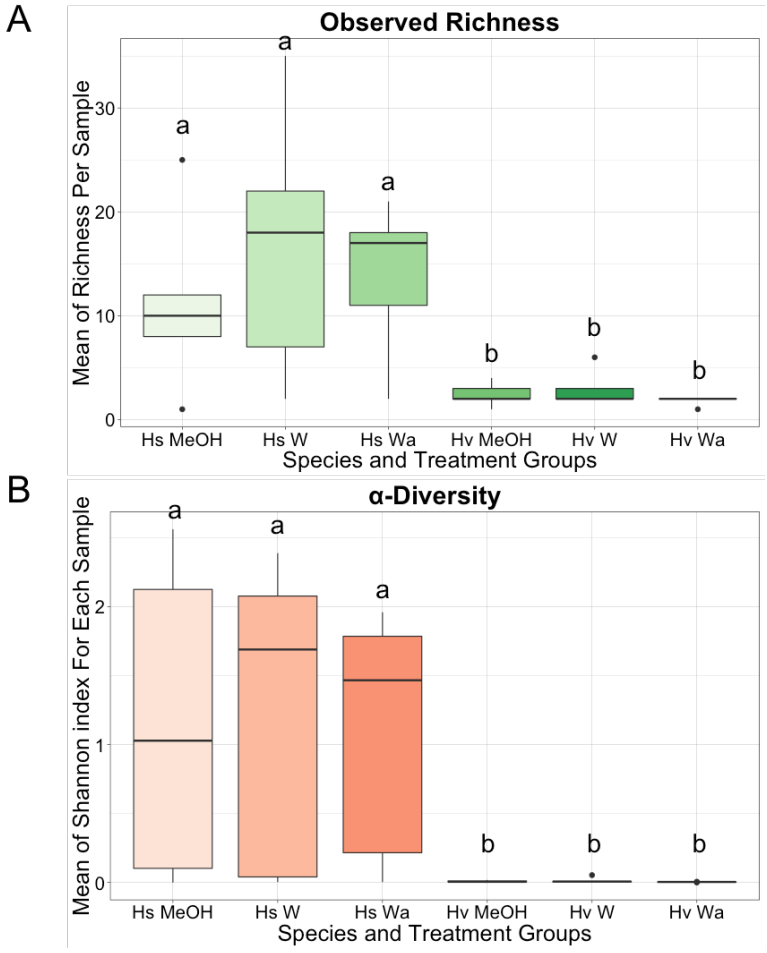

Figure 6 Effect of withanolide extracts and withaferin A on observed richness and diversity of gut bacteria in *H. virescens* and *H. subflexa*. (A) Observed richness per sample is taken as the mean of richness calculated for each gut (n=5). Richness per sample was calculated as the number of families present in the gut of a larvae fed on a given supplemented diet. (B) The α-diversity per treatment was calculated as the mean of the Shannon index for each sample (n=5). Differences of richness ($P < 0.001$) and diversity ($P < 0.005$) between the two species were assessed using a Wilcoxon rank sum test. Effect of treatments were analysed using a Kruskal-Wallis test. Different letters indicates significant differences.

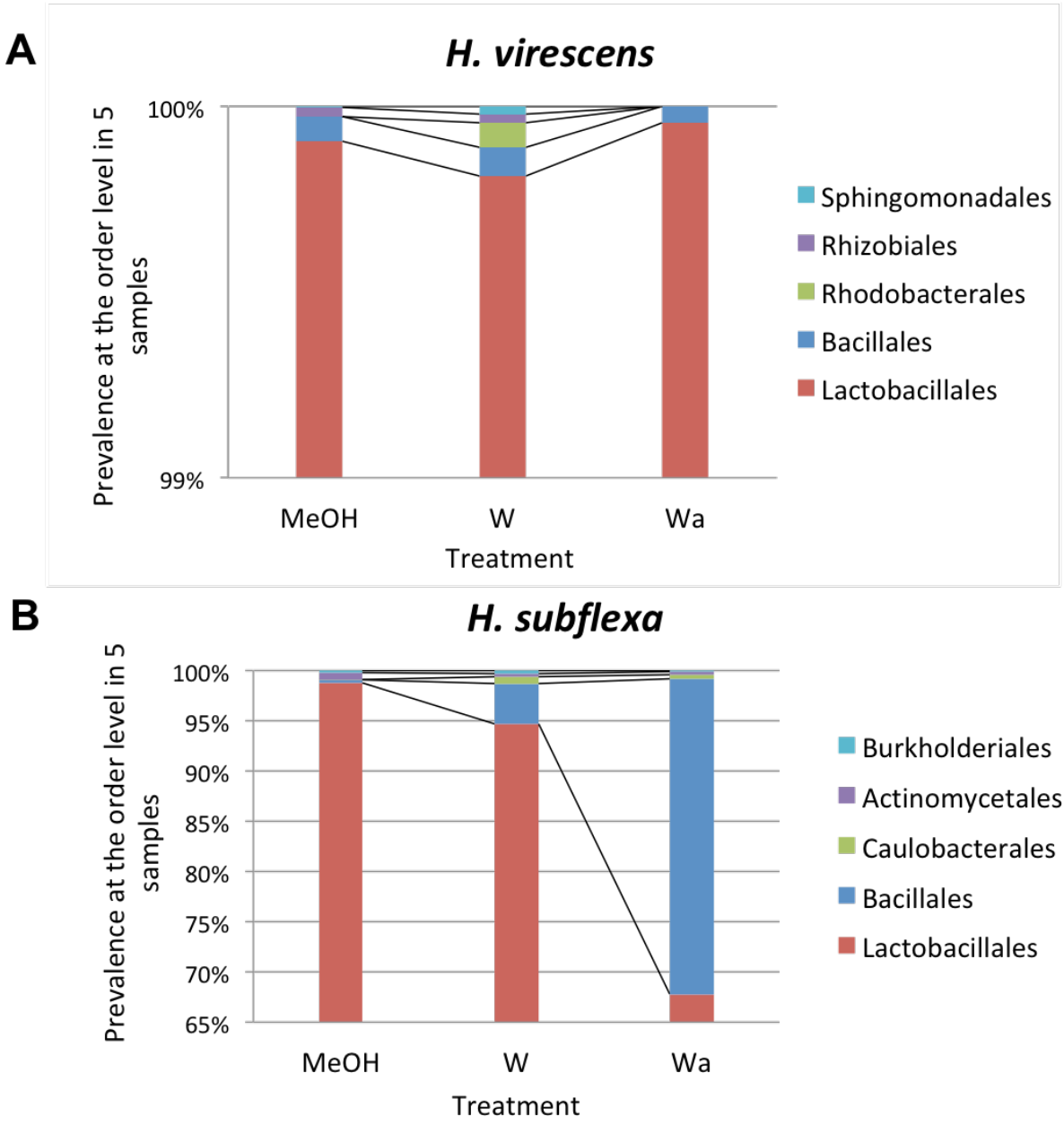

Figure 7 Effect of withanolide extracts and withaferin A on relative abundance of gut bacterial communities in *H. virescens* and *H. subflexa*. Bacterial 16S rRNA gene from gut communities in (A) *H. virescens* and (B) *H. subflexa* third instar larvae were sequenced after feeding for 48h on diet supplemented with a withanolide extract (W), withaferin A (Wa) and methanol (MeOH, as a control). Relative abundances of bacteria were calculated at the family level in each treatment condition (n=5).

Impact of the withanolide extract and withaferin A supplemented diet on the temporal expression of two immune genes.

Expression levels of i-type lysozyme. The second part of the project aimed at revealing if the withanolide extract and withaferin A have an influence on the expression levels of two immune genes in *Heliothis subflexa* and *Heliothis virescens* at five time points over a 24h timeline. The relative expression levels of i-type lysozyme and attacin-1 were obtained by RT-qPCR and the overall results are shown in Figure 8. We showed that i-type lysozyme had very low levels of expression in both species at all time points, not exceeding 0.25 and 3.5 molecules per 1000 molecules of reference gene RPS18 in *H. subflexa* and *H. virescens* respectively. i-type lysozyme was a linear function of time in both species with a stronger relation in *H. subflexa* ($F = 16.2$, $P < 0.001$) than in *H. virescens* ($F = 10.1$, $P < 0.005$). When looking at the expression levels of this gene over time in single treatments, there is a clear increase of expression levels in *H. subflexa* larvae after 24 hours on the withanolide extract ($P < 0.005$, Figure 9, B) and withaferin A ($P < 0.05$, Figure 9, C). However, there was no significant difference at a given time point between the control and the two treatments (Figure 9, D). For instance in *H. subflexa*, even though the mean expressions of i-type lysozyme for both treatments at time point 24h seem differing from the control (non-overlapping error bars, Figure 8, B), the statistical analysis failed to show significant differences ($P = 0.053$, Figure 9, D). It should also be noted that quite surprisingly, i-type lysozyme expression level increases as a linear function of time points in methanol controls of *H. virescens* ($P < 0.005$, Figure 8, A), which makes the effect of the two treatments on the observed increase inconclusive (Figure 10, p. 29).

Expression levels of attacin-1. Attacin-1 was much more expressed than i-type lysozyme but still at lower levels than the reference gene RPS18 (Figure 8). Unlike the lysozyme, attacin-1 was ten times more expressed in *H. virescens* (Mean = 125.2, StdE = 32.95) than in *H. subflexa* (Mean = 12.5, StdE = 3.4) ($t = -7.6$, $P < 0.001$). The expression levels of attacin-1 were not influenced by the treatments over time (in *H. virescens*: ANOVA, $F = 1.9$, $P = 0.175$; *H. subflexa*: ANOVA, $F = 0.22$, $P = 0.63$). After one hour on diet, the mean of *H. virescens'* attacin-1 was higher in the withanolide extract treatment than in the methanol control treatment (Figure 8) but this difference turned out to be not significant ($P = 0.083$, Figure 10, p.29).

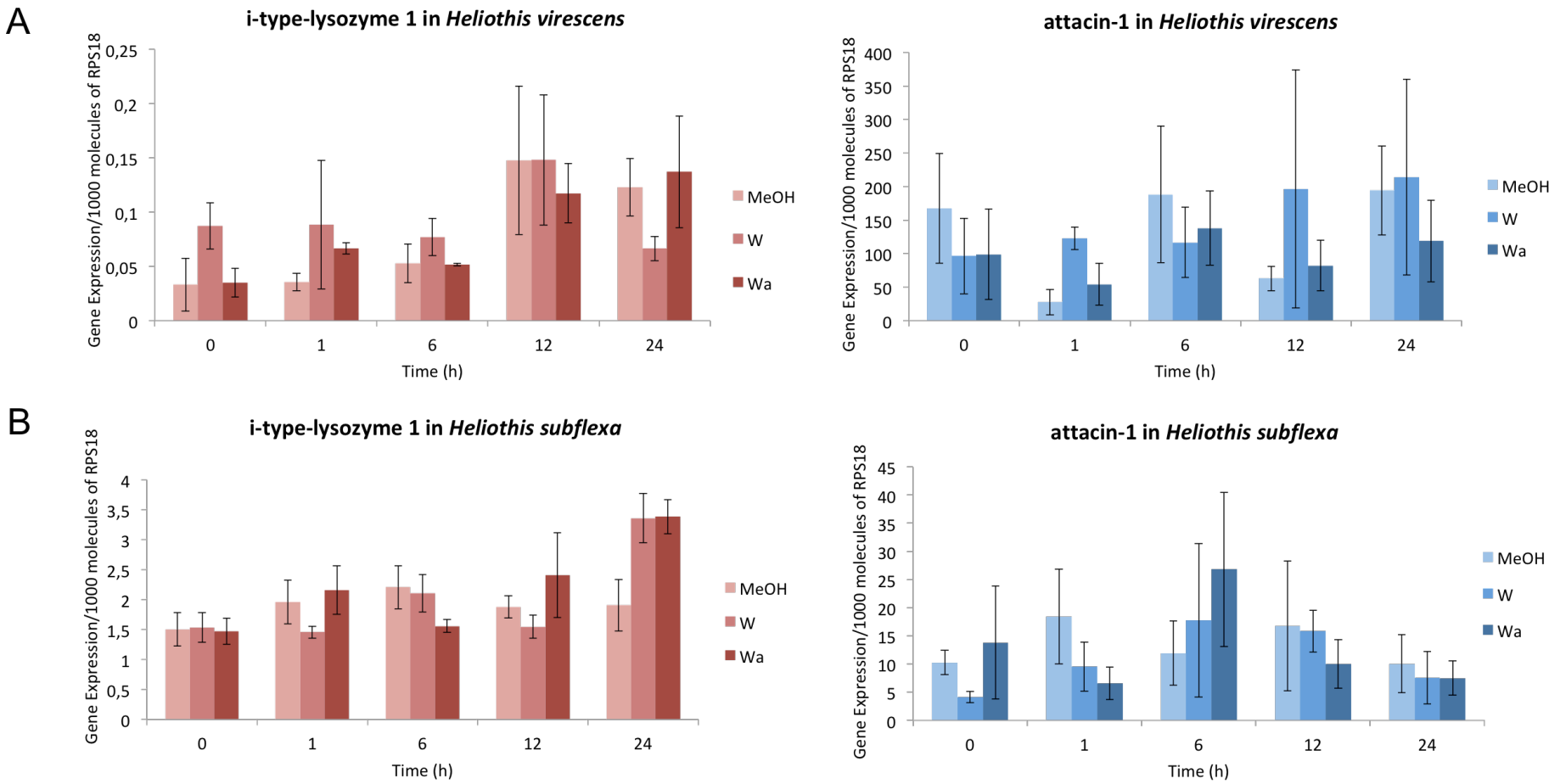

Figure 8 Effect of withanolide extracts and withaferin A on the expression of i-type lysozyme and attacin-1 over a 24 hours timeframe. Gene expression levels of i-type-lysozyme (reds) and attacin-1 (blues) in (A) *H. virescens* and (B) *H. subflexa*. Expression levels of genes of interest are given in numbers of transcripts per 1000 molecules of RpS18 reference gene. Larvae were placed on diet supplemented with either methanol (MeOH, as a control), withanolide extract (W) or withaferin A (Wa) for one, six, 12 and 24 hours after which they were collected and snap frozen in liquid nitrogen for RNA extraction (n=3). When the experiment was started, larvae, which were not exposed to any treatment, were collected and snap frozen. Effect of time points and treatments, used as explanatory variables, on the expression levels were analysed using an ANOVA. i-type lysozyme was a linear function of time in *H. subflexa* ($F = 16.2$, $P < 0.001$) and in *H. virescens* ($F = 10.1$, $P < 0.005$), treatments were not significantly affecting expression levels. attacin-1 expression was not affected by treatments or time points in either of the two species (in *H. virescens*: ANOVA, $F = 1.9$, $P = 0.175$; *H. subflexa*: ANOVA, $F = 0.22$, $P = 0.63$).

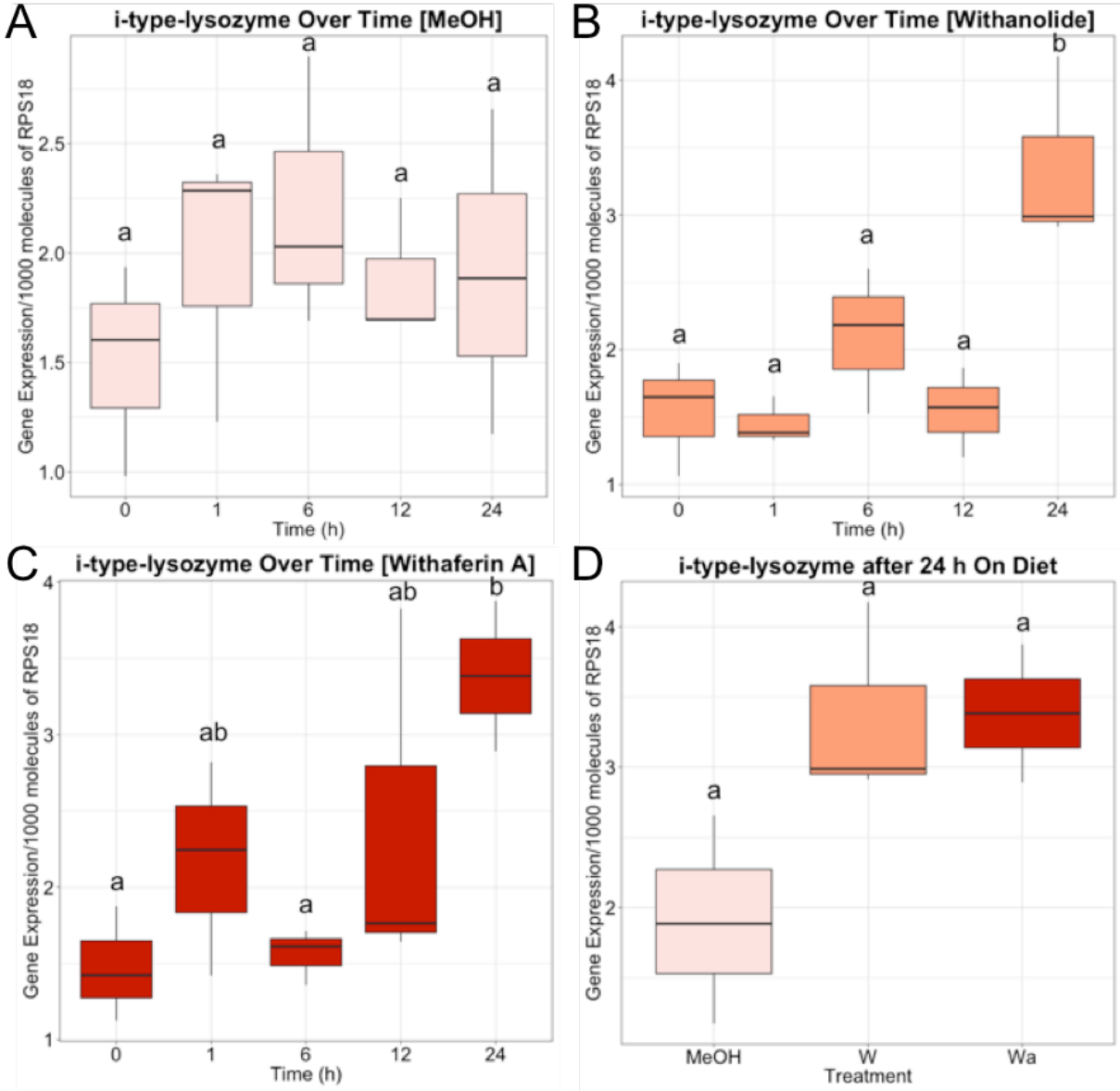

Figure 9 Expression levels of i-type lysozyme in *H. subflexa*. Boxplots showing the expression levels for i-type-lysozyme at each time point for (A) methanol (MeOH, as a control), (B) withanolide extract (W), (C) withaferin A (Wa) and (D) for all treatments at time point t+24h only, where ' t ' represents the beginning of the experiment. Expression levels of genes of interest are given in numbers of transcripts per 1000 molecules of RpS18 reference gene. Multiple comparisons were performed using pairwise t-tests followed by a FDR correction to analyze the effect of treatments or time points separately on the genes expression levels. Different letters above the boxplots indicates significant differences. The bottom and the top of the boxplots represent the 25th and 75th percentile, respectively. The horizontal line represents the median value. The whiskers represent extreme the values.

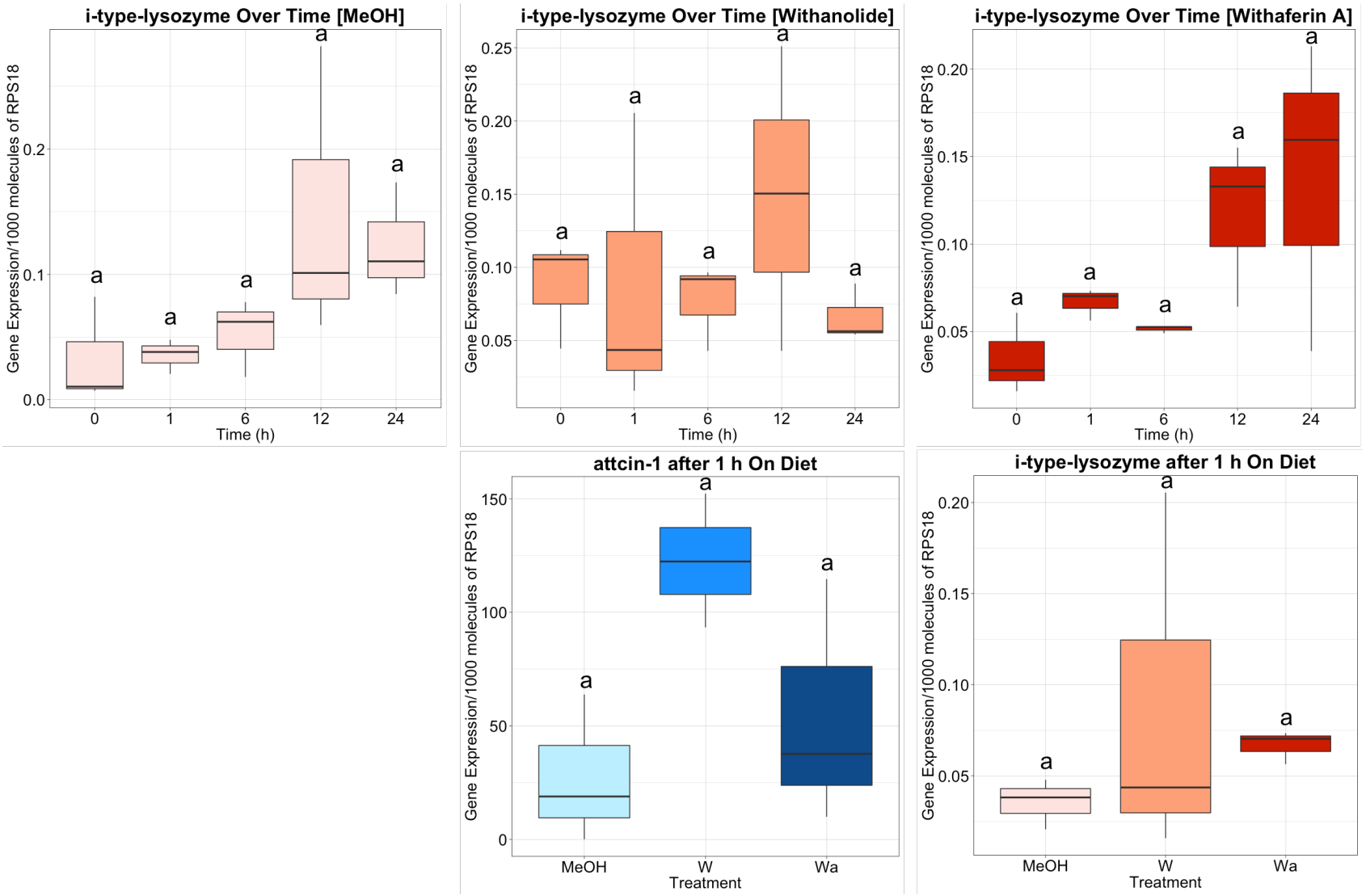

Figure 10 Expression levels of i-type-lysozyme and attacin-1 in *H. virescens*. Boxplots showing the expression levels for i-type lysozyme at each time point for (A) methanol (MeOH, as a control), (B) withanolide extract (W), (C) withaferin A (Wa) and (E) for all treatments at time point t+24h only, where ' t ' represents the beginning of the experiment. (D) Expression levels of attacin-1 for all treatments at time point t+24h. Expression levels of genes of interest are given in numbers of transcripts per 1000 molecules of RpS18 reference gene. Multiple comparisons were performed using pairwise t-tests followed by a FDR correction to analyze the effect of treatments or time points separately on the genes expression levels. Different letters above the boxplots indicates significant differences. The bottom and the top of the boxplots represent the 25th and 75th percentile, respectively. The horizontal line represents the median value. The whiskers represent the extreme values.

DISCUSSION

In this study was assessed the effect of a purified extract of withanolides and withaferin A on two biological aspects of the specialist *H. subflexa* and its close generalist relative *H. virescens.* First, the potential effect of these plant secondary metabolites on gut bacterial communities was examined. Secondly, the effect of the same treatments on the expression levels over a 24 hours time frame of two immune-related genes was evaluated: i-type lysozyme and attacin-1. These two studies were done by feeding assays: artificial diet was supplemented with 100µg.ml^{-1} of treatment solution, from which insects were allowed to feed for 48 hours in the first assay and four time length ranging from one to 24 hours in the second. The concentration of 100µg.ml^{-1} was set according to results obtained by Barthel *et al.* (2016) and natural occurring titers of withanolides in solanaceous plants (Baumann and Meier, 1993).

Effects of the withanolide extract and withaferin A supplemented diets on *H. virescens* and *H. subflexa* gut microbiota.

This study is the first to date to investigate the bacterial gut composition of laboratory-reared *H. subflexa* and to compare the impact of plant secondary metabolites on the gut microbiome of two closely related lepidopteran species, one being a host-plant specialist and the second one a generalist feeding on a broad range of host plants.

The data recovered from the sequencing database BASESPACE was kindly processed by Dr. Shantanu Shukla. A table showing the number of sequence counts per bacterial 16S rRNA gene was obtained from the QUIIME2 analysis. It was used to further analyze the gut microbial composition of both insect species and the impact of the withanolide extract and withaferin A on its diversity.

Some samples contained high amounts of chloroplast sequences. This is not unusual when using universal primers such as 515f/806r (Ghyselinck et al., 2013; Hanshew et al., 2013). The source of chloroplast sequences might have come from the diet, which was a wheat germ and soy flour based preparation (Dry Mix F9772, Frontier Scientific). All non-bacterial sequences were therefore removed from the analysis. The blank-extraction control had an incredibly high amount of *ca.* 50K sequences. The CTAB extraction method for gDNA that was used in this study is known to be a source of contamination, stemming from the use of self-made buffers and high numbers of working steps (Ettenauer et al., 2012; Mitchell and Takacs-Vesbach, 2008), however, ensuring the best sample preservation in terms of DNA quantity (Mitchell and Takacs-Vesbach, 2008). A first attempt to extract genomic DNA from guts with the PowerSoil Kit (MoBio) was performed but too low concentrations of DNA were obtained. Sequences present in higher numbers in the blank control were removed from the gut samples to rule out potential contaminations.

Although the structures of the gut communities were widely divergent between both insect species, no significant effect of the treated diets was observed on intraspecific diversity and richness. *H. virescens* bore a really homogeneous gut community almost exclusively composed of the sole Enterococcaceae family (99.89%). While only four other

families of bacteria shared the remaining 0.11% of the gut community in *H. virescens*, 70 families were present in *H. subflexa*. Low quantities of gut bacteria along with low diversities have been described in Lepidoptera before (Hammer et al., 2017; Mason and Raffa, 2014; Shannon et al., 2001; Staudacher et al., 2017; Vilanova et al., 2016b; Whitaker et al., 2016). This might come from the particular anatomy and physiology of caterpillars gut. Their intestines merely consists of a simple tube with no distinctive separation between the midgut and the for- and hindgut (Figure IV, annexes p. 54)(Appel and Heidi, 2017). Caterpillars are known to be active feeders and the speed at which the food bolus passes through their tract is high (estimated of *ca.* two hours in *M. sexta*) (Brinkmann and Tebbe, 2007). Structures like pouches or diverticula caeca have been shown to be important for the development and settlement of specific symbionts in insects (Voirol et al., 2018), but to date none of these were reported in Lepidopera (Hammer et al., 2017). Moreover, the midgut in Lepidoptera has a strong alkaline pH compared to other taxa, often higher than 10 (Dow, 1992; Johnson and Felton, 1996) with values as high as 12 (Dow, 1984). This makes caterpillar guts a quite hostile environment for bacterial growth (Appel and Heidi, 2017) which might also explain their relatively low richness in symbionts.

This being said, the number of sequences obtained in *Heliothis subflexa* varied greatly among samples. Some had numbers of sequences as low as 388 and 423 (Table I, annexes p. 53) while the highest ranged at 30695 counts. The family tag number used in single pair mating for the rearing was traced for the individual larvae used in the samples (Table II, annexes p. 55). However, there was no pattern of redundant family that could explain the low numbers of sequences. All larvae molted from late second to third instar during the experiment. In insects, the foregut and hindgut are shed during this process, but not the midgut (Engel and Moran, 2013), which is the largest compartment in lepidopteran larvae's gut (Figure IV, annexes p. 54). Furthermore, larvae usually eat their own exuviae after molting (Mira, 2000), behavior by which they ensure the vertical transmission of bacteria from one instar to the next one (Broderick and Lemaitre, 2012; Voirol et al., 2018). In addition, each sample was a pool of genomic DNA originating from three different guts. Yet, molting is an intense process that causes drastic changes in the stable physiology of the feeding larval stage (Klowden, 2013). It is hence not impossible that a delay in the development timing of *H. subflexa* resulted in lower bacterial population levels. Even so, these results hint towards the consideration that *H. subflexa* might not have a resident gut microbiome but rather a transient gut bacterial community or just reflect the gut composition of laboratory reared insects.

Consistent with previous studies, it was found that the genus *Enterococcus* was dominant in both the specialist and generalist insects (Staudacher et al., 2017; Tang et al., 2012; Vilanova et al., 2016b). Bacteria belonging to the genus *Enterococcus* have been associated with the gut microbiome of families such as Noctuidae and Sphingidae in Lepidoptera (Visôtto et al., 2009) and are considered as generalist symbionts as they have been widely encountered in insects (Geiger et al., 2009; Tang et al., 2012; Tholen et al., 1997; Vilanova et al., 2016b). It has also been suggested by some authors that *Enterococcus* species are associated with laboratory populations of insects (Staudacher et al., 2017). This genus was completely absent from their field collected populations and numerous studies

using lab populations as well, reported this genus as being the dominant one in the insects guts (Brinkmann et al., 2008; Broderick et al., 2004; Hammer et al., 2014; Tang et al., 2012). Although a handful of studies using field-collected caterpillars have shown that their insects gut communities were also dominated by *Enterococcus* species (Thakur et al., 2015; Vilanova et al., 2016b), they yet tend to be more diverse and balanced than in field populations. This underpins the importance of using field-collected insects in studies such as the present one, if we were to draw any conclusions about the ecology and biology of insects like *H. subflexa* and *H. virescens*.

Enterococcaceae bacteria represented 99.89% and 84.57% of the whole gut community in *H. virescens* and *H. subflexa* respectively. These results reflect the work of Staudacher *et al.* (2016) who found that Enterococcacea bacteria dominated their lab populations of *H. virescens* reared on chickpea and tobacco by 97.8% and 91.5% respectively. Only 0.03% and 0.11% from *Enterococcus* bacteria in our study could be identified to the species level. More interestingly, these low proportions accounted for only two species that were not shared by the two insects. *Enterococcus casseliflavus* was only present in *H. virescens* (0.03% from total *Enterococcus*) whereas *E. cecorum* was the only representative in *H. subflexa* (0.11%). *E. casseliflavus* has been isolated from lepidopteran larvae before. It was for instance found in the gut of the highly polyphagous pest *Spodoptera litura* (Thakur et al., 2015), as well as in the specialist *M. sexta* (Brinkmann et al., 2008) that feeds on Solanaceae plants. On the other hand, *E. cecorum* has never been associated with insects before and is rather found in bird digestive tracts. It is commonly encountered in chicken and to a lesser extent in cattle and swine (Herdt et al., 2009; Lebreton et al., 2014). As for the remaining unidentified *Enterococcus* bacteria, one could expect *E. mundtii* to be the most abundant representative of its genus (Chen et al., 2016; Tang et al., 2012; Teh et al., 2016)

The prevalence of Enterococcacea bacteria seems at first less strong in *H. subflexa* (84.57%) than in *H. virescens* (99.89%); as it is here taking into account the whole data set (computed data from the two treatment conditions and the control). It is less so when comparing gut relative abundance of bacteria in specific treatment conditions. On methanol-supplemented diet for instance, the prevalence of Enterococcacea was of 98.55% for the specialist. But when fed on withanolide-supplemented diet, Enterococcae proportion was of 93.8% and of only 67.48% on withaferin A-supplemented diet. Simultaneously, relative abundances of bacteria belonging to the order Bacillales ranged from 0.2% in the control, 3.96% when feeding on the withanolide extract and 31.2% on withaferin A. This observation suggests that by being specialized on withanolide-containing plants, *H. subflexa* allows the growth and development of bacteria from the order Bacillales (comprising genera such as *Bacillus*, *Staphylococcus* or *Paenibacillus*), or at least enough for them to start outcompeting Enterococcacea bacteria. In their paper from 2016, Barthel *et al.* observed that *H. subflexa* had higher relative weight gains when reared on withanolide-supplemented diets than its close relative in a development study based on relative weight gain. They suggested that withanolides might play the role of a selective facilitator of essential symbionts, promoting nutrition and digestion in the host. However, this has to be qualified in the light of a closer look at the absolute abundance of Bacillales bacteria in *H. subflexa*: in the withaferin A

samples, only one pool out of five contributed to 81.1% of the 67.48% observed Bacillales (data not shown). So this is still too fragile proof to validate the previous hypothesis. More generally, the analysis of diversity and richness showed that the purified withanolide extract and withaferin A did not affect the gut composition and relative abundance of symbionts in both insects.

Nonetheless, according to personal observations *Heliothis subflexa* larvae seemed healthier and sturdier on treated diets than were *H. virescens* larvae. The difference was notably clear when dissecting the larval gut: the gut tissues in *H. subflexa* were elastic and less fragile than in *H. virescens*. Additionally, most intestines were still containing a thick food bolus when placed in liquid nitrogen, which was less the case in *H. virescens*.

Impact of the withanolide extract and withaferin A supplemented diet on the temporal expression of two immune genes.

The experiment showed that attacin-1 was not affected by the withanolide extract or withaferin A supplementation in any of the two insect species for the studied time frame. On the contrary, i-type lysozyme was significantly more expressed after 24 hours on treated diets in *H. subflexa*, but not in *H. virescens*. Nevertheless, statistical tests showed that the higher gene expression level in the withanolide extract and withaferin A conditions were not significant compared to the methanol control at the same time point, in both species.

Withanolides have well-documented detrimental effects on insect biology. They display immunomodulating effects on many insects species (Castro et al., 2009, 2008b; Garcia et al., 2006; Machado et al., 2006) and are also considered as phytoecdysteroids (Clément et al., 1993; Dinan et al., 1996, 1999, 2001). In *D. melanogaster* for instance, phytoecdysteroids increased phagocytic activity, pathogen encapsulation and the expression of AMPs (Lanot et al., 2001; Meister and Richards, 1996; Sorrentino et al., 2002). Barthel *et al.* investigated the impact of withanolide supplementation on immune indicators such as PO activity and AMP gene expressions in feeding assays on *Heliothis virescens* and *Heliothis subflexa*. Interestingly, they found that after three days of exposure, *H. subflexa* was positively affected by ingestion of withanolide-containing diets. The majority of identified immune genes were upregulated and there was a significant increase in PO activity. Our findings are consistent with the work of Barthel and coworkers, although a direct comparison is not possible because of the differences in exposure time to withanolides. While antimicrobial peptides synthesis is probably common at a low basal level in all insect tracts (Broderick and Lemaitre, 2012), the secretion of these molecules in the hemolymph is not commonplace (Lemaitre and Hoffmann, 2007). Their production and use to defend the entire organism against pathogens is referred to as a “systemic immune response” and is considered as the last set of defenses displayed by insects upon pathogenic challenge (Lemaitre and Hoffmann, 2007). Therefore the timing at which they are upregulated in response to an attack might exceed 24 hours. On the other hand, lysozyme genes are constitutively expressed in insects and are strongly upregulated in response to a bacterial challenge in lepidopteran species (Gillespie and et al., 1997). The present expression assay

suggests that withaferin A and the withanolide extract have the same inducing effect after 24 hours of exposure in bot *H. virescens* and *H. subflexa*.

In the protected environment of the calyx, *H. subflexa* might have been less exposed to pathogenic bacteria leading slowly to a loss in strong immune abilities. A divergence in immune competence might have happened between the two species alongside a divergence in host range. The ancestor of *H. virescens*, by enlarging its dietary choice, might have been exposed to a greater variety of pathogens and developed in this prospect the ability to promptly respond to a wider range of bacterial stresses (Barthel et al., 2014). *H. subflexa*, on the other hand may have lost this immune system potency by feeding on host plants restricted to one genus and in a constantly predictable environment. Does *H. subflexa* rely on withanolides uptaken from *Physalis* plants to keep its immune competence up? The withanolides present in *Physalis* might stimulate on the long term the production of high basal levels of gut-localized AMPs and be responsible, along with their antibacterial properties, for the inconstant bacterial population numbers across *H. subflexa* individuals.

CONCLUSION

This study is the first to investigate the bacterial gut community of a laboratory-reared population of *H. subflexa* and to compare it in different treatment conditions to its closely related relative, *H. virescens*. The treatment conditions, which consisted in a purified withanolide extract from *P. peruviana* and withaferin A, a commercially available withanolide, did not affect the gut richness and diversity of *H. virescens* and *H. subflexa* gut communities. However, withaferin A increased greatly the relative abundance of one bacterial order, the Bacillales, in *H. subflexa*'s microflora. The gut bacterial composition of *H. subflexa* was more diverse and counted 70 different families whereas *H. virescens* counted only five. However the total numbers of counts in *H. subflexa* varied greatly between samples. Both species' gut communities were largely dominated by bacteria belonging to the family Enterococcaceae, with relative abundances ranging to up to 99.89% in *H. virescens* and 84.57% in *H. subflexa*, which is commonplace in laboratory reared population of insects. Moreover, the only two bacteria from the family Enterococcacea identified to the species level were *Enterococcus casseliflavus*, only present in *H. virescens* (0.03% of total *Enterococcus*) and *Enterococcus cecorum*, found only in *H. subflexa* (0.11%). Consistent with other studies these findings are most likely not representative of the gut communities found in wild *H. virescens* and *H. subflexa* populations. Expression levels over time of immune-related genes in response to the withanolide extract and withaferin A showed that the expression of the AMP attacin-1 was not significantly affected by the treatments after 24 hours. The i-type lysozyme, on the other hand, was significantly upregulated after 24 hours but did not differ significantly from the methanol control at any time point.

This work should next be extended to wild populations of both insect species in order to observe the naturally occurring microbiome of *H. subflexa* in comparison to *H. virescens.* This could lead to the identification of a good symbiont candidate that allows *H. subflexa* to feed on *Physalis* plants. Yet there is still a lot of information that needs to be recovered from the gut bacterial 16s rRNA gene sequencing. Specific sequences of some relevant families that were not identified to the species level by QUIIME2 such as *Enterococcus* or within the Bacillales order*,* could be further investigated in other databases to identify what are the species prevailing in *H. subflexa* and *H*. *virescens*. It would be also interesting to look for bacterial enzymatic activities related to the breakdown of withanolides of similar molecules so see if any symbiont could be involved in the detoxification of *Physalis* plants in *H. subflexa*. Another important aspect of the adaptation of *H. subflexa* to *Physalis* plants is the expression profile of detoxification enzymes such as cytochrome P450s or glutathione S-transferases. This could be done in a similar RNAseq study as carried out in the study of Barthel *et al.* 2016.

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ANNEXES

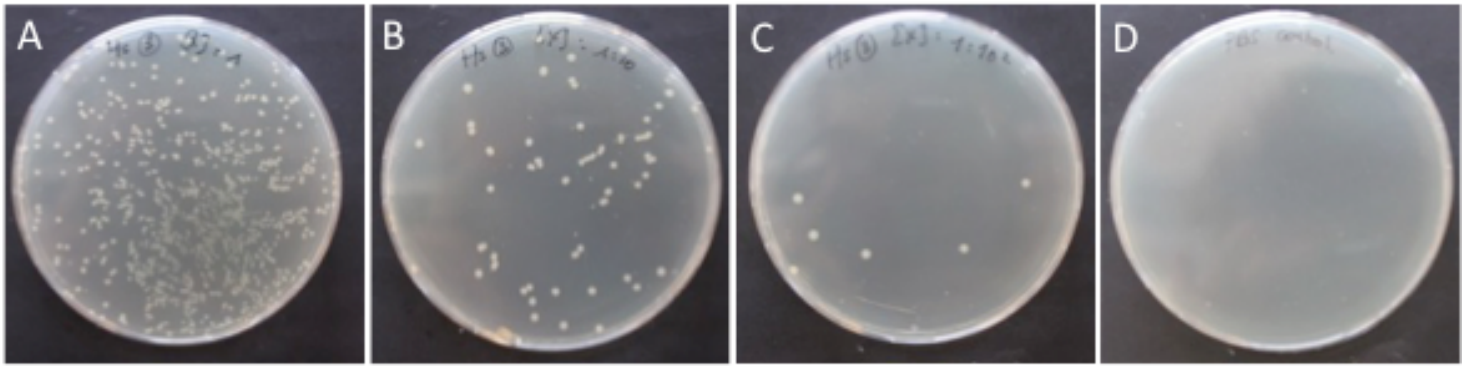

Figure I Bacterial colonies of a homogenized gut from a *H. subflexa*. A single gut was dissected and placed in a tube containing 500µl of PBS prior to be gently grinded with a pestle. The resulting stock homogenate was diluted two times in series (dilution factor: 10X). 150µl of each dilution was plated on an agar Petri dish, which were placed 24 hours at 37°C (A) stock solution (B) dilution 1:10 (C) dilution $1:10^2$ (D) PBS control.

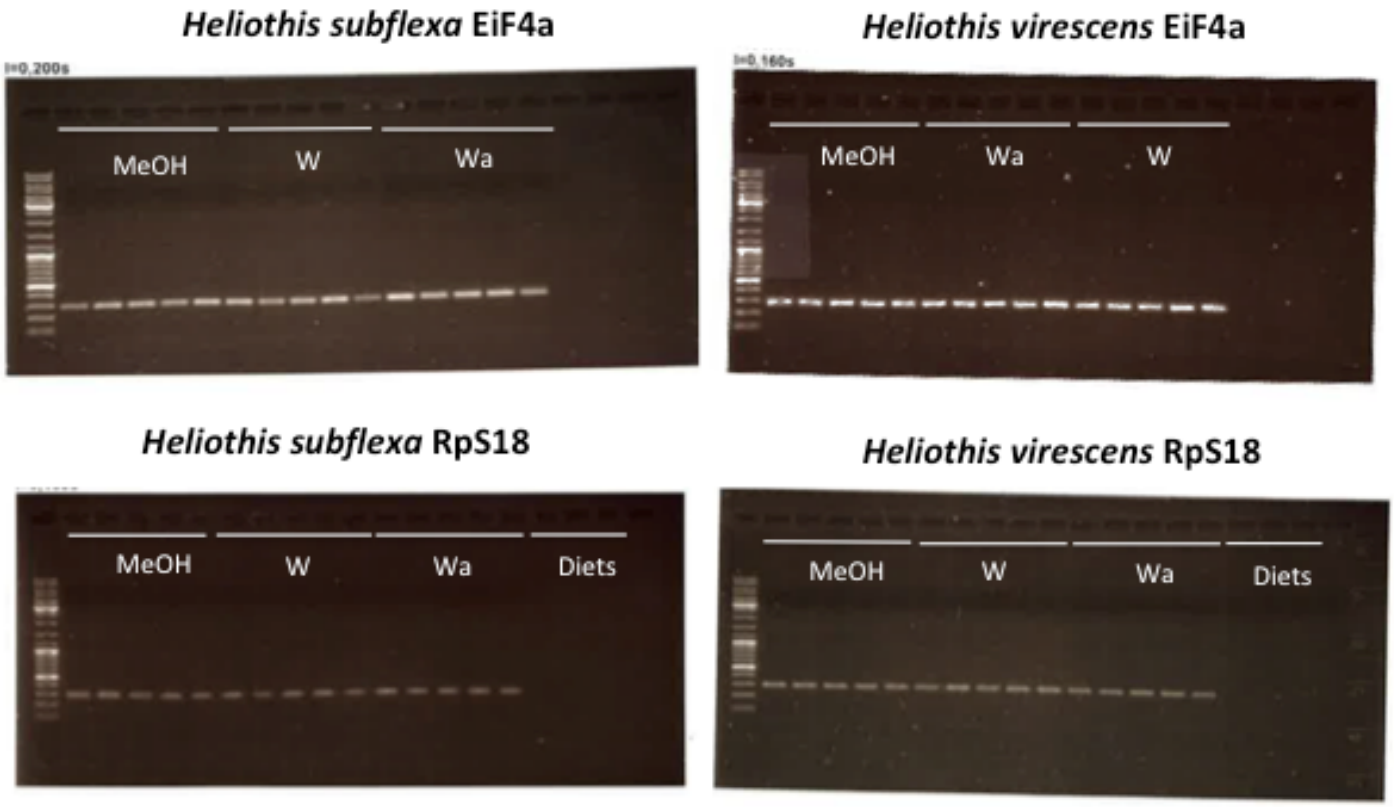

Figure II Genomic DNA quality control. The quality of the genomic DNA of each sample consisting of larval guts (n=3) was checked in both species in electrophoresis assays. Guts were dissected from larvae that had fed on diets supplemented with methanol (MeOH, as a control), withanolide extract (W) or withaferin A (Wa). Two different primer pairs targeting housekeeping genes present in both *H. subflexa* and *H. virescens* were used for the PCR reaction: the eukaryotic translation initiation factor a (EiF4a) and the ribosomal protein S18 (RpS18). Molecular-weight size marker has the form of a 100bp DNA ladder and is located in the leftmost lane.

Table I Number of 16S rRNA gene sequences counts per sample and per species. The total number of sequences obtained by Illumina sequencing for each sample is given in this table. Each pool represents the gDNA from three larval guts. While the numbers of counts in H. virescens are quite constant, numbers of sequences in *H. subflexa* sample varied greatly.

Treatment	Pool number	*H. virescens*	*H. subflexa*
MeOH	1	31982	4469
	2	30902	423
	3	32247	694
	4	31849	565
	5	28002	30251
Withanolide	1	22687	2130
	2	33302	817
	3	24591	20100
	4	24126	1470
	5	30115	25237
Withaferin A	1	30475	388
	2	33617	13881
	3	31028	666
	4	31317	30695
	5	29435	1172

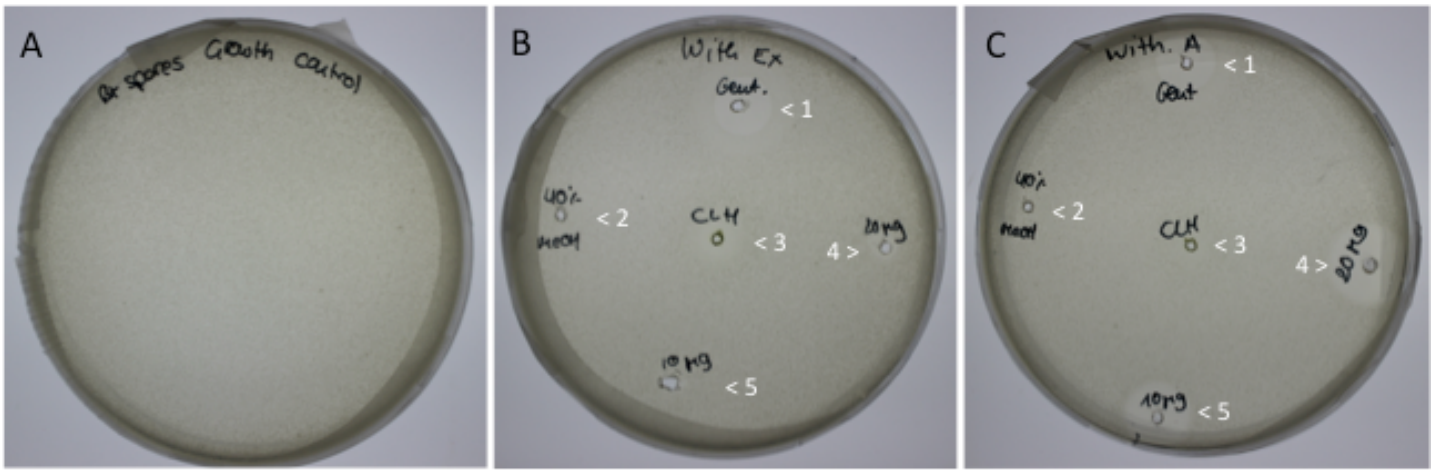

Figure III Antibacterial activity of withanolide extracts and withaferin A against *Bt* spores. Growth inhibition assay showing the antibacterial activity of (B) withanolide extracts (C) withaferin A. Growth inhibition tests were performed with (1) 2µg gentamycin (0.5µg. µl-1). (2) 40% methanol (3) crude P. peruviana leaf homogenate (4) 20µg withanolide extracts in (B) or withaferin A in (C), 10µg. µl-1 and (5) 40µg withanolides extract in (B) or withaferin A in (C), 20µg. µl-1. (A) Growth inhibition assay control.

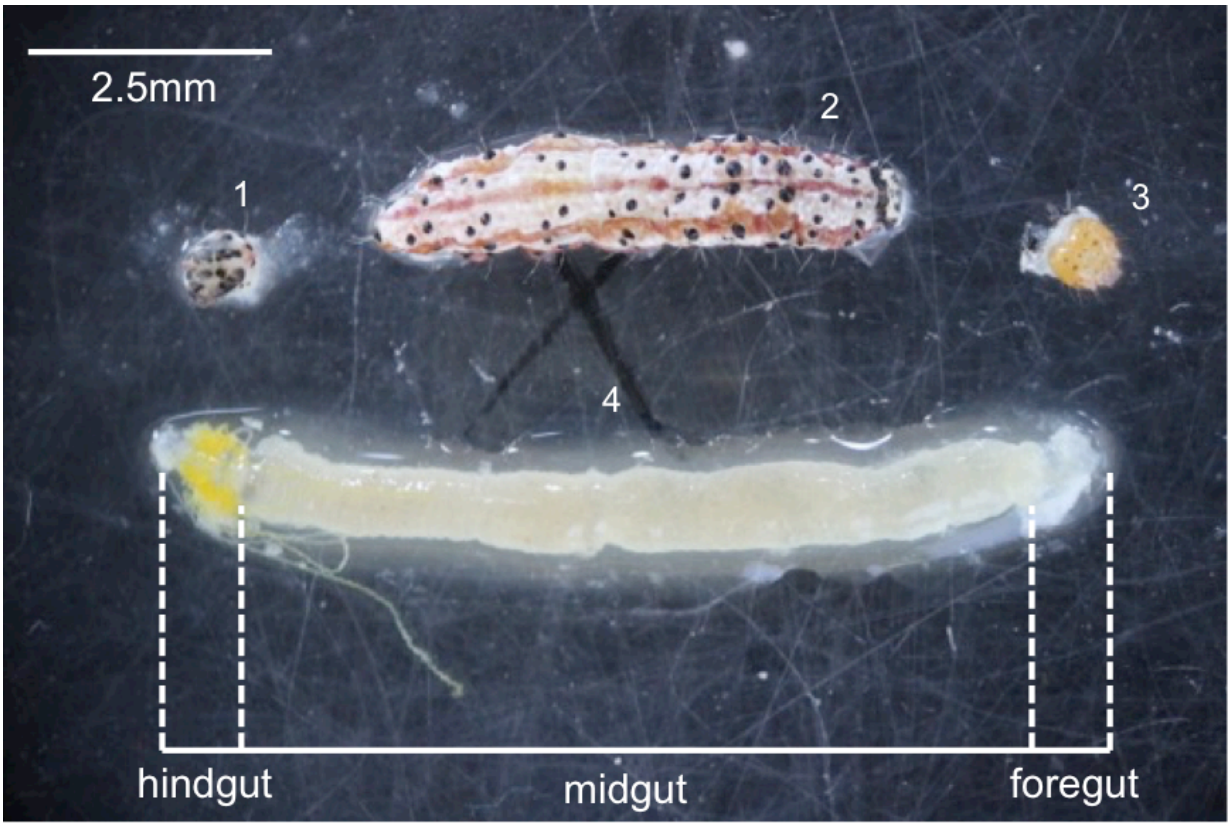

Figure IV Picture of the dissected gut of one *H. subflexa* larvae. The head capsule (3) and the last segment (1) were removed using a pair of dissecting scissors. The larval gut (4) was then simply removed from the outer cuticle (2) using two tweezers.

Table II Summary table showing the composition in larvae of each gDNA sample for microbiome sequencing. Larvae were placed on diet supplemented with methanol (MeOH, as a control), withanolide extract (W) or withaferin A (Wa). After dissection, gDNA from each gut was extract and later pooled (n=3). The family number corresponding to the ascendance of each individual in single pair matings were marked down to know the genetic composition of each sample.

Heliothis virescens

Treatment	Pool	larva ID	Family
MeOH	pool 1	Hv MeOH 3	1509
		Hv MeOH 9	1515
		Hv MeOH 23	1505
	pool 2	Hv MeOH 4	1509
		Hv MeOH 15	1568
		Hv MeOH 31	1508
	pool 3	Hv MeOH 10	1515
		Hv MeOH 6	1509
		Hv MeOH 18	1568
	pool 4	Hv MeOH 11	1515
		Hv MeOH 16	1568
		Hv MeOH 26	1506
	pool 5	Hv MeOH 24	1505
		Hv MeOH 28	1506
		Hv MeOH 32	1508

Treatment	Pool	larva ID	Family
Withanolide	pool 1	Hv W 16	1515
		Hv W 1	1568
		Hv W 2	1509
	pool 2	Hv W 18	1515
		Hv W 22	1505
		Hv W 27	1506
	pool 3	Hv W 20	1515
		Hv W 29	1506
		Hv W 32	1509
	pool 4	Hv W 12	1568
		Hv W 7	1509
		Hv W 25	1505
	pool 5	Hv W 13	1568
		Hv W 26	1505
		Hv W 31	1508

Treatment	Pool	larva ID	Family
Withaferin A	pool 1	Hv Wa 1	1509
		Hv Wa 6	1568
		Hv Wa 25	1508
	pool 2	Hv Wa 3	1509
		Hv Wa 12	1515
		Hv Wa 27	1508
	pool 3	Hv Wa 4	1509
		Hv Wa 19	1505
		Hv Wa 23	1506
	pool 4	Hv Wa 7	1568
		Hv Wa 13	1515
		Hv Wa 16	1505
	pool 5	Hv Wa 9	1568
		Hv Wa 28	1508
		Hv Wa 14	1515

Heliothis subflexa

Treatment	Pool	larva ID	Family
MeOH	pool 1	Hs MeOH 1	1701
		Hs MeOH 15	1703
		Hs MeOH 21	1737
	pool 2	Hs MeOH 3	1701
		Hs MeOH 16	1732
		Hs MeOH 23	1728
	pool 3	Hs MeOH 8	1725
		Hs MeOH 17	1736
		Hs MeOH 24	1748
	pool 4	Hs MeOH 13	1729
		Hs MeOH 18	1739
		Hs MeOH 25	1704
	pool 5	Hs MeOH 14	1729
		Hs MeOH 19	1716
		Hs MeOH 28	1711

Treatment	Pool	larva ID	Family
Withanolide	pool 1	Hs W 2	1729
		Hs W 10	1725
		Hs W 18	1737
	pool 2	Hs W 4	1729
		Hs W 13	1703
		Hs W 19	1716
	pool 3	Hs W 5	1729
		Hs W 14	1732
		Hs W 20	1711
	pool 4	Hs W 8	1728
		Hs W 15	1736
		Hs W 24	1748
	pool 5	Hs W 11	1725
		Hs W 19	1716
		Hs W 26	1701

Treatment	Pool	larva ID	Family
Withaferin A	pool 1	Hs Wa 1	1729
		Hs Wa 14	1703
		Hs Wa 21	1736
	pool 2	Hs Wa 3	1729
		Hs Wa 16	1748
		Hs Wa 23	1704
	pool 3	Hs Wa 4	1725
		Hs Wa 17	1748
		Hs Wa 26	1711
	pool 4	Hs Wa 9	1728
		Hs Wa 18	1748
		Hs Wa 28	1716
	pool 5	Hs Wa 13	1737
		Hs Wa 20	1701
		Hs Wa 24	1704

CTAB gDNA Extraction Protocol

- Add 500µl of TES* buffer and 2 sterile beads to the 2ml tube containing a gut.

- Add 4µl of lysozyme (100mg.ml^{-1})

- Grind tissue in tissue lyser for 5min at 30Hz.

- Incubate at 37°C for 30 min.

- Add 2.5µl of Proteinase K (20 mg.ml^{-1}), vortex 5 sec.

- Incubate 4h at 56°C

    * Warm up CTAB 15 min prior to next step
    * Set the centrifuges to 4°C

- Add 170µl of 5M NaCl to adjust salt concentration to 1.4 M and mix by shaking tubes vigorously by hand for about 30s

- Add 80µl 10% CTAB and incubate at 65°C for 10min.

- Add 750µl chloroform-isoamyl alcohol (24:1) and incubate on ice for 30min while occasionally gently mixing.

- Centrifuge for 10min at 16000rcf at 4°C (max 12000rpm with Ependorf centrifuge 5417 R) and transfer the supernatant in a new clean tube.

- Precipitate with 1 Vol. (*ca.* 550µl) of 100% pre-cooled isopropanol, shake thoroughly (but don't vortex) incubate on ice for 30min.

- Centrifuge for 10min at 15000rcf at 4°C.

- Decant the supernatant, wash with 500µl pre-cooled 70% EtOH, vortex briefly and centrifuge for 10 min at 16000rcf at 4°C.

- Decant EtOH and air-dry at room temperature.

- Dissolve in 90µl of distilled H_2O

* Preparation of TES buffer:
    - 10mM Tris pH 8
    - 10mM EDTA
    - 0.2% SDS (v/v)