Microbial Ecology (2025) 88:33
https://doi.org/10.1007/s00248-025-02535-y

RESEARCH

Parasitoid Calyx Fluid and Venom Affect Bacterial Communities in Their Lepidopteran Host Labial Salivary Glands

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Received: 4 December 2024 / Accepted: 13 April 2025

Abstract

The influence of gut and gonad bacterial communities on insect physiology, behaviour, and ecology is increasingly recognised. Parasitism by parasitoid wasps alters many physiological processes in their hosts, including gut bacterial communities. However, it remains unclear whether these changes are restricted to the gut or also occur in other tissues and fluids, and the mechanisms underlying such changes are unknown. We hypothesise that host microbiome changes result from the injection of calyx fluid (that contain symbiotic viruses known as polydnaviruses) and venom during parasitoid oviposition and that these effects vary by host tissue. To test this, we microinjected *Pieris brassicae* caterpillars with calyx fluid and venom from *Cotesia glomerata*, using saline solution and natural parasitism by *C. glomerata* as controls. We analysed changes in the bacterial community composition in the gut, regurgitate, haemolymph, and labial salivary glands of the host insects. Multivariate analysis revealed distinct bacterial communities across tissues and fluids, with high diversity in the salivary glands and haemolymph. Parasitism and injection of calyx fluid and venom significantly altered bacterial communities in the salivary glands. Differential abundance analysis showed that parasitism affected bacterial relative abundance in the haemolymph, and that *Wolbachia* was only found in the haemolymph of parasitized caterpillars. Altogether, our findings reveal that parasitism influences the host haemolymph microbiome, and both parasitism and injection of calyx fluid and venom drive changes in the bacterial community composition within the host salivary glands. Given that the composition of salivary glands can influence plant response to herbivory, we discuss these results in the broader context of plant-parasitoid interactions.

Keywords Microbiome · Parasitoid symbiont · Haemolymph · Gut · Regurgitant · *Cotesia glomerata* · *Pieris brassicae*

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Introduction

Over the last decade, there has been an increasing interest for the microbial communities of insects [1, 2]. The majority of research efforts have been directed towards exploring three areas: (i) microbes in the reproductive tissues [3], (ii) microbes in the alimentary canal, commonly referred to as the gut, and (iii) microbes in the regurgitant, that mainly comes from the foregut [1, 4]. Studies have unveiled that the composition of the gut microbiome profoundly influences several key aspects of the insect host, including food digestion, nutrient assimilation, performance, and immune response [1, 5, 6]. Notably, the observed effects of the gut microbiome on hosts may be contingent upon species and environmental context [7, 8]. For instance, some studies observed no negative effects of antibiotics on the performance of caterpillars reared under optimal conditions [9, 10], while others reported a negative effect [11, 12]. One hypothesis for these contrasting results is that a portion of the gut microbiome is transient, originating from the diet, passing through the digestive system without establishing residence, and subsequently being excreted with frass [9]. Conversely, another part of the gut microbiota is considered resident, exhibiting greater stability, albeit with significant variations among individuals and species, influenced by genotypes, developmental stages, and habitats [13–17]. There is an ongoing debate surrounding the relative proportions of resident and transient bacteria within the gut of lepidopteran herbivores [1, 18].

In contrast to the examination of gut and regurgitant microbial communities, the analysis of microbial populations inhabiting the haemolymph and labial glands (producing saliva) of lepidopteran insects has received less attention [19, 20]. This may stem from the historical perception that haemolymph, despite its nutrient-rich nature, lacks microbial presence, attributed to the robust immune system of herbivores, including haemocytes [19]. While the role of labial salivary glands in lepidopteran insect larvae has been characterised using proteomics and transcriptomics approaches [21, 22], studies focusing on the microbial community composition remain scarce [20].

Beyond their impact on herbivorous hosts, microbes can also influence their hosts' interactions with organisms from other trophic levels, including plants [23] and parasitic wasps, also known as parasitoids. Endoparasitoids lay one or more eggs inside their host, where they develop until their pupation, ultimately resulting in the host's death [24]. Recent investigations have demonstrated significant differences in the internal (mainly gut) microbiome of parasitized lepidopteran larvae compared to their unparasitized counterparts [25–31]. However, the mechanisms through which parasitoids modify the internal microbiome of their hosts is still unknown. Furthermore, little is known about the effect of parasitism on the microbial communities present in host tissues and fluids beyond the guts [32].

Here, we hypothesise that parasitoid calyx fluid (that contain symbiotic viruses known as polydnaviruses) and venom, which are injected in the host alongside eggs [33, 34], play a pivotal role in inducing changes in the microbiome of parasitized hosts. These substances have been shown to significantly impact the immune system and development of parasitized hosts, facilitating the growth of parasitoid progeny [35, 36]. However, the influence of calyx fluid and venom on the microbial communities of lepidopteran hosts remains unexplored [25, 37]. We further hypothesise that these changes in the host microbiome will vary depending on the tissues and fluids within the caterpillar.

To test these hypotheses, we first microinjected *Pieris brassicae* caterpillars (Lepidoptera: Pieridae) with calyx fluid and/or venom collected from *Cotesia glomerata* parasitoids (Hymenoptera: Braconidae). Then, we analysed the microbial communities present in the gut, regurgitate, haemolymph, and salivary glands of caterpillars. Microbial communities of injected caterpillars were compared with those of untreated control caterpillars and those of caterpillars parasitized with *C. glomerata*.

Materials and Methods

Plants and Insects

In our study, we used wild *Brassica oleracea* plants (population: Kimmeridge), originally collected in Dorset, UK (coordinates 50° 360′ N, 2° 070′ W) [38]. Plants underwent multiplication through open pollination within a field containing diverse wild *B. oleracea* genotypes. The seeds were collected and sown in 2-L pots under controlled greenhouse conditions (18–26 °C, R.H 50–70%). In addition to natural daylight, plants were supplemented with SON-T light (500 μmol; L16:D8; Philips). They were watered every other day and fertilised three times per week. The studied herbivore (*Pieris brassicae*) and parasitoid (*Cotesia glomerata*) were initially collected close to Wageningen University. *Cotesia glomerata* is a gregarious parasitoid that attacks young caterpillars of cabbage butterflies, including *P. brassicae*, whose larvae feed on the host haemolymph [39]. *Pieris brassicae* caterpillars were reared on cultivated cabbage plants (*B. oleracea*, variety: gemmifera cv. Cobelius), while the parasitoids were reared using first instar *P. brassicae* caterpillars, as described before [39].

Parasitism and Injection Treatments

Following individual oviposition of second instar *P. brassicae* caterpillars [40], we dissected the ovaries and venom glands of adult *C. glomerata* in a phosphate-buffered saline (PBS) solution to collect calyx fluid (where symbiotic viruses reproduce) and venom, respectively [41]. Immediately after sampling, organs were placed in Eppendorf tubes on ice, filled with PBS at a concentration of one wasp equivalent (i.e., one wasp organ) per µL of PBS [42]. On the same day, second-instar *P. brassicae* caterpillars were anaesthetized using CO_2 and injected with one of the five treatments using a Femtojet 4i (Eppendorf). The treatments were as follows: (i) PBS (negative control); (ii) calyx fluid; (iii) venom; (iv) calyx fluid + venom, and (v) PBS followed by parasitism by *C. glomerata* (positive control). Each caterpillar received an injection of approximately 0.1 µL of solution, equivalent to what one *C. glomerata* adult injects during a single parasitism event [43]. Because injection is an invasive procedure, the injected caterpillars were first placed in petri dishes with *B. oleracea* cv. Cobelius leaves for 24 h to exclude individuals that would not survive for further experimentation. The following day, the surviving caterpillars (about 100 per treatment) were transferred to 5-week-old *B. oleracea* plants (Kimmeridge variety, ten caterpillars of the same treatment per plant) in a greenhouse. Plants were covered with a mesh bag and put under the same conditions as previously described.

Sample Collection

Ten days after larvae were placed on the plants, we collected the following samples: (i) regurgitate, (ii) haemolymph, (iii) salivary glands, and (iv) gut. Samples were collected using sterile dissection trays placed near a Bunsen burner flame and transferred to 1.5-mL Eppendorf tubes filled with 100 µL of PBS solution with 0.01% Tween 80. For each collection sample (= one replicate), samples from two caterpillars were pooled to reach the following quantities. The regurgitate was sampled using a thin glass capillary (diameter: 1.5 mm) gently pressed against the mouth of the caterpillars to trigger regurgitation. We used a micropipette to collect 10 µL from the glass capillary. Then, a small incision was made in one of the first pairs of the caterpillar abdominal pseudo legs, and 20 µL of haemolymph was collected with a micropipette. Subsequently, the caterpillars were euthanized by separating their head from their body using flame-sterilised forceps. Finally, the salivary glands (made of two long white glands) and full digestive system (gut) were collected. All equipment used to collect samples was changed between caterpillars to prevent contamination. All samples from caterpillars that were parasitized by *C. glomerata* were visually checked to remove any parasitoid larvae. Salivary gland and gut samples were homogenised by adding two glass beads (2-mm diameter) to each tube and placing them in a Bead Ruptor Elite (Omni International, Kennesaw, USA) during 30 s at 30 Hz. All samples were stored at − 80 °C until further analysis. We collected 11 to 13 samples for each combination of caterpillar body tissues and injection treatment.

DNA Extraction and Molecular Analysis

Genomic DNA was extracted from our samples using a DNeasy PowerSoil Pro Kit (QIAGEN), following the manufacturer's instructions with a modification: in the second step of the protocol, the vortex adapter was replaced by two cycles of 30 s at 30 Hz (with a break of 10 s in between) in the TissueLyser II (Qiagen, Hilden, Germany). DNA extractions were conducted over 5 days, following proper protective measures, including the use of a lab coat and gloves. To prevent contamination, pipette tips were changed between samples, and the workspace was regularly cleaned with ethanol. Sample treatments were randomised throughout the process. Ten control samples containing PBS and 0.01% Tween 80 (the solution in which we placed our caterpillar samples) were included to control for reagent contamination. DNA from all samples as well as DNA extraction controls were then used as templates for DNA amplification using the Illumina barcoded primers 515 F and 806R [44] (Table S1), designed according to Kozich et al. [45] (dual index strategy) to amplify the V4 region of the bacterial 16S ribosomal RNA (rRNA) gene. Additionally, six PCR controls, in which the DNA template was replaced with DNA-free water, were included. PCR amplification, library preparation, sequencing, and bioinformatics analysis were performed as previously described in Gloder et al. [28]. Briefly, amplification was carried out in a reaction volume of 40 µL, consisting of 2 µL DNA, 0.5 µM of each primer, 150 µM of each dNTP, 1 × Titanium Taq PCR buffer, and 1 × Titanium Taq DNA polymerase (Takara Bio, Saint-Germain-en-Laye, France) with the following cycling protocol: 94 °C for 120 s, followed by 35 cycles of 45 s at 95 °C, 45 s at 59 °C and 45 s at 72 °C, and a final elongation step of 10 min at 72 °C. The resulting amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics GmbH, South Plainfield, UK) according to the manufacturer's instructions. Subsequently, the concentration of the purified amplicons was measured using a Qubit high-sensitivity fluorometer (Invitrogen, Carlsbad, USA), and each sample was pooled at equimolar concentrations. After ethanol precipitation, the amplicon library was loaded onto a 1.5% agarose gel, and the target band was excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The concentration of the library was measured again after the gel extraction, diluted to 2 nmol, and sent for sequencing at the Centre for Medical Genetics of the University of Antwerp (Antwerp, Belgium) using an Illumina MiSeq sequencer with a v2 500-cycle reagent kit (Illumina, San Diego, USA). DNA sequences were received as demultiplexed FASTQ files, with barcodes and primer sequences removed. Paired-end reads were merged using USEARCH (v11.0.667) to generate consensus sequences [46], allowing no more than ten mismatches in the overlap region. Reads shorter than 250 bp or with a total expected error above 0.05 were discarded. Sequences were then classified into zero-radius operational taxonomic units (zOTUs [47]) using the UNOISE3 algorithm as implemented in USEARCH [48]. The obtained dataset was then decontaminated in R (v3.5.2) (R Core Team 2018) using microDecon (v1.0.2) [49] to remove contaminants based on zOTU prevalence in tissues samples versus the mean of the six PCR controls [50]. The DNA extraction controls were removed from the dataset as they yielded only very low sequence numbers and no additional zOTUs compared to the PCR controls. Additionally, no band was obtained for the DNA extraction controls when loading the samples on an agarose gel, indicating that the DNA extraction kits were free of bacterial contamination. zOTUs occurring below a 0.1% relative abundance threshold in a given sample were discarded prior to further analysis [28, 29]. To standardise sequencing depth across samples, the number of sequences was rarefied to 2000 sequences per sample, and three samples with fewer sequences were excluded from the analysis. The taxonomic origin of each zOTU was determined using the SINTAX algorithm as implemented in USEARCH based on the SILVA Living Tree Project v123. The identity of the most important zOTUs was also verified with a BLAST search in GenBank against type materials. When no significant similarity values were found (< 97% identity), the BLAST analysis was extended to the entire database. Overall, results from the BLAST analysis were consistent with those obtained using the SINTAX algorithm in USEARCH.

Statistical Analyses

Rarefaction curves were generated for each sample using the Phyloseq package in R, presenting the number of observed zOTUs in function of the number of sequences. Using the Phyloseq package in R, we calculated Shannon diversity index and observed zOTU diversity for each sample. The Shannon diversity index considers both species abundance and evenness, and is a common metric for studying community diversity [51]. Higher scores indicate greater diversity, while scores close to 0 suggest low diversity. All analyses were performed using lme4, nlme, and emmeans packages in R [52]. Before analysis, the residuals of Shannon diversity index and observed zOTU diversity were visually checked for normality and homogeneity. We analysed Shannon indexes with a generalised least-square (GLS) model incorporating the different caterpillar tissues and fluids as a variance function to account for heterogeneity in residuals. Observed zOTU diversity was analysed with a generalised linear mixed model (GLMER) following a negative binomial distribution, which is ideal for overdispersed count data. For both models, the type of caterpillar tissue, the injection treatment as well as their interaction were used as fixed factors, and the couple of caterpillars used for each tissue sample was used as a random factor. When significant, Tukey's honestly significant difference (HSD) tests were used to perform pairwise posthoc comparisons between treatments. To visualise bacterial community composition, non-metric multidimensional scaling (NMDS) was used with the Bray–Curtis coefficient as the distance measure in the R software package Vegan. This coefficient was based on Hellinger-transformed relative abundance data of the observed bacteria in each sample. To evaluate differences in bacterial communities according to the caterpillar tissue and injection treatments, a permutational analysis of variance (PERMANOVA) was performed using the 'adonis' function in the Vegan package. When significant, we performed posthoc pairwise analyses (pairwise.adonis). The type of caterpillar tissue/fluid, the injection treatment, and their interactions were treated as fixed factors in the analysis, with significance assessed through 1000 permutations. Finally, a differential abundance analysis using DESeq2 was performed in R [53] to assess differences in zOTU relative abundance. Differential abundance analysis is traditionally used to analyse differential gene expression with RNA-seq data. However, it is increasingly being used in microbiome research to compare zOTUs abundances [54]. First, a filtering step was performed, where zOTUs with average sequence counts below 5 across all samples were excluded. To identify differentially abundant zOTUs relative to the control treatment, a model incorporating all factors (injection treatments and caterpillar tissues and fluids) was executed. The model was adjusted so that the control treatment served as the reference level for each injection treatment, in each tissue type, allowing comparisons to be made against it. zOTUs were classified as differentially abundant if they significantly differed from the control, with a false discovery rate (FDR) below 0.05 and a log2 fold change exceeding 1.0, utilising the apeGLM shrinkage estimator [55].

Results

After quality filtering and rarefying, we retained a total of 624 zOTUs for further analyses. Rarefaction curves approached saturation, indicating that our sequencing depth adequately captured microbial diversity (Fig. S1).

Shannon diversity index and zOTU richness were both significantly different across caterpillar tissues and fluids

($\chi^2 = 682$, df $= 3$, $p < 0.001$ and $\chi^2 = 589$, df $= 3$, $p < 0.001$, respectively) (Fig. 1a, b). We found no effect of the injection treatments on both Shannon diversity index and zOTU richness ($\chi^2 = 7.8$, df $= 4$, $p = 0.1$ and $\chi^2 = 1.6$, df $= 4$, $p = 0.8$, respectively).

The bacterial community composition was significantly different across caterpillar organs and fluids ($F = 135$, df $= 3$, $p = 0.001$) (Fig. 2a). The injection treatments also had a significant effect on bacterial community composition ($F = 2.2$, df $= 4$, $p = 0.027$) (Fig. 2b), with a significant interaction between injection treatments and caterpillar tissues and fluids ($F = 1.5$, df $= 12$, $p = 0.039$).

Further analysis showed that the injection treatments only significantly affected the bacterial community in the salivary glands ($F = 3.2$, $df = 4$, $p = 0.003$) (Table 1). Pairwise PERMANOVA analyses revealed that caterpillars injected with both calyx fluid and venom, as well as those naturally parasitized by *Cotesia glomerata*, had a different bacterial community composition compared to those injected with PBS (negative control) or only venom.

The bacterial community compositions were different among caterpillar tissues and fluids, but relatively similar among caterpillar injections and parasitism treatments (different colours in columns but similar in rows, Fig. 3). Bacterial communities in the guts and regurgitate of caterpillars were dominated by the presence of a member of the genus *Enterococcus* (zOTU1), with a relative abundance (averaged among the five injection treatments) of 99.2% in the guts and 92.8% in the regurgitate. This zOTU was also highly present in the haemolymph (32.2%) and in the salivary glands (20.2%). On the contrary, bacterial communities present in the salivary glands were dominated by the presence of two zOTUs from the *Pseudomonas* genus, with an average relative abundance of 45.8% (zOTU2) and 13.7% (zOTU5). Bacterial communities in the haemolymph were also characterised by the presence of a member of the genus *Flavobacterium* (zOTU4), with an average relative abundance of 19.6%. Strikingly, *Wolbachia* (zOTU9) was only present in the haemolymph of caterpillars naturally parasitized by *C. glomerata* (4.6%) (Fig. 3).

In total, 26 zOTUs were identified as differentially abundant between the negative control and treatments (Fig. 4). The main significant differences were observed in the haemolymph, between negative control and natural parasitism ('CG' injection treatment, 14 zOTUs with different relative abundance) and in the salivary gland, between negative control and injection of calyx fluid and venom ('CF + VNM' injection treatment, 9 zOTUs with different relative abundance). The relative abundance of a member from the Enterobacteriaceae family was significantly reduced in the regurgitate of caterpillars injected with 'CF' and 'CF + VENOM' compared to the

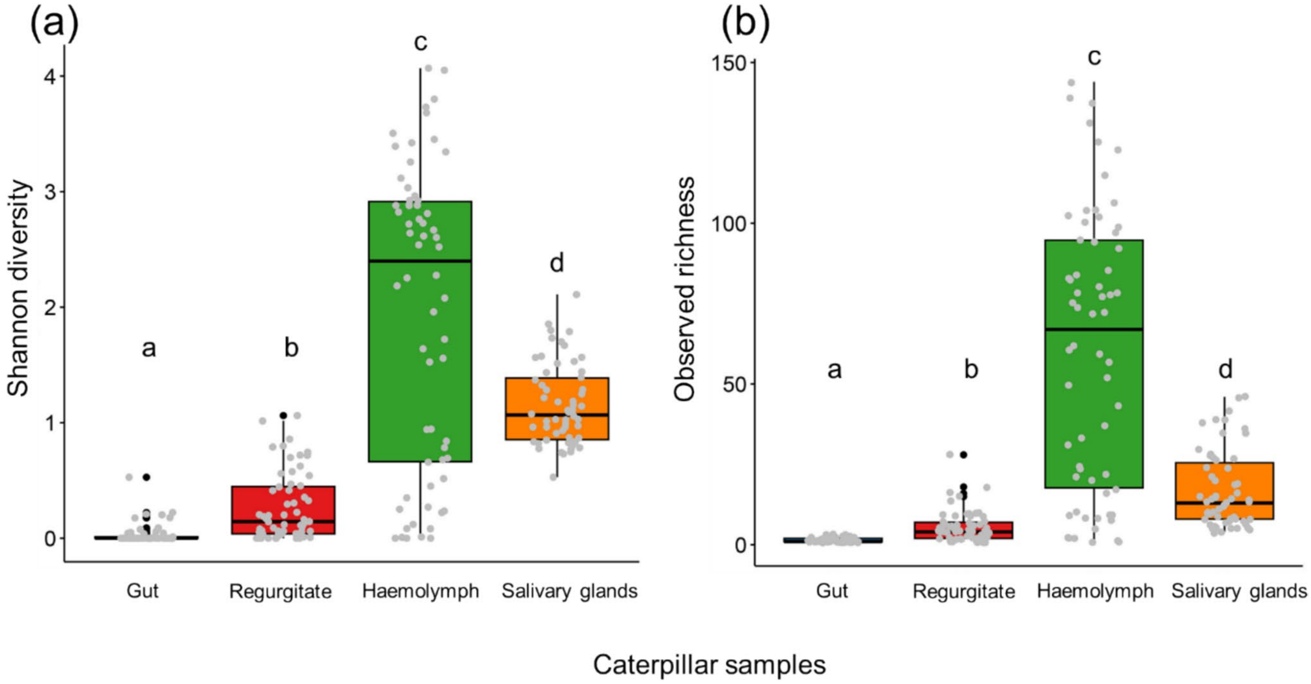


Fig. 1 **a** Shannon index and **b** observed zOTU richness of bacterial communities present in the gut, regurgitate, haemolymph, and salivary glands of *Pieris brassicae* caterpillars. Different letters above the boxplots indicate significant statistical differences among treatments ($p < 0.05$) according to Tuckey's HSD tests. Jittered grey circles represent the raw data (each circle corresponds to one sample), and black circles are outliers (not excluded from the analyses). The middle line within each boxplot shows the median value of the data, while the box itself covers the range between the first and third quartiles. The whiskers extend from the box to the minimum and maximum values within 1.5 times the interquartile range

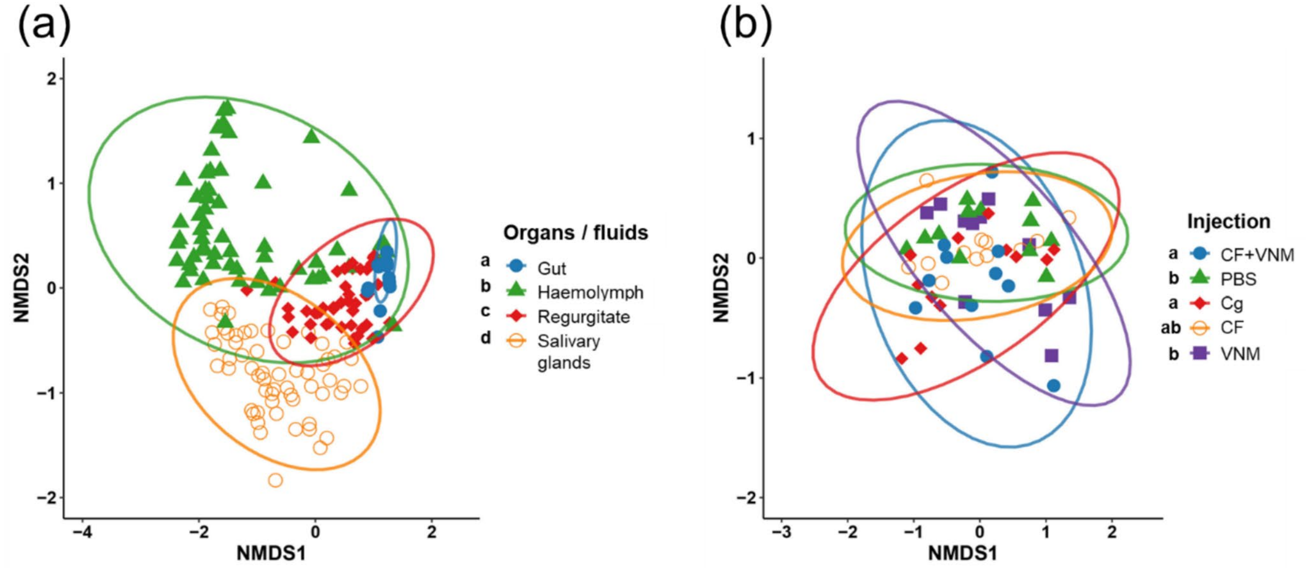


Fig. 2 Non-metric multidimensional scaling (NMDS) ordination plots based on Bray–Curtis distances of Hellinger-transformed relative abundances of bacterial communities. **a** Bacterial community from different tissues and fluids of the caterpillars (gut, haemolymph, regurgitate, and salivary glands). Level of fit between the original dissimilarities and the NMDS representation: goodness of fit =0.104. **b** Bacterial community from the salivary gland of caterpillars exposed to the following injection treatments: 'CF +VNM' =caterpillars injected with *Cotesia glomerata* calyx fluid and venom, 'PBS' =caterpillars injected with phosphate-buffered saline (PBS) solution (control), 'Cg': caterpillars naturally parasitized by *C. glomerata* and injected with PBS, 'CF' =caterpillars injected with *C. glomerata* calyx fluid, and 'VNM' =caterpillars injected with *C. glomerata* venom. Ellipses represent 95% confident intervals around the mean for each treatment (caterpillar tissue sample or injection). Level of fit between the original dissimilarities and the NMDS representation: goodness of fit =0.118. Different letters next to the treatments indicate a significant difference of the PERMANOVA test ($p < 0.05$)

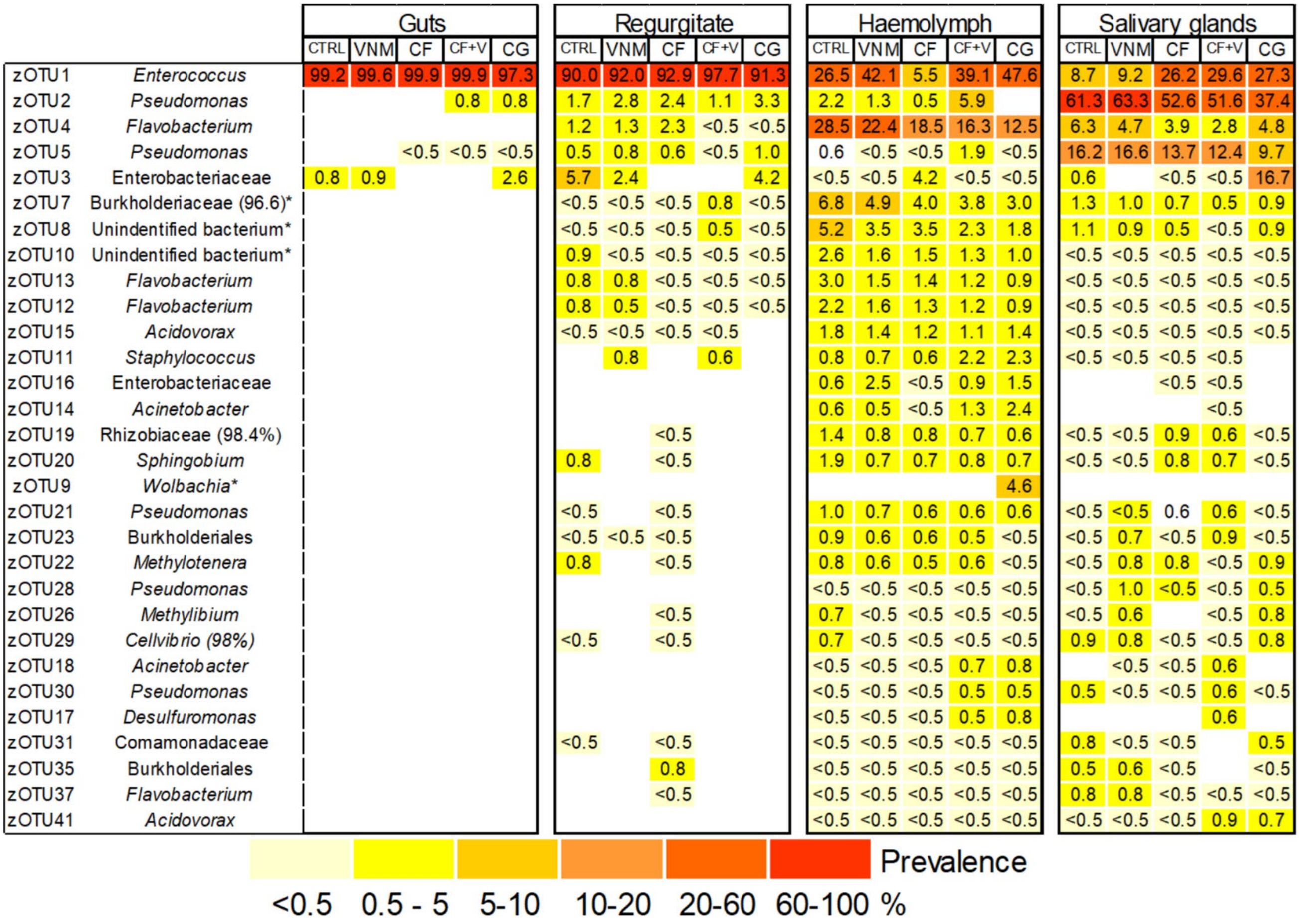
Table 1 Summary statistics of the bacterial community composition (PERMANOVA), Shannon diversity (GLS), and observed diversity (GLMER) used to compare bacterial communities according to the caterpillar samples and injection treatments

	Bacterial community (PERMANOVA)			Shannon (GLS)			Observed (GLMER)	
	F	p		χ^2	p		χ^2	p
Cat.samples	135	0.001	Cat.samples	682	< 0.001	Cat.samples	589	< 0.001
Injection	2.17	0.021	Injection	7.8	0.1	Injection	1.6	0.8
Cat.samples: injection	1.58	0.043	Cat.samples: injection	7.3	0.83	Cat.samples: injection	9.6	0.65
Gut	1.57	0.14						
Regurgitate	1.1	0.38						
Haemolymph	1.23	0.28						
Salivary gland	3.2	0.003						

regurgitant of caterpillars from the negative control treatment, with no effect of natural parasitism.

Discussion

We observed significant differences in the bacterial community compositions present in the gut, oral secretion, haemolymph, and labial salivary glands of *Pieris brassicae*. Permutational analysis of variance, conducted after non-metric multidimensional scaling (NMDS), revealed that both parasitoid maternal factors (calyx fluid and venom) injection, and natural parasitism had a significant impact on the bacterial community composition in the salivary glands of *P. brassicae*. Furthermore, our analysis of differential abundance indicated a significant influence of natural parasitism on the relative abundance of several bacteria in the haemolymph, in comparison to control unparasitized caterpillars,



		Guts					Regurgitate					Haemolymph					Salivary glands				
		CTRL	VNM	CF	CF+V	CG	CTRL	VNM	CF	CF+V	CG	CTRL	VNM	CF	CF+V	CG	CTRL	VNM	CF	CF+V	CG
zOTU1	*Enterococcus*	99.2	99.6	99.9	99.9	97.3	90.0	92.0	92.9	97.7	91.3	26.5	42.1	5.5	39.1	47.6	8.7	9.2	26.2	29.6	27.3
zOTU2	*Pseudomonas*				0.8	0.8	1.7	2.8	2.4	1.1	3.3	2.2	1.3	0.5	5.9		61.3	63.3	52.6	51.6	37.4
zOTU4	*Flavobacterium*						1.2	1.3	2.3	<0.5	<0.5	28.5	22.4	18.5	16.3	12.5	6.3	4.7	3.9	2.8	4.8
zOTU5	*Pseudomonas*			<0.5	<0.5	<0.5	0.5	0.8	0.6	<0.5	1.0	0.6	<0.5	<0.5	1.9	<0.5	16.2	16.6	13.7	12.4	9.7
zOTU3	Enterobacteriaceae	0.8	0.9			2.6	5.7	2.4			4.2	<0.5	<0.5	4.2	<0.5	<0.5	0.6		<0.5	<0.5	16.7
zOTU7	Burkholderiaceae (96.6)*						<0.5	<0.5	<0.5	0.8	<0.5	6.8	4.9	4.0	3.8	3.0	1.3	1.0	0.7	0.5	0.9
zOTU8	Unindentified bacterium*						<0.5	<0.5	<0.5	0.5	<0.5	5.2	3.5	3.5	2.3	1.8	1.1	0.9	0.5	<0.5	0.9
zOTU10	Unindentified bacterium*						0.9	<0.5	<0.5	<0.5	<0.5	2.6	1.6	1.5	1.3	1.0	<0.5	<0.5	<0.5	<0.5	<0.5
zOTU13	*Flavobacterium*						0.8	0.8	<0.5	<0.5	<0.5	3.0	1.5	1.4	1.2	0.9	<0.5	<0.5	<0.5	<0.5	<0.5
zOTU12	*Flavobacterium*						0.8	0.5	<0.5	<0.5	<0.5	2.2	1.6	1.3	1.2	0.9	<0.5	<0.5	<0.5	<0.5	<0.5
zOTU15	*Acidovorax*						<0.5	<0.5	<0.5	<0.5		1.8	1.4	1.2	1.1	1.4	<0.5	<0.5	<0.5	<0.5	<0.5
zOTU11	*Staphylococcus*							0.8		0.6		0.8	0.7	0.6	2.2	2.3	<0.5	<0.5	<0.5	<0.5	
zOTU16	Enterobacteriaceae											0.6	2.5	<0.5	0.9	1.5			<0.5	<0.5	
zOTU14	*Acinetobacter*											0.6	0.5	<0.5	1.3	2.4				<0.5	
zOTU19	Rhizobiaceae (98.4%)								<0.5			1.4	0.8	0.8	0.7	0.6	<0.5	<0.5	0.9	0.6	<0.5
zOTU20	*Sphingobium*						0.8		<0.5			1.9	0.7	0.7	0.8	0.7	<0.5	<0.5	0.8	0.7	<0.5
zOTU9	*Wolbachia**															4.6					
zOTU21	*Pseudomonas*						<0.5		<0.5			1.0	0.7	0.6	0.6	0.6	<0.5	<0.5	0.6	0.6	<0.5
zOTU23	Burkholderiales						<0.5	<0.5	<0.5			0.9	0.6	0.6	0.5	<0.5	<0.5	0.7	<0.5	0.9	<0.5
zOTU22	*Methylotenera*						0.8		<0.5			0.8	0.6	0.5	0.6	<0.5	<0.5	0.8	0.8	<0.5	0.9
zOTU28	*Pseudomonas*											<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	<0.5	<0.5	0.5
zOTU26	*Methylibium*								<0.5			0.7	<0.5	<0.5	<0.5	<0.5	<0.5	0.6		<0.5	0.8
zOTU29	*Cellvibrio (98%)*						<0.5		<0.5			0.7	<0.5	<0.5	<0.5	<0.5	0.9	0.8	<0.5	<0.5	0.8
zOTU18	*Acinetobacter*											<0.5	<0.5	<0.5	0.7	0.8		<0.5	<0.5	0.6	
zOTU30	*Pseudomonas*											<0.5	<0.5	<0.5	0.5	0.5	0.5	<0.5	<0.5	0.6	<0.5
zOTU17	*Desulfuromonas*											<0.5	<0.5	<0.5	0.5	0.8				0.6	
zOTU31	Comamonadaceae						<0.5		<0.5			<0.5	<0.5	<0.5	<0.5	<0.5	0.8	<0.5	<0.5		0.5
zOTU35	Burkholderiales								0.8			<0.5	<0.5	<0.5	<0.5	<0.5	0.5	0.6	<0.5		<0.5
zOTU37	*Flavobacterium*								<0.5			<0.5	<0.5	<0.5	<0.5	<0.5	0.8	0.8	<0.5	<0.5	<0.5
zOTU41	*Acidovorax*											<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.9	0.7

Fig. 3 Bacterial community profiles of two tissues (guts and salivary glands) and two fluids (regurgitate and haemolymph) collected from caterpillars exposed to one of the five injection treatments: 'CTRL' = caterpillars injected with phosphate-buffered saline (PBS) solution, 'VNM' = caterpillars injected with *Cotesia glomerata* venom, 'CF' = caterpillars injected with *C. glomerata* calyx fluid, 'CF + V': caterpillars injected with *C. glomerata* calyx fluid and venom, and 'CG' = caterpillars naturally parasitized by *C. glomerata* and injected with PBS. Bacterial taxa shown represent the 30 most prevalent zOTUs (the full list is shown in Table S2). Each value represents the average relative abundance of the corresponding zOTU. zOTUs were identified through a BLAST search against type materials in the GenBank database. Identifications were performed at genus level; when identical scores were obtained for different genera, identifications were performed at family or order level. When identity percentages were lower than 99%, the percentage of sequence identity with the GenBank entry is given between brackets. Asterisks indicate zOTUs for which no significant similarity (> 97% sequence identity with type sequence) was found. In these cases, the BLAST analysis was performed against the entire GenBank database. Hits with uncultured bacteria are indicated as unidentified bacterium. Colours indicate zOTU prevalence in the sample (white indicates absence)

with minimal effects observed from other injection treatments. Overall, our findings suggest that parasitoid calyx fluid and venom play a role in inducing significant changes in the salivary gland microbiome of herbivorous hosts, while additional factors may contribute to the observed changes in the haemolymph.

Variation in Bacterial Communities Across Tissues

Despite the huge prevalence of *Enterococcus*, known to be common in laboratory-reared caterpillars and matching the zOTU found in previous studies [26, 28], our NMDS analysis showed significant differences in the bacterial community compositions in the gut, regurgitate, haemolymph, and salivary glands of *P. brassicae*. This finding aligns with our expectations, considering the different environments and functions of these tissues and fluids. Notably, the microbiomes of the gut and regurgitate in lepidopteran insects have been extensively characterised in previous studies (reviewed in [1]). However, the presence of bacteria in *P. brassicae*'s haemolymph and salivary glands, whose microbiomes have received minimal attention in lepidopteran insects, is very interesting. Our NMDS analysis revealed marked differences in bacterial community compositions in the haemolymph and salivary glands compared to the gut and regurgitate. Moreover, both Shannon diversity score and observed richness indicated a higher bacterial diversity in the haemolymph and salivary glands compared to regurgitate and gut.

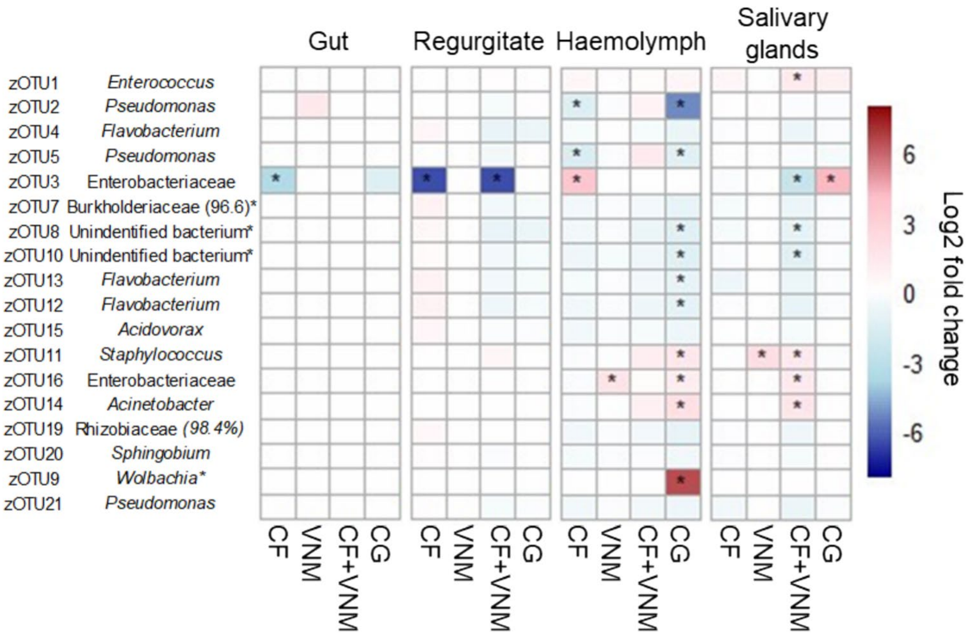


Fig. 4 Fold changes (log2) relative to the negative control treatment (caterpillars injected with PBS) for the relative abundance of bacteria present in the gut, regurgitant, haemolymph, and salivary glands of caterpillars injected with *Cotesia glomerata* calyx fluid ('CF'), *C. glomerata* venom ('VNM'), both calyx fluid and venom ('CF + VNM') and naturally parasitized by *C. glomerata* as well as injected with PBS ('CG'). Bacteria with a relative abundance significantly higher (red) or lower (blue) than those present in the control injection treatment (injection of phosphate-buffered saline solution) are indicated with an asterisk ($p < 0.05$). zOTUs were identified through a BLAST search against type materials in the GenBank database. Identifications were performed at genus level; when identical scores were obtained for different genera, identifications were performed at family or order level. When identity percentages were lower than 99%, the percentage of sequence identity with the GenBank entry is given between brackets. Asterisks indicate zOTUs for which no significant similarity (> 97% sequence identity with type sequence) was found. In these cases, the BLAST analysis was performed against the entire GenBank database. Hits with uncultured bacteria are indicated as unidentified bacterium

Interestingly, a higher bacterial diversity was also found in the salivary glands of mosquitoes, compared to gut [56]. While insect haemolymph is nutrient-rich with a near-neutral pH, it is well-defended by the host immune system, creating a harsh environment for bacteria. Although microbes in the haemolymph of healthy insects may be underreported, only a few microbial taxa are generally thought to persist in this extreme habitat [19]. Our results, among the first to characterise the microbiome of lepidopteran insect haemolymph and salivary glands [57], underscore the potential significance of bacteria inhabiting these fluids and tissues, assuming that these bacteria were alive and in sufficient abundance. This high bacterial diversity may result from vertical transmission, which has been observed for gut bacteria [58]. However, these results should be interpreted with caution, as amplicon screens can yield false positives, and salivary glands may have been contaminated with bacteria from the haemolymph during dissection.

Effects of Injection Treatments

The NMDS analyses did not reveal any significant effect of *C. glomerata* parasitism or injection treatments on the bacterial community composition in the gut, regurgitate, and haemolymph of *P. brassicae*. These results contrast with several recent studies that have demonstrated a significant effect of parasitism on the gut microbiome of lepidopteran hosts [25, 27, 30, 31]. These studies used different biological systems, such as *Melitaea cinxia* parasitized by *Hyposoter horticula* [25], *Diatraea saccharalis* parasitized by *Cotesia flavipes* [27], *Spodoptera frugiperda* parasitized by *Cotesia marginiventris* [30], and *Plutella xylostella* parasitized by *Cotesia vestalis* [31], which could explain the discrepancy. Notably, they only used solitary parasitoids, which could be responsible for a stronger effect on the gut microbiome, compared to our study with a gregarious parasitoid. This hypothesis remains, however, to be tested.

We observed a significant effect of both natural parasitism by *C. glomerata* and microinjection of *C. glomerata* calyx fluid and venom on the bacterial community composition in *P. brassicae* salivary glands. Parasitoid maternal factors, such as polydnavirus and venom, are well-documented for their ability to impair the host immune system [36]. Therefore, we hypothesise that parasitoid maternal factors indirectly affect the bacterial community composition in *P. brassicae* salivary glands by altering the host immune system [25].

The microbiome of salivary glands in herbivorous insects is of particular interest as this organ produces saliva that comes into contact with plants, where effectors are recognised and can induce a specific plant defensive response against herbivory [59, 60]. Several studies have shown that both caterpillar oral secretions [61] and saliva [62] can affect plant defensive responses. Previous research has demonstrated that parasitized herbivores induce different plant responses than their unparasitized counterparts [63, 64]. Furthermore, these changes have been attributed to parasitoid maternal factors [41] that affect the salivary glands of the parasitized hosts [55]. The altered plant response may then be used by other insects to find parasitized hosts, such as hyperparasitoids [55, 65], or avoid them, such as competing parasitoid wasps or adult herbivores looking for parasitoid-free oviposition site. It has been proposed that parasitoids use polydnaviruses and venom to indirectly manipulate plant responses, increasing the fitness of their progeny [66]. This could explain their effect on the host salivary gland microbiome. However, recent studies do not support this hypothesis [64, 67], suggesting that parasitoid-induced plant responses may result from the significant physiological changes in parasitized hosts.

Our results offer new insights into the mechanisms of parasitoids indirect effects on plant responses to herbivory, suggesting that parasitoid maternal factors (polydnavirus and venom) modify the microbiome of salivary glands, which in turn modulate plant-induced responses. Over the past decade, an increasing number of studies have investigated how microbes associated with insects can affect plant responses [68–70]. For example, laboratory-reared *Helicoverpa zea* fed a diet containing several bacterial isolates from the oral secretions of field-collected *H. zea* triggered a significantly higher PPO (polyphenol oxidase activity) response in tomato plants compared to plants damaged by uninoculated laboratory-reared larvae [23]. Other studies [30, 71, 72] have elucidated mechanisms by which bacteria can indirectly mediate plant–insect interactions. They highlighted that gut-bacteria of *H. zea* and *Spodoptera frugiperda* trigger salivary elicitors (e.g., protein glucose oxidase) in labial salivary glands, subsequently modulating tomato- and maize-induced responses against herbivory.

Parasitism Affects Haemolymph Microbiome, with Limited Influence of Maternal Factors

The differential abundance analyses confirmed that *C. glomerata* calyx fluid and venom significantly impact the bacterial composition in the salivary glands of *P. brassicae*, although only minor differences were observed for naturally parasitized caterpillars. The reason for this discrepancy probably lies in the method, that only reveals high changes in the bacterial abundance. It is also possible that we have microinjected higher quantities of maternal factors compared to natural parasitism. Additionally, this analysis revealed a significant effect of natural parasitism on the relative abundance of several bacteria in the haemolymph of *P. brassicae* compared to unparasitized individuals. However, minimal effects of injection treatments were detected on the relative abundance of bacteria in the haemolymph. These findings suggest that the majority of the microbiome changes in the haemolymph due to parasitism are not attributed to parasitoid maternal fluids. Alternatively, these changes could be induced by virulence factors produced by teratocytes [73, 74], or by infection with new bacteria during oviposition. For example, *Wolbachia* was detected only in the haemolymph of parasitized *P. brassicae*, indicating transmission by *C. glomerata* and confirming previous findings [28, 29]. It is also abundant in *C. glomerata* larvae developing in parasitized *P. brassicae* [28]. These bacteria manipulate host reproduction to enhance their transmission [17] and can benefit hosts by conferring resistance to insecticides and viruses [75, 76]. Recent studies suggest that *Wolbachia* alters the body odour of parasitized caterpillars, providing reliable cues for hyperparasitoids to locate hosts [26]. Previous studies [26, 28, 29] showed that *P. brassicae* caterpillars parasitized by *C. glomerata* exhibit a different internal bacterial community composition compared to unparasitized caterpillars, but did not differentiate between internal tissues and fluids. Our current study suggests that these observed differences in the microbiome of parasitized *P. brassicae* were mainly due to changes in the haemolymph and salivary glands, although potential changes in the gonads caused by *Wolbachia* may have been overlooked. This result confirms that, contrary to previous findings, the main effect of parasitism on the host internal bacterial community composition is not always found in the gut.

Conclusion

Our work confirms previous studies in showing that parasitism has significant effects on the microbes present in the parasitized host. It should be noted that we only measured the bacterial microbes in caterpillars, but parasitism can affect other microbes. A previous study showed that parasitism also affects fungi communities in *P. brassicae* [29]. Parasitism can also affect how non-symbiotic viruses multiply in their hosts [77]. Contrary to several studies that showed a significant effect of parasitism on the host gut microbiome, we only found an effect on the salivary glands and haemolymph. Furthermore, our results provide unique insights into the mechanisms underlying these changes by revealing an effect of adult parasitoid calyx fluid (with polydnavirus) and venom on the bacterial community composition in the salivary glands of their lepidopteran host. This mechanism could explain how parasitoids indirectly affect plant response to herbivory: maternal factors affect the bacteria in the salivary glands, which enter in contact with the wounded plant and induces an altered response. More work is needed to confirm this hypothesis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-025-02535-y.

Author Contribution MACC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing—original draft, Writing—review & editing. GG: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing—review & editing. MEB: Conceptualization, Investigation, Formal analysis, Writing—review & editing. SK: Investigation, Writing—review & editing. CV: Investigation. SC: Data curation, Formal analysis, Software. AC: Conceptualization, Writing—review & editing. BL: Funding acquisition, Resources, Writing—review & editing. EHP: Conceptualization, Funding acquisition, Supervision, Project administration, Writing—review & editing.

Funding This work was finantially supported by the Netherlands Organization for Scientific Research (NWO) ALW Open Programme (ALWOP.368) to E.H.P and by the Flemish Fund for Scientific Research (FWO; G.0961.19 N) to B.L.

Data Availability The datasets generated during the current study are available in the supplementary material.

Declarations

Ethics Approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

Competing interests The authors declare no competing interests.

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