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# Microbiome diversity and composition in *Bemisia tabaci* SSA1-SG1 whitefly are influenced by their host's life stage

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ABSTRACT

Within the *Bemisia tabaci* group of cryptic whitefly species, many are damaging agricultural pests and plant-virus vectors, conferring upon this group the status of one of the world's top 100 most invasive and destructive species, affecting farmers' income and threatening their livelihoods. Studies on the microbiome of whitefly life stages are scarce, although their composition and diversity greatly influence whitefly fitness and development. We used high-throughput sequencing to understand microbiome diversity in different developmental stages of the *B. tabaci* sub-Saharan Africa 1 (SSA1-SG1) species of the whitefly from Uganda. Endosymbionts (*Portiera*, *Arsenophonus*, *Wolbachia,* and *Hemipteriphilus* were detected but excluded from further statistical analysis as they were not influenced by life stage using Permutational Multivariate Analysis of Variance Using Distance Matrices (ADONIS, $p = 0.925$ and Bray, $p = 0.903$). Our results showed significant differences in the meta microbiome composition in different life stages of SSA1-SG1. The diversity was significantly higher in eggs (Shannon, $p = 0.024$; Simpson, $p = 0.047$) than that in nymphs and pupae, while the number of microbial species observed by the amplicon sequence variant (ASV) was not significant (n(ASV), $p = 0.094$). At the phylum and genus levels, the dominant constituents in the microbiome changed significantly during various developmental stages, with *Halomonas* being present in eggs, whereas *Bacillus* and *Caldalkalibacillus* were consistently found across all life stages. These findings provide the first description of differing meta microbiome diversity in the life stage of whiteflies, suggesting their putative role in whitefly development.

## 1. Introduction

Bacteria are free-living organisms that thrive in the environment while often interacting symbiotically with higher organisms in various ways (Wells and Varel, 2011). For the case of insects, a wide variety of bacteria are associated with them in different insect body parts that, in turn, influence their host's physiology, ecology, and evolution. Examples of this relationship include the influence on insect fitness, survival, immune response, and fertility. The insect gut harbours a wide range of bacteria that aid in balancing insect nutrition, especially in sap-sucking insects, as they have a diet rich in sugar content but deficient in amino acids. Furthermore, symbiotic microorganisms recycle nitrogen, supply essential amino acids and cofactors, digest significant polymers and make these elements available to the host. These processes contribute to improving the overall fitness of the insect host (Potrikus and Breznak, 1981; Douglas, 2009; Watanabe and Tokuda, 2010).

Various factors affect the relationship between insect microbiome composition and insect fitness, including environmental changes, age, and developmental stages of the insect life cycle. Bacteria use the egg stage to serve as the perfect location for vertical transmission to the subsequent development stage, depending on insect needs. The egg's microbiome often performs essential functions such as providing oxygen and nutrients, and the development from whitefly egg to adult is affected by the microbiome (Zhao et al., 2019).

The insect genus *Bemisia* is one of the most significant pests of crop plants in tropical and subtropical areas (Bellows et al., 1994). The

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whitefly, *B. tabaci* (Hemiptera: Aleyrodidae), was formerly thought to be a highly diverse single species with many morphologically similar biotypes that varied mainly in fecundity, pesticide resistance, and the ability to transmit viruses (Wang et al., 2010; Zang et al., 2006; Guo et al., 2013). However, more recently, phylogenetic analyses and mating experiments have demonstrated that *B. tabaci* is a cryptic species complex of more than 40 species (Tay et al., 2017). In Uganda, thirteen species of the *B. tabaci* complex are endemic, including the Sub-Saharan Africa 1 (SSA1-SG1) (Mugerwa et al., 2018).

Over the past 20 years, Uganda's production of food, vegetables, and cash crops has been severely affected by *B. tabaci* species (Wokorach et al., 2019). They feed on the phloem sap in plants and directly injure them. They also excrete honeydew, which leads to the formation of sooty moulds, thus reducing plant photosynthesis and crop yields (Omongo et al., 2012), and the *B. tabaci* adults are also major vectors of viruses. Several hundred plant viruses belonging to the genera *Ipomovirus, Carlavirus, Crinivirus, Torradovirus,* and *Polerovirus* (Maruthi et al., 2017; Menzel et al. 2011; Polston et al. 2014; Amari et al. 2017; Costa et al., 2020) are transmitted by *B. tabaci*, with the majority (more than 320 species) belonging to the genus *Begomovirus* (Fiallo-Olivé and Navas-Castillo, 2019; Mar et al., 2017).

In eastern Africa, outbreaks of cassava mosaic disease (CMD, begomoviruses) and cassava brown streak virus disease (CBSD, ipomoviruses) epidemics are commonly associated with an abundance of whitefly populations (> 200 whitefly adults on the top five leaves of cassava plants) (Mugerwa et al., 2021; Legg et al., 2014; Colvin et al., 2004). Several biotic and abiotic factors were pointed out to explain these outbreaks (Macfadyen et al., 2018; Ally et al., 2019), among which a recent study investigated the potential role of the microbiome diversity on outbreaking whitefly samples from the CMD epidemic zones in Uganda (El Hamss et al., 2022). This study analysed the endosymbiont communities by comparing SSA1-SG1 samples before (1997) and 20 years after the outbreaks (2017), showing a significant increase in the abundance and diversity of those endosymbionts in the 2017 outbreaking populations.

*Bemisia tabaci* goes through six life stages, starting from the egg, three nymphal stages, pupae, and then adults (Byrne and Bellows, 1991; Basu, 1995). Females lay whitish-yellowish eggs that hatch into nymphs. The first instar nymph, also called the "crawler", feeds on the lower surface of the leaf and becomes sessile after losing its legs in the subsequent moult (Bellows et al., 1994; Byrne, 1999). The fourth stage nymph is called the 'pupa' and is characterised by a yellowish colour and red eyes. The SSA1-SG1 adults hatch from the nymphs after about 22 days from egg-laying to continue the life cycle (Mugerwa et al., 2019).

The presence and activity of the associated microbiome is considered to influence the developmental processes of insects (Weiss and Aksoy, 2011). Throughout the different life stages, microbiome bacteria are either attached to the gut wall or colonised the insect host as free-living organisms, usually scattered all over the body; this type are also called symbionts (Feldhaar, 2011). Other bacteria, also called endosymbionts, can live in the insect's specific organs (Raina et al., 2015; Shi et al., 2018). Organs have specialised bacteriocyte cells, which help keep the bacteria isolated and protected (Raina et al., 2015). Thus, an endosymbiont is a specific type of symbiont that lives inside the host organism's body (Raina et al., 2015).

In both unfertilised or fertilised eggs, bacterial communities establish and gradually change as the embryo and larva develop. In many insects, eggs initially become infected by bacteria from the environment or passed on during mating (Otti, 2015). The structure and composition of these communities are influenced by the host's metamorphosis (Majumder et al., 2020; Nobles and Jackson, 2020). For example, in the wandering larva of *Leptinotarsa decemlineata*, the phyla Proteobacteria, Tenericutes, Firmicutes, and Bacteroidetes dominated in the larvae and adults. In contrast, the phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were dominant in the pupae (Kang et al., 2021). The makeup of the bacterial community depends on the insect's life stage, suggesting the fulfillment of specific metabolic activities during insect development (Alonso-Pernas et al., 2017). The bacterial content may also be shaped by dynamic interactions throughout the life stages and could present significant variations in terms of diversity and composition between different nymphal stages, as has been shown in mosquitoes (Coon et al., 2014; Wang et al., 2011). Many bacteria can be found in egg capsules and subsequently lost after hatching (Fukatsu and Hosokawa, 2002). In agricultural pests such as *S. littoralis,* the different bacteria genus *Enterococcus*, *Pantoea,* and *Citrobacter* were abundant and active in their early instars, whilst *Clostridia* increased in late-instars (Chen et al., 2016), suggesting their critical function at specific life stage.

In this study, we conducted an analysis of the microbiome sequences generated from each whitefly, focusing on two distinct levels. Firstly, we examined the endosymbionts, which reside intracellularly within the whitefly. Secondly, we investigated the remaining bacteria, often referred to as the meta microbiome. No previous information is available regarding the composition change of the whole microbiome during the different life stages of *B. tabaci* species. We hypothesised that different bacterial communities are associated with different life stages. In this study, we used 16 S rDNA sequences to analyse the microbiome in SSA1-SG1 *B. tabaci* life stages collected from the same cassava leaves in Uganda in 1997. Here we worked on the same whiteflies as in the previous study (El Hamss et al., 2022), but we investigated other bacteria instead of only endosymbionts. The meta microbiome found and described in this work could have positive, negative, and neutral effects on the insect host and, thus, should be analysed separately. This whitefly is an interesting insect model due to its economic and agricultural importance.

## 2. Materials and methods

### 2.1. Whitefly sampling

A total of 65 eggs, nymphs, and red-eyed pupae about to emerge into adults were collected from young cassava leaves in farmer's fields in Uganda in 1997 (Table S1 and Fig. 1). Cassava leaves were then conserved in plastic bags and stored at − 80 ℃ at NRI, University of Greenwich, UK. Sampling was conducted in cassava fields located between the towns of Mityana, Kampala, and Masaka districts and coincided with whitefly outbreaks at the sampling time. The distance between sites was up to 5 km, as previously described (El Hamss et al., 2022).

#### 2.1.1. DNA extraction

First, leaves having eggs and nymphs were removed from the − 80 °C freezer and placed onto sterile Petri dishes. Sterile needles were used to collect whitefly eggs and nymphs using a binocular microscope and then transferred into 1.5 mL sterile centrifuge tubes. About 60 mL of Chelex buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added to the centrifuge tube, and the sample was manually ground (Walsh et al., 1991; Ghosh et al., 2015; El Hamss et al., 2021). Samples were incubated at 58 °C for 1.5 h before denaturation at 96 °C for 10 min. After centrifuging the samples at 13,000 rpm, the supernatant was kept at − 20 °C. Both DNA concentration and purity were determined with a NanoDrop 2000 UV (Thermo Scientific, UK), and the DNA quality was determined with 1% agarose gel electrophoresis. Partial mitochondria DNA was amplified using a mitochondrially encoded cytochrome c oxidase I (mtCO1) marker, and the generated PCR product of each sample was sequenced (Ally et al., 2019). The V4-V5 hypervariable regions of the bacterial 16 S rDNA gene of DNA identified as SSA1-SG1 were subsequently amplified with the primers F- (GTGCCAGCMGCCGCGG) and R- (CCGTCAATTCMTTTRAGTTT) (El Hamss et al., 2022, 2021; Wang et al., 2019a) by a thermocycler PCR system (Applied Biosystems, UK). Each reaction contained 3 μl of DNA template, 1x reaction buffer, 2.5 μM each primer, 10 mM dNTPs, and 1.0 U of DreamTaq DNA polymerase (Thermo Scientific, UK). The PCR conditions were an initial

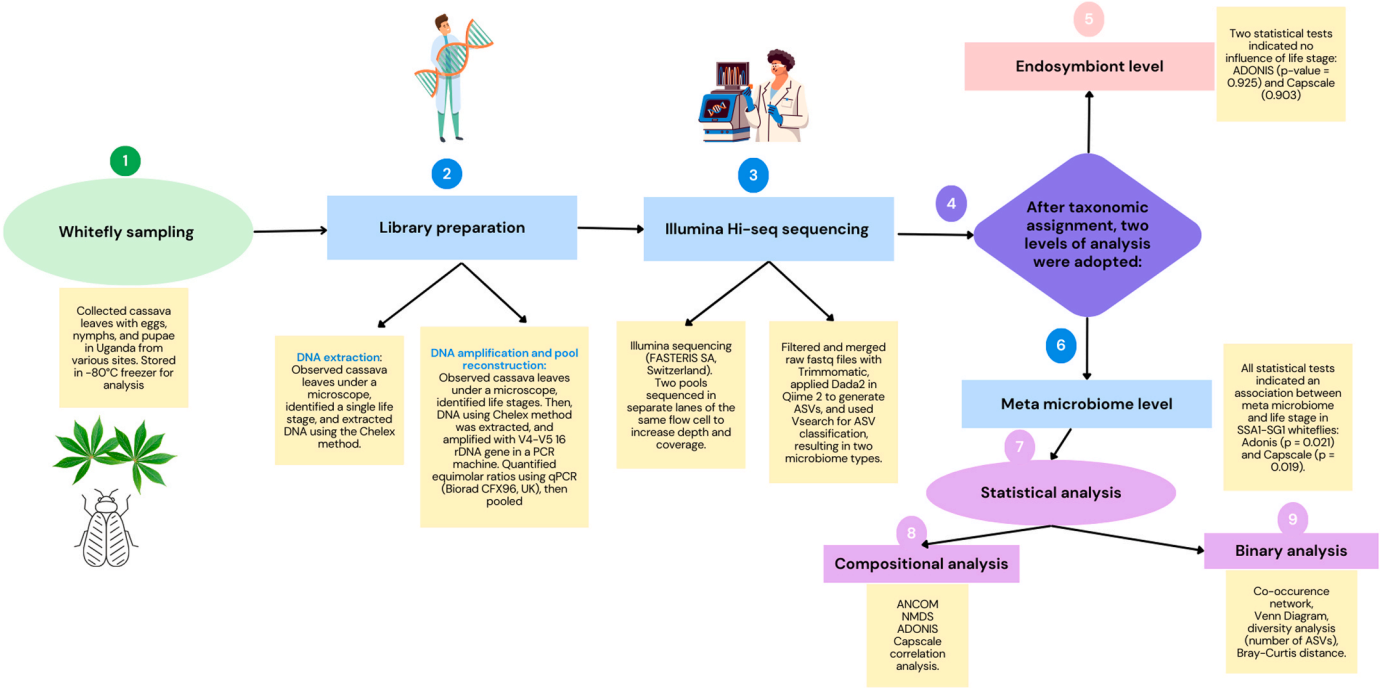


**Fig. 1.** Schematic representation showing steps involved in meta microbiome analysis.

denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 15 s and extension at 72 °C for 50 s, and a final extension at 72 °C for 5 min. Duplicates of each sample were pooled prior to purification using a gel extraction kit (NucleoSpin, Macherey-Nagel, Switzerland) according to the manufacturer's instructions and quantified with PicoGreen DNA quantification assay kit (Thermo Scientific, UK) in a qPCR machine (Biorad CFX96, UK).

### 2.2. Illumina HiSeq sequencing and data analysis

Amplicons obtained from each whitefly sample were pooled in equimolar concentrations for Illumina sequencing (FASTERIS SA, Switzerland). A composite sample with this pool of combined equimolar ratios was subjected to a spin column purification using the same kit, and then the pool was quantified using Nanodrop. Another pool prepared with the same samples was also sequenced in a different lane but in the same flow cell to increase the depth of sequencing and the coverage of samples. Illumina sequencing results generated paired-end (PE) sequence data that were merged into a single sequence based on their location in the V4-V5 region of the 16 s rDNA gene.

Unique barcodes were attached to primer sequences at both ends to separate the samples. Raw fastq files were then filtered and merged by Trimmomatic using the following parameters: (i) the read tail was truncated when four consecutive bases had a low quality $< 15$; (ii) sequences with reads longer than 60 bp were kept and merged according to their overlap, with no more than 2 bp mismatches; and (iii) the sequences of each sample were separated according to barcodes (exact match) and primers (allowing 2 nucleotide mismatches in the seed) and reads below 60 bp bases were removed. The DADA2 plugin (Callahan et al., 2016), which was implemented in the QIIME2 program version 2018, was adopted to perform a second filtering operation on the sequences (Bolyen et al., 2018). Filtering operations included the "Denoise and dereplicate paired-end sequences" to enable additional read quality filtering and trimming, chimera filtering, denoising, and connecting paired reads as previously described (El Hamss et al., 2022, 2021). DADA2-generated amplicon sequence variants (ASVs) were clustered using the VSEARCH consensus taxonomy classifier (Rognes et al., 2016). Taxonomic assignment of ASVs was analysed using the SILVA database. ASVs with their generated taxonomic groups were merged to form operational taxonomic units (OTUs) and were clustered together with a 97% similarity cutoff. Sequences ranging from accession number OR184995 to OR185264 have been deposited in the GENEBANK database. These sequences were analysed at two distinct levels: the endosymbiont level and the meta microbiome level. At the meta microbiome level, we included all bacteria that have not yet been localized within the whitefly body.

Venn diagram at different taxonomic levels was constructed in the microbiome package (Lahti et al., 2017; Shetty and Lahti, 2019) to identify core microbiome membership associated with each life stage of the SSA1-SG1 whitefly. The Venn diagram method was used based on Abundance–Occurrence (Neu et al., 2021) in each taxonomic level, starting from phylum down to the genus level. Bacterial taxa occurring in at least 50% of the samples of each group (i.e., egg, nymph, or pupae) with a minimum relative abundance threshold of 0.03% were considered differential taxa. Taxa with occurrences less than 50% prevalence and a relative abundance of other taxa under 0.03% were discounted from core membership. Subsequently, two (shared and unique) core bacteria groups were identified. Genera occurring in at least 50% of eggs, nymphs, and pupae were considered "shared." Sample interactions were then represented with Venn diagrams. The core microbiome is steadily associated with a particular whitefly stage. Those genera identified with at least 100 reads per each sample type were selected and were considered "unique." Unique genera under 100 reads were discounted. The overall workflow was illustrated in Fig. 1.

### 2.3. Statistical analyses and comparison of meta microbial communities

First, samples rank abundance curve, and rarefaction curve were measured using the function "specaccum" of the vegan package (Team, 2009) in R (Team, 2020) to see if the number of samples used accurately detected all taxa.

Different tests were adopted to determine life stage-associated microbiome, including diversity, correlation, co-occurrence, and compositional analysis. The diversity parameters of the microbiome of whiteflies at various life stages were tested using one-way analysis of variance (ANOVA). The parameters included the richness measured by

the number of observed ASVs and species evenness measured by Shannon and Simpson indices. The differences between the composition of microbiome in each life stage were visualised using the non-metric multidimensional scaling (NMDS) distance algorithm based on the Bray-Curtis distance. For NMDS analysis and mapping, the beta diversity distance matrix was calculated using the vegan package. Both the Capscale and permutational multivariate analysis of variance (Adonis) functions of the vegan package were used to test the structure of microbial communities according to life stage.

We conducted a bootstrapping analysis with equal weight to life stage and with 10,000 resampled datasets to address potential sample size bias between eggs, nymphs and pupae. This analysis, performed using R software, aimed to assess whether observed distributions were related to life stage or discrepancies in sample size. We used Adonis and Capscale for this evaluation.

Another hierarchical clustering method by unweighted pair group method with arithmetic mean (UPGMA) was performed on a matrix of presence/absence to capture clusters of microbiome per life stage. Analysis of compositions of microbiome with bias correction (ANCOM-BC) (Lin and Peddada, 2020) is a methodology of differential abundance (DA) analysis for absolute microbial abundances, and it was used to examine differences in composition on the relative abundances of taxa observed in each life stage. Co-occurrence network analysis was used to see the existence of specific taxa at specific whitefly stages. The co-occurrence networks were constructed using the probabilistic model of co-occurrence among genera (Veech, 2013) without looking at abundance reads. The Pearson correlation coefficient was estimated, and a heat map (Reimann et al., 2017) was constructed according to the whitefly life stage. This correlation was calculated between each group on their relative abundances of genera. The three correlation matrices between taxa calculated at different life stages were tested using Jennrich test (Jennrich, 1970).

In our study, we conducted Multivariate Analysis of Variance (MANOVA) to assess differences in the whitefly's meta microbiome composition across different life stages. We analysed 34 eggs, 19 nymphs, and 6 pupae samples to determine how specific microbial taxa vary among them. To investigate the relationships between these microbial taxa and life stages while accounting for potential covariates, we performed multiple regression analysis. This method allowed us to explore the dependencies and associations in our data.

## 3. Results

### 3.1. Analysis rationale and hypothesis

In this study, our objective was to investigate the hypothesis concerning the variability of the microbiome in *B. tabaci* SSA1-SG1 throughout its various life stages. To achieve a comprehensive understanding of the whitefly microbiome, we meticulously examined two distinct levels: endosymbionts and the meta microbiome. This separation aimed to gain deeper insights into these discrete and different microbiome communities. Our study results demonstrated that endosymbionts remained remarkably stable across different whitefly life stages, showing no significant influence from developmental transitions. On the other hand, our results showed that the meta microbiome was influenced by the whitefly SSA1-SG1 life stage. By dissecting and analyzing these microbial factions independently, we significantly enriched our comprehension of the intricate whitefly microbiome dynamics.

To rigorously assess and interpret these microbiome variations, we employed various statistical tools, including Bray-Curtis distance, Adonis, and Capscale tests. In addition, we implemented Bootstraping to address potential effect size bias and to ensure the robustness of our conclusions. Our findings revealed that 70–100% of the bootstrapped samples showed very significant results with a p-value less than 0.05. For a comprehensive understanding of the distribution of p-values, Table S2 provides further details, including the mean, median, standard deviation, minimum, maximum, lower bound, and upper bounds at a 95% confidence level.

#### 3.1.1. Effect of life stage on endosymbionts versus meta microbiome composition

The results, obtained through ADONIS (p-value =0.925 and the Bray distance metric (0.903), confirmed that the endosymbionts remained unaffected by the life stages of the whitefly (Fig. S1 and S2). This stability was evident from the earliest stage of the egg, indicating their crucial and consistent presence.

In contrast, the analysis revealed that the meta microbiome present in the whitefly was influenced by the life stage (Figs. S1 and S2). As such, the subsequent results exclusively pertain to the meta microbiome, highlighting their dynamic nature and potential interaction with the environment.

### 3.2. Effect of the whitefly life stage on the meta microbiome diversity

Our study calculated alpha diversity parameters within the meta microbiome of SSA1-SG1 whiteflies at various developmental stages, namely eggs, nymphs, and pupae, collected from cassava leaves in Uganda. This analysis was grounded in three key alpha diversity indices: observed species ASVs, Shannon, and Simpson, as illustrated in Fig. 2A, B, and C. Significant variations in Shannon diversity across whitefly life stages were observed ($p = 0.0024$, Fig. 2A), with distinct differences in species composition and relative abundances being underscored. On average, the highest Shannon index (3.7) was exhibited by eggs, followed by nymphs (Douglas, 2009) and pupae (Douglas, 2009) (Fig. 2A). A similar pattern was displayed by the Simpson index of the meta microbiome community from these whitefly life stages ($p = 0.0047$, Fig. 2B), further reinforcing the relationship between species dominance in the meta microbiome and the respective whitefly life stage. The highest average Simpson index (0.95) was exhibited by eggs, followed closely by nymphs (0.91) and pupae (0.91) (Fig. 2B). A declining trend in the average number of ASVs was observed, decreasing from 52 in eggs to 25 in nymphs and pupae, but it was not significant ($p = 0.094$, Fig. 1C). This indicated a relatively consistent presence of microbial species within the whitefly meta microbiome throughout its developmental stages. A comprehensive perspective on alpha diversity within the whitefly meta microbiome was provided by our study, shedding light on both stable and dynamic aspects of diversity across different life stages. To visually represent the impact of the whitefly life stage on the composition of the meta microbiome community, Multidimensional Scaling (MDS) was employed, as depicted in Fig. 2D. The separation of whitefly stages based on their microbial composition was effectively outlined in Fig. 2D, revealing distinct clustering patterns. Significant differences in meta microbiome communities between whitefly life stages were confirmed by statistical analysis using Adonis ($p = 0.021$) and Capscale ($p = 0.019$), showing the influence of whitefly development on the composition of the meta microbiome community.

### 3.3. Overview of whitefly meta microbiome composition

A total of 4,533,906 high-quality (>Q30) reads were filtered and recovered from 65 whitefly samples. At a sequence variant level, 188 ASVs were obtained and deposited in GeneBank with the accession numbers ranging from OR184995 to OR185264, and their relative frequency per sample was calculated (Fig. 3). Hierarchical clustering dendrogram and bar chart showed the composition of whitefly according to their life stage. The taxa were classified into three phyla and were obtained for each whitefly life stage (Table S1, Fig. 3), and the rank abundance curves of each sample progressively stabilised, showing consistent species distribution (Fig. S3A). The rarefaction curve also reached a plateau, indicating that the tested samples accurately detected all bacteria (Fig. S3B). *Caldalkalibacillus* and Micrococcaceae were the

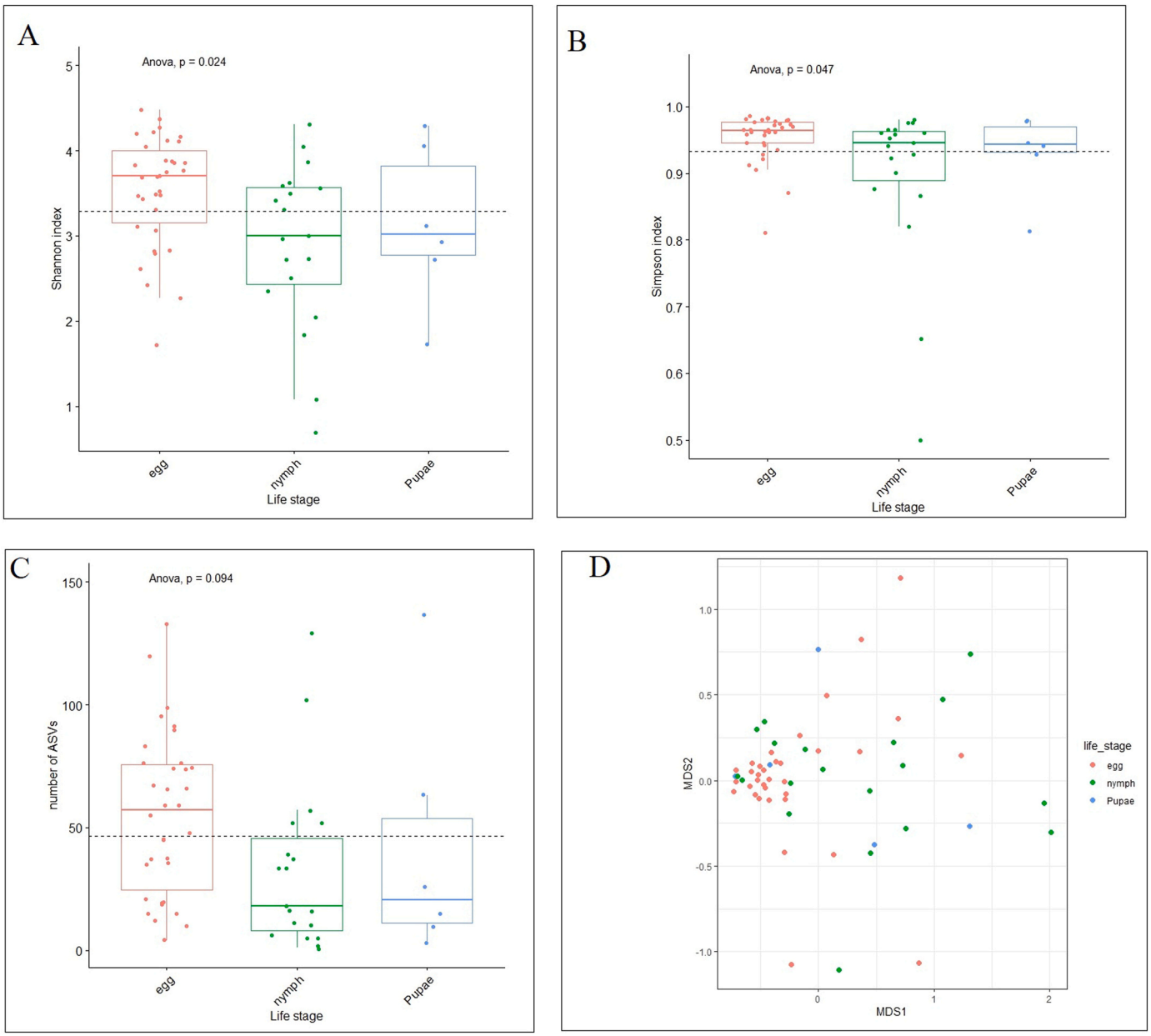


**Fig. 2.** Box plots showing the differences in alpha diversity parameters (Shannon, p = 0.024 (A); Simpson, p = 0.047 (B); and number of identified Amplicon Sequence Variants (ASVs), p = 0.094 (C)) using One-way ANOVA. An ordination plot of the Beta diversity is depicted using Non-metric multidimensional scaling analysis (NMDS) on the Bray–Curtis matrix, with Adonis results showing a p-value of 0.021 and Capscale of 0.019) (D). These analysis were based on 16 S rDNA sequences of the SSA1-SG1 whiteflies. Colored points represent different life stages: eggs (red dots), nymphs (green dots), and pupae (blue dots). Points close to each other indicate a similar microbial composition.

top prevalent taxa, but their relative frequency varied according to the life stage. ANCOM showed significant differences in the composition per life stage (Table S3 and Fig. 3). Six strains of *Caldalkalibacillus* 1–6 were detected at genus level and four strains of Micrococcaceae 1–4 were detected at family level across all stages of *B. tabaci* SSA1-SG1 (Fig. 3).

Of these, *Caldalkalibacillus*.4 and Micrococcaceae4 were exclusively found in pupae, with prevalence rates of 4% ± 0.09% and 3% ± 0.07, respectively (Table 1). *Caldalkalibacillus*.6 prevalence was 0% in nymphs but significantly prevalent in eggs, reaching 1% ± 0.01 and pupae reaching 2% ± 0.04 (Table 1). These distinct microbial prevalence patterns among the different life stages highlight stage-specific microbial associations within the whitefly.

### 3.3.1. *Bacterial correlation across different life stages of the SSA1-SG1 whitefly*

In this study, we simultaneously investigated the prevalence of the top 10 whitefly meta microbiome members across different life stages. Our findings revealed significant variations in the prevalence of Micrococcaceae2, Caldalkalibacillus.3, Caldalkalibacillus.5, *Caldalkalibacillus*.2, *Caldalkalibacillus*.6, Micrococcaceae1, Micrococcaceae3, *Caldalkalibacillus*.4, and Micrococcaceae4 among various life stages, as confirmed by MANOVA (p = 0.009). Individual regression models were used to predict the abundance of these genera within the whitefly meta microbiome at each life stage, with detailed statistical summaries presented in Table S3.

The resulting dendrogram visually depicts clusters of ASVs, with distinct color codes representing their highest and lowest correlation values (Fig. 4). The correlation matrices for each life stage were found to

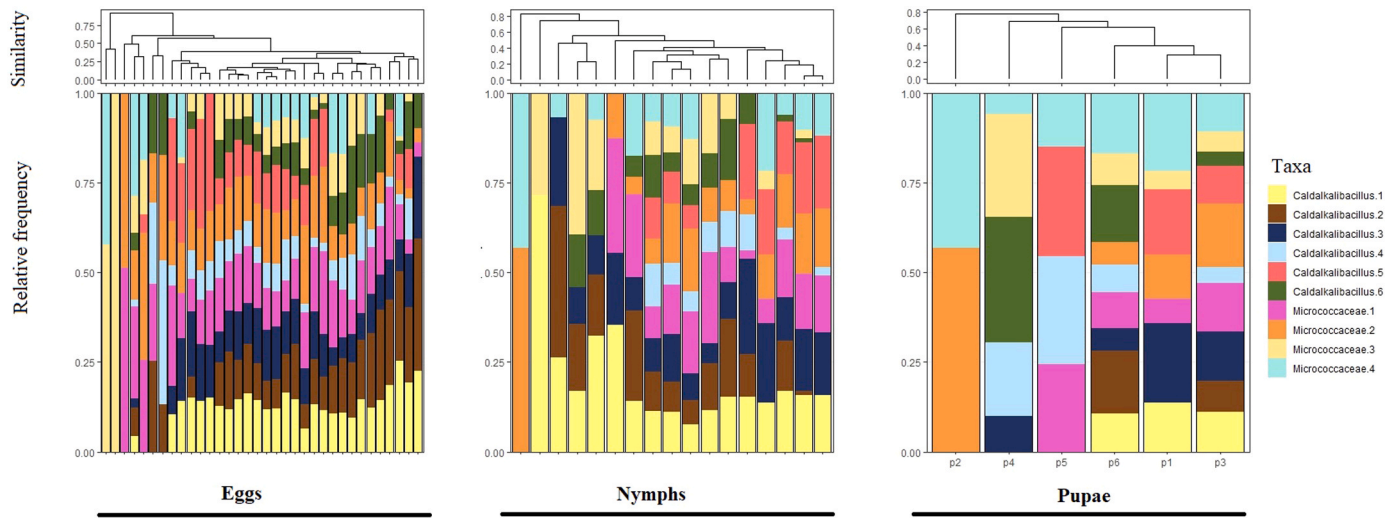


**Fig. 3.** Bacterial composition and relative abundance of 16 S rDNA amplicon sequence variants (ASVs) from B. tabaci SSA1-SG1 whiteflies, presented in dendrogram and histogram formats. Dendrograms were constructed using the unweighted pair group method with the arithmetic mean (UPGMA) on Bray-Curtis dissimilarities, illustrating the evolutionary ties among whitefly samples with similar bacterial compositions. The relative abundance of each sample was depicted by the histogram, with only the top significantly different taxa based on Analysis of Composition of Microbiomes (ANCOM) test depicted in the graph (all adjusted p-values can be found in Table S1).

**Table 1**
Average Relative Frequency of Prevalent ASVs by Life Stage.

| ASVs name | Eggs | Nymphs | Pupae |
|---|---|---|---|
| Caldalkalibacillus 1 | 0.03 ± 0.03 | 0.05 ± 0.04 | 0.07 ± 0.1 |
| Micrococcaceae2 | 0.04 ± 0.03 | 0.04 ± 0.04 | 0.04 ± 0.06 |
| Caldalkalibacillus 3 | 0.05 ± 0.16 | 0.01 ± 0.02 | 0.05 ± 0.08 |
| Caldalkalibacillus 5 | 0.03 ± 0.03 | 0.02 ± 0.03 | 0.03 ± 0.04 |
| Caldalkalibacillus 2 | 0.03 ± 0.03 | 0.02 ± 0.03 | 0.05 ± 0.08 |
| Caldalkalibacillus 6 | 0.01 ± 0.01 | 0 ± 0.01 | 0.02 ± 0.04 |
| Micrococcaceae1 | 0.02 ± 0.03 | 0.03 ± 0.03 | 0.05 ± 0.04 |
| Micrococcaceae3 | 0.01 ± 0.02 | 0.01 ± 0.02 | 0.03 ± 0.03 |
| Caldalkalibacillus 4 | 0 ± 0 | 0 ± 0.01 | 0.04 ± 0.09 |
| Micrococcaceae4 | 0 ± 0 | 0 ± 0 | 0.03 ± 0.07 |

be significantly different, as confirmed by the Jennrich test ($p < 0.0005$). These findings emphasize the dynamic nature of the whitefly meta microbiome across different developmental stages, providing valuable insights into the intricate relationships among its constituent microorganisms.

### *3.3.2. Characterisation of life stage associated microbiome and their interactions*

First, we looked at the existence of a "core" microbiome that is both common to all life stages of the whitefly and unique to each life stage from phylum to genus level. At the phylum level, three taxa were identified in all life stages, including Actinobacteria, Firmicutes, and Proteobacteria (Fig. 5A). At the class level, two were egg-associated taxa, including Alphaproteobacteria and Gammaproteobacteria, whereas Alphaproteobacteria and Bacilli were shared across all life stages (Fig. 5B). Oceanospirillales was an egg-associated bacterial order, whereas Propionibacteriales were present in both eggs and nymphs. Bacillales and Micrococcales orders were shared across all life stages (Fig. 5C). At the family level, Halomonadaceae was an egg-associated family. Propionibacteriaceae occurred in both eggs and nymphs, whereas Bacillaceae and Micrococcaceae were shared across all life stages (Fig. 5D). At the genus level, three bacteria were shared, including *Bacillus*, *Caldalkalibacillus,* and *Caldalkalibacillus* 1 in all life stages, whereas *Halomonas* occurred only in eggs and *Cutibacterium* in eggs and nymphs (Fig. 5E).

The interactions within bacteria in each life stage of the whitefly were also investigated. Three co-occurrence networks were constructed at different life stage levels using the probabilistic co-occurrence model among genera ($p < 0.05$, Fig. 6). The networks only displayed significant connections within bacteria. The co-occurrence of bacteria within the egg network differs from that of the other networks (Fig. 6). Nine bacteria existed together in eggs, including Bacillaceae, *Bacillus*, *Bacillus halodurans*, different strains of *Caldalkalibacillus* 1 and *Caldalkalibacillus*, *Bradyrhizobium*, *Cutibacterium*, *Halomonas*, and Micrococcaceae (Fig. 6). Out of these, three bacteria (Bacillaceae, *Bacillus,* and *Caldalkalibacillus*) were also present in nymphs (Fig. 6), whereas other bacteria were not co-dependent in the nymph stages. *Caldalkalibacillus* 1 and Micrococcaceae co-occurred separately from the other nine bacteria mentioned above in nymphs (Fig. 6). In the pupae network, other bacteria co-occurred, including *Bacillus halodurans* and one strain of *Caldalkalibacillus* (Fig. 6). Microbiome comparisons revealed considerable differences in the bacteria-bacteria associations between the whitefly stages.

## 4. Discussion

The whitefly B. tabaci SSA1-SG1 is a native and invasive species in Uganda, affecting both cassava and other non-cassava plants. The Ugandan species SSA1-SG1 harbours diverse bacteria that influence whitefly development, particularly endosymbionts. Previous studies have extensively discussed endosymbionts harboured by SSA1-SG1 alongside other whitefly species. However, investigations related to the meta microbiome in SSA1-SG1 have been quite limited in Uganda. In the present study, we conducted high-throughput sequencing on Ugandan whiteflies, specifically B. tabaci SSA1-SG1, collected at different life stages to investigate their microbiome diversity. Our objective was to gain a deeper understanding of the dynamics of meta microbiome composition across various life stages and explore their potential impacts on whitefly development and behavior. In the current study, high-throughput sequencing has been employed to explore microbiome diversity in B. tabaci SSA1-SG1. Endosymbionts (Portiera, Arsenophonus, Wolbachia, and Hemipteriphilus) were found but eliminated from statistical analysis since they were not influenced by life stage using Permutational Multivariate Analysis of Variance. SSA1-SG1 meta microbiome differed significantly throughout life stages. The number of meta microbiome species observed by the ASVs was not significant, but eggs had higher diversity than nymphs and pupae. Halomonas was present in eggs, whereas Bacillus and Caldalkalibacillus

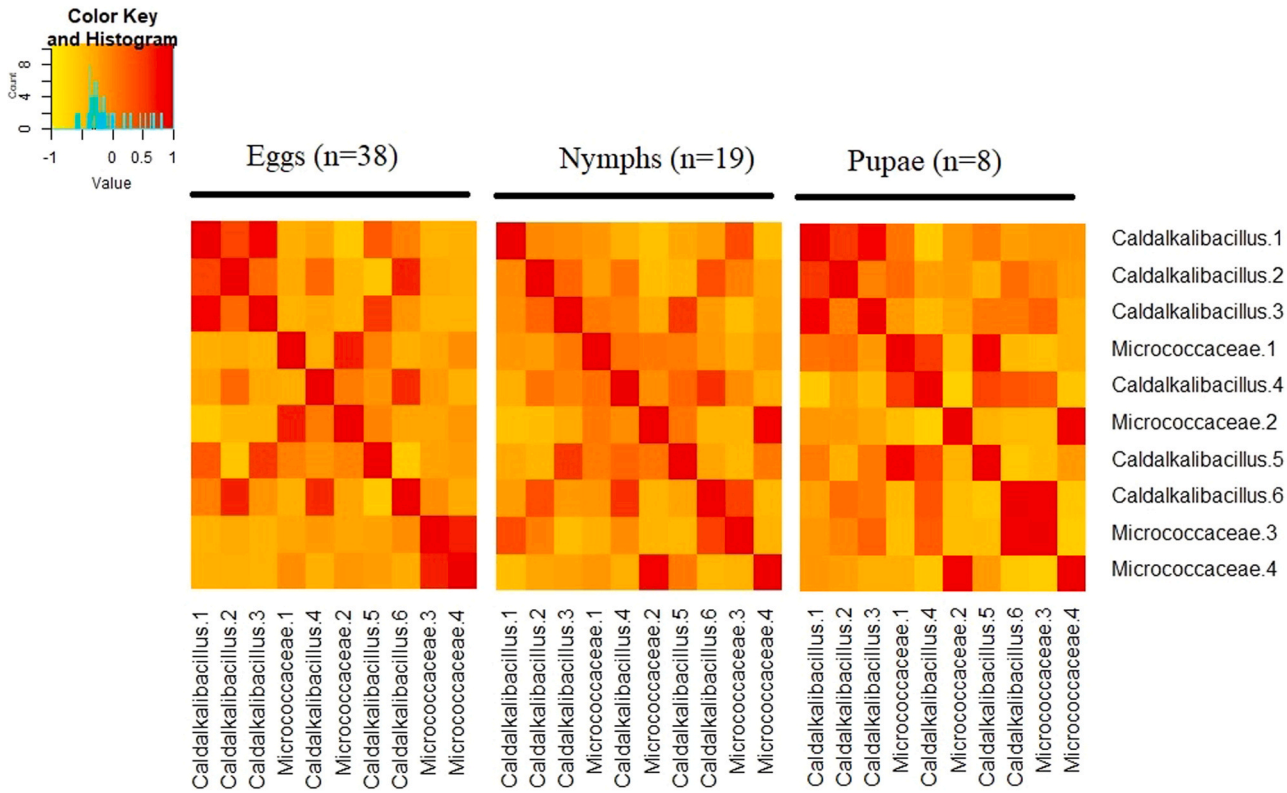


**Fig. 4.** Heat maps of correlation (Pearson) matrices calculated for the relative abundance of the top 10 bacterial amplicon sequence variants (ASVs) for three different life stages of the whitefly B. tabaci SSA1-SG1. The three correlation matrices between taxa calculated at different life stages were significantly different based on the Jennrich test ($p = < 0.0001$). Data was analysed based on 16 S rDNA sequences of the B. tabaci SSA1-SG1 whiteflies.

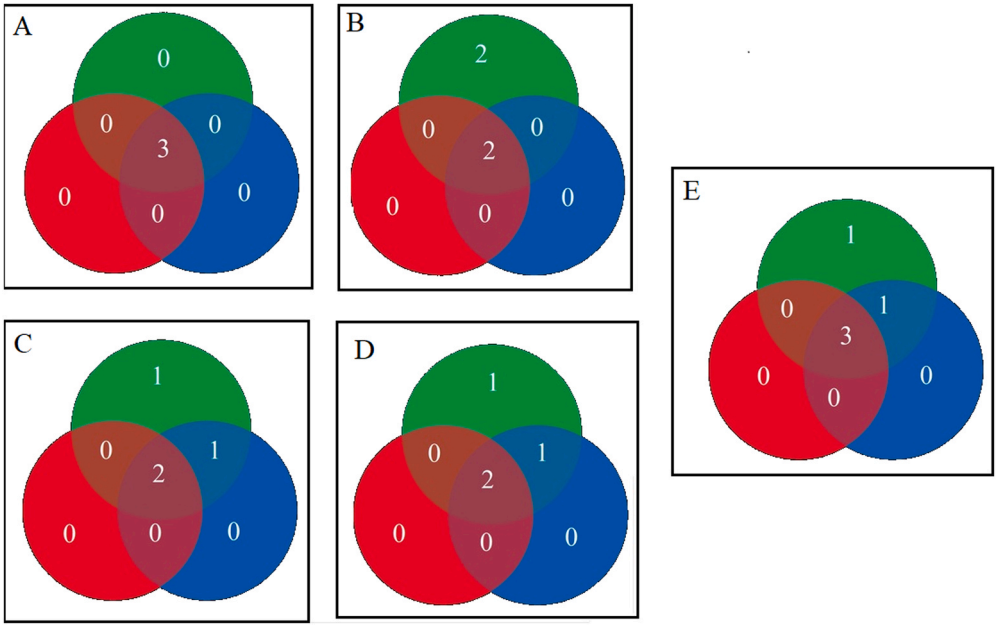


**Fig. 5.** Venn diagrams showing the bacterial amplicon sequence variant (ASV) assemblages detected in whitefly samples from the three tested life stages (egg (green), nymph (blue), and pupae (red)) and across different taxonomic levels (phylum (A), class (C), order (B), family (D) and genus (E)). The Venn diagram method was carried out based on Abundance–Occurrence within each taxonomic level, starting from phylum down to the genus. Bacterial taxa present in at least 50% of the samples within each group with a minimum relative abundance threshold of 0.03% were considered as differential taxa. Data were analysed based on 16 S rDNA sequences of the B. tabaci SSA1-SG1 whiteflies.

were present throughout life. These findings describe whitefly meta microbiome diversity for the first time, suggesting its function in development. This study shed light on the interactions within the meta microbiome of the whitefly, SSA1-SG1. This, in turn, will aid in devising specific management strategies targeting particular stages Other studies also showed that the bacterial composition within the insect's different

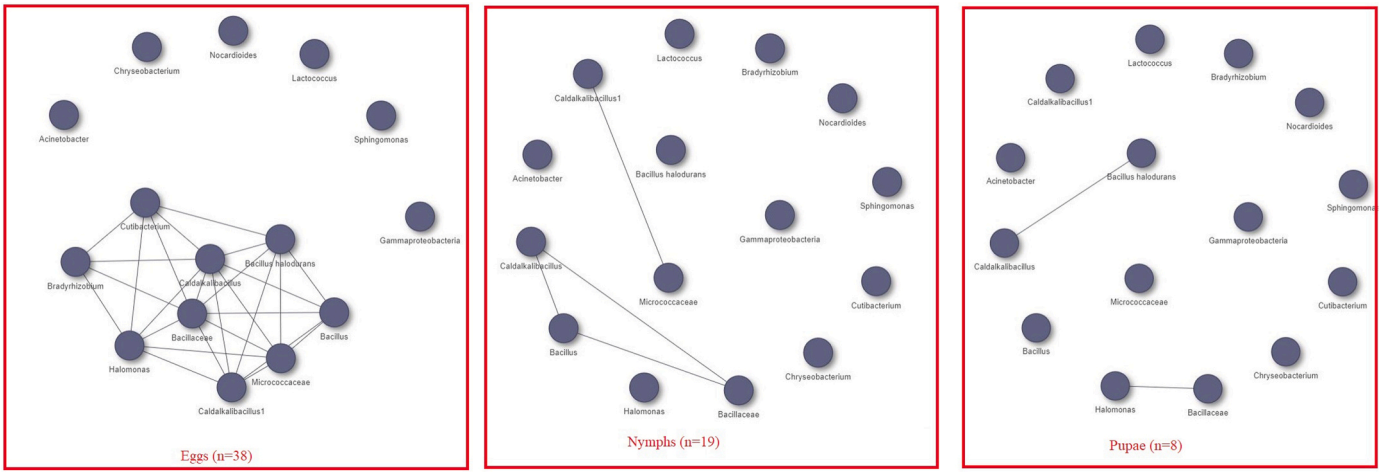


**Fig. 6.** The microbial co-occurrence networks of bacterial associations for each life stage of the tested whitefly (egg, nymph, and pupae). Grey nodes indicated ASVs collapsed at the genus level. The line linking two grey nodes indicated positive interactions. The probabilistic co-occurrence model was used for network construction, and only significant interactions were shown in the network (p-value< 0.05). Data was analysed based on 16 S rDNA sequences of the B. tabaci SSA1-SG1 whiteflies.

developmental stages is influenced by the insect gut's bacterial community, depending on the insect order (Moll et al., 2001; Engel and Moran, 2013; Kowallik and Mikheyev, 2021).

Diversity and taxonomic analyses of 16 S rDNA sequences revealed a high bacterial diversity comprising a total of 188 bacterial ASVs within a *B. tabaci* species, suggesting their complex relationships and potential roles within SSA1-SG1 whiteflies. Similarly, diversity studies in other insects, such as leafhoppers, also identified 249 genera of gut bacterial communities in populations collected from six geographic regions in China (Wang et al., 2019b). A study of the *B. tabaci* Asia II 1, using the V4 region of the 16 S rDNA gene, detected 28 genera (excluding endosymbionts) (Shah et al., 2020). In contrast, in one study carried out across 11 samples of *B. tabaci* Middle-East Asia Minor 1 (MEAM1), they detected three bacterial taxa (Jing et al., 2014), all of which belong to endosymbionts, but other bacteria were not detected. The low bacterial diversity found in other studies could be partly due to the low recovered reads of 5775 (SD = 1882 reads per insect excluding *Portiera*) compared to this study, 76,846 (SD = 2.02E+05). The comparisons of diversity metrics between studies need to be interpreted carefully as different studies use different (i) starting tissues, (ii) methods for DNA release from bacterial cells, (iii) primers, (iv) sequencing methods, and (v) cutoff for distinguishing species (or OTUs) (Jing et al., 2014; Amend et al., 2010).

To provide a comprehensive perspective of the meta microbiome associated with SSA1-SG1, in this study, total meta microbiome assessments were derived from whole insect samples (egg, nymph, and pupae life stages); therefore the reported bacteria may not be restricted to the gut and could have been located outside the digestive tracts. Both putatively helpful and harmful bacterial communities were identified in this study. We report here the first detection of *Caldalkalibacillus* in whiteflies. *Caldalkalibacillus* is a genus belonging to facultative aerobic endospore-forming bacteria. Endospore-forming bacteria are helpful as they produce enzymes and bioactive chemicals to degrade the pollutants, and some of them are used as biopesticides (de Almeida Couto et al., 2015). More studies are required to examine *Caldalkalibacillus* potential effects on the host phenotype and fitness in *B. tabaci* SSA1-SG1.

Bacillus, which was found in all life stages, is a cultivable bacteria and had been previously reported in the other *B. tabaci* species, MEAM1 (Davidson et al., 2000), Asia I, and Asia II 1 (Poddar et al., 2016). *Bacillus* was also previously isolated from six aphid strains belonging to different species, including (i) *Aphis pisum,* (ii) *A. passeriniana,* and (iii) *A. spiraecola* (Grigorescu et al., 2018). Their presence in all whitefly stages may be needed for successful feeding because they are present in adults as well (El Hamss et al., 2021). Their presence may also be a sign of commensalism or parasitism that should be further investigated.

In this study, Halomonas was present in eggs. Halomonas sp. has been previously identified in the gut of other insects, such as the Lepidoptera *Spodoptera exigua* (Gao et al., 2019), an oligophagous aphid (*Toxoptera citricidus*), and the glassy-winged sharpshooter (*Homalodisca vitripennis*) (Guidolin and Cônsoli, 2017; Duan et al., 2022). It is also distributed in the midgut of Mediterranean (MED) *B. tabaci* species collected on sweet potato in Uganda and the Asia II 1 species collected on cotton in China (Wang et al., 2019a), suggesting their wide distribution across insect taxa and host plants. Although the function of *Halomonas* is unknown, it has been proposed that they help improve insect resistance and adaptation to the environment (Dillon and Dillon, 2004). In addition, this study found differences in whitefly microbiome by life stage at a single point in time. Further research that examines changes over time (longitudinal experiment) in controlled conditions should confirm these findings.

We also found in this study Cutibacterium in eggs and nymphs. One study has also reported the presence of *Cutibacterium*-like bacteria in the gut of the yellow fever mosquito (*Aedes aegypti*). Still, the role of these bacteria in the insect's biology is not well understood as research on *Cutibacterium* in insects is scarce. More studies are needed to understand the association of *Cutibacterium* in the insect's development. In this study, the collection of *B. tabaci* SSA1-SG1 specimens coincided with the advent of whitefly outbreaks in Uganda. This whitefly upsurge was estimated at more than 200 adults per top five leaves (Mugerwa et al., 2021; Legg et al., 2014; Colvin et al., 2004). The reasons behind whitefly outbreaks are not yet fully understood, and one explanation for this high whitefly infestation was related to synergistic interactions between whitefly and their microbiome, providing a high fitness advantage to their whitefly hosts. The diverse microbiome and community richness detected from *B. tabaci* SSA1-SG1 in this study may have played a functional role in modulating the behaviour and abundance of the insect during the outbreak.

Overall, we found diverse genera/species of bacteria and different communities that varied according to the host's life stages. This study supports the view that an improved understanding of the roles played by the whitefly microbiome is necessary in order to fully understand whitefly biology, reproduction and behaviour. *Halomonas* was highly associated with eggs, for example, and *Cutibacterium* was also found in eggs and nymphs but not in pupae, suggesting their unique association with the specific life stage. More detailed work is now required to test various hypotheses related to the specific functions played by the

various bacteria in the African cassava whitefly.

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## Author Statement

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## CRediT authorship contribution statement

JC, HD, and MM: conceptualisation. HH: data collection. HH, H-LW, SB, HD, JC, and MM: data analysis and interpretation. HH: writing—original draft. HH, MM, HD, JC, SB and H-LW: writing, review and editing. JC, MM, HD, and CO: funding acquisition. All authors contributed to the article and approved the submitted version.

## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data Availability

Data are publicly available with the accession numbers in the GenBank database.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127538.

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