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Characterization of the microbiome of *Aedes albopictus* populations in different habitats from Spain and São Tomé

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The mosquito microbiome significantly influences vector competence, including in *Aedes albopictus*, a globally invasive vector. Describing the microbiome and *Wolbachia* strains of *Ae. albopictus* from different regions can guide area-specific control strategies. Mosquito samples from Spain and São Tomé were analyzed using 16S rRNA gene sequencing and metagenomic sequencing. *Wolbachia* infection patterns were observed by sex and population. Female mosquitoes were blood-fed, a factor considered in analyzing their microbiota. Results revealed a dominance of dual *Wolbachia* infections, strains A and B, in the microbiome of both populations of *Ae. albopictus*, especially among females. Both populations shared a core microbiome, although 5 and 9 other genera were only present in Spain and São Tomé populations, respectively. Genera like *Pelomonas* and *Nevskia* were identified for the first time in *Aedes* mosquitoes. This study is the first to describe the *Ae. albopictus* bacteriome in Spain and São Tomé, offering insights for the development of targeted mosquito control strategies. Understanding the specific microbiome composition can help in designing more effective interventions, such as microbiome manipulation and *Wolbachia*-based approaches, to reduce vector competence and transmission potential of these mosquitoes.

Keywords Microbiome, *Wolbachia*, Vector competence, Mosquito control, Metagenomics, *Aedes albopictus*

Vector-borne diseases, which account for 20% of infectious diseases worldwide, pose a significant global health threat. Illnesses like dengue fever, yellow fever, chikungunya, and Zika put nearly 3.9 billion people[1] at risk, and effective management and control of these disease vectors are crucial for reducing these risks of transmission.

Aedes albopictus, a mosquito species known for its ability to transmit diseases and for being a nuisance to humans[2], has a worldwide distribution[3] and is responsible for spreading dengue, chikungunya, and Zika viruses[4–6]. Its invasive nature[7] and adaptability to different environments[8] make it a significant threat to human health. Originally from Southeast Asia, *Ae. albopictus* has expanded its range to other continents, facilitated by global trade of used tires, ornamental plants, and road traffic[4,9,10]. Moreover, in recent decades, rapid urbanization has contributed to proliferation of *Ae. albopictus* populations, and this, along with inadequate or absent vector control measures, has further increased the risk of pathogen transmission by this species[11]. The rapid spread of *Ae. albopictus* mosquitoes in São Tomé and Príncipe[12] and Spain[13] underscores the need to understand and manage this species in these regions. São Tomé and Principe, an island nation in the Gulf of Guinea, is particularly vulnerable to vector-borne diseases due to its tropical climate and limited healthcare infrastructure but colonization events should be rare[14]. *Aedes albopictus* was first identified in São Tomé in 2016, although it is probable that the species had inhabited the island for a decade prior to this discovery[12]. In Spain, the first *Ae. albopictus* was detected in 2004, at Sant Cugat del Vallès[15]. Since then, the mosquito's presence has proliferated across the

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country[13,16–18] due to multiple introductions from abroad[19]. This expansion has escalated concerns among public health officials, emphasizing the need for increased surveillance and control measures[20–22].

The host physiology of *Aedes* mosquitoes is heavily influenced by their microbiota, which has been shown to impact various aspects of reproduction, egg production, blood digestion[23–25], regulation of immunity[26], genetic diversity, vector competence, host–pathogen interactions and survival[19,27,28]. For instance, some bacteria can produce antimicrobial peptides that boost the mosquito's immune system, while other might facilitate the digestion of blood meals. Additionally, the presence of certain microbial communities can influence the mosquito's ability to transmit pathogens by either supporting or hindering the development of the pathogens within the mosquito. These factors collectively contribute to the vectorial capacity of mosquitoes and are key for pathogen transmission. Innovative vector control strategies have increasingly focused on manipulating the mosquito microbiome to reduce their capacity for disease transmission[29] and control of populations. One promising approach involves introducing exogenous *Wolbachia* strains into *Ae. albopictus* populations, which has shown potential in disrupting pathogen transmission[30]. However, most of the *Ae. albopictus* populations are already naturally infected with this endosymbiont[31] and different *Wolbachia* strains may exhibit various interactions when coexisting within a host[30]. Describing the native strains of our target-populations is the first step to assess possible interactions between the naturally occurring *Wolbachia* strains and the introduced type.

The genetic background of any given species plays a role in shaping its microbiome[32]. Different populations of mosquitoes may have genetic variations that affect their immune responses, susceptibility to certain microorganisms, and overall interactions with the microbiome. In the same way, different geographical locations may have unique microbial communities in soil, water, and vegetation, and the local climate and temperature can impact both the mosquito and the microorganisms it harbors[33]. Given the significance of understanding and managing vector-borne diseases, this study aimed to examine and to compare the microbiome composition and characterize the presence of *Wolbachia* strains in populations of *Ae. albopictus* from two countries with distinct climatic, habitat, epidemiological, and geographical conditions: São Tomé and Príncipe (Africa) and Spain (Europe). Investigation on the composition of the microbiome and *Wolbachia* strains in *Ae. albopictus* populations from these distinct geographical regions could provide valuable knowledge into potential control measures tailored to the specific problems of each area. By understanding the microbiome composition and the prevalence of different *Wolbachia* strains, researchers can develop targeted strategies, such as microbiome manipulation or *Wolbachia*-based interventions, to reduce the vector competence and transmission potential of these mosquitoes. This information could help develop new vector control strategies and contribute to reducing the global burden of vector-borne diseases.

Materials and methods

Mosquito collection and rearing

Eggs were collected using sixteen ovitraps installed in three distant regions of Spain: Valencia (Paterna), Huesca (Monzón), and Málaga (Rincón de La Victoria), as well as one region in São Tomé, Água Grande (São Tomé). This collection took place between late 2021 (September and October) and early 2022 (April), with the assistance of collaborators, as shown in Fig. 1. The ovitraps were designed as black plastic cups filled with 500 mL of dechlorinated water and lined with a strip of germination paper to provide a substrate for the mosquitoes to lay their eggs. The ovitraps were baited with a small amount of hay infusion (prepared by soaking 50 g of dried hay in 10 L of water for 3 days) to attract gravid female mosquitoes. The ovitraps were strategically placed in shaded

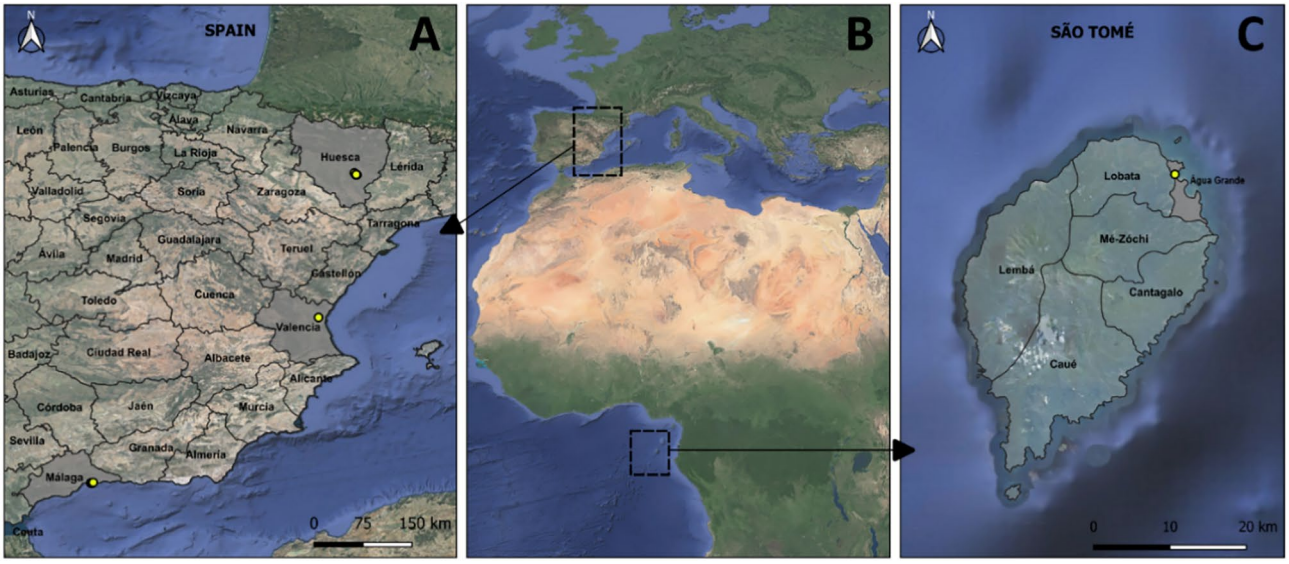

Fig. 1. Map indicating collection sites for *Ae. albopictus*. (**A**) Map of Spain showing the sampling locations in Valencia (Paterna), Huesca (Monzón), and Málaga (Rincón de La Victoria); (**B**) Regional map indicating the relative positions of Spain and São Tomé, per continent. (**C**) Map of São Tomé showing the sampling location in Água Grande. The map was generated with QGIS v3.34 software (https://qgis.org/en/site/).

and sheltered locations near human habitation and vegetation, ensuring optimal conditions for mosquito egg-laying. The germination papers were collected weekly, and new ones were placed in the ovitraps. The collected papers with eggs were transported to the laboratory for further processing.

Four *Ae. albopictus* colonies were subsequently established and maintained in the high-security insectary of the 'In Vivo Arthropod Security Facility' (VIASEF) available at IHMT, between March and May 2022. These colonies were initiated from a parental generation (F0) and maintained until the second generation (F2). Upon hatching in dechlorinated water, larvae were reared under controlled laboratory conditions (temperature: 26 ± 2 °C, relative humidity: 70 ± 5%, photoperiod: 12 h/12 h light/dark) and fed with Tetra Min flakes fish food (Tetra, Melle, Germany). The fish food flakes were processed into powder before being administered to the larvae. Adult mosquitoes were provided with a 10% glucose solution, and females were blood-fed on *Mus musculus* two to three times a week. The process of handling the animals used occurred under supervision and was carried out based on Community Council standards European Union of 24 November 1986 (86/609/EEC) and national legislation in force (Decree-Law 129/92 of June 2nd, Ordinance No. 100/92 of October 23rd). All animal experiments were based on protocols approved by the Direção-Geral de Veterinária, Ministério da Agricultura do Desenvolvimento Rural e das Pescas, Portugal (ID approvals: 023351 and 023355).

DNA extraction

DNA extractions from a total of 199 adult mosquitoes were carried out using two distinct methods, tailored to the specific requirements of our analyses. For the purpose of sequencing the 16S rRNA gene in a subset of 19 female samples, we employed the "NZY tissue gDNA isolation kit" provided by NZYtech, Portugal. Before DNA extraction, the 19 samples were treated with 10% bleach, followed by 70% alcohol, and finally distilled water to sterilize the mosquitoes. These selected samples originated from adult female mosquitoes belonging to the F0 generation, specifically targeted for our 16S rRNA gene sequencing study to provide a detailed microbiome analysis. The selection of these 19 samples was based on ensuring a representative subset that included mosquitoes from both geographic locations (Spain and São Tomé and Príncipe) and aimed to capture any potential differences in microbiota composition between these regions. The limited number of samples for 16S rRNA gene sequencing was due to resource constraints and the need to focus on a manageable subset for high-throughput sequencing and in-depth microbiome characterization.

For the remaining 180 samples, we utilized a ribosomal DNA extraction protocol that was adapted from the methodology described by Collins et al.[34]. This approach was applied to both male and female adult mosquitoes from the F0 and F1 generations to facilitate a comprehensive comparison of *Wolbachia* prevalence and strain distribution across the populations under study.

All DNA extractions were performed on individual mosquitoes, not pooled samples, to ensure accurate representation of each mosquito's microbiome and *Wolbachia* infection status.

To ensure the integrity of our DNA extraction process and to eliminate the possibility of contamination, negative controls were systematically included in each batch of extractions.

16S metagenomic sequencing

The bacterial composition of *Ae. albopictus* microbiota was investigated by sequencing the V3 and V4 regions of the bacterial 16S rRNA gene in 19 samples extracted with the NZY tissue gDNA isolation kit. The sequencing of the amplified products was performed on the Illumina MiSeq platform using v3 chemistry and a 2 × 300 bp paired-end module. Library construction, sequencing, and bioinformatics analysis were performed by Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany). Briefly, bioinformatics pipeline began with the removal of sequences containing ambiguous bases, followed by the identification and exclusion of chimeric reads utilizing the UCHIME algorithm within the VSEARCH package[35,36]. The remaining high-quality sequences were then processed through Minimum Entropy Decomposition (MED), which efficiently partitions the marker gene datasets into Operational Taxonomic Units (OTUs)[37,38]. This method capitalizes on Shannon entropy, selectively considering only the informative nucleotide positions, thus enabling the delineation of sequences based on single nucleotide variances without the interference of random sequencing errors.

After OTU generation, taxonomic assignments were conducted through DC-MEGABLAST alignments against a curated reference database, ensuring the provision of the most specific taxonomic nomenclature to each OTU from the best-matching reference sequences. We applied the QIIME 1.9.1 software package for additional processing of OTUs and their taxonomic classifications, where the abundance data of bacterial taxa were normalized based on lineage-specific gene copy numbers to enhance accuracy[39]. A minimum sequence identity of 70% over at least 80% of the representative sequence was required to consider the sequence as a reference. Further processing of OTUs and taxonomic assignments was performed using QIIME software (version 1.9.1, http://qiime.org/). The abundances of bacterial taxonomic units were normalized using the 16S rRNA Gene Copy Number (GCN) correction. For this, the read numbers assigned to a species were divided by the known or assumed number of marker gene regions[39]. The analytical output comprised a comprehensive suite of files, elucidating the taxonomic structure and diversity within the *Ae. albopictus* microbiota. These outputs included summarized lists of identified taxonomic units per sample, representative sequences for each OTU, and matrices detailing the estimated abundances of OTUs and taxonomic units across samples. Alpha diversity was assessed using Shannon and Simpson diversity indices to evaluate the bacterial diversity within individual samples. Beta diversity was analyzed to compare the bacterial community composition between samples from Spain and São Tomé. These indices were calculated using the QIIME 1.91. software package[36].

All sequences have been submitted to NCBI under the accession number for SRA data PRJNA1028981 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1028981).

Wolbachia genotyping

PCR genotyping of *Wolbachia* infection in 180 samples used *Wolbachia*-specific primers 328Fw and 691Rv for *w*AlbA, and 183F and 691R for *w*AlbB[40]. PCR mix and thermal cycling conditions were standardized. Universal primers *w*sp 81Fw and 691Rv were used to confirm *Wolbachia*-negative samples. Primers and PCR conditions details are provided in Supplementary Table S1.

Statistical analysis

Mixed and single infection rates were calculated by sex and population[41]. Comparisons between mixed and single infections in females and males were performed using the chi-square test. A p-value less than 0.05 was considered statistically significant.

Results

Microbiome composition

To analyze the bacterial communities within *Ae. albopictus* from Spain and São Tomé, we conducted 16S rRNA gene sequencing of the V3–V4 hypervariable regions across 19 individual adult female samples. After pre-processing and quality control, five samples from São Tomé yielded 606.801 clean sequences, and 14 samples from Spain yielded 798.002 clean sequences (Table 1). All these clean sequences were classified into OTUs (Operational Taxonomic Units): 344 from São Tomé and 517 from Spain (Table 1). The OTUs represented different taxonomic levels, and a correction was made to the species found[39] (Supplementary Tables S2 and S3). The Fig. 2 shows shared and non-shared bacterial genera among the populations.

Compared to other studies on the microbiota of mosquitoes from the field, the number of taxa found per sample in our study is relatively low. To ensure that we captured the maximum diversity, rarefaction curves were generated for each sample. These curves, which plot the number of observed OTUs against the number of sequences sampled, indicated that the sequencing depth was sufficient to capture the majority of the bacterial diversity present in our samples (Supplementary Fig. S1). The rarefaction analysis confirmed that our sequencing effort was adequate, as the curves approached an asymptote, suggesting that additional sequencing would likely yield few additional OTUs.

Taxonomic units overview and microbiome diversity analysis

Tables 2 and 3 provide an overview of the taxonomic units identified in samples from São Tomé and Spain. Taxonomic units with readings below 0.1% were categorized as "other." OTU readings analysis showed a predominance of the genus *Wolbachia* in all samples, ranging between 92.4 and 98.8% in São Tomé samples and between 96.1 and 97.5% in Spanish samples.

Shannon and Simpson indices (Table 4) revealed similar bacterial diversity between Spanish and São Tomé populations, as well as among individual samples (Supplementary Table S4).

Wolbachia genotyping

Of the 180 adult mosquitoes analyzed (97 females and 83 males), 178 (98.89%) were infected with *Wolbachia*. Five (2.78%) were infected with the *w*AlbA strain and 34 (18.88%) with the *w*AlbB strain. Mixed infections with *w*AlbA and *w*AlbB strains occurred in 77.22% of the infected mosquitoes (Table 5). Single *w*AlbA infections were

Sample origin	Total number of input sequences	Remaining sequences after processing	Total number of sequences assigned to OTUs/taxa	Number of OTUs assigned to taxa
Spain	798,100	798,002	643,902	517
São Tomé	606,877	606,801	525,776	344

Table 1. Summarized statistics of Spain and São Tomé samples.

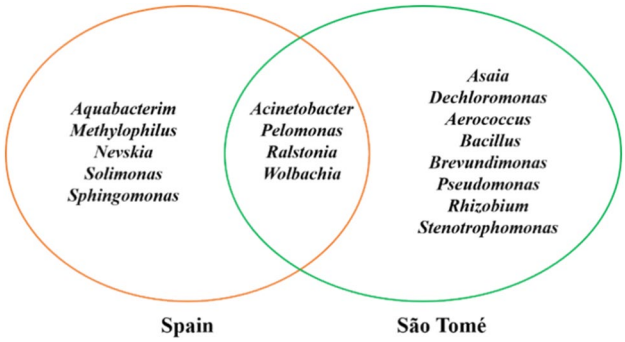

Fig. 2. Venn diagram showing shared and unshared genera between the Spain and São Tomé populations.

Province/district	Sample	Taxonomic unit	Taxonomic level	OTUs reads	Reads	Raw fraction
Água Grande/São Tomé	STPF0.1	*Wolbachia*	Genus	191	101,778	98.1%
		Gammaproteobacteria	Class	1	157	0.2%
		Other	–			1.7%
	STPF0.2	*Wolbachia*	Genus	185	70,626	92.4%
		Asaia sp.	Species	1	1311	1.8%
		Gammaproteobacteria	Class	3	1035	1.4%
		Enterobacteriaceae	Family	1	876	1.2%
		Erwiniaceae	Family	1	242	0.3%
		Enterobacterales	Order	2	195	0.3%
		Acinetobacter	Genus	2	166	0,2%
		Dechloromonas	Genus	1	95	0.1%
		Other	–			2,3%
	STPF0.3	*Wolbachia*	Genus	146	126,113	98.8%
		Other	–			1.2%
	STPF0.4	*Wolbachia*	Genus	202	97,713	98.2%
		Other	–			1.8%
	STPF0.5	*Wolbachia*	Genus	206	73,196	96.7%
		Pelomonas	Genus	1	676	0.9%
		Brevundimonas	Genus	1	170	0.2%
		Pseudomonas	Genus	1	75	0,1%
		Other	–			2.1%

Table 2. Summary of the taxonomic composition of samples from São Tomé.

exclusive to females ($p < 0.05$), while single *w*AlbB infections occurred in both males and females but were more prevalent in males ($p < 0.05$). Mixed infections were more common in females than males (85.57% in females and 67.47% in males, of the total number of infected mosquitoes) ($p < 0.05$). A predominance of mixed infections was observed in all locations, with rates ranging from 62.50 to 82.76%. However, simple *w*AlbB infections were detected only in the three provinces of Spain (Valencia, Huesca, and Málaga) with rates ranging from 13.79 to 31.25% (Table 6) and simple *w*AlbA infections were found only in two provinces of Spain (Valencia and Huesca). In São Tomé, only mixed infections by *w*AlbAeB were found, all in females. Furthermore, it was the only place where negative samples (N = 2) were detected in males.

Discussion

Aedes albopictus microbiome

This study represents the inaugural attempt to provide a comprehensive insight into the composition of the *Ae. albopictus* bacterial microbiome in wild populations originating from Spain and São Tomé through the sequencing of the V3–V4 regions of the 16S rRNA gene. The main phyla identified in the *Ae. albopictus* bacterial microbiome were *Proteobacteria* and *Firmicutes*, consistent with previous studies[42,43]. Both populations exhibited unique bacterial genera (Spain: *Aquabacterium*, *Methylophilus*, *Nevskia*, *Solimonas* and *Sphingomonas*; São Tomé: *Asaia*, *Dechloromonas*, *Aerococcus*, *Bacillus*, *Brevundimonas*, *Pseudomonas*, *Rhizobium* and *Stenotrophomonas*), suggesting a geographic influence on the microbiome[43]. A shared bacterial microbiome was also observed, comprising *Acinetobacter*, *Pelomonas*, *Ralstonia*, and *Wolbachia* genera. In addition to *Wolbachia,* these results suggest the existence of a bacterial microbiome shared among populations of *Ae. albopictus* geographically very distant[44]. The presence of *Pelomonas* and *Nevskia* genera in the *Aedes* genus is a novel finding, and their role in the microbiome of hematophagous insects remains unknown, and further studies are needed to understand their interactions with hosts[45–48]. *Pelomonas* and *Nevskia* are often found in environmental samples, raising the question of whether these bacteria could be externally adhered to the mosquitoes. To address this, we ensured that the mosquitoes were thoroughly cleaned before DNA extraction. The cleaning process involved washing the mosquitoes with a solution of 0.1% bleach followed by 70% alcohol. This procedure is designed to remove external contaminants, thereby ensuring that the DNA extracted and analyzed represents the internal microbiome. Therefore, the presence of *Pelomonas* and *Nevskia* is likely indicative of their role within the mosquito microbiome rather than external contamination.

Consistent with prior research, the bacterial composition of *Ae. albopictus* was predominantly governed by the *Wolbachia* genus[49]. When *Wolbachia* infection is highly dominant, it can "mask" the DNA of other bacteria present[42,50] leading to undetected and unsequenced sequences. Future research may consider individual tissue-based microbiome analysis[51], to ensure that *Wolbachia*, which is naturally abundant in the ovaries, does not interfere with the sequencing data.

This highly dominant infection by *Wolbachia* in both populations may be related to interactions between microbiome components, such as interspecific competition for resources[44,52] but also to laboratory rearing conditions[49]. Research into the microbiota of laboratory and wild *Aedes* mosquitoes has revealed that both are

Province/district	Population	Sample	Taxonomic unit	Taxonomic level	OTUs reads	Reads	Raw fraction
Huesca	Monzón	MZF0.1	*Wolbachia*	Genus	196	39,959	97.0%
			Nevskia	Genus	1	84	0.2%
			Methylophilus	Genus	1	60	0.1%
			Other	–			2.7%
		MZF0.2	*Wolbachia*	Genus	192	42,677	97.8%
			Enterobacteriaceae	Family	1	64	0.1%
			Other	–			2.1%
		MZF0.3	*Wolbachia*	Genus	225	40,718	96.3%
			Acinetobacter	Genus	3	299	0.7%
			Nevskia	Genus	1	58	0.1%
			Comamonadaceae	Family	1	45	0.1%
			Other	–			2.8%
		MZF0.4	*Wolbachia*	Genus	200	45,034	96.9%
			Gammaproteobacteria	Class	1	47	0.1%
			Other	–			3.0%
		MZF0.5	*Wolbachia*	Genus	194	40,367	96.1%
			Nevskia	Genus	1	191	0.5%
			Methylophilus	Genus	1	137	0.3%
			Aquabacterium	Genus	1	109	0.3%
			Comamonadaceae	Family	1	61	0.1%
			Other	–			2.7%
Valencia	Paterna	PAF0.1	*Wolbachia*	Genus	209	45,910	97.4%
			Sphingomonas	Genus	2	223	0.5%
			Other	–			2.1%
		PAF0.2	*Wolbachia*	Genus	202	49,022	97.4%
			Other	–			2.6%
		PAF0.3	*Wolbachia*	Genus	182	41,312	97.4%
			Sphingomonas	Genus	1	49	0.1%
			Pelomonas	Genus	1	46	0.1%
			Other	–			2.4%
		PAF0.4	*Wolbachia*	Genus	166	30,946	96.5%
			Pelomonas	Genus	1	120	0.4%
			Sphingomonas	Genus	1	97	0.3%
			Nevskia	Genus	1	44	0.1%
			Other	–			2.7%
Málaga	Rincón de la Victoria	RCF0.1	*Wolbachia*	Genus	189	49,296	97.5%
			Other	–			2.5%
		RCF0.2	*Wolbachia*	Genus	179	31,177	96.9%
			Other	–			3.1%
		RCF0.3	*Wolbachia*	Genus	177	48,900	97.5%
			Other	–			2.5%
		RCF0.4	*Wolbachia*	Genus	197	44,330	97.3%
			Other	–			2.7%
		RCF0.5	*Wolbachia*	Genus	189	37,996	97.1%
			Other	–			2.9%

Table 3. Summary of the taxonomic composition of samples from Spain.

Population	Shannon (average)	Simpson (average)
Spain	4.229	0.819
São Tomé	4.071	0.773

Table 4. Average of the Shannon and Simpson indices of the Spain and São Tomé populations.

	Infected	*w*AlbA	*w*AlbB	*w*AlbAeB	Total
Females	97 (100%)	5 (5.15%)	9 (9.28%)	83 (85.57%)	97
Males	81 (97.59%)	0 (0.0%)	25 (30.12%)	56 (67.47%)	83
Total infected mosquitoes	178 (98.88%)	5 (2.78%)	34 (18.88%)	139 (77.22%)	180

Table 5. Rate of *Wolbachia* infection in *Ae. albopictus* by sex.

Country	Province/district	Total	Infected	*w*AlbA	*w*AlbB	*w*AlbAeB
Spain	Valencia	16	16 (100%)	1 (6.25%)	5 (31.25%)	10 (62.50%)
	Huesca	116	116 (100%)	4 (3.45%)	16 (1379%)	96 (82.76%)
	Málaga	41	41 (100%)	0 (0.00%)	13 (31.71%)	28 (68.29%)
São Tomé and Príncipe	Água Grande	7	5 (71.43%)	0 (0.00%)	0 (0.00%)	5 (71.43%)

Table 6. Rate of *Wolbachia* infection, *w*AlbA and *w*AlbB, by locality.

primarily characterized by a limited number of phyla[44]. Nonetheless, although the overall microbiota composition is comparable in laboratory-reared and wild mosquitoes, it was observed that the diversity of midgut bacterial communities was more extensive in mosquitoes collected from natural environments. In this study, female mosquitoes were blood-fed, which can substantially modify their microbiota. When compared to other studies on field-collected mosquitoes, the number of taxa found per sample in our study is relatively low. This discrepancy could be attributed to several factors. First, the laboratory rearing conditions, including the controlled diet and environment, may have influenced the microbial diversity, leading to a reduction in taxa compared to field-collected samples. Second, the dominance of *Wolbachia* in our samples might have overshadowed the detection of other bacterial taxa, potentially due to interspecific competition or the high abundance of *Wolbachia* masking other bacterial DNA. In fact, the diversity of female midgut bacteria in *Ae. albopictus* from the laboratory was reduced when compared to females from those from the field[53]. These disparities could be originated from variations in the origin of the blood meal, dietary factors, and habitat[53,54]. Furthermore, recent studies have indicated a more consistent and greater bacterial diversity in breeding water compared to larvae and adults, regardless of sample source, with a notable decrease in the microbial community diversity between the larvae and newly emerged adult mosquitoes[53]. The impact of blood meals and rearing within an insectary environment on the *Ae. albopictus* microbiome warrants thorough investigation and future studies should consider the analysis of larvae's microbiome and the bacterial diversity of the breeding water.

Wolbachia genotyping

To better understand the *Wolbachia* populations naturally present in these *Ae. albopictus*, A and B strains[31,55] were genotyped. Both strains were found to infect the Spanish and São Tomé populations in three different scenarios: mixed infection with both strains, simple infection with *w*AlbA, or simple infection with *w*AlbB. Mixed infection, which is predominant worldwide[56–60] was also the predominant form in this study. Several factors could explain these results, including species susceptibility to infection, facilitation of secondary infections by an active *Wolbachia* infection, or the stable maintenance of dual infections in hosts[61].

In line with previous studies[31,62], the prevalence of mixed infections was significantly higher in females compared to males and this could be due to the maternal transmission[31], ineffective transmission of one strain, or physiological mechanisms allowing *Wolbachia* infection in females but not in males[63]. The exact mechanisms of *Wolbachia* infection and dissemination in mosquito vectors remain unclear[64]. Maternal transmission is a key mechanism for the spread of *Wolbachia*, and high rates of maternal transmission have been documented for mixed infections[31]. However, some studies have identified simple infections in the progeny of females with mixed infections, indicating the possibility of inefficient transmission of one of the strains[63]. This phenomenon could also occur in the transmission from female progenitors to their male offspring, explaining the lower prevalence of mixed infections in males. Physiological differences between male and female mosquitoes also play a role. *Wolbachia* tends to infect the germline cells of the host, persisting in the ovaries of females but not infecting the sperm in males. This differential infection could be due to a tropism of *Wolbachia* for ovarian tissues, allowing stable transmission through the female line, while infection in males is not stable or necessary for the maintenance of the endosymbiont[31,62].

The prevalence of *w*AlbB infection was higher than *w*AlbA, supporting prior findings for European populations[55,57,62]. This inequality is particularly pronounced in males and could be attributed to reduced *w*AlbA density over time[57] or a lower reproduction rate[63,65]. Research has suggested that females may not efficiently transmit *w*AlbA to their progeny, resulting in a reduction in male fertility due to Cytoplasmic Incompatibility (CI)[55].

In this study, males from São Tomé population were not infected by *Wolbachia*. In fact, low *Wolbachia* prevalence has been detected in natural *Ae. albopictus* infections in Cameroon, one of the possible origins of *Ae. albopictus* from São Tomé[66]. Therefore, is not possible to assume that males in this region do not host *Wolbachia*, as infected females were found. Thus, the absence of infected males in São Tomé may be related to the lower prevalence of endosymbiont infection in males and the small sample size (N = 2). This small sample size limits the

ability to draw definitive conclusions and may not accurately represent the population. Cautious interpretation is required, and future studies with larger sample sizes are necessary to validate this finding and provide a more accurate representation of *Wolbachia* prevalence in the male mosquito population from São Tomé.

The number of *Wolbachia*-negative samples was minimal (1.11%), indicating that innate *Wolbachia* infection may provide selective advantages to hosts, such as higher fecundity or hatching rates[67]. Geographic variation in *Wolbachia* infection has been reported[68], but this study did not find significant differences between samples from different locations in Spain, as well as other studies carried out in other locations of this country[60]. An uniform distribution pattern with a predominance of mixed infections has been suggested and supported by prior studies for *Ae. albopictus* populations across Europe[62].

The rapid dissemination and uniformity of distribution in Europe could result from multiple introductions[69,70] in different Mediterranean countries, leading to constant mixed infection of *Ae. albopictus* by *Wolbachia.* Maternal inheritance and cytoplasmic incompatibility may also play a role in promoting the spread of *Wolbachia* infection[71].

Aedes albopictus vector control

To control *Ae. albopictus* populations, one approach involves creating a colony with a stable infection of specific exogenous *Wolbachia* strains that cause Cytoplasmic Incompatibility (CI) and non-viable offspring. The Incompatible Insect Technique (IIT) involves the release of males carrying these *Wolbachia* strains into the environment, leading to a reduction in the target population. This approach has been tested and implemented in *Ae. albopictus*, which naturally hosts two *Wolbachia* strains (*w*AlbA and *w*AlbB). Introducing a third strain, *w*Pip (found in *Culex pipiens molestus*), resulted in a triple-infected colony that effectively decreased the number of *Ae. albopictus* females in the study area[72]. Recent strategies employed *w*Pip transinfection in *Ae. albopictus*, accompanied by the removal of its native *Wolbachia* strains, yielding promising outcomes[73]. These findings underscore IIT's potential as a future tool for *Ae. albopictus* control.

To conclude, this study significantly enhances our understanding of the bacterial microbiome of *Ae. albopictus* populations from Spain and São Tomé. Our study provides valuable insights into the microbiota composition of *Ae. albopictus* populations from Spain and São Tomé, revealing the presence of *Wolbachia* and other bacterial endosymbionts, which establishes a foundational framework for future manipulations and the introduction of new strains for vector control. As the scientific community intensifies its search for innovative and effective methods to combat vector-borne diseases, the insights from this study become increasingly invaluable. Consistent with previous research, we found that the microbiota of *Ae. albopictus* is predominantly governed by the *Wolbachia* genus. The dominance of *Wolbachia* in both populations suggests a stable association, which could be considered a part of the core microbiota of *Ae. albopictus*. The concept of core microbiota refers to the set of microbial taxa that are consistently found across different populations of a host species, regardless of geographical location or environmental conditions. In our study, we identified several bacterial genera that were shared between the Spanish and São Tomé populations, such as *Acinetobacter, Pelomonas, Ralstonia,* and *Wolbachia*. This finding supports the idea that *Ae. albopictus* harbors a core microbiota, which may play crucial roles in the mosquito's physiology, including reproduction, immune regulation, and vector competence. Furthermore, the identification of novel genera like *Pelomonas* and *Nevskia* in *Ae. albopictus* highlights the dynamic nature of the mosquito microbiota and suggests potential new areas of research to explore their roles in mosquito biology and pathogen transmission. The presence of these core and unique bacterial taxa emphasizes the importance of understanding the microbiota composition for developing targeted vector control strategies.

Despite the valuable insights provided by this study, there are several limitations that should be acknowledged. The most significant limitation is the small sample size used for the microbiome analysis and the *Wolbachia* genotype study, particularly for mosquitoes from São Tomé. The limited number of samples may have restricted the detection of the full diversity of the microbiota and *Wolbachia* strains, potentially overlooking important taxa and variations. Additionally, the use of laboratory-reared mosquitoes, although necessary for controlled conditions, might not fully represent the natural microbiota found in field populations due to differences in environmental exposures and diet.

To ensure the robustness of our findings, future studies should aim to include larger sample sizes and consider a broader range of geographical locations. Furthermore, incorporating field-collected mosquitoes and longitudinal sampling could provide a more comprehensive understanding of the microbiota dynamics and their implications for vector competence and control strategies.

While the presence of *Wolbachia* in *Ae. albopictus* is well established, our study contributes to the understanding of *Wolbachia* strain distribution and prevalence in new geographical regions, specifically Spain and São Tomé. We observed a consistent dominance of *Wolbachia* across different populations, reinforcing the stability of this endosymbiont's association with its host. However, we also highlight the presence of dual infections with different *Wolbachia* strains, which could have implications for vector control strategies leveraging *Wolbachia*-induced pathogen interference.

Future research directions to expand upon our findings include examining the impact of the bacterial microbiome on *Ae. albopictus* vector competence, evaluating the potential of CRISPR/Cas9 gene editing in endosymbionts for vector control, investigating paratransgenic strategies, assessing the ecological consequences of microbiome manipulation, and using advanced sequencing technologies for a more detailed understanding of the mosquito microbiome.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI under the accession number for SRA data PRJNA1028981 (temporary submission ID: SUB13907152).SRA records will be accessible with the following link after the release date (2024-11-01): http://www.ncbi.nlm.nih.gov/bioproject/1028981.

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Author contributions

Research design: G.S. and C.A.S.; Mosquito collections: C.A.S., D.B.B., S.D.E.; Molecular genotyping: T.M., G.S.; Data analysis: G.S., T.M., C.A.S.; Manuscript writing: all authors; all authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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