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Farming system shapes the microbiota of *Bactrocera oleae* across Spanish olive regions: consequences for host fitness and pest management

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Abstract

The olive fruit fly (*Bactrocera oleae*) is the most destructive pest of Mediterranean olive production. Although its microbiota is known to influence essential biological traits, the combined effects of farming practices and geographic origin on microbial communities and pest performance remain largely unexplored. This study provides the first high-resolution characterization of the bacterial microbiota of *B. oleae* across four major Spanish olive-growing regions (Jaén, Granada, Málaga and Valencia), comparing organic and conventional orchards. Full-length 16S rRNA sequencing using Oxford Nanopore technology generated between 27,000 and 121,000 high-quality reads per sample. Diversity analyses revealed that organic orchards generally supported higher alpha diversity (richness and evenness), while beta diversity highlighted strong geographic structuring of microbial communities. Organic orchards were enriched in taxa such as *Alcaligenes faecalis* and certain *Enterobacter* species, whereas conventional systems were associated with opportunistic taxa, particularly *Citrobacter braakii* in Málaga. Distinct regional microbial signatures were also detected. Fitness bioassays established clear links between microbiome composition and host performance. Flies from organic orchards exhibited higher longevity, fecundity and larval success than those from conventional orchards. Antibiotic-treated flies showed a strong reduction in reproductive performance, confirming the critical contribution of the microbiota, particularly the obligate symbiont *Candidatus Erwinia dacicola*. Reinoculation with *Ca. Erwinia dacicola* restored fitness, whereas enrichment with conventional-associated opportunistic taxa impaired key performance parameters. These findings demonstrate that farming practices and geography jointly shape the microbiota of *B. oleae*, with measurable consequences for its fitness, and highlight opportunities for microbiome-informed strategies in sustainable olive pest management.

Keywords *Bactrocera oleae*, microbiome diversity, farming system, insect pest fitness, sustainable pest management

Key Messages

- Organic and conventional farming systems induce distinct shifts in the bacterial microbiota of the olive fruit fly *B. oleae* across Spanish olive-growing regions.
- Organic orchards are generally associated with higher alpha diversity (richness and evenness), whereas beta diversity analyses reveal strong geographic structuring; conventional orchards favour opportunistic taxa such as *Citrobacter braakii*.
- Microbiome composition is linked to host performance: flies from organic orchards exhibit higher longevity, fecundity and larval success than those from conventional orchards.
- Experimental disruption of the microbiota strongly reduces reproductive performance, confirming the critical role of *Candidatus Erwinia dacicola*, whereas enrichment with opportunistic taxa impairs host fitness.
- These findings highlight the potential of microbiome-informed approaches for the sustainable management of *B. oleae*.

1. Introduction

Olive cultivation is a cornerstone of Mediterranean agriculture, with major economic, cultural, and environmental importance. According to the International Olive Council (2026), Spain contributed nearly 40% of global olive oil production in the 2024/25 crop year, primarily in the Andalusia and Valencia regions. Despite technological progress and innovations in the olive sector, its development remains constrained by insect pest infestations. The olive fruit fly, *B. oleae* (Rossi), remains the most destructive pest of olives worldwide. Larval feeding inside the fruit causes substantial yield losses and severely reduces olive oil quality through increased acidity and peroxide values (Daane & Johnson, 2010; Medjkouh et al., 2016; Valenčič et al., 2021; Dadras et al., 2022).

For decades, control of *B. oleae* has relied heavily on chemical insecticides. However, the development of insecticide resistance, negative impacts on non-target organisms, environmental contamination, and growing consumer demand for residue-free olive oil have strongly increased the need for more sustainable strategies within Integrated Pest Management (IPM) frameworks (Lantero et al., 2023; Balampekou et al., 2024). In recent years, insect-associated microbiota have emerged as critical modulators of host physiology. They influence pesticide biodegradation (Jaffar et al., 2022), insect reproduction and its consequences for evolutionary biology and sustainable pest management (Abbas et al., 2026), immune activity (Calhoun, 2025), host detoxification and physiological tolerance (Chen et al., 2026),

nutrition (Chabanol and Gendrin, 2024), as well as ecological fitness, health and evolutionary trajectories (Lange et al., 2023; Wang et al., 2025). *B. oleae* has long been associated with microbial symbionts that play essential roles in its biology and ecology (Manousis and Ellar, 1988). Beyond functional roles, the diversity and structure of insect-associated microbiota (alpha and beta diversity) are increasingly recognized as being shaped by regional climatic conditions and farming systems, with significant consequences for host ecology and pest management (Nie et al., 2025; Graber and Moreau, 2026).

Oleicultural management can profoundly shape olive-associated microbial communities. Conventional orchards, characterized by frequent pesticide applications and high inputs of synthetic fertilizers, often exhibit reduced microbial diversity, whereas organic systems tend to promote richer and more balanced bacterial assemblages (Fosso et al., 2026). Moreover, at a deeper level of olive organic farming, biodynamic practices—based on the exclusive use of herbal preparations, organic composts, and the absence of synthetic chemicals—have been shown to favor the presence of entomopathogenic bacteria such as *Bacillus* sp., with potential for biological control (Blibech et al., 2012). Furthermore, because *B. oleae* pupates in the soil, its microbiota is likely influenced not only by the olive phyllosphere but also by the rhizosphere and surrounding soil microbial communities (Dimou et al., 2003; Ortega et al., 2018).

In *B. oleae*, the obligate endosymbiont *Candidatus Erwinia dacicola* is particularly important (Nobre, 2021; Martínez-Sañudo et al., 2024). This symbiont resides in the foregut, where it enables nitrogen recycling and allows larvae to detoxify oleuropein and other secoiridoid compounds present in unripe olives, thereby supporting larval development and female fecundity (Ben-Yosef et al., 2015; Blow et al., 2019; Siden-Kiamos et al., 2025). In contrast, several facultative and opportunistic bacteria belonging to the genera *Citrobacter, Providencia, Morganella* and *Serratia* can become dominant under certain conditions and are frequently associated with reduced host performance (Capuzzo et al., 2005; Ben-Yosef et al., 2015; Siden-Kiamos et al., 2025). Several studies have demonstrated that manipulating the microbiota of *B. oleae* can significantly alter its fitness. Flies rendered axenic in early studies (i.e., deprived of their symbionts under sterile conditions) exhibited drastic reductions in longevity, fecundity, larval survival, and oviposition behavior, while reinoculation with *Candidatus Erwinia dacicola* largely restored these traits (Ben-Yosef et al., 2010, 2014). Moreover, recent studies show that in adults, *Ca. Erwinia dacicola* colonizes the esophageal bulb, midgut and ovipositor, where it actively expresses nitrogen-assimilation enzymes, supporting host nutrition and fecundity (Pantidi et al., 2026). Experimental strategies with antibiotic-treated flies, which strongly reduce but do not completely eliminate microbial communities, have confirmed the critical role of the microbiota in host performance (Sinno et al., 2020). Moreover, the impact of antimicrobial treatments on the olive fly microbiota, particularly its obligate symbiont Ca.

Erwinia dacicola (Maretto et al., 2025), suggest that microbiome shifts induced by farming practices could be exploited for the development of novel, microbiome-informed pest management strategies.

Despite growing interest in insect microbiomes, comprehensive multi-regional studies evaluating the combined effects of farming system (organic versus conventional) and geographic origin on the microbiota of *B. oleae* remain scarce. Most previous research has been limited to single locations and has relied on short-read sequencing technologies, restricting taxonomic resolution and functional interpretation.

The present study addresses these gaps by providing the first high-resolution characterization of the bacterial microbiota of *B. oleae* across four major Spanish olive-growing regions (Jaén, Granada, Málaga and Valencia) using Oxford Nanopore full-length 16S rRNA sequencing. By combining microbiome profiling with controlled fitness bioassays (longevity, fecundity and larval success), we aimed to (i) determine how farming system shapes the structure and diversity of the fly's microbiota, (ii) examine the relationships between microbiome composition and host fitness, and (iii) explore the potential relevance of these findings for microbiome-informed approaches to olive fruit fly management.

2. Materials and Methods

2.1 Sample collection for microbiome analysis

Adult *B. oleae* were collected from naturally infested olive fruits in four major olive-growing regions in Spain: Mengíbar (Jaén), Órgiva (Granada), Álora (Málaga), and Moixent (Valencia) (Figure 1). In each region, samples were taken from both organic and conventional orchards. Approximately 250 infested olives were harvested per parcel during the autumn generation (October–November 2024) and transported to the laboratory in ventilated containers. Fruits were placed in rearing containers with mesh covers and incubated at 25 °C, 70% relative humidity (≈65% during pupation) under a 16:8 h (light:dark) photoperiod until adult emergence. Emerging adults were collected daily. For each replicate, approximately 35 individuals were collected and subsequently stored at −80 °C, stratified by geographic origin and farming system. Four independent biological replicates were generated for each of the eight combinations (4 regions × 2 farming systems), resulting in a total of 32 samples.

2.2 DNA extraction and 16S rRNA gene amplification

Genomic DNA was extracted from individual adult flies originating from the stored replicates (stratified by geographic origin and farming system) using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according

to the manufacturer's instructions, following enzymatic digestion. DNA quantity and quality were assessed with a Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA) using the dsDNA High Sensitivity Assay Kit.

The near full-length bacterial 16S rRNA gene (≈1.5 kb) was amplified with the universal primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Kuske et al., 2006). PCR conditions followed the protocol of El Yamlahi et al. (2025), with slight optimization to reduce non-specific amplification. Amplicons were checked on agarose gels and purified with AMPure XP magnetic beads (Beckman Coulter, USA) prior to library preparation.

Purified amplicons were barcoded with the SQK-NBD114.96 ligation kit (Oxford Nanopore Technologies), pooled, and sequenced on a P2 Solo FLO-PRO114M platform (Oxford Nanopore Technologies) following protocols developed by Secugen (Madrid). This approach yielded long-read, full-length 16S rRNA sequences, enabling high taxonomic resolution.

2.3 Bioinformatic processing and taxonomic classification

Raw Nanopore reads were processed with NanoFilt (De Coster et al., 2018) to retain sequences of 1300–1800 nt and quality scores > Q15. NanoComp (De Coster et al., 2023) was used to evaluate quality statistics. Chimeric sequences generated during amplification were identified and removed with vsearch (Rognes et al., 2016).

Taxonomic assignment was performed using two complementary approaches: 1- EMU (Curry et al., 2022) with its curated full-length 16S rRNA database and 2- usearch_global (USEARCH v11; Edgar, 2010) against the NCBI 16S ribosomal database. Only taxa represented by ≥ 10 reads per sample were retained to minimize spurious assignments. Relative abundances were calculated, and the 20 most abundant taxa were used for downstream community analyses, as rare taxa contributed negligibly to overall structure.

2.4 Relative Abundances and Diversity analyses

Relative abundances were calculated for each taxon, and the 20 most abundant taxa were selected for taxonomic comparisons and visualizations. Alpha diversity was assessed using the original dataset. Observed richness (S) was defined as the number of taxa with non-zero abundance, and the Shannon diversity index ($\boldsymbol{H}'$) was calculated as $\boldsymbol{H}'=-\sum_{i=1}^{S} p_i \ln(p_i)$, where p_i represents the relative abundance of taxon i (Shannon, 1948). Differences in alpha diversity between farming systems and regions were assessed using the Wilcoxon rank-sum test for comparisons

between two groups (Wilcoxon, 1945) or the Kruskal–Wallis test for comparisons involving more than two groups (Kruskal and Wallis, 1952), followed by Dunn's post-hoc test when appropriate (Dunn, 1964).

Beta diversity was assessed using Bray–Curtis dissimilarity calculated from relative abundance data (Bray and Curtis, 1957). Community composition was visualized using non-metric multidimensional scaling (NMDS) ordination (Kruskal, 1964). The effects of farming system, geographic region, and their interaction on bacterial community composition were evaluated using permutational multivariate analysis of variance (PERMANOVA), implemented with the `adonis2` function in the vegan package (Anderson, 2001), with 9,999 permutations. Bray–Curtis dissimilarities between and within regions were additionally compared using Wilcoxon rank-sum tests, where appropriate. Similarity percentage (SIMPER) analysis was performed to identify the taxa contributing most to the observed dissimilarities between groups (Clarke, 1993).

All statistical analyses were performed in R version 4.5.2 (R Core Team, 2025). Alpha- and beta-diversity analyses were conducted using the vegan (Oksanen et al., 2025) and phyloseq (McMurdie and Holmes, 2013) packages. NMDS ordinations were visualized using ggplot2 (Wickham, 2016).

2.5 Fitness bioassays

2.5.1 Sample collection for fitness experiments

To evaluate the functional consequences of microbiome composition on *B. oleae* fitness, two regions exhibiting the strongest microbial contrasts (Álora and Moixent) were selected. During successive field visits, approximately 2 kg of infested fruits were collected on each occasion from organic and conventional orchards in Álora (Aloreña variety, October–November 2024) and Moixent (Lechín and Picual varieties, November 2024–January 2025). Infested fruits, containing larvae at different developmental stages (mainly L2, L3 and pupae), were placed in separate muslin-covered rearing boxes and maintained under controlled laboratory conditions (25 ± 1 °C, 70% relative humidity, (≈65% during pupation), 16:8 h light:dark photoperiod). Rearing boxes from different regions and management systems (organic versus conventional) were kept strictly separated throughout the experiment. Emerged adults were fed with a 15% honey solution sprayed onto sterile cotton pads moistened with distilled water. Emergence was staggered over time amd whenever 60 healthy adults from a given region × management system combination became available, an experimental block was initiated.

2.5.2 Experimental design

Each block consisted of three cages containing 20 flies each (10 males and 10 females) per microbial treatment. A fully factorial experimental design was implemented with the factors Region (Álora vs Moixent), Management system (organic vs conventional) and Microbial treatment (four levels: native control, antibiotic-treated, symbiont-enriched and opportunistic-enriched) (**Table S1**). Each treatment combination was replicated three times, resulting in 24 experimental cages per region and a total of 480 individuals per region. The entire experimental period, from the first fruit collections to the completion of all fitness assays, lasted approximately six months.

2.5.3 Experimental methods

2.5.3.1 Native and antibiotic-treated flies

Native control flies retained their field-derived microbiota. Antibiotic-treated flies were obtained by surface sterilization with 70% ethanol, followed by continuous feeding for 7 days on a sterile 10% sucrose solution containing penicillin (20 µg mL^{-1}) and a 15% honey solution mixed with ampicillin (100 µg mL^{-1}). These antibiotics were selected for their broad-spectrum activity against the Gram-negative bacteria that dominate the gut microbiota of *B. oleae.*

2.5.3.2 Preparation of inoculants

Symbiont homogenates Esophageal bulbs were dissected from field-collected *B. oleae* adults under sterile conditions in phosphate-buffered saline (PBS, pH 7.4). Bulbs (10–20 per preparation) were pooled, homogenized with sterile pestles, and centrifuged at 3000 × g for 5 min to remove debris. The supernatant, enriched in *Ca. Erwinia dacicola*, was resuspended in sterile 10% sucrose solution to a final concentration of ~10^8–10^9 cells mL^{-1}. Concerning opportunistics, pure cultures of *Citrobacter braakii* (strain isolated from Álora, Málaga) and *Morganella morganii* (strain isolated from Moixent, Valencia) were obtained from adult *B. oleae* collected in conventional olive orchards, as described in the Sample collection section. Each strain was streaked on LB agar and incubated at 30 °C for 24 h. A single colony was inoculated into 10 mL LB broth and incubated overnight at 30 °C with shaking (180 rpm). Cultures were harvested at mid-log phase (OD_{600} ≈ 0.6–0.8), centrifuged at 4000 × g for 10 min, washed twice with PBS, and resuspended in sterile 10% sucrose to ~10^8–10^9 cells mL^{-1}, as determined by OD calibration curves.

2.5.3.3 Re-inoculation

Symbiont-enriched flies were re-inoculated with homogenates enriched in *Ca. Erwinia dacicola.* Opportunistic-enriched flies were re-inoculated with pure cultures of *C. braakii* or *M. morganii*. In all cases, bacterial suspensions (~10^8–10^9 cells mL^{-1} in 10% sucrose) were freshly prepared and offered to flies for 72 h.

2.5.3.4 Quantitative PCR (qPCR) verification

Total bacterial load was quantified using the universal 16S rRNA primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′), which amplify a ≈250 bp fragment of the V4 region (Caporaso et al. 2011). Taxon-specific assays were performed to confirm re-colonisation. For *Ca. Erwinia dacicola*, primers EdF1 (5′-CTAATACCGCATAACGTCTTCG-3′) and EdEnRev (5′-CCACCTACTAGCTAATCCC-3′) were used to generate a 91 bp amplicon (Sinno et al. 2020). For *Morganella morganii*, primers MMqF (5′-GCGGCGTGCCTAATACATG-3′) and MMqR (5′-CGGTTGCTCTCGCGAGT-3′) yielded a 179 bp amplicon (Ferrario et al. 2012). For *Citrobacter braakii*, primers were designed from variable regions of the 16S rRNA gene and validated in silico against NCBI databases to ensure species specificity.

Each 20 µL reaction contained 1× SYBR Green Master Mix (Applied Biosystems), 0.3 µM of each primer and 10 ng of template DNA. Cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s, with melt-curve analysis to confirm amplification specificity. Absolute quantification was achieved using standard curves generated from serial ten-fold dilutions of genomic DNA (10^8–10^2 copies per reaction). Curve fitting and efficiency calculations were performed with QuantStudio™ Design and Analysis Software v1.5.1 (Applied Biosystems).

2.5.4 Statistical analysis

Fitness parameters (adult longevity, survival percentage, oviposition rate, F1 emergence rate, and pupal weight) were analysed using two-way analysis of variance (ANOVA), with Region and Microbial treatment as fixed factors. Normality of residuals was assessed with the Shapiro–Wilk test (Shapiro and Wilk, 1965) and homogeneity of variances with Levene's test (Levene, 1960). When necessary, data were log- or square-root-transformed to meet ANOVA assumptions. Significant main effects and interactions were further examined using Tukey's HSD post-hoc tests (Tukey, 1953). In addition, Pearson's correlation coefficients (Pearson, 1895) were calculated to explore relationships between the absolute abundance of key bacterial taxa (*Ca. Erwinia dacicola*, *Citrobacter braakii*, and *Morganella morganii*) and the measured fitness parameters. Statistical significance was set at $\alpha = 0.05$ for all tests.

3. Results

3.1 Sequencing output and quality control

Full-length 16S rRNA gene sequencing on the Oxford Nanopore platform yielded between 27,343 and 121,713 high-quality reads per sample (Table S2). Read quality was highly consistent across samples (mean Q-score 21.3–21.6), with ~77% of reads exceeding Q20. Mean read lengths ranged from 1,510 to 1,549 bp (N50 > 1,540 bp). All samples achieved sufficient sequencing depth, and biological replicates showed good reproducibility.

3.2 Bacterial community composition

Relative abundances were calculated as the proportion of reads assigned to each taxon relative to the total number of high-quality reads in each composite sample. Analysis of the relative abundance profiles revealed that the the bacterial microbiota of *B. oleae* was strongly influenced by both geographic origin and agricultural system (Figure 2). *Alcaligenes faecalis* was the most dominant taxon overall, particularly in Álora (Málaga) and Órgiva (Granada). *Citrobacter braakii* was highly abundant in Álora Conventional, while *Enterobacter quasihormaechei* dominated in Mengíbar Conventional. In Moixent (Valencia), *Morganella morganii* and several *Enterobacter* species were frequently detected.

3.3 Alpha diversity

Organic farming systems exhibited significantly higher Shannon diversity than conventional systems across regions (Wilcoxon rank-sum test, p = 0.00077; Figure 3). Region-specific patterns were clear (Figures 4 and 5). Moixent (Valencia) and Mengíbar (Jaén) showed the highest overall diversity, whereas Órgiva (Granada) had the lowest. The strongest farming system effect was observed in Álora (Málaga), where organic orchards displayed substantially higher Shannon diversity compared to conventional orchards. Detailed alpha diversity metrics are presented in **Table 1.**

These results demonstrate that farming system and geographic origin jointly shape the bacterial communities of *B. oleae*. While organic management generally promoted higher species richness, the effect on Shannon diversity was strongly region-dependent, with Álora showing an opposite pattern likely driven by local ecological conditions.

3.4 Differential abundance of bacterial taxa

Differential abundance analyses highlighted several region-specific responses to farming system (**Table 2**). *Citrobacter braakii* was dramatically less abundant in organic orchards in Málaga (Álora) compared to conventional orchards (fold-change = 0.22, p = 0.001). In contrast, *Enterobacter* spp. were significantly enriched in organic orchards in Valencia (Moixent) (fold-change = 1.80, p = 0.008). In Jaén (Mengíbar), *Alcaligenes* spp. showed higher abundance under

conventional management (fold-change = 0.65, p = 0.012), whereas *Stenotrophomonas* sp. was moderately enriched in organic orchards in Granada (Órgiva) (fold-change = 1.45, p = 0.045).

These results demonstrate that both geographic location and farming system (organic versus conventional) significantly shape the bacterial microbiota of *B. oleae*, with distinct taxon-specific responses across regions.

3.5 Beta diversity and community structure

PERMANOVA analysis demonstrated that bacterial community composition in *B. oleae* was significantly influenced by both geographic region and farming system (**Table 3**). The full model explained 62.83% of the total variation (p = 0.001). Region was the dominant factor, accounting for 25.87% of the variance (p = 0.001), while farming system explained 7.35% (p = 0.026). The Region × Farming System interaction was also highly significant (p = 0.001), indicating that the effect of agricultural management on the microbiota was region-dependent.

Bray–Curtis dissimilarity analyses revealed strong geographic structuring of bacterial communities in *Bactrocera oleae*, with significantly higher dissimilarity between regions than within regions (Wilcoxon rank-sum test, $p < 0.0001$; Figure 6A). Community composition also differed between organic and conventional systems, although the effect size was smaller ($p = 0.0024$; Figure 6B). NMDS ordination (Figure 7) illustrated clear regional clustering, particularly for Málaga samples, and partial separation by farming system. SIMPER analyses identified the taxa contributing most to these patterns (Tables S3–S4). *Citrobacter braakii* and *Morganella morganii* were key discriminators among regions, while *Alcaligenes faecalis* and *Enterobacter quasihormaechei* characterized conventional orchards. Overall, geographic origin was the primary driver of community structure, with farming system exerting a secondary, region-dependent influence.

Table 1. Alpha diversity metrics of the bacterial communities associated with *B. oleae* across regions and agricultural systems (mean ± SD)

Region	Farming System	Total Reads (min–max)	Richness (S)	Shannon Index (H′)	Pielou's Evenness (J′)
Mengíbar (Jaén)	Conventional	21,300 – 55,900	212 ± 38	2.06 ± 0.22	0.48 ± 0.06
Mengíbar (Jaén)	Organic	29,600 – 83,900	245 ± 47	1.78 ± 0.15	0.52 ± 0.05
Álora (Málaga)	Conventional	37,300 – 89,000	225 ± 51	1.79 ± 0.25	0.42 ± 0.07
Álora (Málaga)	Organic	28,900 – 56,200	138 ± 22	0.91 ± 0.17	0.65 ± 0.08
Órgiva (Granada)	Conventional	19,600 – 64,900	118 ± 19	1.68 ± 0.13	0.47 ± 0.04
Órgiva (Granada)	Organic	25,100 – 37,100	187 ± 34	1.68 ± 0.17	0.58 ± 0.06
Moixent (Valencia)	Conventional	33,700 – 45,100	198 ± 42	2.08 ± 0.15	0.55 ± 0.05
Moixent (Valencia)	Organic	28,400 – 70,800	278 ± 61	1.56 ± 0.08	0.62 ± 0.07

Table 2. Differential abundance of major bacterial taxa between organic and conventional farming systems across Spanish olive-growing regions

Taxon	Locality	Fold-change (Org/Conv)	p-value (Wilcoxon)	Significance	Detection method (EMU / USEARCH)
Alcaligenes spp.	Jaén (Mengíbar)	0.65	0.012	*	EMU
Citrobacter braakii	Málaga (Álora)	0.22	0.001	**	USEARCH
Stenotrophomonas sp.	Granada (Órgiva)	1.45	0.045	*	EMU
Enterobacter spp.	Valencia (Moixent)	1.80	0.008	**	USEARCH
Providencia rettgeri	Jaén (Mengíbar)	1.20	0.09	ns	EMU

p-value (Wilcoxon): Statistical test comparing relative abundance between systems.

Significance: Marked as * "Significant" if $p < 0.05$, ** "Highly significant" if $p < 0.01$

Table 3. PERMANOVA results of beta diversity analysis of bacterial communities in *B. oleae* across Spanish olive-growing regions and agricultural systems

Factor	Df	R^2	F	p-value	Significance
Region	3	25.87%	3.257	0.001*	Highly significant
System (Organic vs. Conventional)	1	7.35%	2.381	0.026*	Significant
Interaction	7	–	–	0.001*	Highly significant
Full model	7	62.83%	5.796	0.001*	Excellent

Df = Degrees of freedom, R^2 = Proportion of variance explained, F = F-statistic value, p-value = Probability value indicating statistical significance, * = Significant at $p < 0.05$

3.6 Fitness bioassay analysis across farming practices and regions

The fitness bioassay was performed using flies from Álora and Moixent to determine the functional consequences of the distinct microbial communities observed between organic and conventional olive orchards. Flies from organic and conventional orchards were maintained separately throughout the experiment for all microbial treatments (native control, antibiotic-treated, symbiont-enriched and opportunistic-enriched). Significant effects of Microbial treatment and Region were detected for all fitness parameters (ANOVA, $p < 0.01$). Post-hoc comparisons (Tukey's HSD) revealed consistent differences among treatments **(Table 4)**. Antibiotic-treated flies exhibited the lowest fitness in both regions. Adult survival, longevity, oviposition rate, F1 emergence and pupal weight were all significantly reduced compared with Native controls (Tukey's HSD, $p < 0.05$). Oviposition rate declined by more than 60% and F1 emergence was strongly impaired (4.8–7.3%), confirming the critical contribution of the microbiota to host performance.

Symbiont-enriched flies (exposed to *Ca. Erwinia dacicola*-enriched homogenates) displayed the highest fitness values. Relative to antibiotic-treated flies, this treatment increased adult survival by 41–57%, oviposition rate by 68–89%, and F1 emergence by 3.5–4.8-fold. Performance of symbiont-enriched flies was comparable to, or slightly higher than, that of Native Organic flies, particularly in Álora. Opportunistic-enriched flies showed significantly lower fitness than Native flies, with stronger negative effects in Álora. In this region, opportunistic enrichment reduced adult survival, oviposition rate and F1 emergence more markedly than in Moixent.

Table 4. Fitness parameters of *B. oleae* under different microbial treatments and regions (mean ± SEM)

Treatment	Region	Farming system	Adult longevity (days)	Survival (%)	Oviposition (punctures/♀)	F1 Emergence (%)	Pupal weight (mg)
Native	Álora	Organic	29.8 ± 1.6^{b}	76.5 ± 3.8	27.5 ± 2.0	48.2 ± 3.5	9.6 ± 0.4
Native	Álora	Conventional	23.1 ± 2.1^{c}	60.2 ± 4.5	21.8 ± 1.8	34.7 ± 4.1	8.2 ± 0.5
Native	Moixent	Organic	31.2 ± 1.4^{b}	78.4 ± 3.2	30.1 ± 2.2	51.3 ± 3.7	9.8 ± 0.3
Native	Moixent	Conventional	26.3 ± 1.9^{bc}	69.7 ± 4.1	26.6 ± 1.9	43.1 ± 3.9	9.0 ± 0.4
Antibiotic-treated	Álora	Organic	13.4 ± 2.0^{e}	34.8 ± 4.9	10.1 ± 1.5	5.6 ± 2.1	6.3 ± 0.5
Antibiotic-treated	Álora	Conventional	12.2 ± 2.2^{e}	30.2 ± 5.3	8.3 ± 1.3	4.0 ± 2.3	5.9 ± 0.5
Antibiotic-treated	Moixent	Organic	15.1 ± 1.8^{e}	41.5 ± 4.6	12.4 ± 1.6	8.1 ± 1.8	6.7 ± 0.4
Antibiotic-treated	Moixent	Conventional	14.1 ± 2.0^{e}	37.9 ± 5.0	10.6 ± 1.8	6.5 ± 2.0	6.3 ± 0.4
Symbiont-enriched	Álora	Organic	36.1 ± 1.6^{a}	83.5 ± 3.7	40.2 ± 2.4	65.1 ± 4.3	11.1 ± 0.4
Symbiont-enriched	Álora	Conventional	34.3 ± 1.8^{a}	78.9 ± 4.1	37.0 ± 2.2	59.7 ± 4.7	10.5 ± 0.4
Symbiont-enriched	Moixent	Organic	34.8 ± 1.6^{a}	81.6 ± 3.1	38.4 ± 2.5	61.2 ± 3.8	10.7 ± 0.3
Symbiont-enriched	Moixent	Conventional	33.0 ± 1.8^{a}	78.0 ± 3.3	35.4 ± 2.7	56.2 ± 4.0	10.3 ± 0.3
Opportunistic-enriched	Álora	Organic	20.5 ± 1.9^{d}	52.1 ± 4.5	15.3 ± 1.6	22.4 ± 3.0	7.5 ± 0.5
Opportunistic-enriched	Álora	Conventional	18.1 ± 2.1^{d}	45.1 ± 4.9	12.3 ± 1.4	16.0 ± 3.2	6.9 ± 0.5
Opportunistic-enriched	Moixent	Organic	22.8 ± 1.7^{d}	58.3 ± 4.1	19.7 ± 1.9	29.1 ± 3.3	8.1 ± 0.4
Opportunistic-enriched	Moixent	Conventional	20.2 ± 1.9^{d}	52.5 ± 4.5	16.7 ± 2.1	23.9 ± 3.5	7.5 ± 0.4

Values are presented as mean ± standard error of the mean (SEM) from three independent biological replicates (n = 60 flies per treatment per region). Different letters within the same column indicate significant differences (two-way ANOVA followed by Tukey's HSD test, $p < 0.05$

Native Organic flies consistently outperformed Native Conventional flies across all measured parameters. The magnitude of these differences was greater in Álora than in Moixent, reflecting the stronger microbial contrast between farming systems in this region. Correlation analyses supported these patterns: absolute abundance of *Ca. Erwinia dacicola* was positively correlated with oviposition rate (Pearson $r = 0.78$, $p < 0.001$) and F1 emergence ($r = 0.82$, $p < 0.001$), whereas abundances of *Citrobacter braakii* and *Morganella morganii* were negatively correlated with the same traits ($r = -0.65$ to -0.74, $p < 0.01$).

Overall, these results indicate that the microbiota associated with conventional orchards, characterised by higher relative abundance of opportunistic taxa, is linked to reduced host fitness, whereas the microbiota associated with organic orchards is associated with higher performance of *B. oleae*.

4. Discussion

This study provides the first high-resolution characterisation of the bacterial microbiota associated with *B. oleae* across four major Spanish olive-growing regions, integrating both geographic origin and farming-system variables. By applying full-length 16S rRNA sequencing with Oxford Nanopore technology, we achieved superior taxonomic resolution compared with short-read approaches, enabling robust ecological insights into dominant and region-specific taxa. The results revealed clear geographic signatures, with *Citrobacter braakii* strongly associated with Málaga, *Morganella morganii* enriched in Valencia and Órgiva, and *Clostridioides mangenotii* characteristic of Jaén. At the same time, agricultural management significantly shaped microbial diversity: conventional orchards were enriched in opportunistic taxa, whereas organic systems generally promoted higher richness and evenness, although these effects were region-dependent. The originality of this work lies in its multi-regional sampling design and the integration of ecological and agronomic factors, revealing that microbiome structuring is context-dependent.

The ecological implications of these findings are consistent with previous reports showing that sustainable management systems, such as organic fertilisation, enhance microbial diversity and stability in olive agroecosystems (Ahmad et al. 2026). Organic orchards harboured a higher number of unique bacterial genera, suggesting that reduced chemical inputs and diversified soil management practices favour richer and more heterogeneous microbial communities in soil–plant compartments (Fausto et al. 2019; Melloni and Cardoso 2023). By contrast, conventional orchards exhibited a more homogenised microbial profile, consistent with selective pressures imposed by synthetic fertilisers and phytosanitary treatments. Despite these differences, both systems shared a robust core microbiota, highlighting the dual influence of intrinsic plant-associated factors and external agricultural practices on microbiome assembly.

Importantly, our results extend these ecological patterns to the insect pest itself. Several studies have demonstrated that insect-associated microbiomes are not isolated entities but are shaped by the surrounding plant and soil microbiota (Kakagianni et al. 2023; Dimou et al. 2003; Ortega et al. 2018). In *B. oleae*, the prevalence of *Ca. Erwinia dacicola* in organic orchards and its positive correlation with oviposition and F1 emergence confirm its role as an obligate mutualist, essential for host fitness (Ben-Yosef et al. 2010, 2014, 2015; Blow et al. 2019; Siden-Kiamos et al. 2025). This endosymbiont is strictly vertically transmitted and absent from external compartments such as soil or olive roots, underscoring its intimate co-evolution with the host. Conversely, the enrichment of opportunistic taxa such as *Citrobacter braakii*, *Morganella morganii* and *Providencia sp*. in conventional orchards mirrors their dominance in the plant microbiome of intensively managed systems, suggesting microbial spillover between plant compartments and insect hosts. This supports the hypothesis that insect pests acquire part of their microbiota from the agroecosystem in which they develop, establishing a functional continuum between farming practices, plant-associated communities and insect performance (Engel and Moran 2013; Douglas 2015).

In addition to these agroecosystem and plant–soil influences, our results emphasise the role of regional ecological contexts in shaping microbial diversity. Even within Andalusia, semi-arid Mediterranean areas such as Jaén, Órgiva and Álora differ markedly: Jaén is characterised by vast olive plains, Órgiva by mountainous terrain with natural vegetation (*Cedrus*, *Pinus*, hedgerows), and Álora by mixed agroecosystems where the local “Alorina” olive variety coexists with citrus and fruit trees. By contrast, Moixent in Valencia is less arid, with surrounding forested landscapes. These geographic and climatic contrasts likely contribute to the beta diversity patterns observed, influencing both microbial community assembly and the reproductive performance of *B. oleae*. Differences in olive phenology and insect life cycles across regions may further modulate microbiome composition, underscoring the need for future studies that explicitly evaluate microbial diversity in relation to ecological and biological parameters (Nie et al. 2025; Graber and Moreau 2026).

Taken together, these findings suggest that microbial diversity in *B. oleae* is shaped not only by farming practices but also by regional ecological contexts. Recognising these multi-scale drivers of diversity is essential for developing microbiome-informed strategies that support sustainable pest management in Mediterranean olive agroecosystems.

Our bioassays provided functional validation of these ecological patterns. Native flies originating from organic orchards consistently exhibited higher fitness than those from conventional orchards across all measured parameters, with the magnitude of the difference being greater in Álora. Antibiotic-treated flies showed a strong reduction in adult survival, longevity, oviposition rate and F1 emergence, confirming the critical contribution of the microbiota to host performance

(Sinno et al. 2020; Maretto et al. 2025). Exposure to *Ca. Erwinia dacicola*-enriched homogenates restored fitness and, in some cases, yielded values comparable to or slightly higher than those of native organic flies. Conversely, enrichment with opportunistic taxa typical of conventional orchards (*Citrobacter braakii* or *Morganella morganii*) significantly impaired adult longevity, fecundity and offspring emergence. These results provide experimental support for a causal link between microbiome composition and *B. oleae* fitness, and indicate that farming practices can influence pest performance through their effects on the insect microbiota.

The functional roles of dominant taxa further underscore the ecological significance of these findings. *Ca.* Erwinia dacicola, the obligate endosymbiont of *B. oleae*, provides nitrogen recycling and amino acid supplementation that support female fecundity and larval development (Ben-Yosef et al. 2010, 2014, 2015; Nobre 2021; Martínez-Sañudo et al. 2024). Its consistent presence across samples underscores its central role in olive fly biology. In contrast, opportunistic taxa such as *Citrobacter braakii*, *Morganella morganii* and *Providencia* spp., which were more abundant under conventional management, have previously been associated with reduced performance in tephritid flies (Capuzzo et al. 2005; Salas et al. 2023; Hassan et al. 2020). These findings reinforce our results in *B. oleae*, where antibiotic-treated flies exhibited severely reduced fitness, while symbiont-enriched flies restored or even enhanced biological performance.

Our study aligns with previous work on tephritid–microbial interactions, which showed that supplementation with beneficial bacteria enhances fruit fly performance in sterile insect technique (SIT) programmes (Deutscher et al. 2019). In both contexts, the presence of obligate or facultative symbionts is critical for host survival, reproduction and competitiveness, whereas depletion or replacement by opportunistic taxa leads to reduced fitness. This parallel highlights a functional dichotomy: obligate symbionts consistently enhance host performance, opportunistic taxa impair it, and entomopathogenic bacteria isolated from biodynamic systems (e.g., *Bacillus* sp.) can directly suppress olive fly survival (Blibech et al. 2012; Fosso et al. 2026). This tripartite framework clarifies the ecological roles of insect-associated microbes and opens distinct avenues for microbiome-informed pest management.

From an applied perspective, these findings have implications for the development of microbiome-informed pest management strategies in olive agroecosystems. The identification of *Ca. Erwinia dacicola* as a key positive contributor, and of *Citrobacter, Morganella* and related opportunistic taxa as negative contributors, highlights potential microbial targets. Approaches aimed at reinforcing beneficial symbionts or selectively disrupting detrimental taxa could form components of Integrated Pest Management (IPM) programmes that reduce reliance on broad-spectrum insecticides (Daane and Johnson 2010; Medjkouh et al. 2016; Valenčič et al. 2021; Dadras et al. 2022). Organic and

biodynamic practices appear to conserve microbial diversity and favour a microbiota associated with higher host performance, sometimes even promoting entomopathogenic taxa such as *Bacillus* sp. with biocontrol potential (Blibech et al. 2012; Fosso et al. 2026). Future studies should evaluate the stability of manipulated microbiomes under field conditions, explore tripartite soil–plant–insect interactions, and test the efficacy of microbiome-based interventions at orchard scale. Such work will build on the foundation established here and contribute to the rational design of strategies that support both pest suppression and the long-term sustainability of olive production.

5. Implications

Our findings demonstrate that farming practices shape the microbiota of *B. oleae* with measurable consequences for its fitness. The enrichment of the obligate symbiont *Ca. Erwinia dacicola* under organic management, contrasted with the dominance of opportunistic taxa (*Citrobacter, Morganella, Providencia*) under conventional systems, identifies clear microbial targets. These results open avenues for microbiome-informed strategies aimed at reinforcing beneficial symbionts or disrupting detrimental taxa, thereby reducing pest performance while decreasing reliance on chemical insecticides, particularly in organic and low-input olive systems.

6. Conclusion

This study provides the first high-resolution characterization of the bacterial microbiota of *B. oleae* across four major Spanish olive-growing regions, revealing that both geographic origin and farming system significantly structure microbial communities. Organic orchards generally supported higher bacterial diversity and a microbiota associated with superior host fitness, while conventional systems favoured opportunistic taxa linked to reduced performance. Fitness bioassays confirmed a causal role of the microbiota: antibiotic treatment severely impaired reproductive success, reinoculation with *Ca. Erwinia dacicola* restored fitness, and enrichment with conventional-associated opportunistic taxa reduced key performance parameters.

Overall, this work establishes a clear link between agricultural practices, microbiome composition and pest fitness. By integrating ecological and agronomic perspectives, it provides a scientific foundation for the rational design of microbiome-informed tools that support both pest suppression and the long-term sustainability of Mediterranean olive production.

7. Statements and Declarations

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Author Contributions

I.B. and F.C.S. conceived and designed the research methodology. I.B., F.J.B., and M.R.T. conducted the investigation and performed field collections. I.B. conducted the experiments and performed the molecular procedures and DNA extraction. Oxford Nanopore sequencing was performed by Secugen (Madrid, Spain). I.B. and F.R.C. performed validation. I.B. and F.C.S. conducted software-related tasks and data analysis. I.B. prepared the original draft. M.R.T., F.J.B., and F.C.S. contributed to resources and data curation. I.B., F.J.B., and F.C.S. contributed to writing—review and editing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no relevant financial or non-financial interests to disclose.

Data availability

The datasets generated during the current study are available in the Zenodo repository, (Version 1: 10.5281/zenodo.22784591; Version 2: 10.5281/zenodo.23056189).

Ethics approval

Not applicable. This study did not involve experiments on humans or vertebrate animals

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10. Figures

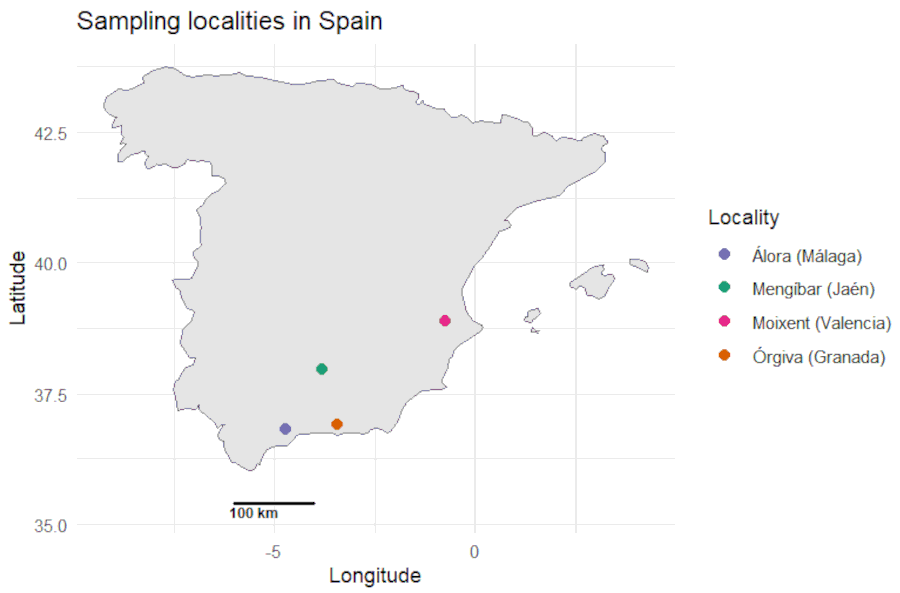

Figure 1. Geographic distribution of the four sampling sites in Spain. Adult *B. oleae* were collected in Mengíbar (Jaén), Órgiva (Granada), Álora (Málaga) and Moixent (Valencia). Each point marks a region where olives were sampled under both organic and conventional farming systems (map created with R version 4.5.2)

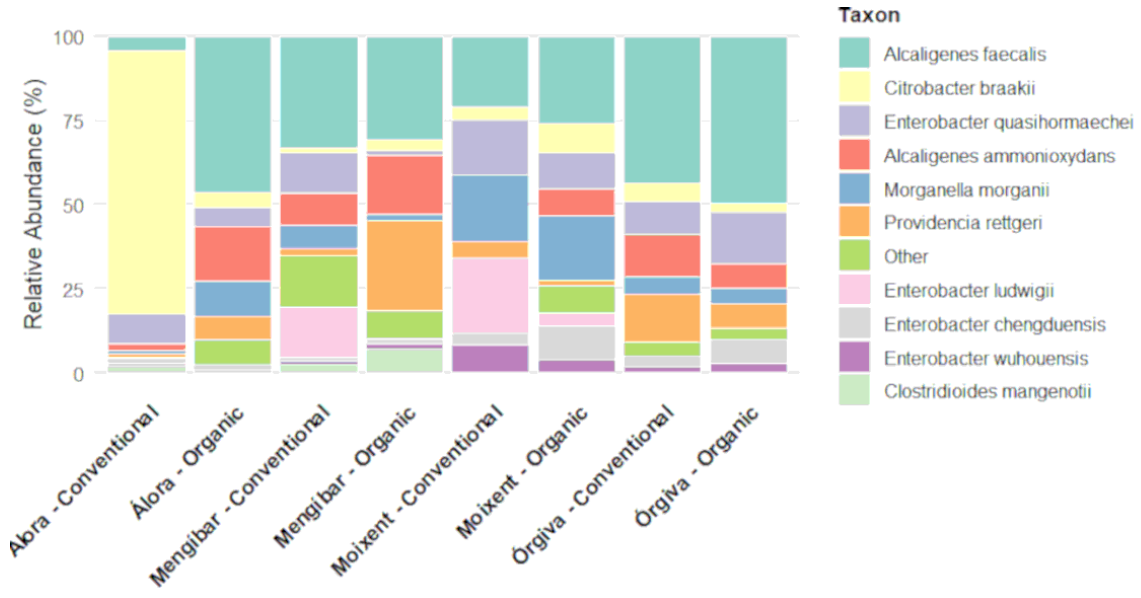

Figure 2. Relative abundance of bacterial taxa in *B. oleae* across four Spanish regions and two farming systems (organic vs. conventional). Bars represent merged biological replicates (n = 4 per condition). (R v4.5.2)

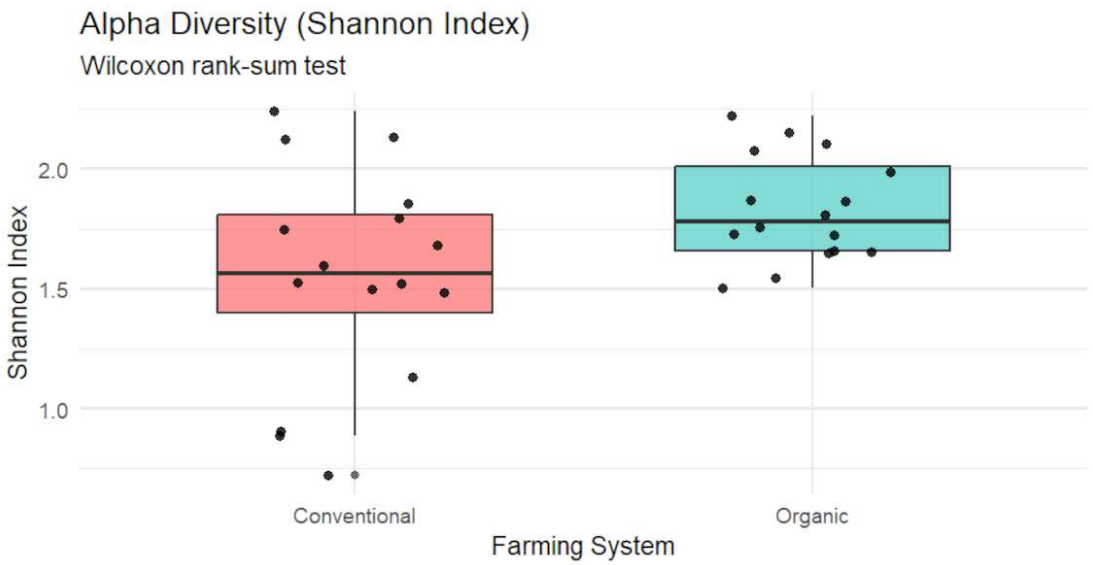

Figure 3. Alpha diversity (Shannon Index) of bacterial communities in *B. oleae* according to farming system (organic vs conventional). Each point represents one composite sample (merged biological replicates). Wilcoxon rank-sum test: p = 0.00077. (R v4.5.2)

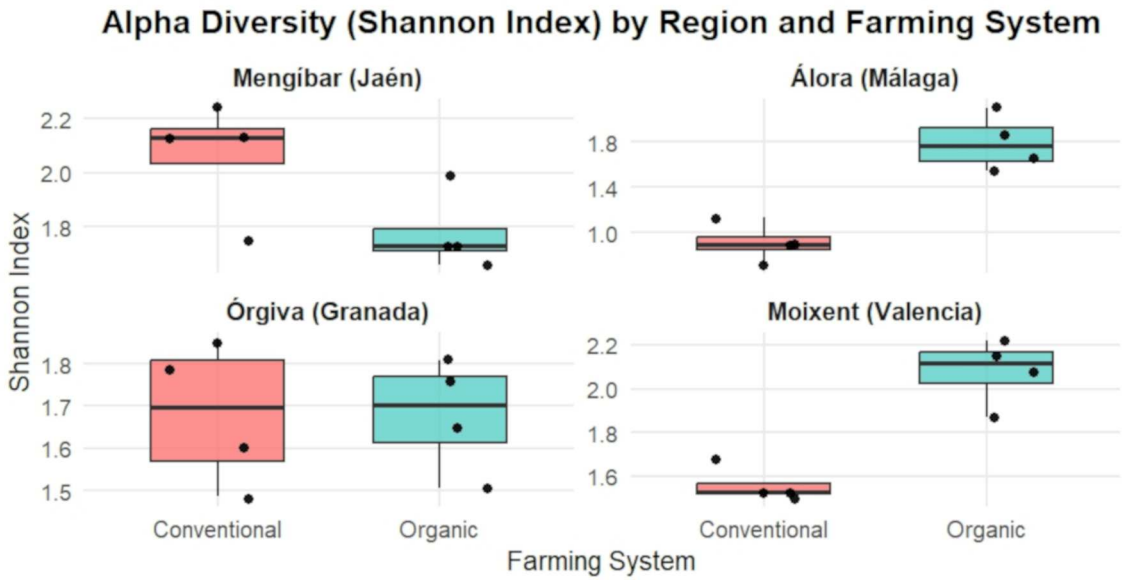

Figure 4. Alpha diversity (Shannon Index) of bacterial communities in *B. oleae* across four geographic regions and two farming systems. Each point represents one composite sample. (R v4.5.2)

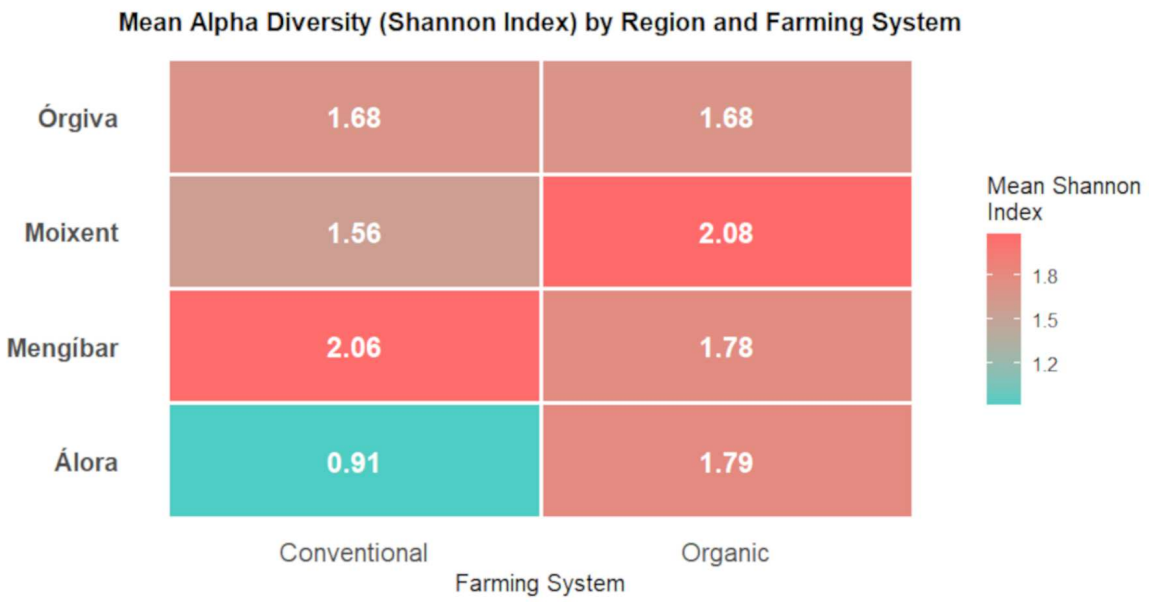

Figure 5. Mean Shannon diversity index of bacterial communities associated with *B. oleae* by geographic region and farming system (organic vs conventional). Heatmap colors represent mean Shannon index values (R v4.5.2)

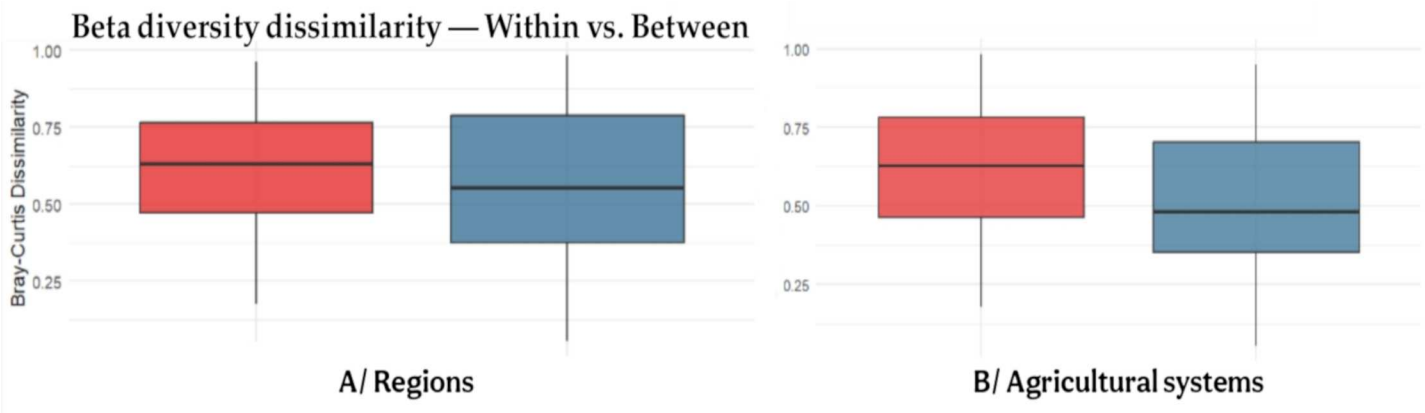

Figure 6. Bray–Curtis dissimilarity within (intra-group, bleu) and between (inter-group, rouge) samples. Comparisons among: (A) geographic regions (Wilcoxon rank-sum test, $p < 0.0001$), (B) Agricultural systems (Conventional vs. Organic; Wilcoxon rank-sum test, $p = 0.00242$). (R v4.5.2)

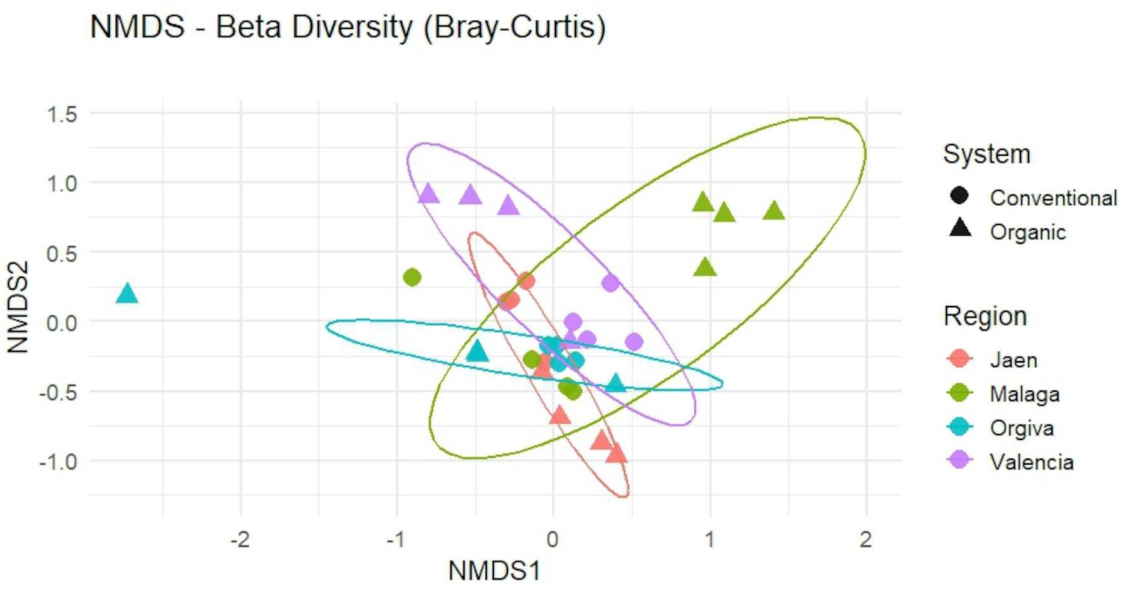

Figure 7. Non-metric multidimensional scaling (NMDS) ordination of bacterial communities associated with *B. oleae* based on Bray–Curtis dissimilarities. (R v4.5.2). Points represent samples, with colors indicating geographic regions and shapes distinguishing farming systems. Ellipses indicate group distributions.

11. Supplementary Materials

Table S1. Experimental design for the fitness bioassays, showing the factorial combinations of region (Álora vs. Moixent), management system (organic vs. conventional), and microbial treatments (native control, antibiotic-treated, symbiont-enriched, opportunistic-enriched), with replicates and number of flies per replicate.

Region	Management system	Microbial treatment	Replicates	Flies per replicate	Total flies
Álora	Organic	Native control	3	20 (10♂ + 10♀)	60
Álora	Organic	Antibiotic-treated	3	20	60
Álora	Organic	Symbiont-enriched	3	20	60
Álora	Organic	Opportunistic-enriched (*Citrobacter braakii*)	3	20	60
Álora	Conventional	Native control	3	20	60
Álora	Conventional	Antibiotic-treated	3	20	60
Álora	Conventional	Symbiont-enriched	3	20	60
Álora	Conventional	Opportunistic-enriched (*Citrobacter braakii*)	3	20	60
Moixent	Organic	Native control	3	20	60
Moixent	Organic	Antibiotic-treated	3	20	60
Moixent	Organic	Symbiont-enriched	3	20	60
Moixent	Organic	Opportunistic-enriched (*Morganella morganii*)	3	20	60
Moixent	Conventional	Native control	3	20	60
Moixent	Conventional	Antibiotic-treated	3	20	60
Moixent	Conventional	Symbiont-enriched	3	20	60
Moixent	Conventional	Opportunistic-enriched (*Morganella morganii*)	3	20	60

Table S2. Sequencing output and quality metrics of full-length 16S rRNA gene reads generated by Oxford Nanopore sequencing from *B. oleae* samples collected across the four Spanish olive-growing regions.

Region	Farming system	Replicate	Number of reads	Throughput (Mb)	Mean read length (bp)	N50 (bp)	Mean quality score (Phred)
Jaén	Organic	1	47,147	71.2	1,512	1,548	21.5
Jaén	Organic	2	82,630	127.4	1,542	1,552	21.4
Jaén	Conventional	1	73,722	112.8	1,530	1,545	21.6
Jaén	Conventional	2	56,709	86.5	1,525	1,550	21.3
Málaga	Organic	1	42,401	64.3	1,517	1,540	21.5
Málaga	Organic	2	80,280	124.1	1,545	1,551	21.4
Málaga	Conventional	1	121,713	186.2	1,530	1,546	21.6
Málaga	Conventional	2	96,046	148.0	1,541	1,550	21.5
Granada	Organic	1	39,138	59.4	1,518	1,542	21.3
Granada	Organic	2	57,322	88.1	1,537	1,548	21.4
Granada	Conventional	1	29,346	44.3	1,509	1,538	21.3
Granada	Conventional	2	27,343	41.2	1,507	1,538	21.3
Valencia	Organic	1	56,567	87.3	1,543	1,551	21.5
Valencia	Organic	2	78,552	121.3	1,545	1,550	21.4
Valencia	Conventional	1	80,230	123.5	1,538	1,547	21.5
Valencia	Conventional	2	62,478	96.4	1,542	1,550	21.4

Note: Values include the number of reads, sequencing throughput (Mb), mean read length (bp), N50 (bp), and mean quality score (Phred). N50 represents the read length at which 50% of the total sequenced bases are contained in reads of that length or longer. Approximately 77% of reads had a quality score above Q20 across all samples.

Table S3. SIMPER Analysis – Key Taxa Driving Differences Between Region

Region Pair	Key Discriminating Taxa	Contribution	p-value	Interpretation
Jaen vs Malaga	*Citrobacter braakii* *Alcaligenes faecalis* *Providencia rettgeri*	High	0.007 ns 0.220	*C. braakii* strongly associated with Malaga
Jaen vs Orgiva	*Citrobacter murliniae* *Clostridioides mangenotii* *Providencia rettgeri*	High	0.001 0.001 0.012	Jaen enriched in *Citrobacter* & *Clostridioides*
Jaen vs Valencia	*Morganella morganii* *Citrobacter murliniae* *Clostridioides mangenotii*	High	0.014 0.001 0.001	Clear separation driven by *Morganella* in Valencia
Malaga vs Orgiva	*Citrobacter braakii* *Alcaligenes faecalis*	Very High	0.001 0.150	*C. braakii* is the main driver (much higher in Malaga)
Malaga vs Valencia	*Citrobacter braakii* *Morganella morganii* *Enterobacter ludwigii*	High	0.004 0.017 0.039	Malaga strongly differentiated by *C. braakii*
Orgiva vs Valencia	*Morganella morganii* *Enterobacter ludwigii* *Enterobacter chengduensis*	High	0.001 0.001 0.001	Valencia enriched in *Morganella* and several *Enterobacter*

ns = not significant

Tableau S4. SIMPER analysis identifying key taxa contributing to dissimilarity between conventional and organic systems

Rank	Taxon	Average Contribution	p-value	Interpretation
1	*Alcaligenes faecalis*	0.149	0.041 *	Significantly higher in Conventional
2	*Citrobacter braakii*	0.128	0.139	Tends to be higher in Organic (Malaga effect)
3	*Alcaligenes ammonioxydans*	0.054	0.075 .	Marginally higher in Conventional
4	*Enterobacter quasihormaechei*	0.042	0.035 *	Higher in Conventional
5	*Providencia rettgeri*	0.052	0.270	No clear difference
6	*Morganella morganii*	0.050	0.132	Tends to be higher in Conventional
7	*Enterobacter chengduensis*	0.023	0.026 *	Higher in Conventional
8	*Stenotrophomonas sp.*	0.006	0.006 **	Higher in Conventional
9	*Enterococcus sp.*	0.006	0.028 *	Higher in Conventional
10	*Clostridium sporogenes*	0.003	0.038 *	Higher in Conventional
11	*Vagococcus fluvialis*	0.001	0.036 *	Higher in Conventional