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Pharmaceutical-contaminated water irrigation impacts the human pathobiome of soil and alters aphid-endosymbiont dynamics☆

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ABSTRACT

The use of reclaimed water for agricultural irrigation presents a sustainable strategy to address water scarcity, yet it may introduce residual pharmaceuticals that persist despite treatment. This study investigates the ecological impacts of irrigation with pharmaceutical-contaminated water on plants, soil microbial communities, aphid life history traits, and aphid-associated bacterial symbionts within the *Cucurbita pepo* - *Aphis gossypii* model system. Plants were irrigated with water spiked with some of the most common antibiotics found in wastewater, including clarithromycin, trimethoprim, clindamycin, sulfamethoxazole, azithromycin, and ofloxacin, as well as the pharmaceuticals venlafaxine and metoprolol. While overall soil microbial diversity remained unchanged, potentially pathogenic bacteria, such as *Afipia*, *Methylobacterium*, *Paracoccus*, and *Saccharopolyspora*, exhibited increased abundance, raising concerns about potential human health risks. However, no significant changes were detected in class 1 integron (a proxy of antibiotic resistance) abundance, suggesting that the concentrations used in this study did not exert sufficient selective pressure. Plant traits, aphid survival and fecundity were unaffected by chemical exposure, yet a reduction in endosymbiont abundance was observed, indicating potential long-term ecological consequences for aphid populations. These findings highlight the need for further research on the long-term effects of pharmaceutical contamination in agroecosystems, particularly regarding its implications for biodiversity, soil health, and human safety.

1. Introduction

The use of reclaimed water for agricultural irrigation presents a promising strategy to mitigate water scarcity pressures in the agricultural sector (Mahjoub et al., 2022; Sunyer-Caldú et al., 2022; Zhao et al., 2022a). Despite its environmental and economic benefits, reclaimed water may still contain residual contaminants, including pollutants, pharmaceuticals, and pathogens, which are not entirely removed during treatment processes.

Among the various contaminants, antibiotics are well-documented in reclaimed water (Zhong et al., 2023). Global antibiotic consumption increased by 16 % between 2016 and 2023, with projections indicating a potential 52.3 % escalation by 2030 (Klein et al., 2024). This increase is largely attributed to domestic use in human medicine, but also to the therapeutic and prophylactic use of antibiotics in livestock and aquaculture. Consequently, antibiotics and their metabolites reach agricultural systems through wastewater discharge and manure application (Thiele-Bruhn, 2003). Municipal wastewater is a major source, often containing residual antibiotics due to human excretion and improper disposal.

In addition to antibiotics, other pharmaceuticals such as venlafaxine and metoprolol are frequently detected in wastewater due to their widespread prescription and incomplete removal during treatment (Lin et al., 2021; Telgmann and Horn, 2024). Venlafaxine, a serotonin-norepinephrine reuptake inhibitor, has been detected in effluents at concentration up to 2.19 μg L^{-1} and in surface waters up to 1.22×10^{-1} μg L^{-1} (Wu et al., 2024; Lajeunesse et al., 2012; Collado et al., 2014; Writer et al., 2013; Schultz et al., 2010; Rúa-Gómez and Püttmann,

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2012). Metoprolol, a β1-selective adrenergic receptor blocker, is also commonly detected, whit concentrations ranging from 6.6×10^{-2} to 8.042 μg L^{-1} in various aquatic environments (Love et al., 2024).

All these pharmaceuticals, together with antibiotic resistant bacteria and antibiotic resistance genes (ARGs), can accumulate in soil and crops irrigated with reclaimed water (Zhao et al., 2022a; Lyu et al., 2022; Trotta et al., 2024a). Indeed, plants can absorb antibiotics and other chemicals from water or soil, leading to their accumulation in edible and non-edible plant tissues (Geng et al., 2022). Although the implications for plant health and human exposure have been studied, the potential ecological effects of these contaminants on insect pests, through plant-mediated exposure, and on soil microorganisms remain poorly understood.

Aphids are important phloem-feeding insect pests and serve as model organisms for agroecological studies (Trotta et al., 2024a; Caccavo et al., 2025; Trotta et al., 2024b). Aphids have evolved in close association with bacterial endosymbionts, which influence their immune response, reproduction, and adaptation to host plants (Leonardo and Mondor, 2006). They share an obligate mutualistic relationship with *Buchnera aphidicola*, an intracellular bacterium that provides essential amino acids and vitamins absent in phloem sap (Douglas, 1998). As a result of a long co-evolution with the aphids, this symbiosis is so integral that neither the aphid nor *B. aphidicola* can survive independently (Baumann et al., 1995; Shigenobu and Yorimoto, 2022). Experimental reduction of *B. aphidicola* density in young aphids using high doses of antibiotics significantly impairs aphid development and reproduction (Douglas, 1998; Douglas, 1996) and negatively affects plant growth, which in turn indirectly affects aphid development and reproduction (Pufal et al., 2019). Aphids also harbour facultative bacterial endosymbionts that enhance their stress tolerance and resistance to biotic pressures (Oliver et al., 2010). Despite their importance, research on the direct effects of environmental antibiotics and other pharmaceuticals on aphids and their symbionts, particularly at the low concentrations, remains limited.

Pharmaceutical residues can enter the soil and the plant via wastewater irrigation, manure application, and runoff from livestock operations, where they persist and also exert a selective pressure on soil microbial community (Pan and Chu, 2017; Cycoń et al., 2019). The impact of environmentally relevant concentrations of pharmaceuticals in wastewater on soil microbial communities and insect pest populations is still largely unknown. This knowledge gap is particularly evident in the context of their intricate interactions with insect-associated bacterial endosymbionts. Antibiotic-induced disruptions may influence insect physiology, potentially affecting host survival, reproductive success, and interspecies interactions.

In this study, zucchini squash (*Cucurbita pepo* L.) plants were irrigated with water spiked with some of the most common antibiotics found in wastewater, including clarithromycin, trimethoprim, clindamycin, sulfamethoxazole, azithromycin, and ofloxacin, as well as the pharmaceuticals venlafaxine and metoprolol. The eight chemicals selected for this study were chosen based (i) on environmental relevance, (ii) analytical feasibility, (iii) inclusion in the EU Watch list at least once, (iv) bioaccumulation and (v) toxicity, following Castaño-Trias et al. (Castaño-Trias et al., 2024). These pharmaceuticals were added at a concentration of 0.125×10^{-1} μg L^{-1} each, reflecting typical antibiotic levels found in wastewater treatment plants and the highest concentrations measured in surface waters (Sanseverino et al., 2018). Furthermore, the cotton-melon aphid, *Aphis gossypii* Glover, one of the most economically significant pests of cucurbit crops worldwide, capable of causing severe yield losses (Blackman and Eastop, 2007; Herron and Wilson, 2017), was also included in the experiment as model organism.

This study examines the bottom-up ecological effects of pharmaceutical contaminants in irrigation water on plants, soil microbial communities, and insect herbivores, addressing a critical knowledge gap. We first investigated the effect of antibiotic contamination in irrigation water on the soil microbial community, hypothesizing that antibiotics, even at low concentrations, could disrupt microbial activity by selectively inhibiting the growth of specific taxa, thereby altering microbial diversity, composition, and functional dynamics within the soil ecosystem. We then investigated the influence of pharmaceutical-contaminated irrigation water on the shoot biomass and leaf development of zucchini plants and on life history traits of *A. gossypii*. In parallel, we conducted a quantitative analysis of changes in the bacterial endosymbiont community of the aphids. We also investigated potential transgenerational impacts of pharmaceutical exposure on aphid performance. Given the close link between aphid development, reproduction, and endosymbiont function, microbial disturbances caused by contaminants may not manifest within a single generation. To account for potential delayed effects, the experiment included a second generation of *A. gossypii*.

Our goal is to provide an integrated, ecologically based evaluation of the influence of trace-level pharmaceutical contaminants on below- and above-ground biota. Our approach expands current ecotoxicological frameworks by linking plant-mediated exposure to microbial dynamics and insect physiology within agricultural systems.

2. Materials and methods

2.1. Contaminated water preparation

A combination of the most important and common antibiotics found in municipal wastewater was used (Sanseverino et al., 2018; Liu et al., 2023). The pharmaceutical compounds utilized in this study included the antibiotics clarithromycin, trimethoprim, clindamycin, sulfamethoxazole, azithromycin, and ofloxacin, as well as the two pharmaceuticals venlafaxine and metoprolol. All compounds were obtained as analytical standards from Sigma-Aldrich (Steinheim, Germany) and had a purity level of at least 98 %, as stated in the certificate from the supplier. Stock solutions were prepared by dissolving each compound in methanol and stored at 4 °C in the dark to maintain stability throughout the experiment. A working standard solution (1 mg L^{-1}) was then prepared by diluting the stock solution with ultrapure water. The irrigation water (tap water) was subsequently spiked with an appropriate volume of the pharmaceutical stock solution. Each of the eight pharmaceuticals was added individually at a concentration of 0.125 μg L^{-1}, resulting in a total combined concentration of 1 μg L^{-1} in the irrigation water. This concentration is well below the solubility limits in methanol for all compounds and is consistent with the values reported in the effluents (Castaño-Trias et al., 2023). This concentration was chosen to represent the upper range of levels typically found in wastewater and surface waters that are environmentally relevant. These values are based on international monitoring studies (Liu et al., 2023; Zhang et al., 2021) and align with prior ecotoxicological evaluations of reclaimed water quality in agricultural settings. While not derived from site-specific measurements, this concentration provides a realistic exposure scenario for addressing potential ecological effects under controlled conditions. A solvent control consisting of tap water with methanol alone (0.02 % v/v) was included in the experiments as a control treatment. The plants were watered several times during the experiment, so the amount of pharmaceuticals utilized increased over time, although a low degree of degradation or mineralization cannot be excluded.

2.2. Plants and insect rearing

Germinated zucchini seedlings of the cultivar "San Pasquale" were sown in cylindrical pots (12 cm height × 15 cm upper diameter) containing 1500 cc of synthetic soil (40 % peat, 40 % perlite, 10 % sand, and 10 % clay, pH 8.2, EC (μS/cm) 572 (Trotta et al., 2024b)). The pots were placed in a climatic growth chamber set to 22 ± 1 °C, with 65 ± 5 % relative humidity, and a 16:8 h light/dark (L/D) photoperiod. Synthetic soil was used to maintain highly controlled conditions, including soil texture, nutrient availability, moisture content, and pH, while minimizing background microbial complexity, the presence of pesticides, fertilizers, or soil-borne pathogens. Immediately after germination, plants were irrigated with either tap water (control) or tap water supplemented with pharmaceuticals. Three days after transplanting, each plant received 50 mL of the NPK nutrient solution "Piante verdi" (NKP: 7-3-6 + Fe and microelements, Compo®) at a 3 % concentration; this treatment was repeated one week later. In the third week, each plant was fertilized with 1 g of "Concime Sprint" (NPK (S): 20-10-10 (8), FertilCompany, Ragusa, Italy).

The *A. gossypii* strain used in this study was collected in August 2022 from a zucchini field near Potenza, Italy (40°34′N, 15°45E) (Forlano et al., 2022). The aphids were reared in the laboratory on zucchini plants at room temperature with a 16:8 h L/D photoperiod, without chemical treatments. Experiments were conducted using *A. gossypii* dark green apterous parthenogenetic females. To eliminate maternal and grandmaternal effects, approximately 100 adult virginoparae females were placed on a host plant and allowed to reproduce for 24 h. The newly born nymphs were then reared on the plant until reaching adulthood, at which point the adults were removed. This process was repeated for two successive generations and independently applied to each experimental replicate.

2.3. Experimental design

To ensure adequate replication, the experiments were conducted in four separate sessions, each 7 days apart. Within each session, five individual zucchini plants were randomly assigned to either the control or the contaminated water treatment, resulting in four independent biological replicates per treatment group for the aphid survival assay (total of 20 plants per treatment). One replicate (five plants) was excluded from the analysis after the aphid survival measurements due to a malfunction of the climate control system that generated temperature fluctuations, which compromised the reliability of subsequent data. Three independent biological replicates per treatment group were available for the subsequent aphid fecundity, real-time quantitative PCR (qPCR), and metagenomic experiments (Fig. 1).

Zucchini plants were grown for approximately two months prior to the aphid experiments and soil sampling. During this period, irrigation was surface applied and carefully managed to maintain soil moisture between 40 % and 90 % of the water-holding capacity. Each pot received the same volume of water (100 mL) at each irrigation event, while irrigation intervals were adjusted according to plant growth stage to maintain consistent soil moisture conditions. The volume of water applied at each watering event was recorded to calculate the total chemical dosage. In the control treatment, plants were irrigated with tap water with methanol alone, while in the experimental treatment, tap water was supplemented with a mixture of pharmaceuticals at a concentration of 1 μg L^{-1} (coded as "Contaminated water"). Following transplanting, each plant received a total of 1700 mL of experimental water over 17 irrigation events spanning 60 days, resulting in a total exposure of 1.7 μg of pharmaceuticals per treated pot. Control plants received the same volume of water without chemical addition (Fig. 1).

2.4. Composition of the soil bacterial community and detection and quantification of ARGs and the *intI1* gene

Sixty days after transplanting, three soil subsamples of approximately 30 g each were collected from different locations within each individual pot to capture within-pot spatial variability. These subsamples were then combined to form one composite sample per biological replicate (approximately 90 g). No pooling across pots was performed, and each pot was maintained independently as a separate

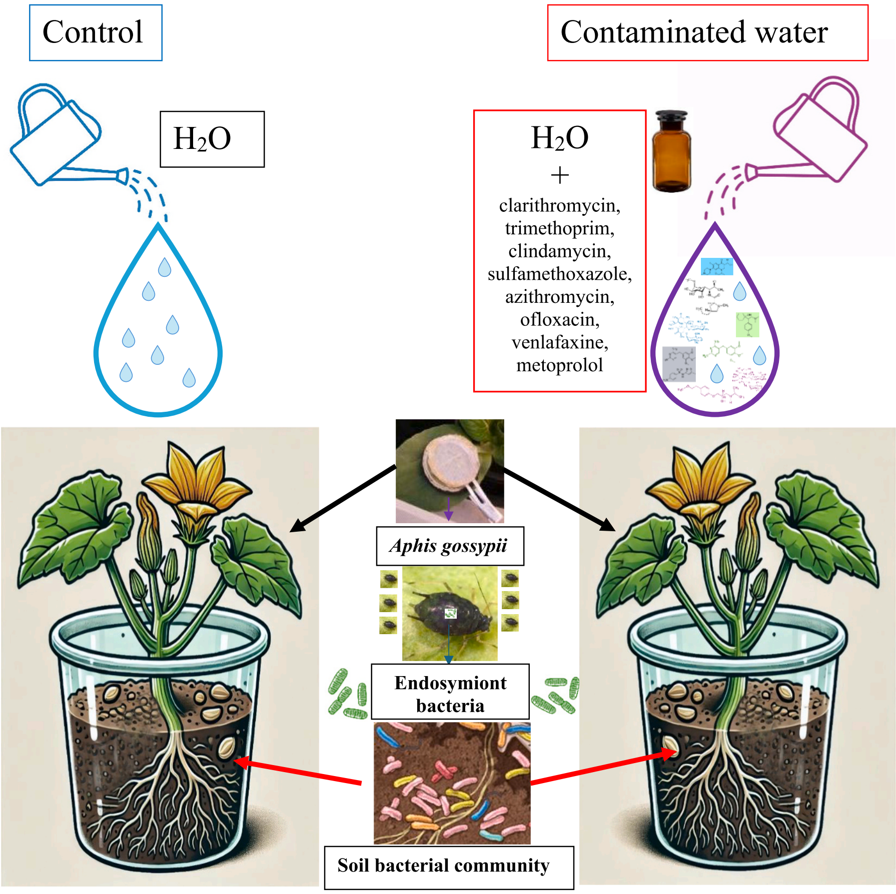

Fig. 1. Schematic representation of the experimental design.

biological replicate. This approach ensured representative coverage of the rhizosphere without compromising replicate integrity and was applied to both the control and treated plants, resulting in three independent representative soil samples per treatment.

DNA was then extracted from each of these six independent soil samples (0.25 g each) using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with a final elution volume of 50 μL. An aliquot of DNA samples was shipped under controlled conditions to an external company (IGA Technologies, Udine, Italy) for 16S rRNA gene amplicon sequencing. The hypervariable regions V3-V4 of the 16S rRNA gene were sequenced on an Illumina NovaSeq 6000 platform, using the universal bacterial primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Herlemann et al., 2011). The sequences were processed using the DADA2 pipeline, as indicated in the online tutorial (https://benjjneb.github.io/dada2/tutorial.html). Briefly, the raw reads were quality filtered and trimmed. Then, they were merged and merged sequences used to obtain unique Amplicon Sequences Variants (ASVs). ASVs were annotated against the Silva database for taxonomic assignation. Raw reads were deposited in the NCBI database and are accessible under project n° PRJNA1247779.

Another DNA aliquot was used to investigate the presence of selected ARGs and class 1 integrons by real-time PCR. Only genes detected in at least one sample were subsequently quantified. The selected ARGs, *erm*B and *qnr*S, are associated with resistance to macrolide-lincosamide-streptogramin and quinolones respectively. These genes were chosen due to their clinical relevance, as they are classified among the high-risk ARGs for human health (Zhang et al., 2021). Class 1 integrons were selected as a proxy for antibiotic resistance in the environment and as key contributors to the global dissemination of ARGs, as previously proposed (Ghaly et al., 2020). The qualitative and quantitative Real Time PCR assays were carried out using the RT thermocycler CFX Connect (Bio-Rad, Hercules, USA) (Barraud et al., 2010). The total reaction volume was 20 μL, containing 2 μL of DNA template, 0.5 μM of each primer (Supplementary Table 1), 10 μL of SsoAdvanced universal SYBR Greensupermix (Bio-Rad, Hercules, USA), and filtered and autoclaved MilliQ water (Merck Millipore, Germany), to reach the final volume. The amplification programs were 95 °C for 30 s, followed by 35 cycles of 95 °C for 10 s, annealing temperature (reported in Supplementary Table 1) for 30 s and 72 °C for 10 s. The specificity of the reaction was verified by melting peak analysis, as previously described (Di Cesare et al., 2015), and further checked after agarose gel electrophoresis run. Standard calibration curve was prepared as described in Di Cesare et al. (Di Cesare et al., 2015) and each DNA sample was assayed in duplicates. For normalizing the abundance of the *intI*1 gene (the only gene detected among those tested), the 16S rRNA gene was also quantified using the primer set Bact1369F (CGGTGAATACGTTCYCGG) and Prok1492R (GGHTACCTTGTTACGACTT), with an annealing temperature of 55 °C. The limit of quantification of the reactions, determined as proposed by Bustin et al. (Bustin et al., 2009), was 22.5 and 500 gene copies μL^{-1}, for *intI*1 and 16S rRNA gene, respectively. The mean value ± standard deviation of the efficiency and R^2 were 91.4 ± 0.7 and 0.998 ± 0.002, respectively. *intI*1 gene abundances were expressed by dividing the gene copy number by the corresponding 16S rRNA gene copy number.

2.5. Aphid life history traits and plant performance

Two 8-day-old adults *A. gossypii*, reared under the previously described optimal conditions, were transferred to a leaf and enclosed in a 2-cm diameter clip cage (Supplementary Fig. 1), where they were allowed to reproduce for one day. Two clip cages were placed on the adaxial sides of two fully developed leaves on each plant. After one day, the adults were removed and all nymphs were discarded except for two per cage, which were left to develop into adults.

The nymphs that developed on these plants were used to assess nymph-to-adult survival. After six days, clip cages were inspected, and the number of surviving adults was recorded. Aphid fecundity of these individuals was determined by counting and removing newborn nymphs at 24 and 48 h, with fecundity estimated as the mean number of nymphs produced per aphid per day.

To explore the potential transgenerational effects of exposure to pharmaceuticals, nymph-to-adult survival and fecundity after 48 h were measured in a second generation of aphids reared under identical conditions. The second generation consisted of late progeny from the first generation. In order to evaluate the potential effects of pharmaceutical exposure on plant performance, fresh shoot biomass and the number of green, fully developed leaves were measured at the end of the experiment. A total of six plants were measured, two for each of the three biological replicates. Immediately following the harvest, the fresh biomass (expressed in grams) was recorded using an analytical balance. In addition, green leaf counts were conducted visually.

2.6. Total expression of the aphid associated bacterial community

qPCR was performed to evaluate the impact of antibiotics in irrigation water on the gene expression of aphid endosymbiont bacteria. Following the fecundity test, live adult aphids were collected from each of the five plants within each replicate, pooled, and subjected to three independent RNA extractions per treatment group. The aphids from each replicate were placed directly into 200 μL of 0.5 % Tris-Borate-EDTA (TBE) buffer and then frozen.

RNA was extracted from whole aphids using the NucleoZOL reagent (Macherey-Nagel) with a protocol specifically optimized for this matrix. RNA quantification was performed using a NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The RNA was subsequently reverse transcribed into cDNA using the FIRE Script® RT cDNA Synthesis Mix (Solis Biodyne).

For the qPCR assay, the Power SYBR Green PCR Master Mix (Applied Biosystems®, Waltham, MA, USA) was used, following the protocol described in De Fabrizio et al. (De Fabrizio et al., 2024). Amplification was carried out in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems), and data were analyzed using 7500 Software v2.3 (Applied Biosystems).

Endosymbiont abundance can vary among individual aphids and between samples due to multiple factors related to aphid body size, including host plant quality, sampling timing, and environmental fluctuations. To quantify endosymbiont levels in each sample, we used qPCR to determine the ratio of symbiont genome copies to host genome copies (Chong and Moran, 2016; Gao et al., 2023). The bacterial endosymbiont 16S rRNA gene expression was measured using the primers 5′-ACTCCTACGGGAGGCAGCAGT-3′ and 5′-TATTACCGCGGCTGCTGGC-3′ (Clifford et al., 2012), while the aphid 18S rRNA gene expression was assessed with the universal ss5 and ss3 primer pairs 5′-GGTGATCCTGCCAGTAGTCATATGCTTG-3′ and 5′-GATCCTTCCGCAGGTTCACCTACGGAAACC-3′.

Relative endosymbiont abundance was calculated using the ΔCt method with whole DNA from individual aphids, where:

$$\Delta Ct = Ct_{16S\ rRNA} - Ct_{18S\ rRNA}$$

This approach compensates for variations in aphid cell number and RNA extraction efficiency. The $2^{-\Delta\Delta Ct}$ method was then used for the analysis of qPCR data on the relative abundance of aphid endosymbionts between aphids reared on control plants and those exposed to contaminated water (Livak and Schmittgen, 2001).

2.7. Statistical analysis

For the statistical analysis of 16S rRNA gene amplicon sequencing data, two independent datasets were examined: (i) the whole bacterial community and (ii) a subset representing the pathobiome (i.e., the total content of potentially pathogenic bacterial genera), based on the list of established pathogenic bacteria provided by Bartlett et al. (Bartlett et al., 2022).

For both datasets, species richness (alpha diversity) was calculated as the number of different ASVs or potentially pathogenic genera per sample. Beta diversity was assessed using the Bray-Curtis dissimilarity index, generating a dissimilarity matrix that was subsequently used to visualize sample composition through hierarchical linkage analysis. Differences between control and pharmaceutical-treated samples were then evaluated within this experimental framework.

To analyze richness, a negative binomial generalized linear model (NB-GLM) was applied, as richness represents count data. Beta diversity differences were assessed using PERMANOVA on the Bray-Curtis dissimilarity matrix. Additionally, we determined, at genus level, the bacteria whose abundances differed between the treatments and controls, by conducting a differential abundance analysis (DAA). Genera were considered as differentially abundant only if Benjamini-Hochberg adjusted p-values were significant ($p < 0.05$).

For the potentially pathogenic bacterial genera, total abundance per sample was calculated and analyzed using a generalized linear model (GLM) with a quasipoisson error distribution and a log link function. The same GLM approach was also used to analyze the abundance of the *intI*1 gene.

Nymph-to-adult survival data (categorized as dead or alive) were analyzed using a generalized linear mixed-effects model (GLMM) with binomial errors and a logit link function. Differences between the two experimental treatments were assessed using type II Wald chi-squared tests in an analysis of deviance. In this model, “water treatment” (two levels: Control and Pharmaceuticals) was included as the main fixed factor, while “replicate nested within treatment” was included as a random effect.

To meet the assumption of normality (Shapiro-Wilk test), fecundity data were $\ln(x + 1)$-transformed. The transformed fecundity values were analyzed using a linear mixed-effects model (LMM) fitted with restricted maximum likelihood (REML). For the data of the first generation, “Water treatment” and “reproductive interval” (two levels: 24 h and 48 h) were included as fixed factors, while “replicate nested within treatment” was included as a random effect. The model initially included the main effects and their interaction term, and a backward stepwise approach was used to sequentially remove non-significant effects, ensuring the selection of the most parsimonious model. For the analysis of fecundity in the second generation, since data were available for only one reproductive interval, “Water treatment” was included as a fixed factor and “replicate nested within treatment” as a random effect. The same LMM was applied to analyze plant fresh shoot biomass and the number of fully developed leaves per plant. These variables met the assumption of normality based on the Shapiro–Wilk test. The significance of the difference in endosymbiont abundance between treated and control aphids, measured as $2^{-\Delta\Delta Ct}$, was assessed using the 95 % confidence intervals (CI) of the mean fold-change, with the null hypothesis stating that this value did not differ from 1 (i.e., no effect of the contaminated water).

All the statistical analyses were made in the R environment v 4.4.1 (R Core Team, 2024) with the packages lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), vegan (Oksanen et al., 2025), MASS (Venables and Ripley, 2002), and ANCOMBC (Lin and Das Peddada, 2020).

3. Results

3.1. Bacterial community composition and detection and abundance of ARGs and class 1 integrons

After trimming, filtering, and merging the raw reads, we retrieved 6333 ASVs, identifying 238 bacterial families and 447 genera (Supplementary Table 2). The most abundant family across all the samples was Oxalobacteraceae, followed by Pirellulaceae and Devosiaceae (Supplementary Table 2). At genus level, *Massilia*, a potentially pathogenic bacterium, exhibited the highest abundance, followed by *Devosia* and *Pseudarthrobacter* (Fig. 2A; Supplementary Table 2). DAA showed that *Methylobacterium-Methylorubrum*, *MM2*, and *Methylotenera* were the three bacterial genera significantly more abundant in the treated samples (Fig. 2C). The pathobiome was characterized at the genus level using amplicon sequencing. Although this approach provides an overview of potentially pathogenic bacterial genera in the soil, it does not allow for the assessment of functional genes or virulence factors. That would require shotgun metagenomics or additional molecular analyses. Within the pathobiome, *Massilia*, along with *Arthrobacter* and *Brevundimonas* showed the highest abundance (Fig. 2B; Supplementary Table 3). Whole bacterial community richness was comprised between 1973 and 2749 different ASVs (mean value 2463), for the controls, and between 1935 and 2656 (mean value 2379.7), for the treated samples, showing a trend toward a decrease with the treatment. Pathobiome alpha diversity ranged from 24 to 29 potentially pathogenic bacterial genera (mean value 26.3), for the controls, and from 27 to 32 (mean value 29.7), for the treated samples (Fig. 3). Neither difference was statistically significant (NB-GLM: $\chi^2 = 0.1$, d.f. $= 1$, $P = 0.767$ for community richness; $\chi^2 = 0.6$, d.f. $= 1$, $P = 0.440$ for pathobiome richness). After linkage hierarchical analysis, both in the case of the whole bacterial community and pathobiome, samples clustered together only limitedly based on the treatment (Supplementary Fig. 2). Dissimilarity indexes did not exceed 0.56 value, for the whole bacterial community, and 0.34 value, for the pathobiome, thus pointing out a rather uniform composition of samples (Supplementary Fig. 2).

PERMANOVA analysis revealed that the treatment factor explained 17.8 % of the bacterial community composition and 28.5 % of the pathobiome composition. The total abundance of the pathobiome did not significantly differ among samples (GLM: $\chi^2 = 1.2$, d.f. $= 1$, $P = 0.265$); however, treated samples showed a tendency to accumulate potentially pathogenic genera (Fig. 3).

When examining individual potentially pathogenic genera, five demonstrated a significantly differential response to the treatment: *Afipia*, *Methylobacterium*, *Paracoccus*, and *Saccharopolyspora* were significantly more abundant in the treated samples (GLM: $\chi^2 \geq 4.7$, d.f. $= 1$, $P \leq 0.0303$) (Fig. 4). Conversely, *Aureimonas* showed significantly higher abundance in the control samples (GLM: $\chi^2 = 11.1$, d.f. $= 1$, $P = 0.0008$), although it is important to note that *Aureimonas* was detected with only one read in two of the three control samples (with zero reads in the third control and the treated samples) (Fig. 4).

Among the tested genes only *intI*1 was positive, thus it was further quantified and statistically analyzed and no significant differences were observed among the samples (GLM: $\chi^2 = 0.1$, d.f. $= 1$, $P = 0.716$) (Supplementary Fig. 3).

3.2. Plant performance

Throughout the experiment, the plants were visually inspected and no disease symptoms were observed in any of the treatments. This indicates that the enrichment of potential pathogens in the soil microbiome did not impact the health of the plants under controlled laboratory conditions. Plant performance did not differ significantly between the control and pharmaceutical treatment groups, as measured by fresh shoot weight (mean ± E.S.: control, 24.6 ± 1.1 g; contaminated water, 25.5 ± 1.5. LMM: $F_{1,10} = 0.23$, $P = 0.64$) and number of green leaves (mean ± E.S.: control, 8 ± 0.26; contaminated water, 7.7 ± 0.33. LMM: $F_{1,10} = 0.62$, $P = 0.45$). These results indicate that the pharmaceutical mixture used in the present study did not induce deleterious effects on the growth or visible development of zucchini plants.

3.3. Aphid survival, fecundity, and endosymbiont abundance

For the first generation, nymph-to-adult survival was not affected by the water treatment (mean ± E.S.: control, 90 ± 3.2 %; contaminated water, 85 ± 4.1 %. GLMM: $\chi^2 = 0.9$, d.f. $= 1$, $P = 0.34$), indicating that aphids reared on plants irrigated with the pharmaceuticals had similar

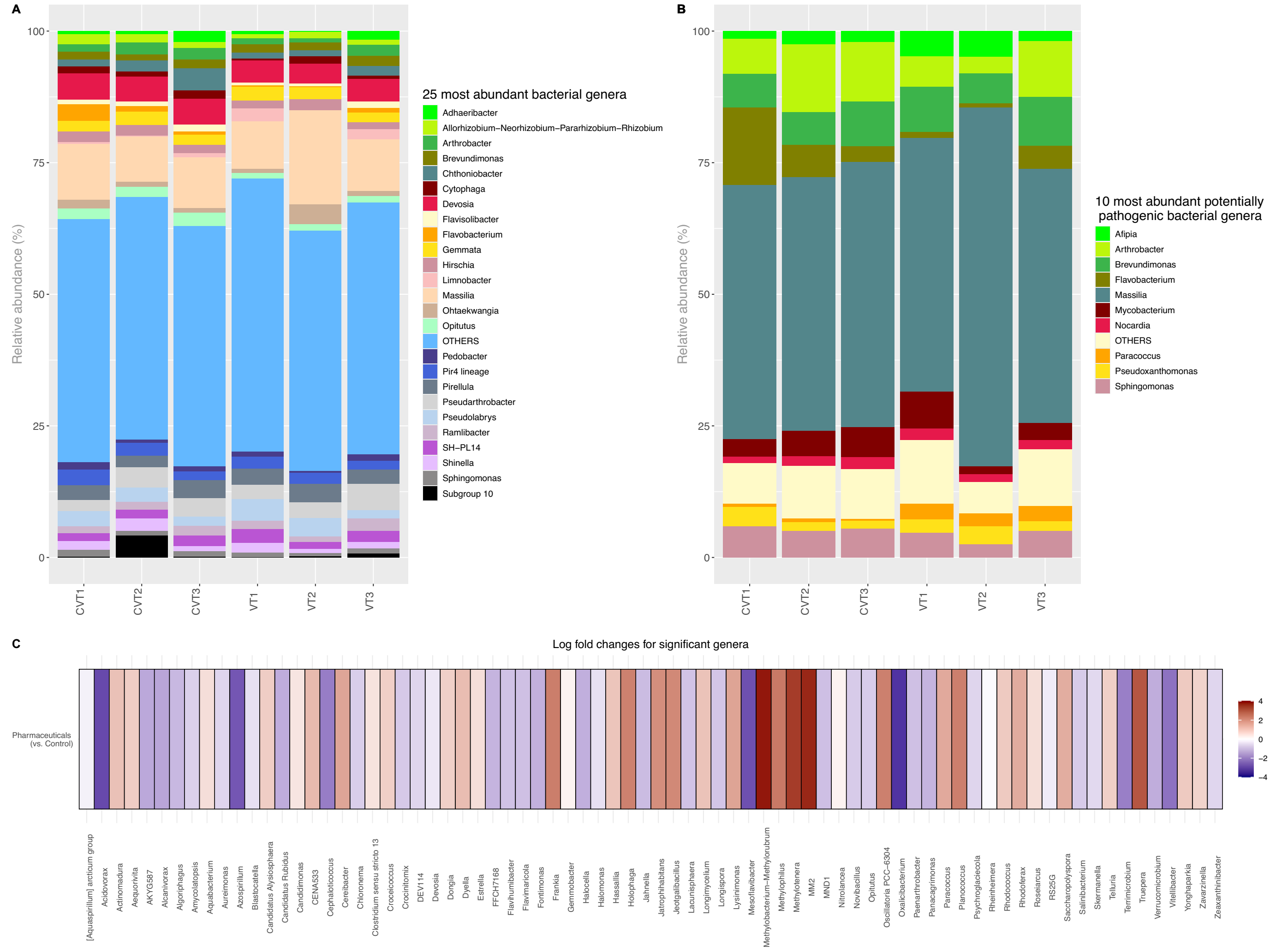

Fig. 2. Abundances of bacterial genera per sample. A) 25 most abundant bacterial genera and B) 10 most abundant genera associated to potential pathogens. Abundances are expressed as percentage. C) Differential abundance analysis as determined by the ANCOM-BC method, displaying the log fold change in abundance (pharmaceuticals vs. control) for the genera with a significant difference among samples.

survival rates compared to those on control plants (Supplementary Fig. 4). Aphid age significantly affected fecundity during the first generation (Supplementary Fig. 5), with low nymph production on the first day and a marked increase 48 h after reaching adulthood compared to 24 h (LMM: $F_{6,278} = 13.14$, $P < 0.001$). Water treatment and its interaction with reproductive intervals were excluded from the analysis as they were not significant, indicating that aphids on contaminated-water irrigated plants produced the same number of nymphs as those on control plants.

The survival and fecundity patterns observed in the second generation of aphids were consistent with those of the first generation. There were subtle shifts in performance, but no significant differences were found between the control and pharmaceutical treatments for survival (mean ± E.S. second generation: control, 87.5 ± 6.5 %; contaminated water, 83.3 ± 7.1 %. GLMM: $\chi^2 = 0.166$, d.f. = 1, $P = 0.68$). Since nymph production was previously found to be very low in the first generation, fecundity in the second generation was assessed after 48 h. Aphids reared on contaminated plants produced a similar number of nymphs after 48 h compared to those on control plants (mean ± E.S.: control, 4.48 ± 0.17; contaminated water, 4.13 ± 0.1. LMM: $F_{1,82} = 2.4$, $P = 0.123$).

Irrigation with contaminated water resulted in a statistically significant reduction in the relative abundance of endosymbionts in aphids reared on treated plants compared to controls. This result was supported by a mean fold-change (measured as $2^{-\Delta\Delta Ct}$) of 0.257 ± 0.2387 (mean ± 95 % CI), where the confidence interval excluding 1 confirms a significant decrease in the endosymbiotic bacterial population.

4. Discussion

This study provides insight into the bottom-up effects of irrigation with pharmaceutical-contaminated water on soil microbial communities, plant-insect interactions, and aphid-associated bacterial symbionts in the *Cucurbita pepo* - *Aphis gossypii* model system. Because irrigation was surface-applied, pharmaceuticals were taken up by plant roots and translocated to aerial tissues, resulting in indirect aphid exposure via plant tissues rather than through direct contact with contaminated water. While previous studies have demonstrated that plants can absorb and accumulate antibiotics (Pan and Chu, 2017; Jalloul et al., 2021; Christou et al., 2019; Azanu et al., 2016), their ecological consequences for soil microbiota and insect pests remain poorly understood. All experiments were conducted under fully controlled laboratory conditions to minimize environmental variability. This design, in line with the scope of the study, aimed to investigate specific mechanistic responses under controlled exposure conditions rather than reproduce the complexity of field environments. Variability among replicates was consistently low, and treatment effects when present, were clearly detectable. However, this controlled framework

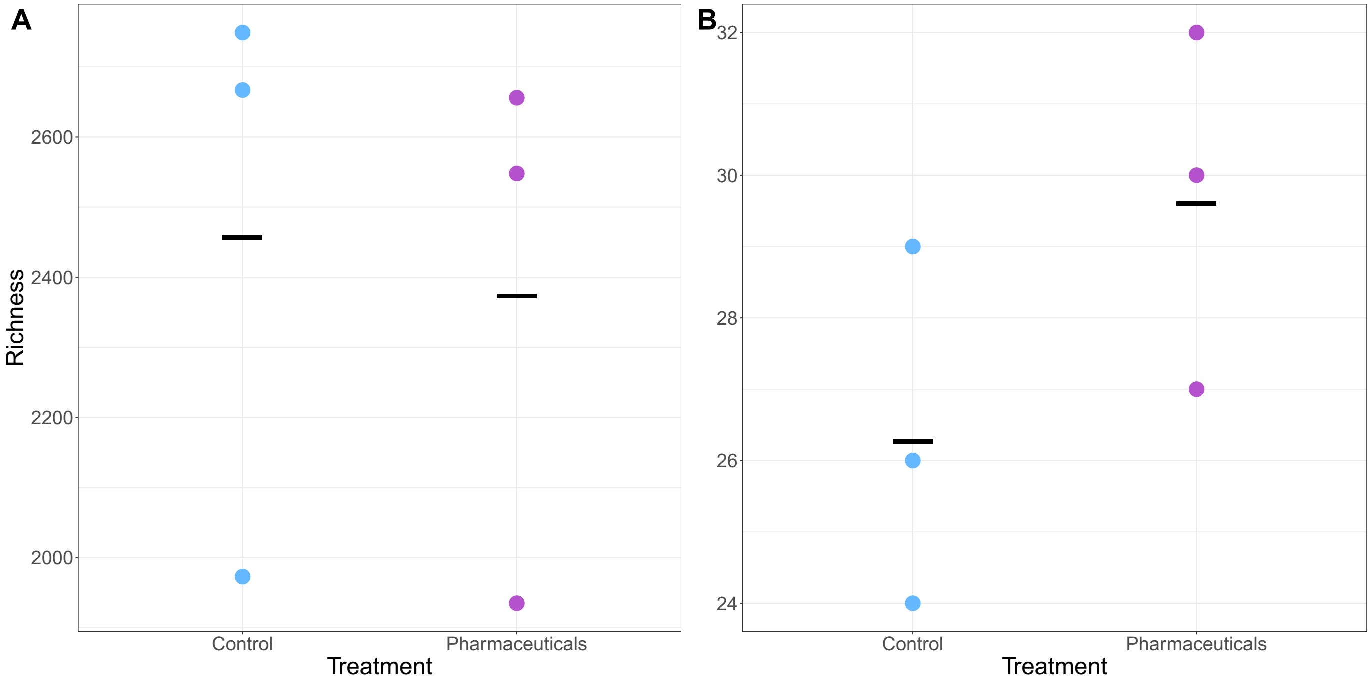

Fig. 3. Richness. Number of the different ASVs, for A) the whole bacterial community, and genera, for B) the pathobiome, according to the treatment. The single replicate is depicted as a dot; the thick black line represents the mean value of the observations.

cannot capture the full spatial, temporal, and environmental heterogeneity of real agricultural systems and should be viewed as a mechanistic step toward more complex field-based investigations. Overall, our findings highlight subtle yet ecologically relevant compositional changes induced by exposure to very low pharmaceutical concentrations.

Irrigation with water spiked with common antibiotics and pharmaceuticals found in wastewater altered the composition of the soil bacterial community, with differential responses among taxa. Notably, *Methylobacterium* and *Methylotenera* increased in relative abundance. *Methylobacterium* is an opportunistic pathogen mainly infecting immunocompromised patients (Truant et al., 1998), with human diseases mostly occurring in hospital environments due to its resistance to temperature, disinfection processes, and its ability to produce biofilm (Kovaleva et al., 2014). *Methylotenera* was previously identified as a possible host of ARGs in environmental samples (Guan et al., 2023; Kim and Yoo, 2024; Weng et al., 2022). These findings could be mainly due to the presence of antibiotics in the contaminated water, but the influence of venlafaxine and metoprolol may also have contributed to these shifts by affecting microbial metabolism, biofilm formation, and interspecies interactions (Liu et al., 2022). Several genera associated with opportunistic pathogenicity (*Afipia*, *Methylobacterium*, *Paracoccus*, and *Saccharopolyspora*) showed higher abundances, suggesting potential selective pressure favoring these taxa. The first genus includes *A. felis*, a species reported as the cause of rare cases of human cat scratch disease (Lührmann et al., 2001). Along with other species such as *A. broomeae* and *A. clevelandensis*, it is potentially relevant to human disease (Sawada et al., 2025). *Paracoccus* includes species such as *P. yeei*, recognized as an opportunistic pathogen (Szuplewska et al., 2024). Finally, *Saccharopolyspora* includes *S. rectivirgula*, a species responsible for the main cause of farmer's lung disease (Barrera et al., 2014). These compositional shifts, especially the rise in potential pathogens in agricultural soils, may pose long-term risks. If these taxa persist or proliferate with repeated exposure, concerns may arise regarding the sustainability and safety of using reclaimed water in food production systems without targeted treatments.

Although functional gene profiling was not performed, the shifts in potentially pathogenic genera observed in this study provide insight into the soil pathobiome under exposure to environmentally relevant concentrations of pharmaceuticals. Concerns regarding persistence and potential mobilization of ARGs remain (Berendonk et al., 2015; Manaia, 2017). However, the lack of significant variation in class 1 integron abundance (Ghaly et al., 2020) suggests that antibiotic concentrations used in this study were insufficient to induce measurable ARG selection, consistent with previous studies (Gillings et al., 2015). Previous investigations have shown that antibiotic residues in soil can progressively alter microbial composition, with implication for nutrient cycling and plant health (Cycoń et al., 2019; Thiele-Bruhn and Beck, 2005). The modest shifts observed in our study align with reports of soil microbial sensitivity to low-dose antibiotic exposure (DeVries et al., 2015; Zhao et al., 2022b; Zhao et al., 2022c), highlight the importance of long-term monitoring to determine whether repeated exposure may amplify such effects over time.

Consistently with the controlled conditions of the experiment, no disease symptoms were observed in plants, indicating that microbial shift did not translate into visible plant health impacts during the study period. Nonetheless, functional and virulence-focused studies are needed to fully assess the long-term ecological and agronomic risks. Aphids are known to be sensitive to direct exposure to a wide range of antibiotics (Griffiths and Beck, 1974; Mittler, 1971), due to disruption of the metabolism of their endosymbiotic bacteria (Douglas, 1998); yet, plant-mediated exposure remains poorly explored (Pufal et al., 2019). In the present experiment, plant traits and aphid life history traits were not directly affected by irrigation with contaminated water. Nymph-to-adult survival remained unchanged between treatments, indicating that any direct pharmaceutical effect or indirect effects on aphid development through plant-mediated changes were negligible. Similarly, aphid fecundity was not significantly different between treatment groups. This finding is important because it provides insights on the potential plant-mediated trophic transfer of pharmaceuticals in terrestrial food webs, even when contaminated irrigation water is used to simulate a realistic exposure pathway. To explore potential delayed or cumulative effects, we included a second generation of aphids in the experiment. Their survival and fecundity patterns were similar to those of the first generation, suggesting that low-dose pharmaceutical exposure does not immediately impair fitness across the first two generations. However,

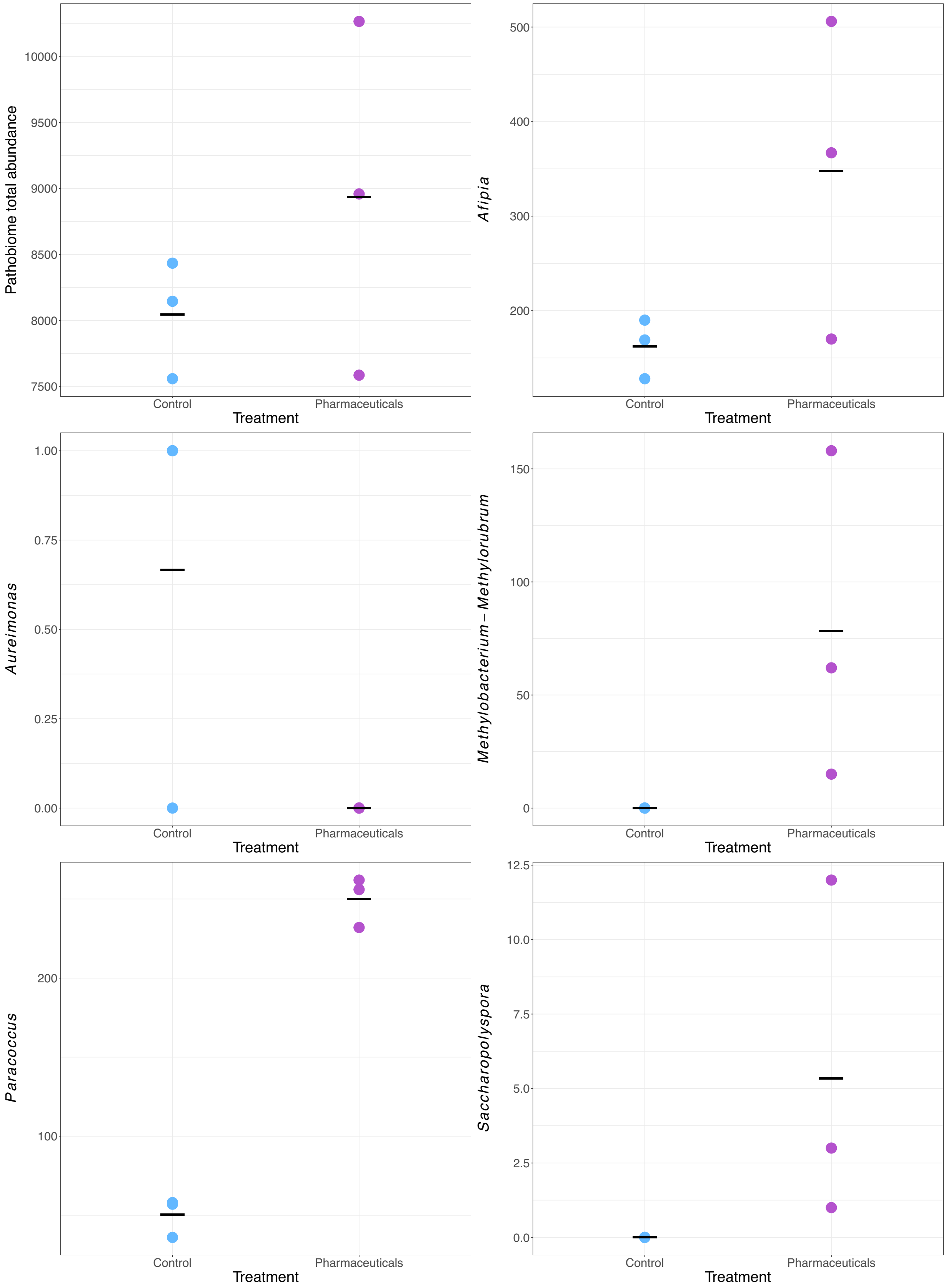

Fig. 4. Pathobiome abundance. Total abundance and abundance of the genera that significantly differed under the experimental conditions. The abundances are expressed as number of reads per replicate. The single replicate is depicted as a dot; the thick black line represents the mean value of the observations.

long-term field studies are required to detect cumulative or context-depended effects.

Interestingly, a reduction in the total expression of aphid endosymbionts was observed in response to pharmaceutical treatment. Although pharmaceutical residues were not quantified in plant tissues, prior studies demonstrate plant uptake and translocation of such compounds, without necessarily producing visible phenotypic changes (Pan and Chu, 2017; Jalloul et al., 2021; Christou et al., 2019; Azanu et al., 2016). Therefore, the observed reduction in the abundance of the *A. gossypii* endosymbiont is plausibly linked to indirect exposure via plant uptake. Although we do not have estimated of the composition of secondary bacteria within the aphid strain used in the present study, it is reasonable to assume that the reduction in the expression of endosymbiont bacteria is largely accounted for by *B. aphidicola*, since it is the most abundant endosymbiont in *A. gossypii* (Xu et al., 2023; Xu et al., 2020). Endosymbionts such as *B. aphidicola* are not merely passive residents of the aphid microbiome. Rather, they are essential for nutrient provisioning, thermal tolerance, and even resistance to pesticides (Zytynska and Weisser, 2016). Therefore, a reduction in symbiont expression might impair aphid resilience over time, particularly when facing concurrent stressors like heat waves or parasitoid pressure, even though this reduction did not result in significant changes to the survival or fecundity of second-generation aphids in our experiment.

However, we observed a non-significant downward trend. The apparent stability of aphid fitness despite the reduction in endosymbiont abundance may indicate the activation of compensatory or buffering mechanisms that maintain essential physiological functions. Such mechanisms could involve metabolic plasticity or shifts in nutrient provisioning. These effects could become biologically significant under field conditions where aphids are exposed to diverse environmental stressors over extended periods and plants receive substantial volumes of contaminated irrigation water.

In general, the findings of this study align with the idea that microbial disruptions are an early warning signals for ecological stress, often preceding observable phenotypic effects. A key insight from this experiment is the apparent decoupling of visible fitness traits, such as survival and fecundity, from internal physiological responses in *A. gossypii.* The fact that aphid development and reproduction remained stable despite a significant reduction in endosymbiont gene expression suggests a form of latent vulnerability that could have profound ecological implications.

As for aphid-related traits, short-term exposure to environmentally realistic concentrations of pharmaceuticals does not always produce visible stress symptoms in plants, measured as plant fresh biomass and green leaf number. However, this does not exclude the possibility of more subtle physiological disruptions such as oxidative stress, hormonal imbalances, and altered metabolism that could impact plant performance in the long-term or in field conditions. As for insect phenotype, when plant health is the only indicator, many ecological consequences of environmental contamination remain hidden. Our findings highlight the need to integrate plant performance data with below- and aboveground biological responses, as soil microbiota and insect symbionts may be more sensitive indicators of environmental disturbance than plant morphology alone.

Importantly, this study extends the concept of pharmaceutical pollution to terrestrial food webs, demonstrating indirect exposure routes through plant-mediated trophic and symbiotic pathways. The ability of low-dose, environmentally relevant concentrations of pharmaceuticals to reach and influence herbivorous insects via plant uptake requires a reassessment of ecological risks evaluation frameworks beyond aquatic systems.

5. Conclusion

This study is based on controlled experiments which provide an initial insight into how environmentally relevant concentrations of pharmaceuticals may affect soil-plant-insect systems. Future research should expand upon this framework by conducting field studies and implementing site-specific monitoring to capture spatial and temporal variability more accurately in contaminant exposure. However, our results suggest that irrigation with environmental concentrations of antibiotics and pharmaceuticals induces shifts in the composition and abundance of the pathobiome and also affects aphid-associated endosymbionts. We acknowledge that the concentration of pharmaceuticals can vary substantially depending on geographic location, seasonal fluctuations, and wastewater treatment technologies. Nevertheless, generalized monitoring data is essential for refining ecological risk assessments and for developing an evidence-based approach to managing reclaimed water use in agroecosystems. Although no immediate trophic level disturbances were detected, the alteration of endosymbiotic communities could have long-term implications for aphid populations and their ecological interactions. Changes in symbiont abundance, composition, or function may influence aphid performance and, consequently, their population dynamics and the success of their natural enemies. Parasitoids developing within less suitable hosts and predators such as coccinellids feeding on prey of reduced nutritional quality could both experience altered fitness, potentially reshaping higher trophic dynamics within agroecosystems. Such effects could ultimately influence the efficiency and stability of biological control within integrated pest management programs, where even subtle shifts in aphid–natural enemy interactions may impact pest suppression outcomes. Given the increasing reliance on reclaimed water for irrigation, it is essential to understand its ecological implications. Future studies should expand the range of pharmaceuticals and ARGs assessed, explore potential correlations between aphid reproductive responses and oxidative stress in host plants, and examine whether prolonged exposure exacerbates microbial shifts, affects the fitness of insects (both pests and beneficials) across multiple generations, or promotes the development of antibiotic resistance in environmental microbiota. By integrating microbial ecology with plant-insect interactions, our results underline the importance of the wider ecological implications of pharmaceutical contamination, highlighting its potential impacts on human, plant, and soil health, as well as agricultural sustainability.

CRediT authorship contribution statement

Monica Brienza: Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Raffaella Sabatino:** Writing – original draft, Visualization, Supervision, Methodology, Investigation. **Roberto Rosamilia:** Writing – review & editing, Methodology, Formal analysis. **Giulia Borgomaneiro:** Writing – review & editing, Methodology, Data curation. **Oussama Baaloudj:** Writing – review & editing, Methodology, Formal analysis. **Andrea Di Cesare:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Donatella Battaglia:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Paolo Fanti:** Writing – review & editing, Methodology, Formal analysis. **Vincenzo Trotta:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 from OpenAI in order to review the language (English). After using this tool/service, the authors reviewed and edited the content as needed, taking full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106769.

Data availability

The amplicon sequencing data were deopisited in a public database and the Project Number is reported in the manuscript.

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