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Occurrence of antibiotics in Lettuce (*Lactuca sativa* L.) and Radish (*Raphanus sativus* L.) following organic soil fertilisation under plot-scale conditions: Crop and human health implications

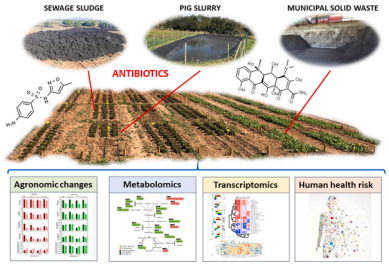
V. Matamoros [a,b,*], M. Escolà Casas [a,b], S. Mansilla [a,b], Đ. Tadić [a,b], N. Cañameras [a,b], N. Carazo [a,b], J. Portugal [a,b], B. Piña [a,b], S. Díez [a,b], J.M. Bayona [a,b]

[a] Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain
[b] Department of Agri-Food Engineering and Biotechnology DEAB-UPC, Esteve Terrades 8, Building 4, Castelldefels, Spain

HIGHLIGHTS

- Plant antibiotic uptake depends on the compound and the plant variety.
- The occurrence of antibiotic metabolites increases following soil fertilization.
- Antibiotics do not affect plant agronomics, metabolomics, or transcriptomics.
- Plant dopamine content can be used as tracer of organic fertilization.
- The presence of antibiotics does not pose a human health risk.

GRAPHICAL ABSTRACT



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ABSTRACT

Recent studies have demonstrated the crop uptake of antibiotics (ABs) from soils treated with AB-carrying fertilisers. However, there is a lack of plot-scale studies linking their effects at the agronomic and metabolomic/transcriptomic level to their impact on human health. This paper assesses the plant uptake of 23 ABs following two productive cycles of lettuce and radish cropped with sewage sludge, pig slurry, the organic fraction of municipal solid waste, or chemical fertilisation under plot-scale conditions (32 plots spanning 3-10 m^2 each). AB uptake by plants depended on both the vegetable and the AB class and was higher in radish than in lettuce edible parts. Levels ranged from undetectable to up to 76 ng/g (fresh weight). Repetitive organic fertilisation resulted in an increase in the concentration of ABs in lettuce leaves, but not in radish roots. Significant metabolomic and transcriptomic changes were observed following soil fertilisation. Nevertheless, a human health risk assessment indicates that the occurrence of ABs in lettuce or radish edible parts does not pose any risk. To our knowledge, this is the first holistic plot-scale study demonstrating that the use of organic fertilisers containing ABs is safe for crop security and human health.

* Corresponding author at: Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain.
E-mail address: victor.matamoros@idaea.csic.es (V. Matamoros).

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1. Introduction

In view of projected global population increase, agricultural yield needs to be improved in a sustainable way to meet the future worldwide food demand in the context of extreme climate events (Fukase and Martin, 2020). In fact, the UN has already recognised food security as the second sustainable development goal for 2030 (Pérez-Escamilla, 2017). Amongst other strategies, the safe and sustainable reuse of organic and waste-based fertilisers in agriculture to cope with nutrient demand is central to achieve food security. In the context of the EU's Circular Economy strategy, the safe use of manure, sewage sludge, and urban organic waste as fertilisation products has attracted increasing attention. However, the load of antimicrobial agents such as antibiotics (ABs) (e.g. tetracyclines, fluoroquinolones, sulphonamides, etc.) or biocides in biosolids and/or manure (Xie et al., 2018); (You, 2020) also raises concerns regarding the environmental, agricultural, and human health implications of the spread of these compounds in agri-ecosystems. Several studies have shown that these compounds induce co- or cross-resistance to ABs in bacteria (Murray et al., 2019). In fact, antimicrobial resistance (AMR) is amongst the biggest threats to global health, food security, and development today and is estimated to be responsible for 700,000 human deaths per year worldwide, a figure that will swell to 10 million if urgent action is not taken, according to WHO estimates (WHO, 2019).

The occurrence of ABs in organic fertilisation products depends on the source and treatment of the fertiliser. Their concentrations can range from undetectable to up to 331 mg/kg in fresh weight (fw) in the case of chlortetracycline in pig manure (Wohde, 2016). The presence of ABs such as tetracycline, sulphonamides, and fluoroquinolones in vegetables following soil fertilisation with manure or biosolids has already been reported (Zhao et al., 2019; Pan and Chu, 2017; Tasho and Cho, 2016). Nevertheless, most of these studies have been performed under controlled conditions (greenhouse-hydroponics) with model plants using spiked irrigation waters or manure, usually at non-environmentally relevant concentrations. Whilst this information is considered useful for understanding the uptake mechanisms of contaminants by plants, it cannot be used for human risk assessment. Furthermore, current pot/laboratory-scale studies indicate that the presence of ABs in agriculture may have the potential to change plant phenotype (e.g. biomass production, shoot and root growth) and physiological (e.g. nitrogen metabolism, oxidative metabolism, and photosynthesis) profiles (Rocha et al., 2021; Liu, 2009). In fact, the presence of organic pollutants triggers an oxidative plant stress response and, consequently, plant transcriptomic, metabolic, and morphological changes (Mansilla, 2021). Recently published greenhouse studies on lettuce crops cultivated with different fertilisation products suggest a low plant uptake of ABs due to the high content of organic matter in the fertilisation products, which would reduce plant uptake of ABs and, therefore, their impact on plant morphology and metabolism (You, 2020; Margenat, 2020). Nevertheless, there is no evidence of how real field-scale conditions, soil, and repetitive fertilisation strategies may affect the contaminant load in crops and human health.

Currently, only 5% of bio-waste is recycled and used as fertilisers. However, recent estimates indicate that this figure could be increased, replacing up to 30% of mineral fertilisers. According to Regulation (EU) 2019/1009, marketed organic fertiliser products will have to demonstrate that they meet those requirements, as well as limits for organic contaminants, microbial contaminants, and physical impurities, before affixing the CE marking (European Commission, 2016). It is thus very important to determine the potential human health implications of using these fertilisation products in agriculture under real practices.

The present study aims to assess the impact of ABs due to the repetitive soil fertilisation on crops and human health under plot-scale conditions. Two different vegetables (lettuce and radish) were selected according to their edible parts (leaf vs root). Soil fertilisation with pig slurry (PS), sewage sludge (SS), and the organic fraction of solid municipal waste (OFSMW) were compared with control conditions using chemical fertilisation (CF). To this end, the concentration of 23 ABs (sulphonamides, fluoroquinolones, lincosamides, benzylpyrimidine, and tetracyclines), including 6 AB transformation products (TPs), were measured in fertilisers, soil, and crops throughout two productive cycles. Additionally, changes in the crop phenotype and metabolic and transcriptomic profile were followed and the human health risk was estimated.

2. Material and methods

2.1. Plot-scale studies

The study was performed in an agricultural experimental station belonging to the Polytechnic University of Catalonia (Agròpolis, Viladecans, Spain) from April 2019 to March 2020. A 500-m^2 outdoor plot was used. The soil had a loam-clay texture (40% sand, 35% silt, 25% clay), a pH of 8.4, and an electrical conductivity of 0.24 dS/m. The total organic carbon content was 1.27%, and the total nitrogen content (Kjeldahl) was 0.09% of the soil dry weight. The Olsen phosphorus concentration was 0.033 g/kg, whereas the K^+, Ca^{2+}, Mg^{2+}, and Na^+ cations were 0.344, 7.014, 0.362, and 0.091 g kg^{-1} of the soil dry weight, respectively. The selected field plot had not been used for agricultural purposes for at least the last 10 years.

The fertilisation products were SS (primary and secondary sludge with anaerobic digestion collected from a domestic WWTP), OFMSW (municipal organic food waste composted with pruning residues of an approximate particle size between 2 and 30 mm), PS (pig slurry), and CF (chemical fertiliser). Further details on the physico-chemical characteristics of the fertilisation products are shown in Table 1-Supplementary Material (SM).

The experimental set up was as follows: 16 plots (4 treatments x 4 replicates) measuring 5 ×2 m for growing lettuce vegetables (*Lactuca sativa* L. cv. Maravilla de Verano) and another 16 plots (4 treatments x 4 repetitions) measuring 3 ×1 m for growing radish vegetables (*Raphanus sativus* L). For each assessed vegetable, 4 plots were amended with SS, another 4 with PS, 4 more with OFMSW, and the final 4 with CF as a control (Fig. 1 and Fig. 1-SM). Randomised block design was applied to minimise the effect of soil heterogeneity. The dose of fertilisation product added per plot was calculated to ensure the same quantity of total ammoniacal nitrogen in all treatments, as previous studies have shown it to be the limiting nutrient (Margenat, 2020). Fertilisation products were added to the soil 4 days before the vegetables were

Table 1
Concentration of parental antibiotics (in ng/g fw) in fertilisation products (n=3).

	SS		PS		OFMSW
	1st	2nd	1st	2nd	
Sulfacetamide	<LOD	<LOD	<LOD	<LOD	<LOD
Trimethoprim	<LOD	<LOD	<LOD	<LOD	<LOD
Lincomycin	29±7	27±7	51012 ±1109	1172 ±54	<LOD
Sulfadiazine	39±2	49±9	19±1	34±4	<LOD
Sulfapyridine	32±3	23±5	<LOD	<LOD	<LOD
Sulfathiazole	18±2	5±1	3±1	<LOD	<LOD
Sulfamethazine	<LOD	<LOD	<LOD	<LOD	<LOD
Ciprofloxacin	3539 ±866	462±21	238±25	534±71	<LOD
Ofloxacin	1930 ±138	<LOD	1649±210	<LOD	<LOD
Enrofloxacin	87±10	1709 ±398	115±45	214±15	<LOD
Sulfamethizole	26±5	6±1	9±2	<LOD	<LOD
Sulfamethoxazole	28±4	<LOD	<LOD	<LOD	<LOD
Oxytetracycline	<LOD	<LOD	358±10	44±2	<LOD
Tetracycline	153±27	80±15	10±2	<LOD	<LOD
Doxycicline	154±9	60±16	346±43	1506 ±68	<LOD

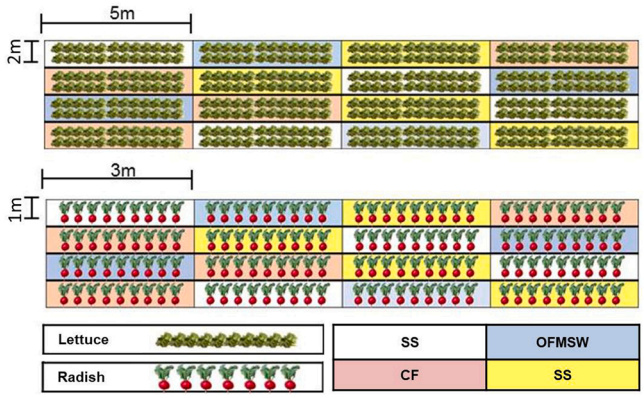

Fig. 1. Experimental design diagram showing the plots located at the Experimental station of Agropolis (UPC, Castelldefels). The fertilisation with pig slurry (PS), sewage sludge (SS), and the organic fraction of solid municipal waste (OFSMW) were compared with control conditions using chemical fertilisation (CF)

planted to assess the worst-case scenario. The plants were watered by drip irrigation with well water to ensure high quality (electrical conductivity of 1220 μS/cm and a pH of 8.0). Two productive cycles with repetitive amendment of the soil plots with the same fertilisation products were carried out. The first productive cycle for lettuces lasted from April to May 2019 (42 days), and the second one from November 2019 to March 2020 (116 days). In the case of the radish plants, the first productive cycle was from April to May 2019 (37 days), and the second from November 2019 to January 2020 (68 days). The average temperature during the first productive cycle was of 16 ºC and 14 ºC for the lettuce and radish respectively, compared to 11 ºC and 9 ºC, respectively, in the second cycle. A heavy rainstorm (Gloria Storm) took place 15 days before harvesting during the second productive cycle, flooding all the lettuce plots for 24 hours. The radish crops were not affected by this event as they were harvested earlier.

2.2. Sampling strategy

Fertilisation product samples were collected directly from the fertiliser sources just before the soil was amended. For each of the above-mentioned plots (n=32), 10 vegetable plants were randomly harvested, taking care to avoid the border effect. The edible parts of the vegetables (leaves for lettuces and roots for radishes) were then assessed for general quality parameters, and the remaining part was homogenised to determine the antibiotics.

For the metabolomics, 5 middle- and old-stage leaves from 5 lettuce plants from each plot were collected using an 8-mm leaf punch disk and were immediately frozen in liquid nitrogen (5 leaf samples per plot) following a previously described sampling strategy for studying plant metabolomics (Hurtado, 2017). Samples were stored at −80 °C until analysis. Immediately after metabolic sampling, lettuces were harvested, weighed, and measured for leaf height and number of leaves.

For the transcriptomics, 2 middle-stage leaves from 8 lettuce plants from each plot were collected. Leaf samples were cut using an 8-mm leaf punch (3 discs per leaf) and deposited in a 2-mL microcentrifuge tube, containing 1 mL RNAlater Solution (Ambion). The samples were first incubated at 4 ºC to allow the RNAlater Solution to thoroughly penetrate the tissues and then stored at −80 °C until analysis.

2.3. Soil and fertilisation product characterisation

The chemical characterisation of the soil and the different fertilisation products was carried out at the Eurofins agri-environmental accredited laboratory using certified methodologies (https://www.eurofins.es/). Detailed descriptions of the physico-chemical characteristics of each fertilisation product and the soil are given in Table 1-SM. The predominant available nitrogen form in all the fertilisation products was ammoniacal nitrogen. The fertilisation products applied to the soil were calculated following the ammoniacal nitrogen concentration, 10 g/m^2 for lettuce and 8 g/m^2 for radish. The amount of fertilisation products per radish crop was as follows: 3 L of PS per m^2, 2.3 kg of SS per m^2, and 2.8 kg of OFMSW per m^2. Per lettuce crop, the amounts were as follows: 4 L of PS per m^2, 3 kg of SS per m^2, and 4.5 kg of OFMSW per m^2. The amount of nutrients added along with chemical fertiliser added was as follows: 59 and 47 g of N per m^2, 9.2 and 6.9 g of P_2O_5 per m^2, and 34 and 17 g of K_2SO_4 per m^2 for lettuce and radish, respectively. Electrical conductivity of the soil after the second productive cycle was as follows: 0.27±0.09 dS/m for CF soil, 0.29±0.03 dS/m for PS fertilised soil, 0.26±0.04 dS/m for OFMSW fertilised soil, and 0.27±0.11 dS/m for SS fertilised soil, whereas pH ranged from 7.0 to 7.2.

2.4. Antibiotic content in fertilisers and vegetables

The chemicals and reagents employed are listed in the SM section. Antibiotics were determined in fertilisation products using a previously described methodology (Berendsen et al., 2015). Their determination in lettuce leaves and radish roots depended on the antibiotic family. For sulphonamides, fluoroquinolones, lincosamides, and benzylpyrimidine, a methodology previously described by Tadić et al. (2019) was employed. For tetracyclines, the methodology consisted of extraction using 10 mL of McIlvaine buffer pH=4.0 containing 0.1 M of EDTA (two cycles were required). In the SPE step, 6 mL of H_2O was used as a wash step. Elution was then performed with 4 mL of methanol and ethyl acetate (50/50, v/v). Finally, the samples were filtered, reconstituted, and injected in the same way as the method described above. The analytical methodologies, including the analytical quality parameters, are detailed in the SM section (Table 2-SM, 3-SM and 4-SM).

2.5. General plant parameters

The lettuce and radish phenotypes were measured for each scenario. The length and number of lettuce and radish leaves were measured at the end of each productive cycle. Chlorophyll content in leaves and weight were measured in situ. Chlorophyll was gauged using a chlorophyll-meter (Opti-Sciences, Hudson, NH, USA). Lipid and sugar content were measured as described elsewhere(Margenat, 2018).

2.6. Plant metabolomics

Briefly, 10 mg of homogenised lettuce material was transferred to an Eppendorf tube, and 400 μL of methanol was added. The samples were then vortexed, sonicated, and centrifuged with methanol, chloroform, and water following a previously described methodology (Hurtado, 2017). The extracts were vacuum-dried and derivatised with methoxyamine in pyridine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS). Finally, the extracts were filtered through a 0.22 μm pore size filter and spiked with triphenylamine (TPhA) as an instrumental standard. 2 μL of sample was injected into the GC-Orbitrap system (Q Exactive GC, Thermo Scientific, Bremen, Germany), which was operated in the full-scan mode and equipped with a 30 m × 0.25 mm I.D., 0.20 μm film thickness Sapiens-X5.MS coated with 5% diphenyl 95% dimethylpolysiloxane from Teknokroma (Sant Cugat del Vallès, Spain). Further details on the chromatographic and mass spectrometry, as well as the data processing, are described in the SM section.

2.7. Gene expression analyses

For the RNA extraction, leaf samples were homogenised using a TissueLyser LT (QIAGEN) sample disruptor. After lysis, an RNeasy Plant

Table 2
Concentration of antibiotics and their transformation products in lettuce leaves and radish roots according to the fertilisation product and productive cycle. Notice that lettuces fertilised with OFMSW and CF are not included since antibiotics were not detected.

	Lettuce leaves (ng/g fw)				Radish roots (ng/g fw)			
	SS		PS		SS		PS	
	1st	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*
8-Hydroxyquinoline*	nd	nd	0.9±0.7	0.9±1.4	nd	nd	0.4±0.4	0.8±0.9
Sulfacetamide	nd	nd	nd	nd	5.3±5.1	nd	nd	nd
Trimethoprim	nd	nd	nd	nd	nd	nd	0.40±0.1	0.5±0.2
Hydroxyl-trimethoprim*	nd	nd	nd	nd	nd	nd	nd	nd
Decarboxyl-ofloxacin *	nd	2.3±1.2	nd	7.2±7.1	nd	nd	nd	nd
Ofloxacin Methyl Ester*	nd	nd	nd	nd	nd	nd	nd	nd
Lincomycin	nd	0.2±0.1	24±15	0.3±0.1	nd	0.3±0.2	0.6±0.5	0.3±0.2
Sulfadiazine	nd	nd	nd	nd	nd	nd	2.0±0.8	1.1±0.5
Sulfapyridine	nd	nd	nd	nd	nd	nd	nd	nd
Sulfathiazole	nd	nd	nd	nd	nd	nd	nd	nd
Sulfamethazine	nd	nd	nd	nd	nd	nd	nd	nd
Keto-trimethoprim*	nd	nd	nd	nd	nd	nd	nd	0.3±0.2
Ciprofloxacin	nd	4.9±3.2	nd	5.4±2.4	0.7±0.6	nd	6.0±4.7	3.6±2.5
N-desmethyl Ofloxacin*	nd	1.5±1.1	3.0±2.5	2.3±1.9	nd	nd	1.0±1.2	2.1±1.9
Ofloxacin	nd	nd	nd	nd	nd	nd	1.0±0.7	1.1±0.9
Ofloxacin Ethyl Ester*	nd	nd	nd	nd	nd	nd	0.8±0.7	0.8±0.7
Enrofloxacin	nd	nd	nd	nd	8.9±7.8	nd	7.1±4.5	7.1±5.2
Sulfamethizole	3.4±2.0	8.8±1.7	nd	9.9±3.8	nd	4.4±3.2	nd	7.8±8.1
Sulfamethoxazole	nd	nd	nd	nd	nd	nd	nd	nd
N-Acetyl-sulfamethoxazole*	nd	nd	nd	nd	nd	nd	nd	nd
Oxytetracycline	0.5±0.4	nd	0.5±0.4	0.6±0.4	57±21	76±17	49±22	68±11
Tetracycline	nd	0.2±0.1	0.4±0.2	0.2±0.3	nd	0.4±0.2	nd	0.3±0.1
Doxycycline	nd	nd	nd	nd	1.2±1.1	0.9±0.9	2.5±2.2	0.6±0.5
Frequency of detection	**9%**	**26%**	**22%**	**35%**	**22%**	**22%**	**48%**	**61%**

*Transformation products.

Mini Kit (QIAGEN) was used for total RNA extraction following the procedure provided by the vendor. Briefly, samples were centrifuged in QIAshredder columns to remove insoluble material and reduce the lysate viscosity. Ethanol was added to the purified lysates to allow selective binding of RNA to the RNeasy kit (QIAGEN) columns. On-column DNase I digestion was performed (RNase-free DNase set, QIAGEN), and the RNA was eluted in RNase-free water. The yield and purity of the total RNA were assessed spectrophotometrically in a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the RNA integrity was examined in an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE).

For quantitative real-time PCR (qRT-PCR), first-strand cDNAs were synthesised from 4 μg of DNAse I-treated total RNA (pools of 2 leaves, 2 μg each), obtained from 8 biological replicates, using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics) following the manufacturer's instructions.

Oligonucleotide primers were designed and validated to obtain a reliable gene expression analysis by quantitative real-time PCR (qRT-PCR). Some primers were designed using PrimerExpress (Applied Biosystems) software, whilst others were taken from Borowski (2014). The stress genes and primers used for the qRT-PCR are shown in Tables 9-SM and 10-SM, respectively. The qRT-PCR reactions were performed in triplicate in a Roche LightCycler 480, using 50 ng of cDNA, 300 nM of each primer, and the LightCycler 480 SYBR Green I Master (Roche Diagnostics). PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of a denaturation step at 95 °C for 15 s, and an annealing/extension step at 60 °C for 1 min. A final dissociation curve was generated to verify that a single product was amplified. Series of 10-fold dilutions of cDNA covering were used to generate the standard curves to calculate the efficiency of the primers. Reactions in the absence of template and in the absence of enzyme were also included as negative controls. PCR products were sequenced (Capillary Sequencing Service, CRAG, Barcelona) to confirm that they corresponded to the intended genes by using online databases (GenBank, Blast, etc.).

Relative expression values of the different genes were calculated from the threshold cycle (Ct) following the ΔΔCt method (Pfaffl, 2001) using *EIF4A1* as an internal reference control gene.

2.8. Human health risk

The hazard quotient (HQ) was calculated using the estimated daily intake (EDI, μg/kg/day) and the acceptable dose intake (ADI) approach, as described in Margenat (2019):

$$EDI = \frac{DI \times C_M}{BW}$$

where DI (g/day) and BW (kg) are the daily intake of the edible part of vegetables and body weight, respectively. In this case, CM (μg/g) indicates the 95th percentile value for the concentration of each AB in vegetable edible parts. The DI for fresh vegetables in Spain used in the calculations was taken from the EFSA's Comprehensive Food Consumption Database (87 g of fresh lettuce per day for adults and 22 g of fresh radish per day). The HQ is the ratio between the EDI and threshold levels considered to be acceptable daily intakes (ADIs). The ADI (μg/kg/day) values used were taken from a list based on microbiological and toxicological endpoints compiled by Wang (2017). For AB transformation products, the ADI value of the parental compound was considered when no data were available. The ADIs for the ABs detected in the lettuce samples are listed in Table 6-SM. The THQ is estimated as the sum of the individual HQs. If the THQ is less than 1, the risk is generally deemed to be acceptable.

2.9. Data analysis

Differences between agronomical parameters or AB content in vegetables were determined by the Kruskal-Wallis test using IBM SPSS v25 and R software. MetaboAnalyst 5.0 (http://www.metaboanalyst.ca) was used to perform (agglomerative) hierarchical cluster analysis of the occurrence of ABs in the vegetable samples with the hclust function in package stat using the Euclidean distance measure and Ward clustering algorithm (the heat map was shown in addition to the dendrogram as a visual aid). As for the metabolic analysis, a final matrix containing 64

feature/metabolite peak areas from each sample was uploaded to the MetaboAnalyst 5.0 server http://www.metaboanalyst.cafor data analysis (Table 5-SM). The data were then normalised by the sum of areas, and Pareto scaling was applied. Subsequently, volcano plots of each of the treatments vs the control (CF) were used to pinpoint features with a fold change (FC) of at least 1.5 and t-test thresholds (p-value) less than 0.05. Volcano plots were further generated with VolcaNoseR (Goedhart and Luijsterburg, 2020). The selected features were used to conduct pathway analysis with a library of *Arabidopsis thaliana* (ath, KEGG organisms abbreviation). The selected over-representation analysis method was the hypergeometric test, and the node importance measure for topological analysis was relative betweenness centrality. The MetaboAnalyst 5.0 pattern search function was used to measure the correlation between metabolites and concentrations of ABs in lettuce samples. To do this, a correlation analysis was performed of the 64-feature peak-area matrix against the sum of AB concentrations found in each lettuce sample, with a Pearson's r distance measure.

Significant differences in the mean values of gene expression amongst the samples were determined by one-way ANOVA followed by Tukey's post-hoc test using R (R Development Core Team (2008) R: A Language and Environment for Statistical Computing). Heat map and hierarchical clustering were performed in R using the heatmap package (https://CRAN.R-project.org/package=pheatmap).

3. Results and discussion

3.1. Occurrence of antibiotics in fertilisation products

Table 1 shows the chemical composition of the different fertilisation products examined in this study. The abundance of ABs depends on the fertilisation product, but also on the sampling moment (first or second productive cycle). In this regard, the frequency of detection of ABs was greater in SS fertiliser (11/15) than in PS (10/15), whereas none of the studied ABs were detected in the case of OFMSW. The most abundant ABs in the fertilisation products (>1,000 ng/g fw in at least one productive cycle) were lincomycin, ciprofloxacin, ofloxacin, enrofloxacin, and doxycycline. For PS lincomycin was the most abundant (51,012 ng/g fw in the first productive cycle), whereas for the SS fertiliser the most abundant ABs were ciprofloxacin (3,539 ng/g fw), ofloxacin (1,930 ng/g fw in the first productive cycle), and enrofloxacin (1,709 ng/g fw). These results are consistent with previous studies showing that lincomycin is an AB employed for veterinary purposes (Mehrtens et al., 2021). Fluoroquinolones have been detected in SS in a wide range of concentrations, reaching up to 12,858 ng/g dw for ciprofloxacin and 6,712 ng/g dw for ofloxacin (Mejías et al., 2021).

3.2. Plant uptake of ABs

Table 2 shows the concentration of ABs in vegetables following the different soil fertilisation strategies. Although 12 parental ABs were detected in the fertilisation products, only between 2 and 9 were identified in crops following PS and SS fertilisation. Moreover, 6 AB transformation products (TPs) were identified. The concentration of ABs in vegetables fertilised with OFMSW and CF was always below the limit of detection, which is consistent with the fact that they were not identified in these fertilisation products. This is also consistent with previous greenhouse pot studies in which no ABs were detected following amendment with OFMSW or CF (Margenat, 2020). The frequency of detection of ABs in vegetables amended with SS and PS increased from the first to the second productive cycle (from 16 to 31% and from 35 to 42% on average for lettuce and radish, respectively), whereas the frequency of detection was higher in radish roots than in lettuce leaves (39 vs 24%). The frequency of detection of ABs was higher in vegetables amended with PS than those amended with SS (23 vs 39%), which is consistent with the relative AB concentration in both fertilisers (Table 1).

The concentration of ABs ranged from undetectable to 76 ng/g in fw in radish edible parts following SS fertilisation. Oxytetracycline showed the highest concentration in radish roots (63 ng/g in fw, on average), whereas sulphamethizole and lincomycin were the most abundant ABs in lettuce leaves (5.6 and 6.1 ng/g in fw, on average). These results are in line with previous studies which found that oxytetracycline accumulated in radish roots but not in lettuce leaves (Youssef et al., 2020). The high occurrence of oxytetracycline in radish roots following PS and SS fertilisation might be explained by its high concentration in these fertilisation products (Table 1). Nevertheless, since oxytetracycline was not detected in SS, its occurrence in the radish roots following SS fertilisation can only be explained by the presence of antibiotic conjugate forms which would deconjugate following soil fertilisation (Kinney and Heuvel, 2020). The average concentration of ABs for both vegetables grown with SS and PS between the first and second productive cycle increased slightly, by 17% (paired t-test, p-value >0.05). Therefore, the repetitive soil fertilisation did not significantly increase plant uptake of ABs (Cycoń et al., 2019), which can be explained by soil biodegradation of ABs between the first and second productive cycle (9-10 months). Previous studies[30] showed higher AB concentration for vegetables in winter than in summer; in those cases, however, the amount of PS applied in the cold season was much greater than in the warm season, whereas in the present study it was the same.

The concentration of ABs in radish roots (3.7 ng/g fw, on average) was more than three times greater than in lettuce leaves (1.1 ng/g fw, on average). This is consistent with previous studies suggesting that the most relevant factor in plant uptake of pollutants is the vegetable species selected (Tadić, 2021), and that the edible parts are different – roots vs leaves in radish and lettuce crops, respectively. A recent review study indicates that plant uptake of contaminants of emerging concern depends on plant species, but also on crop compartments. In this sense, the concentration of compounds was higher in roots than in leaves, and greater in leaves than in aerial fruits (Christou, 2019). Similarly, heat-map hierarchical clustering analysis grouped samples according to the abundance of ABs (Fig. 2). The results clearly differentiate between radish and lettuce samples. Specifically, the results indicate that of the 16 ABs identified in the vegetables, 10 were accumulated in radish roots, whereas 6 were predominately identified in lettuce samples. In summary, the heat map shows that the concentration of ABs in edible parts of radish and lettuce depends not only on the antibiotic but also on the studied vegetable. These results are consistent with previous studies indicating that AB uptake by plants from soil amended with animal manure depends on both the AB and the plant (Bassil et al., 2013).

The occurrence and abundance of ABs was higher than in previous studies carried out in a greenhouse pot study with the same fertilisation products and soil (You, 2020; Margenat, 2020) This might be related to the longer growing time and, thus, greater biodegradation of the organic matter contained in the fertilisers, which may enhance the release of ABs and, thus, their uptake by plants (Quaik, 2020). The abundance of ABs was in the same concentration range as in other field-scale studies where manure was used as fertiliser (Zhao et al., 2019; Hu et al., 2010), but greater than that observed in studies in which vegetables were irrigated with reclaimed water (Liu et al., 2020), suggesting that organic fertilisation could be a greater source of ABs than reclaimed water.

As for the occurrence of AB TPs, trimethoprim and sulfamethoxazole TPs were not detected in crop edible parts, whereas decarboxyl-ofloxacin was mainly detected in lettuce leaves during the second productive cycle and N-desmethyl-ofloxacin in lettuce and radish fertilised with PS. Furthermore, the concentration of ofloxacin TPs was higher than that of the parental compound, which was below the quantification limits, suggesting considerable ofloxacin metabolisation. These results are at variance with previous studies conducted by Tadić (2021) suggesting that the concentration of the ofloxacin parental compound was greater than its TPs in lettuces irrigated with reclaimed water. This difference may be because fertilisation products are applied before vegetables have been planted in the soil, whereas reclaimed water is

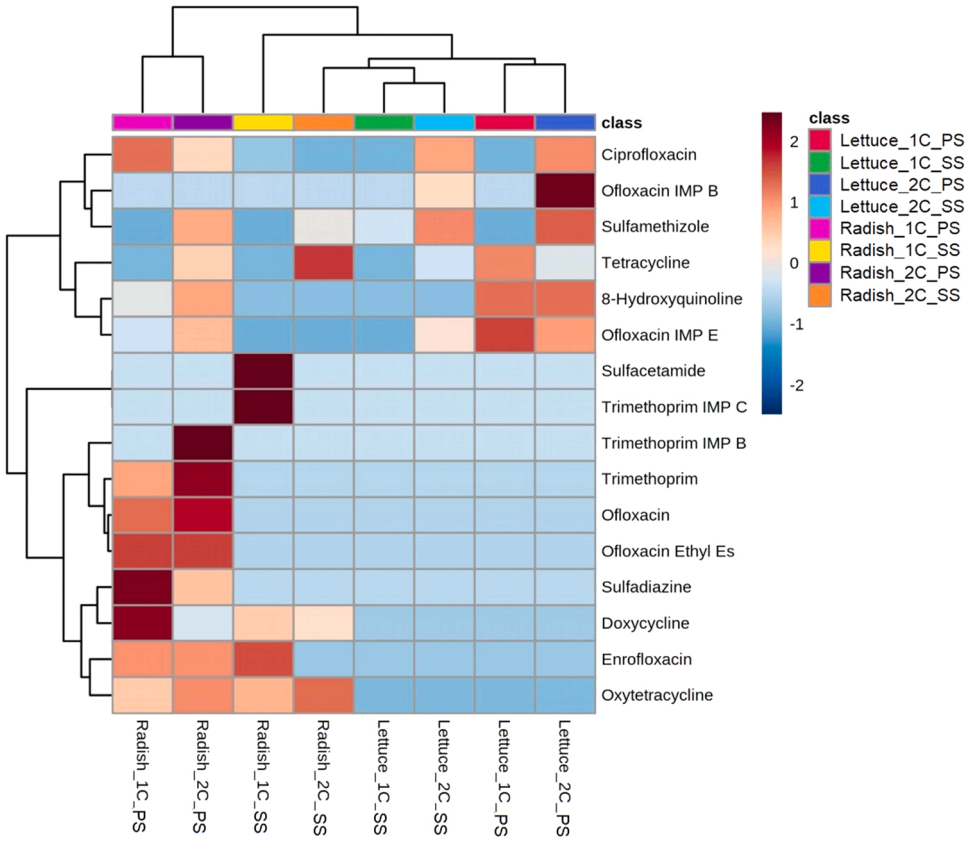

Fig. 2. Heat-map of the abundance of antibiotics in lettuce and radish edible parts. Clustering result shown as heat-map (distance measure using euclidean and clustering algorithm using ward.D).

added continuously, indicating that ofloxacin is more likely to be transformed and metabolised when it is applied as a fertiliser than when it originates from reclaimed water. Finally, the present results indicate that ofloxacin TPs accumulate in lettuce leaves, whereas the ofloxacin parental compound accumulates in radish roots. Leaves may be the predominant compartment of AB metabolisation and, thus, of TP accumulation in vegetables (Malchi et al., 2014).

3.3. Plant agronomic changes

The agronomic parameters of the vegetables grown in the soil amended with the different fertilisation products changed compared to those fertilised with CF (Fig. 3). The number and length of leaves differed between the crop species, but also between seasons and were greatest in lettuce in the warmer (first) productive cycle. The effect of fertilisation products on leaf number and length was only statistically observed for lettuces fertilised with the OFMSW (p-value <0.05); the other fertilisers resulted in leaves similar in number and length to the CF used as a reference. Fresh weight ranged from 200 to 650 g and from 10 to 45 g for lettuce and radish edible parts, respectively. These values are consistent with previous studies for lettuces grown in outdoor plots for commercial use (Margenat, 2017). No seasonal pattern was observed for lettuce (p-value >0.05), but radish crops fertilised with SS and PS showed greater fresh/dry weight in the first productive cycle than in the second (p-value <0.05). Two different reasons may explain this fact. First, radish roots may be affected by the greater concentration of OTC in the first productive cycle for SS and PS, as OTC has recently been shown to have a positive effect on root growth, but a negative effect on shoot growth (Li et al., 2020; Zhang, 2021). Second, radishes may be more sensitive to nutrients than lettuce, and higher temperatures result in a greater nutrient release/bioavailability from organic fertilisers.

Chlorophyll content was greater in lettuce than in radish leaves (p-value <0.05), whereas a seasonal trend was observed only for radish, with the content being greater in the second productive cycle (colder and with a shorter day length) (p-value <0.05). This is consistent with previous studies by Stautas et al. (2011) who found that the lowest concentration of pigments was observed for radish under longer daily photoperiods, regardless of temperature and treatment. Lettuce leaves showed a lower chlorophyll content in crops fertilised with OFMSW during the warm (first) productive cycle compared with the other fertilisation products (p-value <0.05). The negative effect of OFMSW on most of the studied agronomic parameters cannot be related to nutrient availability as the content of nutrient bioavailable forms (ammonia or nitrate) was similar in all cases (Table 1), suggesting other scenarios. Hydric stress is the most likely reason, as the amount of compost made of 2-30 mm pruning residues added to the soil was very high, increasing soil porosity and water percolation (Luna et al., 2018). This is also consistent with a reduction in chlorophyll content and crop yield under drought stress in plants (Mafakheri et al., 2010; Efeoğlu et al., 2009).

Overall, the agronomic results are similar to those found in previous greenhouse studies (You, 2020; Margenat, 2020), suggesting that the greater presence of ABs in SS and PS fertilisers, as well as in lettuce and radish edible parts grown with those fertilisers, does not negatively affect crop morphology. Nevertheless, the greater abundance of certain ABs in crops following SS and PS fertilisation in the first productive cycle correlated positively with the fresh/dry weight of radish roots. A positive effect of ABs such as OTC on radish root growth cannot be disregarded. Given that the effect of ABs may depend on vegetable species, future studies should address this knowledge gap under real agricultural practices.

3.4. Plant metabolic changes

The volcano plot highlighted 24 metabolites with different levels

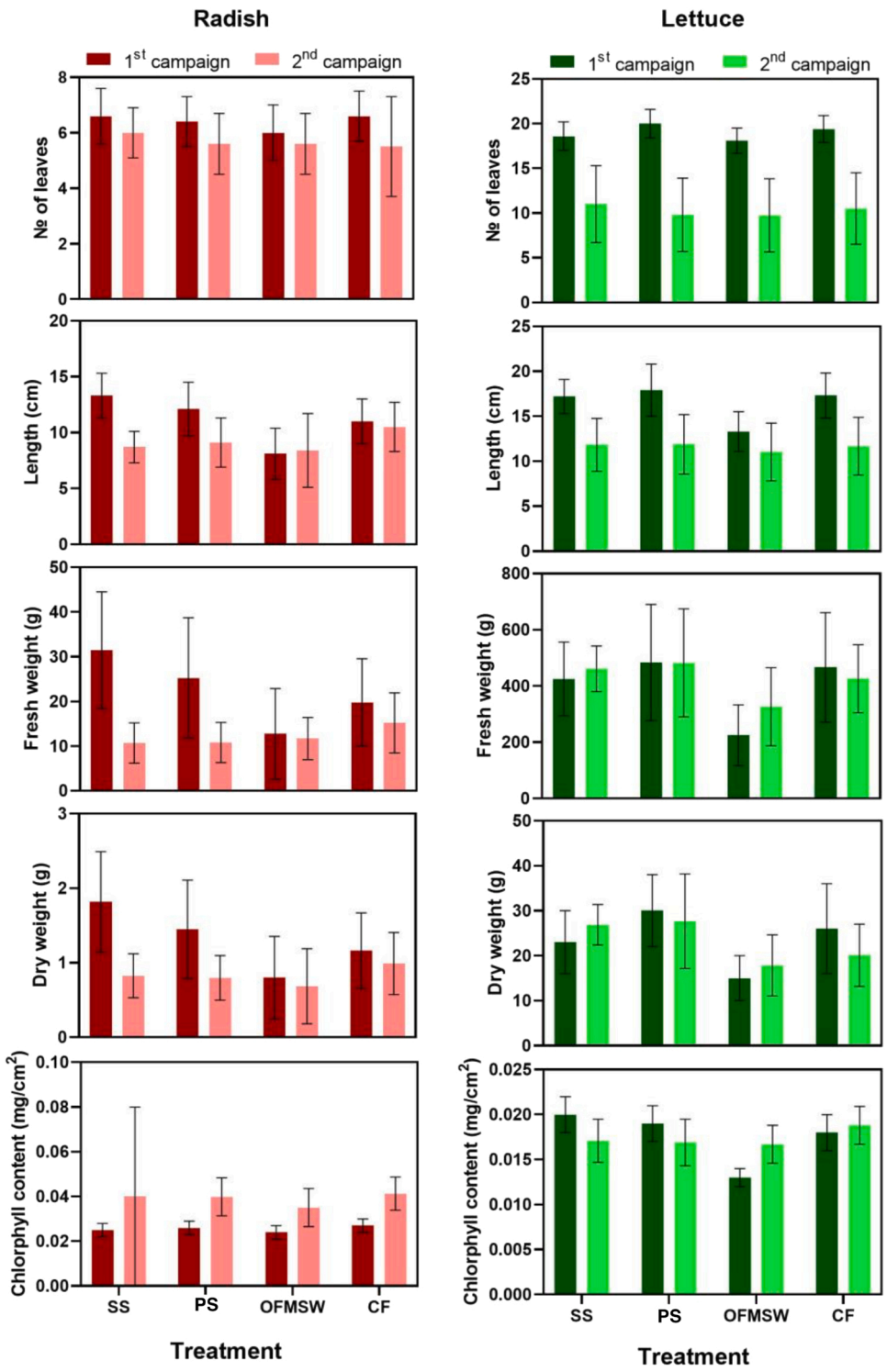

Fig. 3. Agricultural crop parameters following the soil fertilisation with SS, PS, OFMSW and CF (used as control for comparison).

compared to the control lettuce vegetables fertilised with CF (Fig. 4 and Table 7-SM). The compounds included sugars, organic acids, amino acids, and alcohols. The use of different fertilisation products resulted in considerable metabolic changes compared to lettuces fertilised with CF. SS and PS were grouped and differed from lettuces fertilised with OFMSW. This is consistent with recent greenhouse studies showing that the low ammoniacal and nitrate content of this fertiliser results in changes in the metabolic profile (Matamoros, 2021). Nevertheless, in this case the ammoniacal content was the same for all the fertilisation products. Hence, the differences need to be explained by other mechanisms.

Lettuces grown with OFMSW showed a greater number of changed metabolites (24) than lettuces grown with CF, whereas only 5 and 4 metabolites changed following fertilisation with SS and PS, respectively. The amounts of dopamine in lettuces following fertilisation with SS, PS, and OFMSW were higher than in those fertilised with CF. Dopamine can promote plant growth in various stressful environments and could enhance plant tolerance to drought, salt stress, and nutrient deficiency

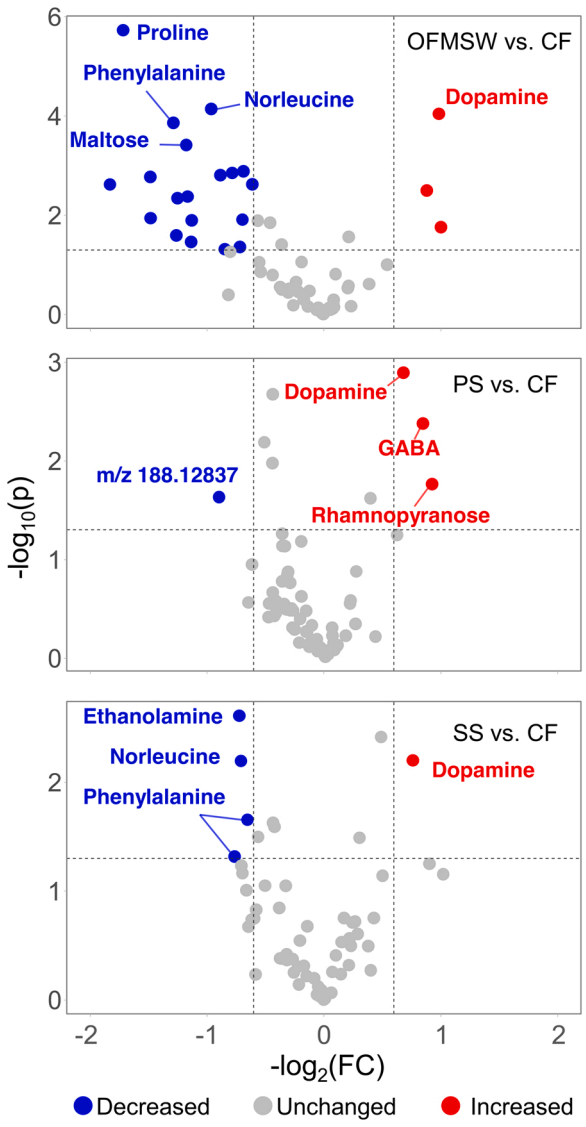

Fig. 4. Volcano plot of lettuces grown in the three different treatments (OFSMW, SM and SS) versus the chemical fertiliser (control) labeling the 5 top significant features. FC (Factor Change) threshold has been set to 1.5 and p-value has been set to <0.05. Both axes presented in a logarithmic scale.

(Liu, 2020). The present results thus indicate that dopamine could be used as a metabolic lettuce biomarker of the soil application of fertilisation products. GABA and rhamnopyranose were more abundant following PS and OFSMW fertilisation, but not in lettuces fertilised with SS. This may be related to nutrient availability, in keeping with the possible role of GABA as a signal molecule in plants, as well as roles in plant response to stress and in the carbon:nitrogen (C:N) balance (Fromm, 2021). Conversely, the abundance of phenylamine and norleucine in lettuce leaves decreased following SS and OFSMW fertilisation, but not PS. This is in keeping with the fact that the gene for phenylalanine ammonia-lyase (PAL), a gateway enzyme in the biosynthesis of various phenolic compounds, is activated by a number of biotic and abiotic stresses (Oh et al., 2009). Finally, many metabolites (21), such as amino acids (phenylalanine and norleucine, proline, serine, alanine, and isoleucine), sugars (maltose and rhamnose), and nucleotides (adenine and guanine), were less abundant following fertilisation with OFMSW, and only 3 increased (dopamine, GABA, and rhamnopyranose). Altogether, this indicates that lettuces fertilised with OFMSW are exposed to greater stress conditions than those fertilised with other fertilisers, which is also in line with the lower weight and length of these lettuces (Fig. 3). OFMSW is a compost with a high C:N ratio, which has been described to cause nitrogen immobilisation and, thus, nitrogen deficiency in plants (Guo, 2012).

Therefore, the use of either of the assessed fertilisation products resulted in a plant metabolism stress, as denoted by the dopamine increase, compared to control plants fertilised with CF. Plant metabolic correlation analysis indicated no link between metabolic changes and the abundance of ABs (correlation analysis test, p-value >0.05).

Fig. 5 and Table 8-SM show that lettuce fertilisation with OFMSW affected several metabolic pathways (8), whereas specific stress metabolites such as dopamine or GABA synthesis were affected following SS (2) and PS (3) fertilisation. Some of the altered pathways are involved in amino-acid metabolism, such as aminoacyl-tRNA biosynthesis, and phenylalanine metabolism, but also in the tricarboxylic acid cycle, such as alanine, aspartate, and glutamate. Therefore, fertilisation with OFMSW mainly affected pathways involved in amino-acid biosynthesis, which would be consistent with low nutrient bioavailability or water stress, whereas the other fertilisation practices affected specific pathways.

3.5. Plant gene expression

Fig. 6A shows changes in gene expression in lettuces fertilised with CF, OFMSW, SS, or PS based on reference and stress gens (Table 9-SM and Table 10-SM). In response to the different fertilisers, changes were observed in the expression of genes encoding aquaporins, reactive oxygen species (ROS) scavengers, enzymes regulating osmolyte synthesis, and chaperones. This is in line with the fact that lettuce and radish crops are sensitive to salt and drought stress (Kurunc, 2021). The highest gene expression values were observed in lettuces fertilised with CF and OFMSW, indicating greater stress conditions than in SS- or PS-fertilised ones. Accordingly to the OFMSW composition (basically composed by pruning resides of 2-30 mm) and the fact that soils fertilised with PS and SS increase water holding capacity due to their high OM content (Hussein, 2009), we suggest lower water retention in soils fertilised with CF and OFMSW. Salinity stress was not considered since there were no differences in soil salinity after the second productive cycle between the different assessed plots (Table 11-SM). The negative effects of the drought of CF fertiliser could be overcome by enhancing adaptative responses to oxidative stress, such as reactive species (ROS) scavengers (*LGalDH*, *MnSOD*) and chaperones (*HSP70*), resulting in osmotic-stress tolerance (Mansilla, 2021). The plasma membrane intrinsic protein 2 (*PIP2*) gene was significantly induced following fertilisation with OFMSW (Fig. 6 A). This gene is known to encode a water-channel aquaporin and its up-regulation promotes drought stress tolerance (Singh et al., 2020), which is compatible with the agronomic properties observed in the lettuces fertilised with OFMSW (see above). No significant up-regulation of stress-responsive genes was seen in lettuces fertilised with PS, whilst SS induced significant up-regulation of 4-hydroxyphenylpyruvate dioxygenase (HPPD) (Fig. 6 A). HPPD catalyses the formation of vitamin E (tocopherol) and homogentisic acid, the precursor of plastoquinone, preventing plant bleaching (Borowski, 2014).

Hierarchical cluster analysis differentiated two clearly defined groups of samples, based on their gene expression patterns (Fig. 6B). The first group consisted of lettuces fertilised with CF and OFMSW (mainly positive correlations, in red), whilst the second clustered lettuces fertilised with SS and PS (predominantly negative correlations, in cyan). Transcriptomic data indicate that changes in the expression of genes involved in plant stress responses are independent of the occurrence of ABs (Table 2), in keeping with metabolomic analyses (Fig. 5).

Given that not all the transcriptome changes are reflected at the phenotype level, correlations between the metabolome and transcriptome are rather complex (Figs. 5 and 6). The reduction of phenylalanine levels following fertilisation with OFSMW and SS (Fig. 5) is, for example, independent of changes in *PAL* gene expression (Fig. 6 A). The

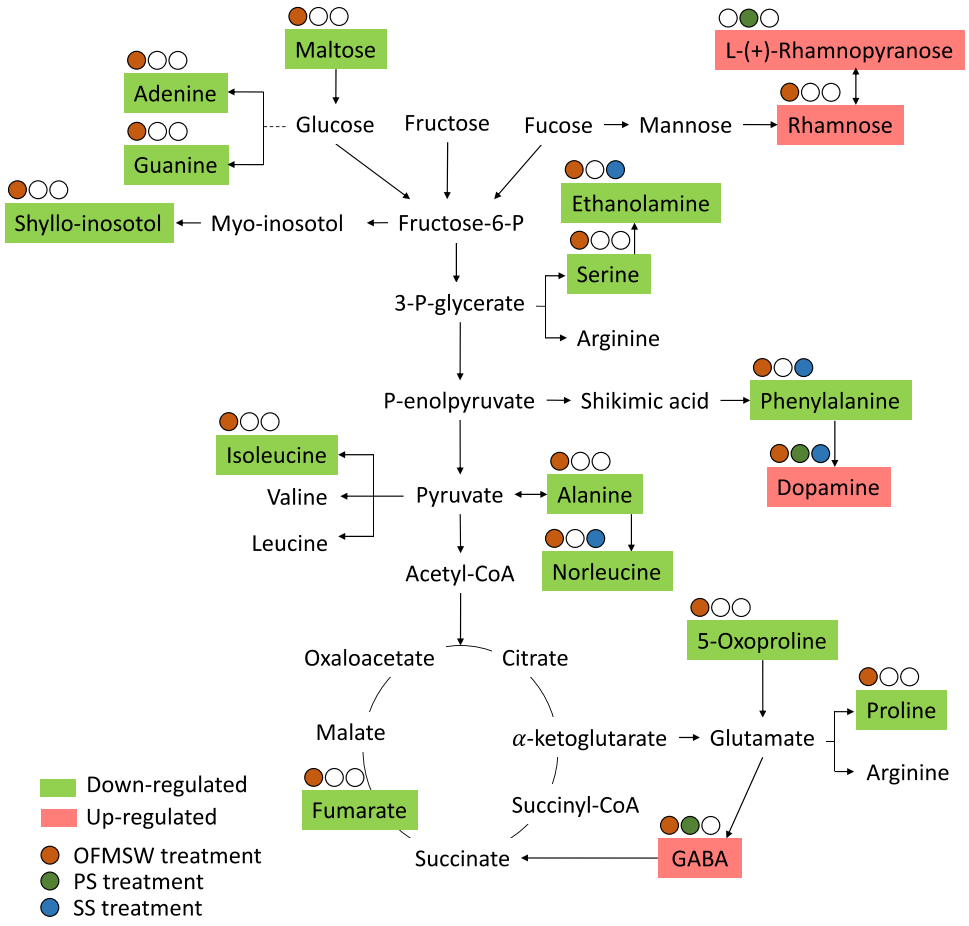

Fig. 5. Metabolic pathway of the metabolites that significantly changed in each of the treatments according to the volcano plot.

multilevel regulation of biological processes (transcriptional and translational, amongst others) is the most likely reason hampering, in many cases, a direct link between a concrete transcript and a metabolite.

Fig. 6C shows a correlation map between the concentrations of different metabolites and expression levels of the analysed genes. Only metabolites significantly correlated with the expression of at least one gene are included (cyan asterisks). Hierarchical clustering identified different groups of metabolites according to their mutual correlation with the expression patterns of stress-responsive genes. A first group of metabolites shows significant positive correlations with the *HSP70*, *MnSOD,* and *MIPS1* genes (Fig. 6 C, cyan asterisks and red squares) and includes the sugars lactose, maltose, and glucose, as well as niacin, and pentenoic and butanedioic (succinic) acids. In general, those molecules are known positive regulators of the aforementioned genes, acting in response to drought stress (Keunen et al., 2013; Valluru and Van den Ende, 2011), which reinforces the idea that hydric stress was a leading driver of the phenotypic, metabolic, and transcriptomic variability related to the use of the different fertilisers.

3.6. Human health implications

Human health risk was assessed taking into consideration the HQ, as well as the legislated values for ABs in foodstuffs established by the pertinent EU Council Regulation. The AB concentration levels in lettuce and radish edible parts (Table 2) were less than the MRLs established for animal tissues or food samples by EU regulations (The European Commission, 2010), ranging from 100–500 μg/kg for quinolones, 100 to 600 μg/kg for tetracyclines, and 100 μg/kg for sulphonamides, depending on the foodstuff. Nevertheless, as oxytetracycline was detected at a concentration of up to 70 μg/kg in radish edible parts following SS and PS fertilisation, very close to the EU guideline limits, future studies should pay more attention to edible root vegetables.

Table 3 shows that the HQ for each individual compound and the THQ for all the studied ABs are well below 0.1. Ciprofloxacin was the AB of greatest concern, as it had the lowest ADI. Nevertheless, the HQ was always less than 0.1. Similarly, with regard to the occurrence of ABs, the repetitive application of SS and PS fertilisation products increased the human health risk for lettuce (THQ from 0.00017-0.0069 to 0.064-0.084), but not for radish (THQ from 0.01-0.03 to 0.01-0.02). The first amendment with fertilisation products resulted in a greater risk for radish, but the subsequent addition of both fertilisers increased the human health risk of lettuce consumption to values higher than those observed for radish.

These values are consistent with previous greenhouse studies on lettuces amended with SS and PS. In those studies, ciprofloxacin was the AB showing the greatest HQ, with THQ values up to 0.16 following PS amendment (Margenat, 2020). These results indicate that whilst field-scale studies normally differ from greenhouse studies, they help us estimate the human health implications. Similarly, Tadić (2021) observed that field-scale studies using lettuce crops irrigated with reclaimed water resulted in maximum estimated HQ values of less than 0.01.

The human health risks regarding the abundance of ABs are not remarkable, given that AB concentrations were much lower than those listed in the EU directive for foodstuffs (the calculated HQ was <0.1).

4. Conclusions

This study showed that the use of different fertilisation products under current agricultural practices at field scale results in AB uptake by

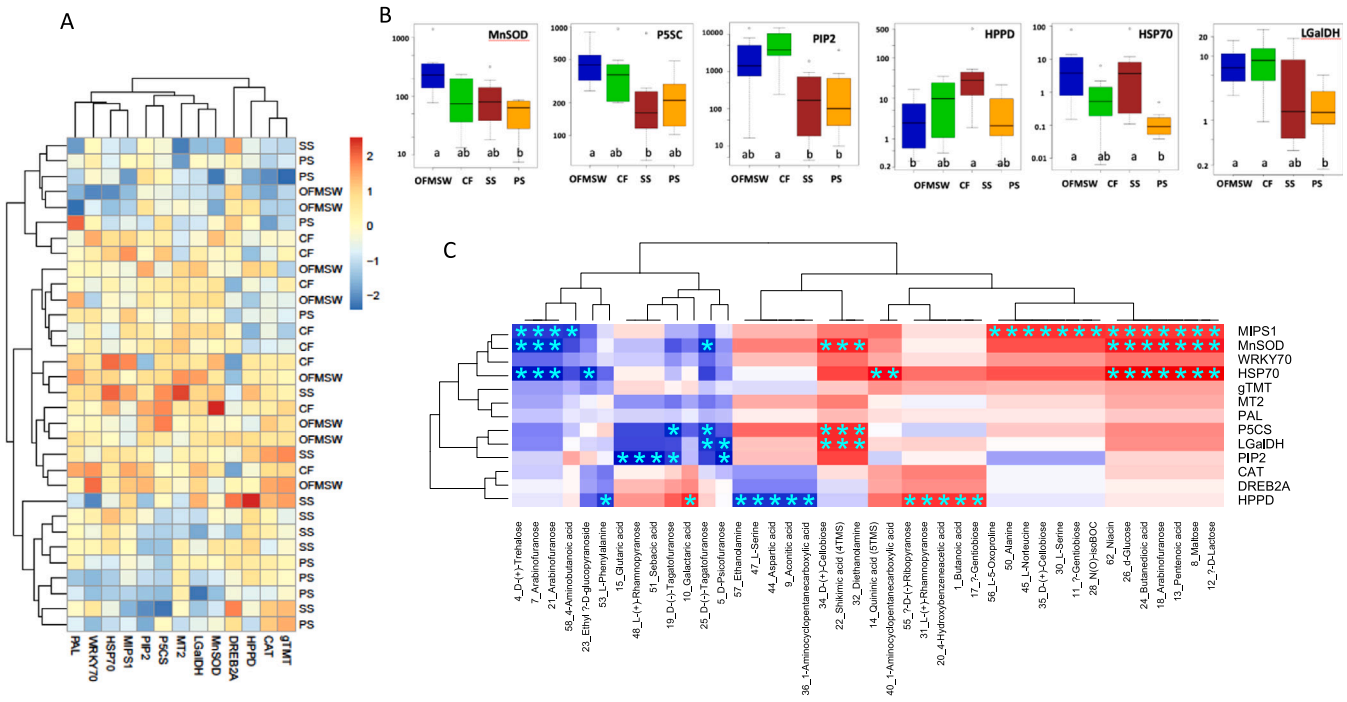

Fig. 6. Analysis of stress gene expression in lettuce leaves. (A) Heatmap plot for hierarchical clustering of the samples, based on gene expression patterns of 13 selected stress-responsive genes in leaves from lettuces fertilised with FC, OFSMW, SS or PS. Genes with similar behavior were grouped by hierarchical clustering. Red and cyan squares indicate positive and negative correlations, respectively (B) Boxplots comparing the expression of different genes in the four groups of samples. Lowercase letters at the bottom of the graph correspond to statistically different groups of data, calculated by ANOVA followed by Tukey's post hoc analysis. Only genes with significant differences among the groups are represented. Boxes cover the data distribution in the second and third quartiles, whiskers show the total distribution and a thick black bar within the boxes represent the median. Data are expressed as mRNA copies of the different genes per 1000 mRNA copies of EF4A, note the logarithmic scale on the Y-axis. C) Correlation map (Spearman's) between metabolites levels (average values for each sample group) and gene expression values. Red and blue sectors represent positive and negative correlations, respectively, cyan asterisks represent significant correlations (fdr, p<=0.05).

Table 3
Individual HQ and cumulative HQ for each of the detected AB in radish or lettuce edible parts according to the different fertilisation strategies. Data from vegetables fertilised with OFMSW and CF are not shown because none of the studied ABs was detected.

	Lettuce leaves				Radish roots			
	SS		PS		SS		PS	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
8-Hydroxyquinoline	-	-	4.3E-05	6.6E-05	-	-	5.8E-06	1.4E-05
Trimethoprim	-	-	-		-	-	5.2E-05	6.9E-05
Decarboxyl-ofloxacin *	-	1.6E-03	-	5.4E-03	-	-	-	-
Lincomycin	-	3.8E-05	5.0E-03	4.0E-05	-	1.3E-05	3.6E-05	1.5E-05
Sulfadiazine	-	-	-	-	-	-	4.0E-05	2.4E-05
Ciprofloxacin	-	6.1E-02	-	7.6E-02	2.9E-03	-	2.2E-02	1.3E-02
N-desmethyl Ofloxacin	-	9.2E-04	1.2E-03	1.5E-03	-	-	2.4E-04	4.0E-04
Ofloxacin	-	-	-	-	-	-	1.8E-04	2.0E-04
Ofloxacin Ethyl Ester*	-	-	-	-	-	-	1.6E-04	1.6E-04
Enrofloxacin	-	-	-	-	-	-	5.3E-04	5.7E-04
Sulfamethizole	1.5E-04	9.8E-05	-	1.1E-04	-	1.9E-05	-	4.8E-05
Sulfamethoxazole	-	-	-	-	-	-	-	-
Oxytetracycline	2.2E-05	-	3.5E-04	5.2E-04	8.5E-03	9.6E-03	7.9E-03	8.5E-03
Tetracycline	-	1.1E-04	2.7E-04	1.3E-04	-	8.9E-05	-	3.5E-05
Doxycicline	-	-	-	-	-	2.2E-04	5.4E-04	1.2E-04
Total HQ	**1.7E-04**	**6.4E-02**	**6.9E-03**	**8.4E-02**	**1.1E-02**	**9.9E-03**	**3.2E-02**	**2.3E-02**

*Transformation products

plants, but does not pose a human health risk.

The following conclusions can be drawn:

- Plant AB uptake depends on the compound and the plant variety, with concentrations ranging from undetectable to 76 ng/g in fw for oxytetracycline in radish roots. Repetitive fertilisation did not significantly increase the average AB concentration in edible vegetable parts.
- The use of OFMSW resulted in changes in lettuce and radish at the agronomic level, whilst the other fertilisers did not affect crop morphology or chlorophyll content compared with CF. The presence of ABs did not correlate with changes in crop morphology.
- The metabolic analysis highlighted that dopamine, a metabolite involved in the plant stress response, can be used as a crop tracer of fertilisation, but OFMSW resulted in a greater number of metabolic changes compared to the CF and the other assessed fertilisers. No

correlation was observed between metabolite changes and AB occurrence in lettuce leaves.

- Lettuce transcriptomic data indicate that fertilisation with OFMSW up-regulates the *PIP2* aquaporin gene, suggesting hydric stress, in keeping with the metabolomic analysis and agronomic parameters. This hypothesis would also explain the general higher expression of several stress-responsive genes in lettuces fertilised with CF.
- AB occurrence in vegetables repeatedly fertilised with SS or PS does not pose any potential human health risk (cumulative HQ <0.1), although further analysis is needed to understand long-term exposure to ABs in the human gut.

The present results indicate that using fertilisation products such as SS and PS in agriculture results in plant uptake of ABs, but plant morphological and metabolic changes are small, at least compared with the changes observed in lettuce fertilised with OFMSW. Plant uptake of ABs seems to be vegetable-specific. Therefore, future studies should include a larger number of crops, monitoring plant transcriptomic and metabolomic changes upon antibiotic exposure under controlled conditions.

CRediT authorship contribution statement

V. Matamoros: Conceptualization, Writing – original draft, Funding acquisition. **M. Escolà Casas:** Data curation, Formal analysis. **S. Mansilla**: Investigation. **Đ. Tadić:** Formal analysis. **N. Cañameras**: Investigation. **N. Carazo**: Investigation. **J. Portugal**: Writing – review & editing. **B. Piña**: Writing – review & editing. **S. Díez**: Review. **J.M. Bayona**: Writing – review & editing, Funding acquisition.

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.

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Novelty statement

This study assesses the impact of repetitive soil fertilisation (pig slurry, sewage sludge, and municipal compost) on crops (root and leaf edible parts) and human health under plot-scale conditions due to the occurrence of ABs. Furthermore, the occurrence of AB metabolites on crops, and the plant transcriptomic and metabolomic changes have been monitored for the first time following soil fertilisation. This expands the current knowledge on this topic as most of the previous studies were done under pot or greenhouse conditions. Therefore, the results will be relevant for scientific community, farmers and society.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129044.

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