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1 **Ecotoxicological impact of the antihypertensive valsartan on earthworms, extracellular**
2 **enzymes and soil bacterial communities**

3

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18

19 **Abstract**

20 The use of reclaimed water in agriculture represents a promising alternative to relieve
21 pressure on freshwater supplies, especially in arid or semiarid regions facing water scarcity.
22 However, this implies introducing micropollutants such as pharmaceutical residues into the
23 environment. The fate and the ecotoxicological impact of valsartan, an antihypertensive drug
24 frequently detected in wastewater effluents, were evaluated in soil-earthworm microcosms.
25 Valsartan dissipation in the soil was concomitant with valsartan acid formation. Although
26 both valsartan and valsartan acid accumulated in earthworms, no effect was observed on
27 biomarkers of exposure (acetylcholinesterase, glutathione S-transferase and carboxylesterase
28 activities). The geometric mean index of soil enzyme activity increased in the soils containing
29 earthworms, regardless of the presence of valsartan. Therefore, earthworms increased soil
30 carboxylesterase, dehydrogenase, alkaline phosphatase, β -glucosidase, urease and protease
31 activities. Although bacterial richness significantly decreased following valsartan exposure,
32 this trend was enhanced in the presence of earthworms with a significant impact on both alpha
33 and beta microbial diversity. The operational taxonomic units involved in these changes were
34 related to four (Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes) of the eight
35 most abundant phyla. Their relative abundances significantly increased in the valsartan-
36 treated soils containing earthworms, suggesting the presence of potential valsartan degraders.
37 The ecotoxicological effect of valsartan on microbes was strongly altered in the earthworm-
38 added soils, hence the importance of considering synergistic effects of different soil
39 organisms in the environmental risk assessment of pharmaceutical active compounds.

40

41 **Keywords:** Pharmaceuticals, ecotoxicology, microbial ecotoxicology, soil

42

43 **Capsule:**

44 Valsartan accumulates in earthworms but has no effect on earthworm enzyme activities. It

45 dissipates in the soil where four bacterial phyla increase in valsartan-spiked soils.

46 **Introduction**

47 Within the context of the global change, water resource shortage is becoming a critical issue
48 in arid and semi-arid areas such as the Mediterranean basin, one of the main vegetable and
49 fruit producers for all European consumers. To cope with this shortage, the European Union
50 proposed wastewater reuse for agricultural irrigation and aquifer recharge (European
51 Commission, 2015). A guideline describing the minimum quality requirements of wastewater
52 and the risks of wastewater reuse for crop irrigation grown on arable soils was published in
53 2017 (Alcalde-Sanz and Gawlik, 2017). However, information on the possible
54 ecotoxicological effects of wastewater-borne micropollutants on soil-borne organisms and the
55 supported ecosystem functions is obviously lacking.

56 Valsartan (an antagonist of the angiotensin II type-1 receptor) is one of the most highly
57 prescribed antihypertensive drug for high blood pressure treatment (Sörgel et al., 2019). It
58 reduces the risk of fatal and nonfatal cardiovascular events such as strokes, myocardial
59 infarctions, and complications when heart attacks result from abrupt increases in blood
60 pressure (Andresen et al., 2017; Criscione et al., 1995; Jugdutt, 2006). Like other
61 pharmaceuticals, valsartan is excreted via the urine and feces, reaches the sewer systems, and
62 is transported to wastewater treatment plants (WWTPs) to be fully or partially removed
63 through a combination of processes (Auvinen et al., 2017; Bayer et al., 2014; Kovalova et al.,
64 2012; Margot et al., 2013). Valsartan and its main transformation product valsartan acid are
65 poorly eliminated by conventional WWTPs (Gurke et al., 2015). Both are frequently detected
66 at relatively high concentrations (a few μg per L for valsartan and up to 150 ng per L for
67 valsartan acid) in WWTP effluents (Batt et al., 2008; Bayer et al., 2014; Botero-Coy et al.,
68 2018; Gurke et al., 2015; Kostich et al., 2014; Santos et al., 2013). Discharge of WWTP
69 effluents in the rivers leads to the contamination of surface waters and other aquatic
70 environments with valsartan (Godoy et al., 2015; Kasprzyk-Hordern et al., 2009, 2008;

71 Klosterhaus et al., 2013; Nödler et al., 2013). The effects of frequently found pharmaceuticals
72 in wastewater on soil microorganisms (Barra Caracciolo et al., 2015; Gallego and Martin-
73 Laurent, 2020) and terrestrial invertebrates (Carter et al., 2020, 2014; Carter and Kinney,
74 2018) have been previously documented. On aquatic organisms, valsartan showed no acute
75 toxic effect on the freshwater alga *Desmodesmus subspicatus* at concentrations up to 120
76 mg·L⁻¹ (Bayer et al., 2014), and a non-observed-effect concentration (NOEC) of 12.5 mg·L⁻¹
77 and a lowest-observed-effect concentration (LOEC) of 25 mg·L⁻¹ on sea urchin (*Lytechinus*
78 *variegates*) (Yamamoto et al., 2014). To our knowledge, only two publications have
79 addressed the effects of candesartan and losartan, two antihypertensive sartans, in the gut
80 microbiome in rats (Robles-Vera et al., 2020; Wu et al., 2019) and no information is available
81 as to its ecotoxicity to terrestrial macro- and micro-organisms.

82 Earthworms and microorganisms represent the largest part of the living biomass in soils. They
83 support essential soil functions that contribute to soil health and ecosystem services (Barrios,
84 2007; Blouin et al., 2013; Costanza et al., 1997; Delgado-Baquerizo et al., 2020; Hanajík,
85 2016; Pulleman et al., 2012). Earthworms are commonly considered as soil engineers (Jones
86 et al., 1994). Their continual burrowing and feeding activities modify the physicochemical
87 and biological properties of soils where they bore galleries and thus increase soil macro
88 porosity. Earthworms also disperse microorganisms in the bulk soil through their continual
89 burrowing activity (Yang and van Elsas, 2018). Bioturbation thereby modifies bacterial and
90 fungal habitats and leads to important changes not only in their abundance, composition and
91 diversity but also in the functions they support such as the biodegradation of pesticides and
92 other organic pollutants (Bart et al., 2019; Kersanté et al., 2006; Sanchez-Hernandez et al.,
93 2015). Due to their sensitivity to pollutants (Pelosi et al., 2013), soil-dwelling earthworms
94 such as *Lumbricus terrestris* are used as bioindicators of environmental contaminants (Fründ
95 et al., 2011; Rodríguez-Castellanos and Sanchez-Hernandez, 2007; Solé, 2020). The

96 measurement of biochemical markers in earthworms as a screening method to estimate the
97 bioavailability and toxicity of soil pollutants arouses more and more interest (Sanchez-
98 Hernandez, 2006). Some of these biological indicators have indeed been used to assess
99 genotoxicity, oxidative stress, and alterations in the metabolic profile of earthworms exposed
100 to pharmaceutical-contaminated soils (Dong et al., 2012; Lin et al., 2012; McKelvie et al.,
101 2011). Despite these ecotoxicological studies, information on the role of earthworms in
102 pharmaceutical dissipation in soils is scant, even though they stimulate soil microbial
103 proliferation (Brown et al., 2000; Dempsey et al., 2013).

104 Soil microorganisms are the most abundant and diverse organisms in terrestrial ecosystems
105 (Fierer and Jackson, 2006; Locey and Lennon, 2016; Pedrós-Alió and Manrubia, 2016). They
106 fulfill an impressive range of functions that support biogeochemical cycles, contribute to food
107 supply and water quality, regulate greenhouse gas emissions, and participate in soil
108 detoxification processes. Loss of microbial diversity may alter multiple soil functions and
109 services, with tremendous consequences on the global ecosystem (Delgado-Baquerizo et al.,
110 2017, 2016; Philippot et al., 2013; Wagg et al., 2014). On the one hand, pollutants such as
111 pesticides, pharmaceuticals and personal care products (PPCPs) can indirectly affect soil
112 microorganisms by inhibiting enzymes involved in key microbial processes (Feld et al., 2015;
113 Puglisi et al., 2012), so that the survival of certain microbial guilds and associated functions is
114 compromised (Karpouzias et al., 2014; Romdhane et al., 2016). On the other hand, repeated
115 exposure to pollutants may induce the emergence of microorganisms able to metabolize them
116 to obtain nutrients and energy (Arbeli and Fuentes, 2007; Devers et al., 2008; El-Sebai et al.,
117 2005). In this context, soil microorganisms represent relevant bioindicators of soil quality
118 (Thiele-Bruhn et al., 2020). Thanks to the latest developments in microbial ecotoxicology, a
119 diverse toolbox can be used to assess the impact of pollutants on the abundance, composition,
120 diversity and activity of soil microbial populations by means of -omics methodologies

121 (Gallego et al., 2019; Storck et al., 2018). In parallel, the use of soil extracellular enzymes has
122 long been used in environmental risk assessment of soil contamination and deterioration (Rao
123 et al., 2014; Sanchez-Hernandez et al., 2018, 2017). Soil enzymes, mainly produced by
124 microorganisms and plant roots, catalyze multiple biochemical processes that drive nutrient
125 cycles, organic matter decomposition, and organic pollutant breakdown (Burns et al., 2013;
126 Caldwell, 2005; Sanchez-Hernandez et al., 2015). Soil enzyme activities are also altered by
127 pharmaceuticals (Cycon et al., 2016; Molaei et al., 2017), so they can be used as indicators of
128 soil disturbance, together with other microbial parameters (e.g., those derived from diversity
129 assessment). Taken together, these studies suggest that the proliferation of microorganisms
130 induced by earthworms could reduce the impact of pharmaceuticals on soil functioning.
131 Therefore, we hypothesized that adding anecic earthworms (*L. terrestris*) to agricultural soil
132 could be an eco-friendly strategy to alleviate the toxicity of pharmaceuticals and reduce their
133 potential uptake by edible plants.

134 The aims of this study were to i) evaluate the environmental fate of valsartan in agricultural
135 soils added with *L. terrestris* or not under a predicted worst-case scenario, and ii) assess the
136 ecotoxicological impact of valsartan on earthworms and soil microorganisms. Dissipation of
137 valsartan, the production of its main transformation product – valsartan acid – and its
138 bioaccumulation in earthworms were monitored in valsartan-treated soils using liquid
139 chromatography-high resolution mass spectrometry (LC-HRMS). The ecotoxicological
140 effects of valsartan were assessed by measuring earthworm biomarkers (weight changes and
141 enzyme activities) and assessing bacterial composition and diversity (by MiSeq sequencing of
142 16S rRNA amplicons) and soil microbial activity (by measuring extracellular enzyme
143 activities). This study shows the interest for a multilevel framework based on the microbiota
144 and the macrofauna, and their interactions for the environmental risk assessment of
145 pharmaceuticals on soil borne organisms.

146

147 **2. Materials and Methods**

148 2.1 Chemicals

149 High-purity valsartan (VST) for a reference standard was purchased from Sigma-Aldrich
150 (98% purity, St. Louis, MO, USA). High-purity (mostly 90%) valsartan acid (VSA) and
151 deuterated compounds (d3 and d4 for VST and VSA, respectively) were obtained from
152 Toronto Research Chemicals (Toronto, ON, Canada). LC-MS grade acetonitrile (ACN)
153 ($\geq 99.9\%$), methanol (MeOH) ($\geq 99.9\%$), water and di-sodium hydrogen phosphate dehydrate
154 ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were purchased from Merck (Darmstadt, Germany). Citric acid
155 monohydrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) and ethylenediaminetetraacetic acid anhydrous (EDTA) ($\geq 99\%$)
156 were supplied by Sigma-Aldrich (St. Louis, MO, USA), while ammonium fluoride was
157 bought from Fisher Chemical (Fisher Scientific SL, Madrid, Spain). The Original non-
158 buffered (OR) QuEChERS extraction salts kit (4g MgSO_4 + 1g NaCl) and the buffered
159 European EN 15662 kit (EN) (4g MgSO_4 ; 1g NaCl; 1g sodium citrate; 0.5 g disodium citrate
160 sesquihydrate) were obtained from BEKOlut GmbH & Co. KG (Hauptstuhl, Germany). Oasis
161 PRiME HLB Cartridges (3 cc, 150 mg) were purchased from Waters (Waters Corporation,
162 Milford, MA, US). Individual standard stock solutions (at a concentration of $1,000 \mu\text{g} \cdot \text{mL}^{-1}$)
163 and stock solutions of isotopically labeled compounds (at $1,000 \text{mg} \cdot \text{L}^{-1}$ too) used as internal
164 standards (IS) were prepared in MeOH and stored at $-20 \text{ }^\circ\text{C}$. The mixtures containing VST
165 and VSA (at $2 \mu\text{g} \cdot \text{mL}^{-1}$) and the working IS solution (VST-d3 and VSA-d4, $2 \mu\text{g} \cdot \text{mL}^{-1}$) for
166 analysis and calibration were prepared by diluting adequate volumes of the individual stock
167 solutions with MeOH. All these solutions were stored at $-20 \text{ }^\circ\text{C}$.

168

169 2.2 Soil characteristics and experimental setup

170 Pristine soil (loam, pH 8.5, total organic matter 2.91%, organic carbon content 1.68%, total
171 nitrogen 0.171%) never exposed to pesticides before was collected from the Parc Agrari of
172 Llobregat (Barcelona, SP). The soil was placed without sieving in 1.5-L pots containing 1.5
173 Kg dw of soil, equivalent to 1 L in volume. Four different treatments were tested in triplicate:
174 a) earthworm- and VST-free soil (CTRL), b) soil added with earthworms (EW), c) soil treated
175 with VST (VST) and d) VST-treated soil with earthworms added (VST+EW). VST dissolved
176 in 10 mL of methanol was sprayed over the soil (1% v/v) and left 3 hours to allow methanol
177 to evaporate to reach a final concentration of $5 \text{ mg}\cdot\text{kg}^{-1}$ (equivalent to $11.5 \text{ }\mu\text{mol}\cdot\text{kg}^{-1}$). The
178 same quantity of methanol was sprayed onto the CTRL soils. Soil humidity was then adjusted
179 to 22-25% with sterile water and kept constant throughout incubation. The soil was
180 thoroughly mixed to facilitate VST homogenization. Then, ten earthworms (*Lumbricus*
181 *terrestris*, Linnaeus, 1758) from commercial vermiculture (Decathlon) were added per pot.
182 All earthworms were kept in clean soil for 7 days before the start of the experiment for
183 acclimation. They were fed with organic oats placed on top of the soil throughout incubation.
184 The experiment was carried out in a chamber, at 15°C with a 12h photoperiod (photosynthetic
185 active radiation (PAR) = $60 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The pots were covered with a thin mesh that
186 allowed light in and prevented worms from escaping.

187 Homogeneous samples of the soil column (about 30 g) were collected 0, 7, 14 and 21 days
188 after the start of the experiment and stored at -20°C until use. At each sampling time, two or
189 three earthworms per pot were collected for each condition and treated as described below.

190 2.3 Soil and earthworm sample preparation; pharmaceutical extraction

191 For each soil sample, 10 g of wet material were dried overnight under a fume hood, ground in
192 a mortar and sieved at 2 mm pore size. Pharmaceuticals were extracted following a modified
193 QuEChERS procedure. In a single extraction step, 10 g of soil (dry weight equivalent) were
194 mixed with 3 mL of acetone and 50 μL of IS mix in a 50-mL centrifuge tube to obtain a final

195 concentration of 10 ng·g⁻¹. Samples were then vortexed for 2 minutes at 2,500 rpm using a
196 BenchMixer XLQ QuEChERS Vortex (Benchmark Scientific, Sayreville NJ, US) and left
197 under the hood at room temperature overnight, to let the solvent evaporate. The following
198 day, the samples were hydrated by adding 8 mL of EDTA-McIlvaine buffer (pH = 4) (ESM),
199 vortexed, and left to rest for one hour before extraction. Ten mL of acetonitrile were added to
200 each hydrated sample, and each sample was vortexed 2 minutes at 2,500 rpm. The OR
201 QuEChERS salts kit was emptied into the sample and immediately hand shaken for 30
202 minutes to prevent salt agglomeration. Then, all the samples were vortexed for another 2
203 minutes at 2,500 rpm using a BenchMixer XLQ. Finally, the tubes were centrifuged for 10
204 minutes at 4,000 rpm and 4°C, and 1 mL of the supernatant was transferred to a 2-mL glass
205 vial and evaporated under gentle nitrogen flow at 24°C using a TurboVap® LV (Biotage AB,
206 Uppsala, Sweden) until total dryness, and then reconstituted with 1 mL of water/MeOH
207 (90:10, v/v) for injection.

208 Earthworm samples were extracted according to the following procedure (Montemurro et al.,
209 2021). Briefly, 0.5 g of freeze-dried earthworm powder were rehydrated in 8 mL of pure
210 water in 50-mL falcon tubes that were vortexed and left on the bench for half an hour.
211 Thereafter, 50 µL of IS solution were added (final concentration 20 ng·g⁻¹) to the sample that
212 was vortexed (2,500 rpm, 2.5 min) and incubated for 30 minutes at room temperature. Then,
213 10 mL of ACN were added to the samples, vortexed for 2 minutes at 2,500 rpm, added to the
214 EN QuEChERS salt kit and immediately hand shaken to prevent agglomeration of the salts.
215 All the samples were vortexed another time, centrifuged (4,000 rpm, 10 min, 4 °C), and each
216 supernatant was recovered and cleaned up by gravity with the Oasis PRiME HLB Cartridge to
217 remove co-extractives from the matrix. Finally, 1 mL of purified extract was transferred into
218 an injection vial, evaporated under a gentle nitrogen flow at room temperature to total dryness
219 and recovered in 1 mL of water/MeOH (90:10, v/v).

220 All detailed information on the LC-MS/MS methodology is described elsewhere
221 (Montemurro et al., 2021)

222

223 2.4 Earthworm biomarkers

224 Earthworms were individually placed in petri dishes and left for 48 hours in a 15°C incubator
225 to empty their gastrointestinal tracts and avoid interference with enzyme measurements. Then,
226 they were frozen in liquid nitrogen and kept at -80°C until use. Whole animals were
227 individually ground with a Mills MM400 mixer for 1.5 min at 28c·s⁻¹ frequency using 50-mL
228 stainless steel capsules submerged in liquid nitrogen to obtain a homogenous powder; 0.3 g of
229 tissue per worm were collected for biomarker analysis, and the remaining tissue was used for
230 pharmaceutical analysis. For biomarker analysis, each 0.3 g of worm powder was mixed at
231 1:5 (w:v) with a solution of 20 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid
232 (EDTA) (pH 7.4) and sonicated 3 times for 10 sec. Each homogenized sample was
233 centrifuged (10,000 g, 20 min, 4°C), and then the supernatant was collected and stored at -
234 80°C until use.

235 Acetylcholinesterase (AChE, EC 3.1.1.7) activity was determined according to Ellman et al
236 (1961). Briefly, 25 µL of earthworm homogenate were added to acetylthiocholine (ATC; 1
237 mM) and dithiobisnitrobenzoate (DTNB; 0.180 mM). The product was quantified at 412 nm,
238 using $\epsilon=1.36 \cdot 10^4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

239 Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured following the method
240 described by Habig et al. (1974). In short, 25 µL of reduced glutathione (1 mM) was mixed to
241 1-chloro-2,4-dinitrobenzene substrate (1 mM). The reaction was monitored continuously for 5
242 min at 340 nm and quantified using $\epsilon=9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

243 Carboxylesterase (CE, EC 3.1.1.1) activity was measured using different substrates: 1 mM p-
244 nitrophenyl acetate (4NPA) and p-nitrophenyl butyrate (4NPB) according to Hosokawa and
245 Satoh (2002), and 0.25 mM 1-naphthyl acetate (1NA), 1-naphthyl butyrate (1NB) and 2-
246 naphthyl acetate (2NA) according to Mastropaolo and Yourno (1981). The appropriately
247 diluted sample was mixed with the respective substrates in 50 mM phosphate buffer (pH 7.4).
248 The formation of the product (4-nitrophenolate or 1-naphthol at 405 nm or 235 nm,
249 respectively) was measured in triplicate at 25°C using a TECAN infinite 200 microplate
250 reader. Extinction coefficients (ϵ) of $1.8 \cdot 10^4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $2.34 \cdot 10^4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ were used to
251 calculate enzyme activities (in $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) representing the hydrolysis of
252 nitrophenyl esters and naphthyl-derived esters, respectively.

253 The total protein content was determined by the Bradford method (1976) using the Bradford
254 Bio-Rad Protein Assay reagent. A series of ten-fold dilutions of bovine serum albumin
255 solution (BSA; $0.05\text{-}0.5 \text{ mg} \cdot \text{mL}^{-1}$) was prepared to establish a standard curve and quantify
256 total proteins in the homogenates.

257

258 2.5 Soil enzyme analysis

259 Potential extracellular enzyme activities (carboxylesterase, β -glucosidase, urease, and alkaline
260 phosphatase) were measured in soils according to Sanchez-Hernandez et al. (2018). Briefly,
261 wet soils were mixed in distilled water (1:50, w/v) using an orbital shaker (50 rpm for 30 min)
262 and the enzymes activities were measured by discontinuous colorimetric methods.

263 Carboxylesterase was measured according to Sanchez-Hernandez et al., (2017) using 1-
264 naphthyl butyrate (2.5 mM final concentration): the hydrolysis product (1-naphthol) was
265 revealed with Fast Red and measured at 530 nm.

266 Alkaline phosphatase and β -glucosidase activities were assayed according to Popova and
267 Deng (2010) using 4-nitrophenyl phosphate and 4-nitrophenyl β -D-glucanopyranoside (4 mM
268 final concentration, respectively), as the substrate of the reactions. For both enzymatic
269 reactions, the product (4-nitrophenolate) was measured at 405 nm after 4h reaction.

270 Urease activity was determined following the procedure by Schinner et al. (1996) using urea
271 (80 mM) as substrate: formed ammonium was measured at 690 nm after 1h of reaction

272 Protease activity was measured according to Schinner et al. (1996). Soil suspensions were
273 incubated for 2 h in the presence of 2% w/v casein (substrate): formed aromatic amino acids
274 were measured at 700 nm with the Folin-Ciocalteu's phenol® reagent (Sigma-Aldrich).

275 Dehydrogenase activity was measured according to von Mersi and Schinner (1991) using
276 iodonitrotetrazolium chloride as substrate: after 1 h of reaction, formed iodonitrotetrazolium
277 formazan (INTF) was extracted with ethanol and N,N-dimethylformamide (1:1, v/v), and
278 measured at 464 nm.

279 All enzyme assays were performed in quadruplicate and using 96-well bottom flat microplates
280 (alkaline phosphatase, β -glucosidase, carboxylesterase), 1.5-ml microfuge tubes (urease,
281 protease), or 10-ml tubes (dehydrogenase). Calibration curves were constructed with the
282 reagents 4-nitrophenol (alkaline phosphatase, β -glucosidase), 1-naphthol (carboxylesterase),
283 INTF (dehydrogenase), urea (urease), and tyrosine (protease), dissolved in the reaction
284 mixture of the corresponding enzyme assays. Enzyme activities were expressed on a basis of
285 dry mass.

286

287 2.6 Enzyme indexes

288 The functional diversity of the selected soil enzymes was assessed using the geometric mean
289 index (GMean) (Lessard et al., 2014) calculated as follows:

290
$$GMean = \left(\prod_{i=1}^n y_i \right)^{\frac{1}{n}}$$

291 Where y_i is the mean value for each enzyme activity, and n is the total number of soil
292 enzymes.

293

294 2.7 Composition and diversity of the soil bacterial community

295 Soil DNA was extracted using the Power soil DNA isolation kit (Qiagen, Germany) and
296 quantified with the Quant-iT™ PicoGreen® dsDNA Assay kit (Invitrogen, France) according
297 to the manufacturer's recommendations. Bacterial community composition and diversity were
298 determined by Mi-Seq sequencing of 16S rRNA amplicons generated with a two-step PCR as
299 described in (Gallego et al., 2020). Sequences were deposited in the GenBank and submitted
300 to the sequence read archive (SRA) under the accession numbers PRJNA630861:
301 SRR11727342-SRR11723789. Bacterial α -diversity indices describing richness (Chao1),
302 evenness (Simpson reciprocal) and relatedness (PD whole tree) were calculated based on
303 rarefied tables (37,000 sequences per sample) (Suppl. Table S1 and Figure S1). In total, after
304 de-multiplexing and removal of low-quality raw sequence reads, amplicon sequencing
305 generated 8,883,345 high-quality sequences with an average sequence length of 464 ± 11 bp
306 (Suppl. Table S1). Using a threshold at 94% nucleotide sequence identity, these sequences
307 were grouped in 25,515 different OTUs.

308 UniFrac distance matrices were also computed to detect changes in microbial community
309 composition. Constrained analysis of principal coordinates (CAP) of OTUs on weighted and
310 unweighted UniFrac distance matrices was also performed and plotted. The relative abundance
311 of the different bacterial phyla was also determined using a comparative bar chart.

312

313 2.8 Statistical analyses

314 All statistical analyses were performed in R (<http://www.r-project.org>). The normality of the
315 data and residuals was checked using Shapiro Wilk's test ($p > 0.05$) and the homogeneity of
316 variances was verified using Levene's test ($p > 0.05$). Inverse and log-10 transformations of the
317 data were performed when necessary. Two-way ANOVA followed by Tukey's test (using
318 time and treatments as factors) and pairwise Student's t-test were used to determine
319 differences. For the statistical analysis of earthworm biomarkers, each value was calculated as
320 the mean ($n=2$ or $n=3$) of the individual values from earthworms from a same pot. To detect
321 significant differences in community structure among sample types, permutational
322 multivariate analysis of variance (PerMANOVA) was used on weighted and unweighted
323 Unifrac distance matrices using the Adonis function in R "vegan" package (Oksanen et al.,
324 2018). Significant differences in OTU abundance between the different treatments were
325 detected using the "dds" function in R DESeq2 package (Love et al., 2014). The relative
326 abundances of the selected OTUs were then visualized using the Interactive Tree of Life
327 (iTOL) webserver (Letunic and Bork, 2011)

328

329 **3. Results**

330 3.1 Soil and earthworm valsartan and valsartan acid concentrations

331 VST and VSA were monitored in soil and earthworms (Figure 1). VST concentrations
332 decreased over time in both the worm-added and worm-less soil microcosms. VST
333 concentrations in the worm-less microcosms were significantly lower at the end of the
334 incubation period than on d0 ($p=0.002$). VSA was detected barely above the detection limit
335 ($0.07 \pm 0.009 \mu\text{mol kg}^{-1}$) at the beginning of the experiment, and significantly increased
336 throughout incubation in both worm-added and worm-less microcosms ($p=0.00004$).

337 Valsartan and VSA were detected in earthworms after 7 days of exposure. VST
338 concentrations increased throughout incubation, and reached $92.3 \pm 13.3 \mu\text{mol kg}^{-1}$ after 21
339 days of incubation. This was almost one order of magnitude higher than the applied VST
340 concentration ($11.5 \mu\text{mol kg}^{-1}$). As for the VSA concentration, it reached $5.9 \pm 2.2 \mu\text{mol kg}^{-1}$
341 after 21 days of incubation. This seems to indicate that VST is bioaccumulated by earthworms
342 ($\text{BAF}_{\text{VST}} = 20.50 \pm 32.60$ and $\text{BAF}_{\text{VSA}} = 4.51 \pm 3.72$, bioaccumulation factor (BAF)
343 calculated as total VST or VSA ($\mu\text{g}\cdot\text{g}^{-1}$) in the earthworms at 21 days/ total VST or VSA
344 ($\mu\text{g}\cdot\text{g}^{-1}$) in the soil at 21 days).

345

346 3.2 Earthworm biomarkers

347 First, we assessed a possible effect of time and incubation on earthworms by measuring
348 selected biomarkers in VST-free soils. Differences in body weight ($p > 0.12$), AChE ($p = 0.807$),
349 GST ($p = 0.909$) and most of CE activities (4NPA, 4NPB, 1NA, 2NA) ($p \geq 0.06$) remained non-
350 significant throughout the 21 days of incubation (Suppl. Figure S2).

351 Then, we assessed the potential sublethal toxic effects of VST on earthworms. VST did not
352 significantly affect AChE ($p = 0.578$) or GST ($p = 0.56$) activities throughout incubation (Figure
353 S3). However, CE activity responded to VST exposure in a different way depending on the
354 substrate used in the enzyme assay, even though the response of this biomarker was not
355 statistically significant ($p > 0.09$). In non-exposed earthworms, a decrease trend was observed
356 for CE activity measured with acetate-derived esters substrates (4NPA, 1NA and 2NA),
357 whereas a slight increase in CE activity was detected with butyrate-derived esters substrates.

358

359 3.3 Soil enzyme activities

360 Extracellular enzyme activities remained unchanged during incubation in both control
361 (earthworm- and VST-free) soils and VST-treated soils ($p>0.09$) (Suppl. Figure S4 and Suppl.
362 Figure S5). However, a significant increase in carboxylesterase ($p=0.01$), alkaline
363 phosphatase ($p=0.0002$), β -glucosidase ($p=0.002$) and urease ($p=0.007$) activities was
364 observed after 14 days of incubation in the VST+EW soils as compared to the control soils
365 (Figure S5). Alkaline phosphatase activity significantly increased in non-treated EW soils
366 compared to control soils after 14 days ($p=0.003$) and 21 days ($p=0.003$). The VST+EW and
367 EW soils did not display significant differences in any enzyme activity after 14 days of
368 incubation ($p\geq 0.97$). In addition, protease activity was significantly higher in the VST+EW
369 and EW soils after 21 days as compared to the control soils ($p=0.02$ and $p<0.05$, respectively).
370 Interestingly, after 21 days of incubation, β -glucosidase activity was significantly higher in
371 the VST+EW soil microcosms than in all other microcosms that produced similar activity,
372 suggesting a synergistic effect of earthworms and VST on β -glucosidase activity ($p<0.02$).
373 Based on all our measurements, the geometric mean index (GMean) was calculated to assess
374 the effect of earthworms and VST on soil enzyme activities (Figure 2). After 14 and 21 days
375 of incubation, the GMean was significantly higher in the EW soil microcosms, whether
376 treated with VST or not, than in the EW-less microcosms. Therefore, the presence of
377 earthworms promoted soil enzyme activities ($p<0.04$ and $p<0.04$, respectively).

378

379 3.4 Soil bacterial community composition and diversity

380 Bacterial community diversity was assessed by calculating several α and β -diversity indices.
381 Firstly, to assess a possible time effect, α and β -diversity indices from control soil samples at
382 different incubation times were analyzed statistically. The differences in α -diversity indices
383 describing richness (Chao1, $p=0.84$) and relatedness (PD whole tree, $p=0.05$) over time were

384 non-significant. However, the differences in α -diversity evenness indices (Simpson's
385 reciprocal index, $p=0.02$) between d7 and d0 were significant, indicating a time effect (Table
386 S2). The differences in all α -diversity indices after d7 were non-significant ($p>0.05$). β -
387 diversity analysis using canonical analysis of principal coordinates (CAP) revealed significant
388 differences in bacterial community composition in the control soil over time ($p=0.001$ and
389 $p=0.003$ for weighted and unweighted Unifrac distance matrices, respectively). The bacterial
390 community of the control soil microcosms on days 7, 14 and 21 was separated along CAP1
391 (accounting for 39.3% and 13.1% of explained variance, respectively) (Figure S6a and S6b).

392 The effect of VST on soil microbial diversity was assessed on d0 and throughout incubation.
393 On d0, no significant difference was observed in α and β -diversity indices between the worm-
394 less microcosms treated or untreated with VST ($p>0.1$) (Table S2 and Figure S7). Exposure to
395 VST significantly decreased Simpson reciprocal index throughout incubation ($p=0.01$), but
396 did not modify the α -diversity Chao1 and PD whole tree indices ($p=0.09$ and $p=0.06$) (Figure
397 3 and Table S3).

398 Overall, the presence of earthworms decreased α -diversity indices, but only Simpson
399 reciprocal index was significantly lower after 14 and 21 days as compared to the worm-less
400 control ($p=0.001$ and $p=0.02$, respectively) (Figure 3). In the VST+EW microcosms, α -
401 diversity decreased as compared to the control microcosms, with significant differences after
402 7 days for Simpson reciprocal index ($p<0.0002$) and after 14 days for the other two indices
403 ($p=0.04$ for Chao1 and $p<0.0005$ for PD whole tree).

404 VST had a significant effect on bacterial β -diversity throughout incubation. CAP ordinations
405 explained 92.3 and 43.5% of the variance (48.84 and 33.52% on the first axis and 15.18 and
406 5.03% on the second axis for weighted and unweighted Unifrac distance matrices,
407 respectively) (Figure 3 and Figure S8). The bacterial community composition of the EW
408 microcosms unexposed to VST was clearly separated from the control along CAP1. In

409 response to VST exposure, the bacterial composition of the EW-less microcosms was
410 separated from the control along CAP2. Similarly, VST exposure led to changes in the
411 bacterial composition of the EW-less microcosms. Recovery of the bacterial composition
412 following exposure to VST was observed along CAP2 in both EW and EW-less microcosms
413 over time.

414 The effect of VST and earthworms on soil bacterial diversity was also assessed.
415 Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, Chloroflexi,
416 Gemmatimonadetes, Verrucomicrobia and Firmicutes were the 8 most abundant bacterial
417 phyla in the soil samples. They represented altogether up to 98% of bacterial abundance. The
418 relative abundance of Proteobacteria ($p=0.003$), and Acidobacteria ($p=0.01$) significantly
419 increased in the control microcosms, while the abundance of Actinobacteria initially
420 significantly decreased ($p=0.01$) but the difference was no longer found after 7 days ($p>0.06$)
421 (Figure S6). Exposure to VST significantly increased the abundance of Proteobacteria after 7
422 days of incubation ($p<0.00002$) (Figure S0), and also significantly decreased the relative
423 abundance of Bacteroidetes ($p=0.004$) after 7 days and of Actinobacteria ($p=0.0001$) and
424 Acidobacteria ($p=0.0001$) after 14 days as compared to the control. Interestingly, the relative
425 abundance of Proteobacteria was significantly higher in the VST+EW microcosms than in the
426 control microcosms on d7, and remained so throughout incubation ($p<0.03$).

427 To further evaluate the effect of VST and earthworms at a lower taxonomic level, the OTUs
428 represented in at least half of the samples (a total of 8,974 different OTUs) were analyzed
429 using the DESeq2 R package. Three hundred and fifteen OTUs ($p=1\cdot 10^{-9}$) were selected as
430 being involved in the differences among treatments. The relative abundances of these
431 discriminant OTUs were then visualized using an iTOL phylogenetic tree (Figure 4). Most of
432 them were affiliated to 4 of the most 8 abundant bacterial phyla (Proteobacteria,
433 Bacteroidetes, Actinobacteria, and Firmicutes). Their relative abundances were strongly

434 affected in different ways. For instance, OTUs belonging to Gammaproteobacteria and
435 Bacteroidetes, highly represented in the EW microcosms, significantly increased throughout
436 incubation under VST exposure. The relative abundance of OTUs related to
437 Betaproteobacteria was higher in the VST microcosms. It also increased importantly in the
438 EW microcosms unexposed to VST over time. Furthermore, the relative abundance of OTUs
439 related to Firmicutes, Tenericutes, Actinobacteria, Verrucomicrobia, Deltaproteobacteria and
440 Alphaproteobacteria increased in the VST+EW microcosms over time.

441

442 **4. Discussion**

443 The fate and ecotoxicological effect of VST and its main transformation product VSA were
444 investigated in a soil-earthworm microcosm experiment under a predicted worst-case
445 scenario. The scenario included a continuous input of VST (irrigation with treated
446 wastewater), soil fertilization with VST-contaminated biosolids, a high soil organic matter
447 content and a low degradation rate. To date, the environmental fate of VST had only been
448 evaluated in municipal biosolids applied to a soil used in greenhouse experiments (Sabourin et
449 al., 2012) and in river samples (Nödler et al., 2013). VST was measured in the soil and
450 earthworm samples by combining an extraction method based on QuEChERS and LC-HR/MS
451 analyses. VST concentrations significantly decreased throughout incubation in both EW and
452 EW-less control soil microcosms as compared to d0. In parallel, VSA was formed, and its
453 concentrations increased over time. The absence of VSA at the beginning of the experiment
454 indicates that all the recovered VSA resulted from VST transformation. Interestingly, at the
455 end of incubation, a significant increase in the VSA concentrations was observed in both EW
456 and EW-less soils. It was slightly lower in the EW soils, probably due to uptake by
457 earthworms. After 7 days of exposure, both VST and VSA were detected in earthworms. By
458 the end of incubation, earthworms contained 16 times more VST than VSA, and almost 10

459 times more VST than the VST concentration initially spiked in the soils. Therefore,
460 earthworms bioaccumulated VST (Pearson et al., 2000; Zhang et al., 2009). In line with these
461 results, Bergé and Vuillet (2015) showed that pharmaceuticals could be taken up from soils
462 and accumulate in earthworms. Then, they can contaminate higher trophic levels of the food
463 chain via bioaccumulation and biomagnification processes (Shore et al., 2014).

464 Although VST and VSA accumulated in earthworms, AChE, GST, and CE activities
465 remained unchanged. Pesticides and other organic pollutants can inhibit AChE, leading to
466 acetylcholine accumulation and malfunctioning of the nervous system of earthworms (Caselli
467 et al., 2006; Saint-Denis et al., 2001). Under our conditions, VST and VSA did not seem to be
468 neurotoxic to *L. terrestris*. Similarly, GST activity can inactivate a broad range of xenobiotics
469 and endogenous metabolites via conjugation, and thus favor their excretion (LaCourse et al.,
470 2009). GST activity increased in earthworms exposed to pesticides (Aly and Schröder, 2008;
471 Łaszczyca et al., 2004; Maity et al., 2008), but did not increase following exposure to VST in
472 our conditions. This suggests that *L. terrestris* might not have inducible GST enzymes, as
473 observed by Stokke and Stenersen in *Eisenia andrei* earthworms after exposure to poisonous
474 secondary plant metabolites (Stokke and Stenersen, 1993). Similarly, CEs play a key role in
475 detoxification processes, but did not respond to VST exposure. Keeping in mind that CE
476 activity varies in different earthworm tissues (Sanchez-Hernandez et al., 2009), possible
477 activity changes in a given tissue might have been hidden by our analyses carried out on
478 whole organisms.

479 The impact of VST, VSA and earthworms on soil microbial activity and community
480 composition was assessed on a range of soil enzymes. Carboxylesterase, alkaline phosphatase,
481 β -glucosidase and urease activities increased in VST+EW soils. Among them, β -glucosidase
482 and carboxylesterase activities are involved in carbon cycling. β -glucosidase more particularly
483 catalyzes the final stages of cellulose degradation (Turner et al., 2002). VST may favor

484 microbes possessing β -glucosidase and likely to use predigested cellulose provided by
485 earthworms as a nutrient source. This trend was not observed in EW-less soil microcosms
486 where enzyme activities were similar in both VST and control treatments.. However, enzyme
487 activity in EW soils was still higher than in EW-less soils, indicating that soil enzyme activity
488 was mainly driven by the activity of earthworms. This was further confirmed by the geometric
489 mean index, which increased after 14 and 21 days in the EW microcosms exposed or
490 unexposed to VST. In line with these results, earthworms increased soil enzyme activities in
491 chlorpyrifos-polluted soils, but did not favor the dissipation of the insecticide (Sanchez-
492 Hernandez et al., 2018, 2017, 2015).

493 To further assess the impact of VST and earthworms on soil microbial communities, α -
494 diversity and β -diversity of the soil bacterial community were estimated. While Chao 1 and
495 PD whole tree indices remained constant, Simpson reciprocal index significantly decreased in
496 the EW microcosms, and to a greater extent in those exposed to VST as compared to their
497 respective controls. Interestingly, VST combined to EW significantly decreased Simpson
498 reciprocal index after 7 days, and Chao 1 and PD whole tree indices after 14 days. This
499 suggests that both EW and VST modified the relative abundance of certain OTUs in the short
500 term, and that bacterial community composition significantly changed over time when they
501 were combined. In line with these observations, richness estimated using Chao 1 and Shannon
502 bacterial diversity indices significantly decreased in response to exposure to the
503 anticonvulsant carbamazepine in agricultural soils (Thelusmond et al. 2016). However, the
504 synergistic effect of EW and VST on the soil bacterial community may not only be the result
505 of the simple addition of the effects of the active ingredient and of the changes of soil habitat
506 resulting from EW activity. Part of the synergistic effect might be mediated by the gut
507 microbiota of the EW which is known to modify the abundance, diversity and activity of soil
508 microorganisms at least in their biostructures (Kersanté et al., 2006). Nonetheless this

509 hypothesis cannot be tested with our dataset because we only sampled the soil and not the
510 biostructures formed by EW.

511 CAP ordination analysis based on weighted Unifrac distance matrix further confirmed α -
512 diversity observations by showing that the soil bacterial community composition changed in
513 response to VST, to earthworms, or to their combination as compared to the control. The
514 effect of earthworms on bacterial community composition has already been observed:
515 bacterial populations can be selected by adapting to the microenvironments created by
516 earthworm activity (Kersanté et al., 2006). To our knowledge, this is the first report showing
517 that VST modifies bacterial community composition. This is in accordance with two studies
518 reporting significant changes in the microbial composition of soil microcosms amended with
519 different pharmaceuticals and personal care products (PPCPs) such as diclofenac,
520 carbamazepine, triclocarban and triclosan (Thelusmond et al., 2019, 2018). The bacterial
521 community composition of the VST microcosms (whether with earthworms added or not)
522 moved closer to their respective controls over time, suggesting partial recovery from VST
523 exposure. Similar recovery of the bacterial community was observed in soils exposed to
524 leptospermone and was related to decreased bioavailability of this bioherbicide (Romdhane et
525 al., 2016), in line with our observations.

526 Sequencing of 16S rRNA amplicons generated from our soil microcosms revealed that most
527 OTUs were affiliated to Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria,
528 Chloroflexi, Gemmatimonadetes, Verrucomicrobia and Firmicutes, as previously reported in a
529 range of soils exposed to pesticides and other PPCPs (Gallego et al., 2019; Shen et al., 2019;
530 Thelusmond et al., 2016). The relative abundance of OTUs affiliated to Proteobacteria
531 significantly increased in the VST microcosms, as observed in agricultural soils exposed to
532 diclofenac, carbamazepine and triclocarban (Thelusmond et al., 2018). This suggests that
533 OTUs belonging to this phylum may benefit from exposure to pharmaceuticals in different

534 ways: directly by using them as a carbon source or an energy source for their growth, or
535 indirectly by growing in niches made available by VST-susceptible bacteria. Increased
536 relative abundance of Proteobacteria has also been observed in soil microcosms harboring
537 earthworms probably because fast-growing bacteria such as Proteobacteria can use labile
538 organic compounds released by earthworms (Bernard et al., 2012; de Menezes et al., 2018;
539 Furlong et al., 2002; Gong et al., 2018). On the contrary, the relative abundances of OTUs
540 affiliated to Bacteroidetes, Actinobacteria and Acidobacteria significantly decreased in the
541 VST microcosms as compared to the control, indicating a toxic effect of VST to the OTUs
542 affiliated to these phylotypes. The relative abundances of Bacteroidetes and Actinobacteria
543 also decreased in soils exposed to carbamazepine (Thelusmond et al., 2016), KBr (Bech et al.,
544 2017), or polluted with thiabendazole (Papadopoulou et al., 2018). Keeping in mind that the
545 abundance of Acidobacteria is correlated to the soil pH (Mukherjee et al., 2014), VST might
546 have slightly modified the soil pH and thereby decreased the abundance of OTUs related to
547 this phylum.

548 Further analyses at a lower taxonomic level led to the identification of 315 OTUs mostly
549 responsible for the shifts observed in the soil bacterial community composition. OTUs
550 belonging to Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Bacteroidetes,
551 Firmicutes, Tenericutes, Actinobacteria and Verrucomicrobia significantly increased in the
552 VST+EW microcosms, reinforcing the observations at the phylum level. In this context,
553 several organisms belonging to Gammaproteobacteria (e.g. *Serratia*, *Raoultella*, *Klebsiella*,
554 *Pseudomonas*), Firmicutes (e.g. *Bacillus*), Alphaproteobacteria (e.g. *Sphingomonas*,
555 *Sphingopyxis*, *Labrys*) and Actinobacteria (e.g. *Rhodococcus*, *Mycobacterium*) have been
556 showed to degrade or co-metabolically transform different pharmaceuticals such as ibuprofen,
557 diclofenac, paracetamol, or triclosan. This may indicate the presence of putative VST
558 degraders among these phylotypes. On the other hand, OTUs affiliated to Betaproteobacteria

559 significantly increased in the EW microcosms untreated with VST. This is in line with the
560 abundance of bacteria related to Betaproteobacteria found in the intestine and casts of
561 earthworms (Singh et al., 2015; Singleton et al., 2003).

562 5. Conclusions

563 The results of the present study indicate that neither earthworms nor soil enzyme activities
564 were affected by VST. Although this pharmaceutical and its metabolite accumulated in
565 earthworms, only slight changes in the soil bacterial diversity and composition were observed
566 after VST spiking in the soil microcosms. Interestingly, VST combined with earthworms
567 enhanced the soil microbial activity, had a strong negative impact on soil bacterial richness
568 and a transient effect on soil bacterial composition. Further studies are needed to ascertain
569 whether this synergistic effect between VST and earthworms was due to direct effect on soil
570 bacterial community and/or indirect effect on earthworm's gut microbiota, which might have
571 affected both the soil bacterial enzyme activity and richness.

572 The risks associated with pharmaceutical exposure in agricultural systems due to wastewater
573 reuse has mainly focused on the fate and plant uptake of single pharmaceuticals to evaluate
574 their implications in food chain transfer and human exposure. Little is known in complex
575 agroecosystems where multiple living receptors are involved. Our findings underscore the
576 pressing need for complex environmental-scale experiments so as to address the synergistic
577 effects of different organisms and accurately assess the environmental risks of active
578 pharmaceutical compounds.

579

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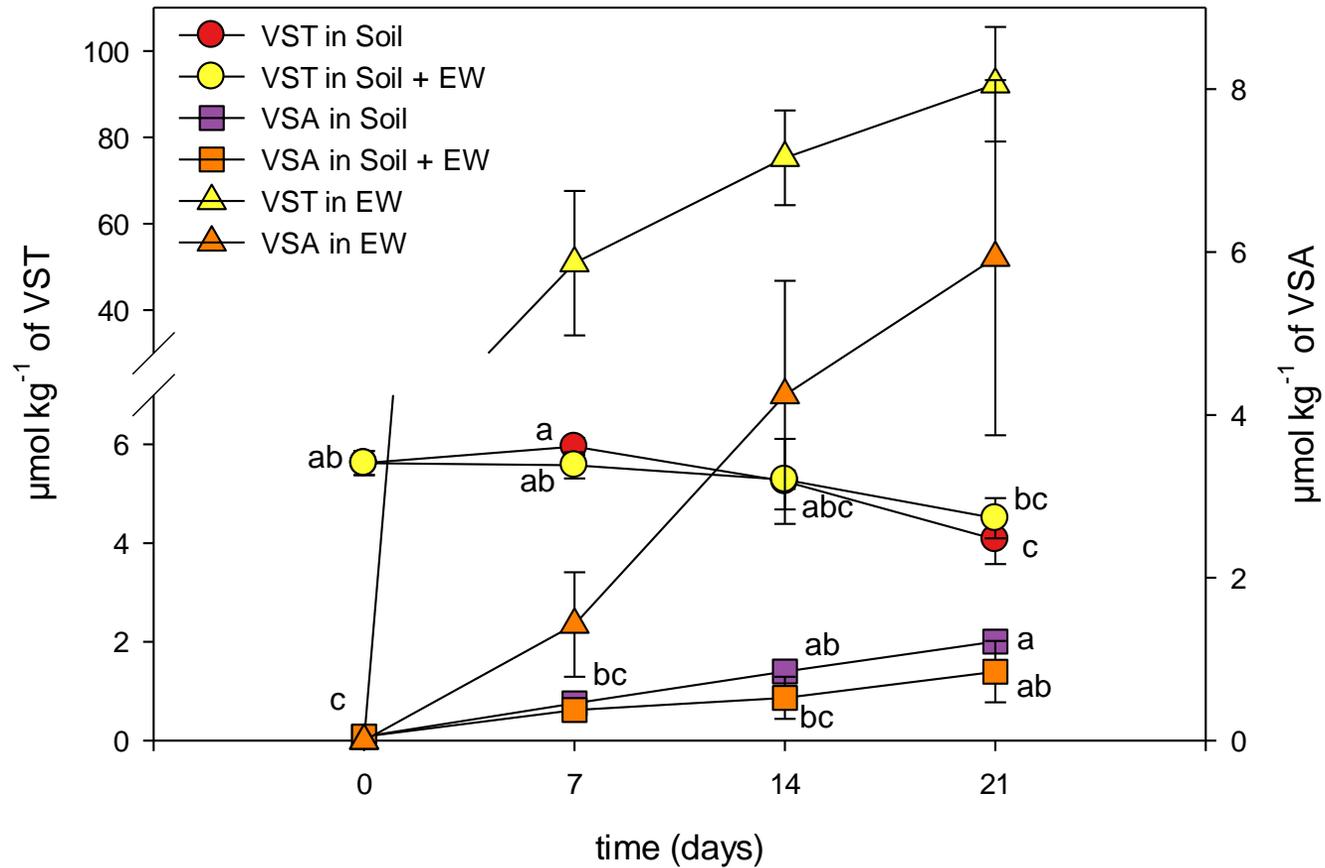


Figure 1. Fate of valsartan (VST) and valsartan acid (VSA) in soil and earthworms (EW). Each value is the mean of three replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed. Values indicated by different letters are significantly different.

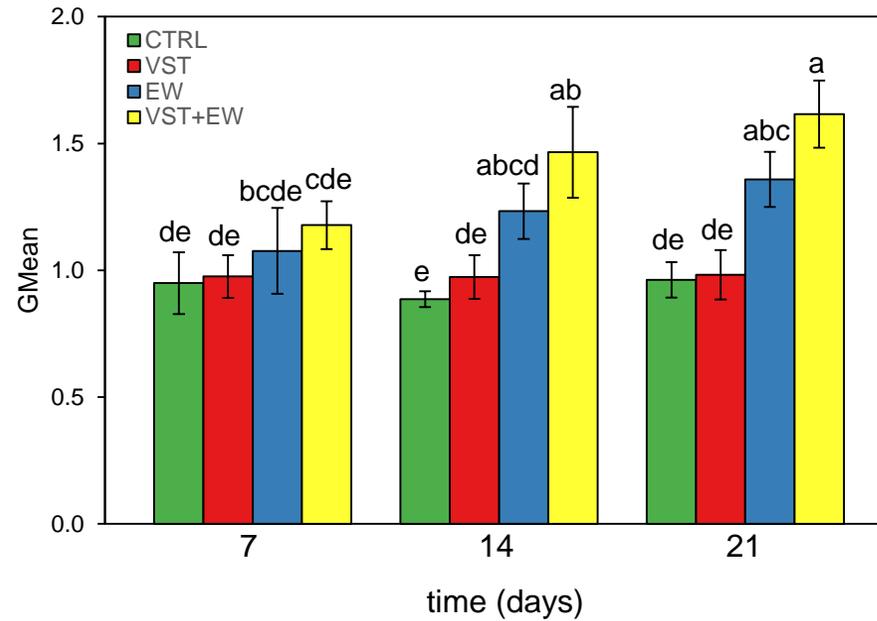


Figure 2. Geometric mean index (GMean) of soil enzymes in microcosms treated with valsartan (VST) or not (CTRL), with earthworms (EW) added or not (CTRL) at various time points (7, 14 and 21 days). Each value is the mean of three replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed. Values indicated by different letters are significantly different. ANOVA was performed on log-transformed data.

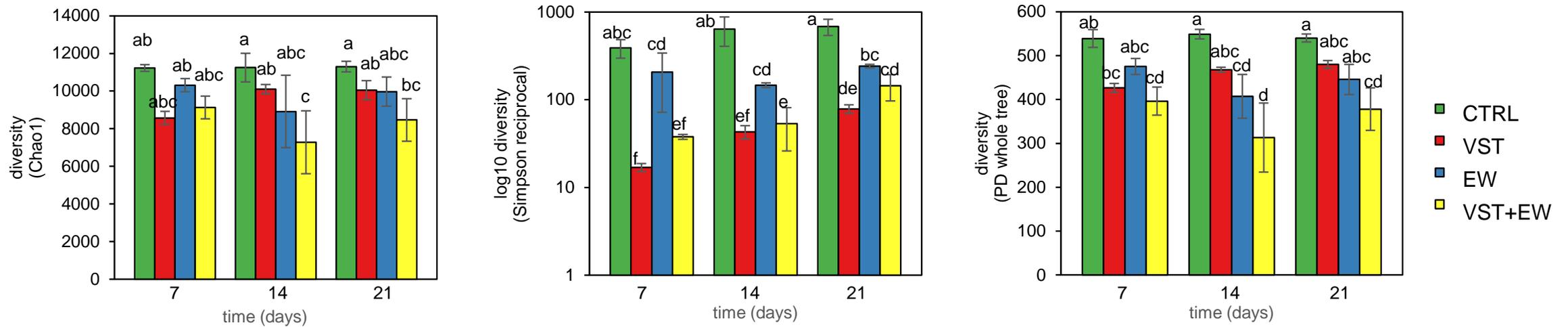


Figure 3. α -diversity indices estimated in soils with earthworms added or not (control), treated with valsartan or not (control). Each value is the mean of three replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed for each diversity index. Values indicated by different letters are significantly different.

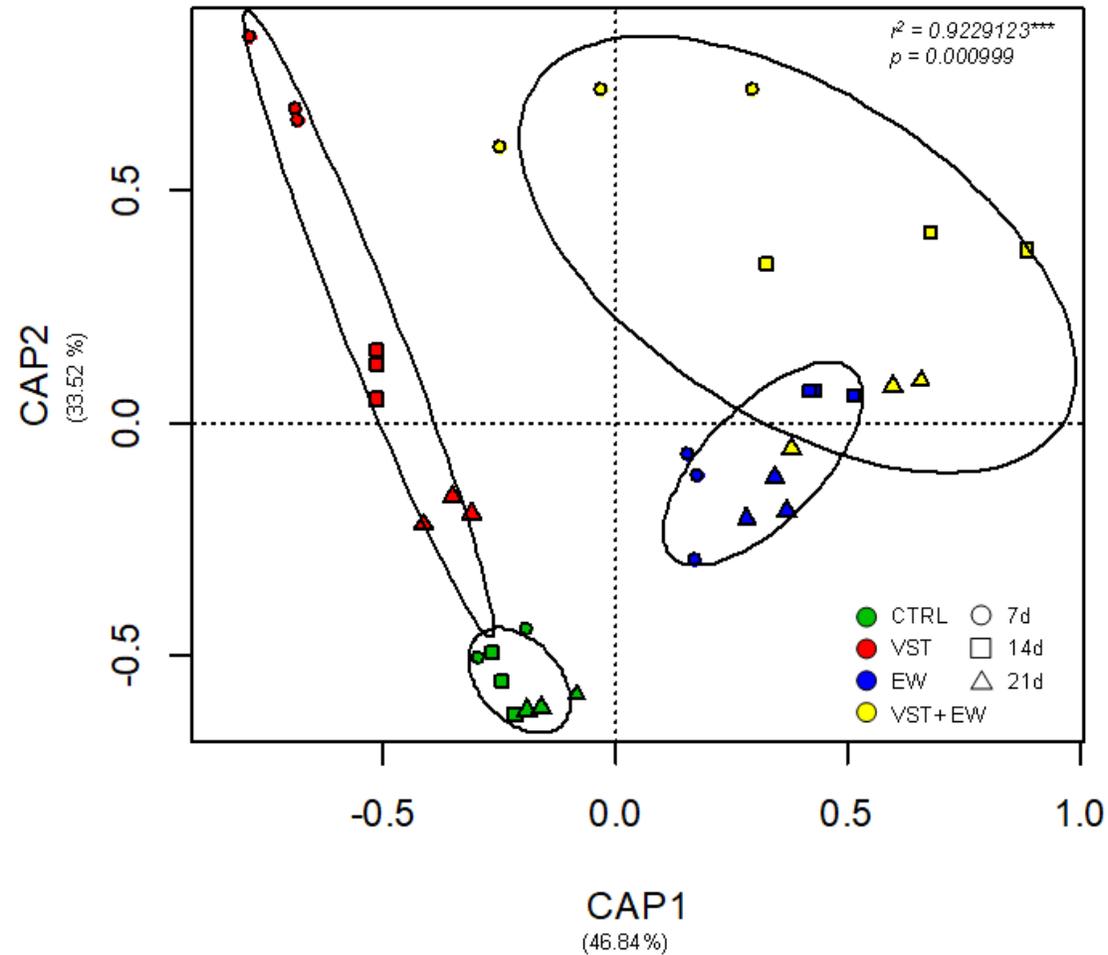


Figure 4. Bacterial β -diversity analysis of bacterial communities in soils treated with valsartan (VST) or not (CTRL), with earthworms (EW) added or not (CTRL) at various time points (7, 14 and 21 days). The first two axes of the CAP using weighted UniFrac distance matrix of 16S rRNA sequences are shown. The variance explained by each axis is given as a percentage. The triplicates of each treatment are represented by the same color.

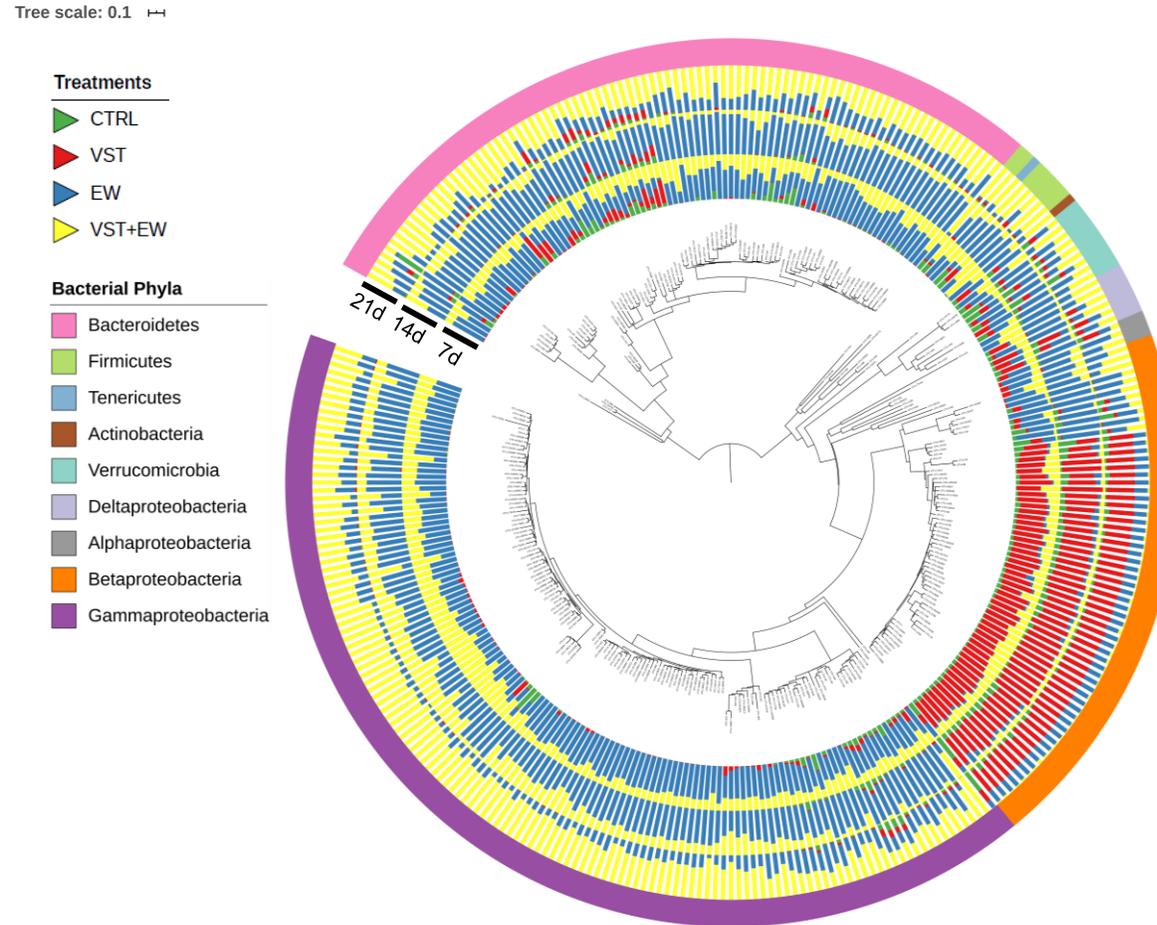
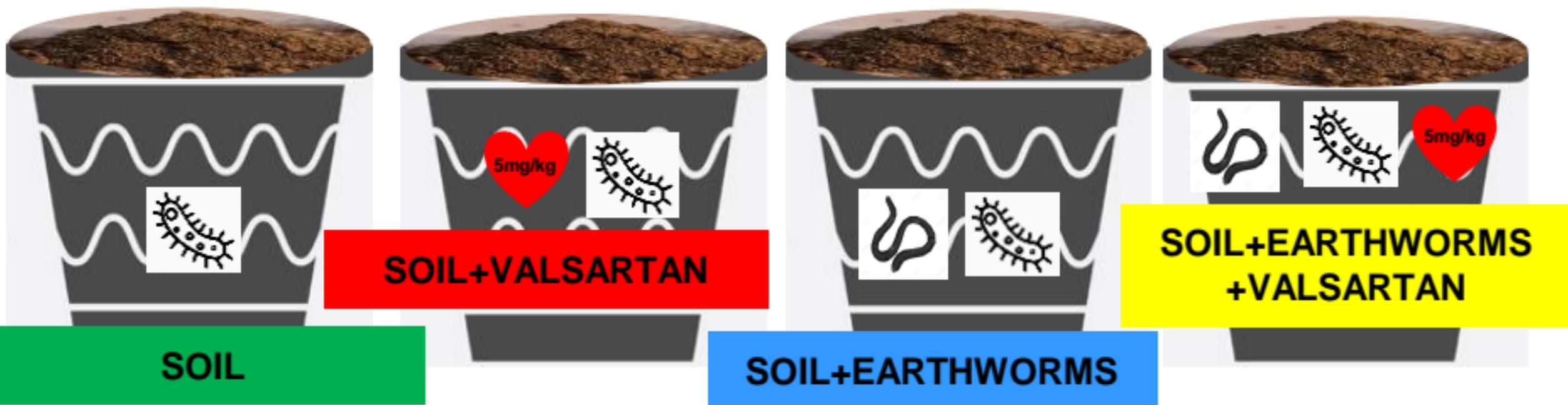


Figure 5. Phylogenetic relationships and distributions of selected 16S rRNA OTUs using DESeq2 after 7, 14 and 21 days of incubation in control (CTRL) and treated (VST) soils, with earthworms (EW) added or not (CTRL). The relative abundance of each OTU under each treatment and at each time point is represented by bar plots. Affiliation to the main microbial groups (at the phylum and class levels) is indicated in the outer circle by different colors.



SOIL

SOIL+VALSARTAN

SOIL+EARTHWORMS

SOIL+EARTHWORMS
+VALSARTAN

VALSARTAN in SOIL and EARTHWORMS (QuEChERS LC-HR/MS)

Effects on EARTHWORMS and SOIL ENZYMES

Effects on SOIL MICROBES (High Throughput sequencing)