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1 **Presence of antimicrobial residues in edible pig tissues after**
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3 **sulphadimethoxine/trimethoprim**

4 Cristina Santos-Santórum Suárez, Pascal Sanders, Agnès Perrin-Guyomard, Michel Laurentie,
5 Jean-François Taillandier, Marie-Pierre Lagree, Murielle Gaugain[§] & Alexis Viel*[§]

ANSES-Fougères Laboratory (French National Agency for Food, Environment and
Occupational Health & Safety), 10 B, rue Claude Bourgelat – Javené, CS 40608, 35306
Fougères Cedex, France

6 * corresponding author: E-mail address: alexis.viel@anses.fr

7 § These authors share last authorship

8 **Abstract**

9 When manufacturing oral premixes, cross-contamination may occur between medicated and
10 non-medicated feed leading to the exposure of food-producing animals to low concentrations
11 that may result in antimicrobial residues in edible products. This project was designed to
12 assess the level of antibiotic residues in edible pig tissues, faeces and plasma after exposure
13 for 12 days to feed contaminated with sulphadimethoxine/trimethoprim (2 % of the
14 therapeutic dosage). Our results show that sulphadimethoxine concentrations can significantly
15 exceed the maximal residue limits (MRL) in liver ($618 \pm 96 \mu\text{g/kg}$) and kidneys (510 ± 73
16 $\mu\text{g/kg}$), and that putative metabolites can also be detected in the liver. Based on tissue and
17 plasma data, a pharmacokinetic model was developed to assess which contamination rates
18 would not result in residue concentrations above the MRL. Our simulations show that the

19 contamination rate should remain below 0.2% of the therapeutic dose, i.e. far lower of the
20 tolerated contamination rate.

21 **Keywords:** antibiotic, residues, cross-contamination, Feed, LC-MS, pharmacokinetics

22 **1. Introduction**

23 The use of antimicrobials in veterinary medicine has been decreasing in Europe over the last
24 decade. However, their administration to prevent or treat infectious diseases is still
25 widespread. The combination of sulphonamide/trimethoprim are among the most often used
26 antibiotic family in pig production, as they are classified in category D of the European
27 Medicines Agency (EMA) categorisation of antibiotics (EMA, 2019) that promotes to use
28 them in priority compared to the other antibiotic classes present in categories A to C.

29 In order to protect public health, the European Union has established maximum residue limits
30 (MRLs) for all pharmacological active substances. These MRLs are the maximum authorised
31 levels of these substances in edible products of animal origin (Commission Regulation (EU)
32 N°37/2010). In order to comply with these limits, withdrawal periods are applied to food-
33 producing animals, i.e. minimum times between the last administration of a veterinary
34 medicine and the collection of edible products from this animal (Regulation (EU)
35 N°2019/06).

36 Several routes of administration of antibiotics exist in veterinary medicine, but the oral route
37 for medication is widely used via premixes (EMA, 2022). These are veterinary medicines
38 intended to be added to feedstuff, which is thus known as medicated feed (Regulation (EU)
39 2019/4). However, medicated feed is usually manufactured on the same production lines used
40 for non-medicated feed. This situation can lead to contamination, i.e. the uncontrolled
41 presence of antimicrobials in animal feedstuff (Borràs et al., 2011; Stolker et al., 2013). This
42 carry-over, which has already been reported on several occasions (Gaugain et al., 2020;

43 Lynas, Currie, McCaughey, McEvoy & Kennedy, 1998; Przeniosła-Siwczyńska et al., 2020;
44 Robert, Brasseur, Dubois, Delahaut & Gillard, 2016), seems to be unavoidable because it
45 depends on several factors and can take place not only at the feed mill, but also during
46 transport or storage on the farm (Borràs et al., 2011; Filippitzi, Sarrazin, Imberechts, Smet &
47 Dewulf, 2016). Although no contamination limits have been established by European
48 legislation, the French Ministry of Agricultural and Fisheries published good manufacturing
49 practices (GMPs) setting maximum contamination rates (French Ministry of Agriculture and
50 Fisheries, 2007). These GMPs established that the first batch of non-medicated feed following
51 a medicated one should not be contaminated by more than 5% of the therapeutic dose, and the
52 following batches by no more than 1%.

53 When cross-contamination occurs, it will result in exposure of animals to low antibiotic
54 concentrations, which may involve several risks. Firstly, there may be toxic effects in animals
55 exposed to substances not intended for certain animal species, such as lincomycin in cows or
56 some ionophores in pigs (McEvoy, 2002). Secondly, as antibiotic concentrations in
57 contaminated feed are low, animals may be exposed to subtherapeutic concentrations. This is
58 known to increase the selection of resistant bacteria, which may then spread to humans or to
59 the environment (Bacanlı & Başaran, 2019; Holman & Chénier, 2013; Marshall & Levy,
60 2011; Sandegren, 2014; Santos-Santórum Suárez et al., 2023). Furthermore, when there is
61 uncontrolled exposure of animals to antibiotics due to cross-contamination in feed, the
62 concept of withdrawal periods is not applicable, therefore antimicrobial residues could be
63 present in animal products at concentrations close or higher than the authorised MRL
64 (Cristina Santos-Santórum Suárez et al., 2022). This could be a risk for consumers, indeed,
65 exposure to antibiotics can cause allergic reactions, perturbation of the intestinal microbiota
66 and even blood disorders. Metabolites of some veterinary medicinal products are also known
67 to cause undesirable side effects (Baynes et al., 2016). Moreover, just as in animals, exposure

68 of consumers to antimicrobial residues through food increases the risk of developing
69 antibiotic resistance in the gut microbiota (Bacanlı & Başaran, 2019; Baynes et al., 2016).
70 The purpose of this study was to assess the risk of presence of antibiotic residues in edible
71 animal products after exposure of pigs to a simulated cross-contaminated feed with
72 sulphadimethoxine/trimethoprim (SDMT/TMP). Within the sulphonamide family,
73 sulphadimethoxine has a good oral bioavailability in pigs, as well as a long half-life, which
74 supports the possible presence of residues in tissue in case of feed cross-contamination
75 (Mengelers et al., 2001). That is why the combination of SDMT/TMP were the selected
76 antibiotics for our study. Plasma and tissue data were used to develop a population
77 pharmacokinetic model capable of predicting the contamination rate that would not result in
78 SDMT residues above the MRL. The potential presence of SDMT metabolites was also
79 studied, as the consumer may be at risk not only from the parent compound but also the N-
80 acetylated metabolite, which has been associated with renal toxicity (Kishida & Furusawa,
81 2004).

82 **2. Materials and methods**

83 **2.1. *In vivo* experimental study**

84 **2.1.1. Animals**

85 The animal study was approved by the French Committee on Animal Research and Ethics
86 (referral number: APAFIS#29960-2021021816313832 v2). This experimental study was
87 conducted on eight pigs, as pork is the most frequently consumed meat in Europe (OECD,
88 Food, & Nations, 2020). The animals were 8-week-old female pigs weighing between 20.5
89 and 22.6 kg at the beginning of the study. They were all housed in individual boxes and given
90 non-medicated feed for an acclimation period of 7 days.

91 A central catheter was then implanted under general anaesthesia and aseptic conditions as
92 described elsewhere (Viel et al., 2023). Briefly, pigs were first sedated with an IM injection of

93 ketamine at 20 mg/kg (Imalgène, Merial, Lyon, France) and xylazine at 2 mg/kg (Rompun,
94 Bayer, Loos, France). After intubation, pigs were kept anesthetized by inhalation with
95 isoflurane 2.5% (IsoFlo, Zoetis, Malakoff, France). After surgical opening of the neck, a
96 catheter was implanted in one of the jugular veins. A recovery period of 24-48 h was allowed
97 before the experiment began.

98 The animals were fed using an organic non-medicated feed (UFAB, Lamballe-Armor, France)
99 and had unlimited access to water throughout the experiment. The feed composition is
100 presented in Supplementary Table 1.

101 **2.1.2. Drug administrations to mimic a cross-contaminated feeding**

102 The two antibiotics administered in combination were SDMT/TMP. From day 0 (D0), in
103 order to simulate a cross-contaminated feeding at a tolerable rate for French GMPs (French
104 Ministry of Agriculture and Fisheries, 2007) of 2% of the recommended therapeutic dose (i.e.
105 14.9 mg of SDMT/kg of feed and 3.2 mg of TMP/kg of feed per day), SDMT/TMP premix
106 combination (Trimethosulpha V, Dopharma, Vair-sur-Loire, France) was used as follows:

107 - The total feed intake was of 50 g/kg body weight/day. The animals were weighed once a
108 week in order to adapt the feed quantity and the SDMT/TMP amounts to add in the
109 contaminated feed.

110 - The individual daily dose of premix was calculated (i.e. 0.75 mg/kg body weight for SDMT
111 and 0.16 mg/kg body weight for TMP) and split into two administrations at 8 a.m. and 4 p.m.
112 each day.

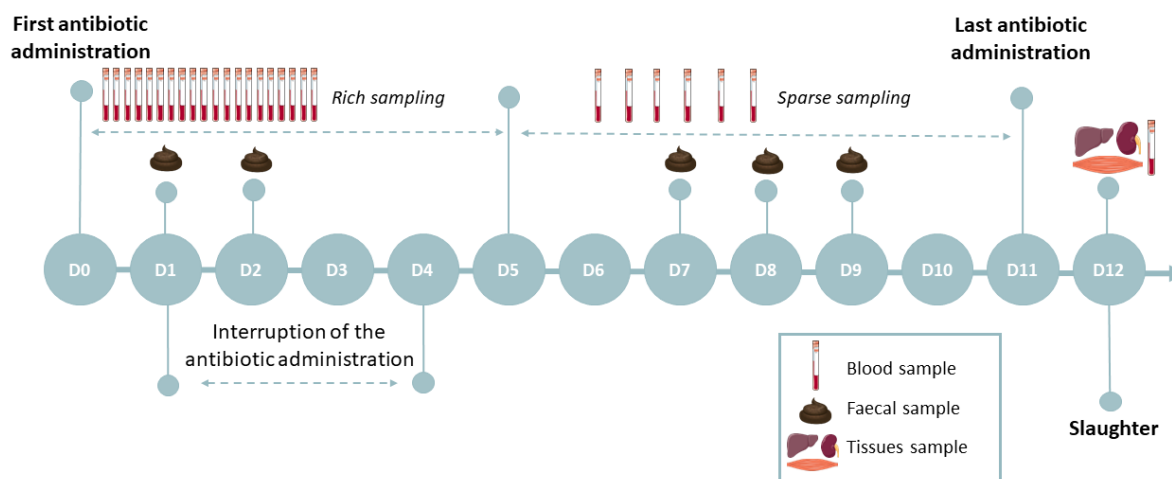
113 - To ensure that the whole antibiotic amount was ingested, the premix was given first within a
114 small quantity of feed and the pigs were closely monitored before giving the animals the rest
115 of the (antibiotic-free) ration.

116 The antibiotics were administered for 12 days (from D0 to D11) except after the second
117 administration on D1 (i.e. after four administrations since initiation), when administration of

118 the antimicrobial was interrupted for two days and then restarted on D4 (see Figure 1). The
119 purpose of this temporary stoppage was to closely monitor the shape of the decrease in plasma
120 concentrations of SDMT/TMP in order to develop a robust PK model (see 3.4). Finally, the
121 animals were slaughtered by electronarcosis followed by exsanguination on D12 (i.e. between
122 17 and 19h after the last administration).

123 **2.1.3. Blood, faecal and tissue samplings**

124 From D0 to D4, 1.5 ml of blood were sampled 22 times for each animal (rich sampling).
125 Then, in order to verify that the plasma steady-state was eventually achieved, three to five
126 samples were collected from six out of the eight pigs from D5 until the end of the study
127 (sparse sampling) (see Figure 1). The blood samples were kept in heparinized tubes and
128 centrifuged (3000g for 10 min) within 60 min after sampling. Then, the plasma was collected
129 and stored at -18°C until analysis. During the study, individual faeces were also collected
130 from the floor between four and five times (between two and three times a week, see Figure
131 1) and stored at -80°C for further analysis. The faecal and plasmatic sampling was done
132 taking into account ethical recommendations as well as data from a pilot study carried out
133 previously (Cristina Santos-Santórum Suárez et al., 2022).The slaughter of the animals
134 allowed a last blood sample to be taken. Liver, kidney and muscle samples from the loin and
135 leg were also collected and stored at -80°C for liver and -18°C for kidney and muscle, until
136 analysis.



137

138

Figure 1. Timeline of the in vivo experiment with the sampling details

139 2.2. Sample preparation and analysis

140 2.2.1. Chemicals and reagents

141 Methanol for high-performance liquid chromatography, Optima liquid chromatography/mass
 142 spectrometry grade methanol, ethylenediaminetetraacetic acid disodium dihydrate
 143 (Na₂EDTA) and citric acid monohydrate were obtained from Fisher Scientific (Illkirch-
 144 Graffenstaden, France). Formic acid and disodium hydrogen phosphate were purchased from
 145 Merck (Fontenay-sous-Bois, France) and dimethyl sulfoxide (DMSO) from VWR (Fontenay-
 146 sous-Bois, France). Standards of SDMT, TMP and trimethoprim-D9 were purchased from
 147 Sigma-Aldrich (Saint-Quentin-Fallavier, France) and sulphadimethoxine-D6 from Cluzeau
 148 Info Labo (Sainte-Foy-La-Grande, France).

149 2.2.2. Samples extraction

150 All samples were processed as described previously in order to extract and quantify SDMT
 151 and TMP (Cristina Santos-Santórum Suárez et al., 2022) . Briefly, after addition of SDMT-D6
 152 and TMP-D9 as internal standards, liquid-liquid or solid-liquid extraction with acetonitrile
 153 was used for plasma, liver, muscle and kidney samples, followed by an evaporation under
 154 nitrogen and resuspension with water. For faeces, after adding McIlvaine/EDTA buffer, a
 155 solid-phase extraction (SPE) was performed using water for washing the columns and

156 methanol for eluting the molecules. Finally, an evaporation under nitrogen followed by
157 resuspension in water was done.

158 **2.3. LC-MS/MS analysis for the quantification of SDMT and TMP**

159 Faeces, plasma, liver, kidney and muscle extracts were analysed by LC-MS/MS as previously
160 described (Cristina Santos-Santórum Suárez et al., 2022) in order to quantify SDMT and TMP
161 concentrations. The LC-MS/MS system consisted of an Accela Pump and a Thermo Pal
162 autosampler coupled with a TSQ Vantage triple quadrupole mass spectrometer from Thermo
163 Scientific (Villebon-sur-Yvette, France). A Kinetex Biphenyl column manufactured by
164 Phenomenex (100 x 2.1 mm, 2.56 mm) was used for chromatographic separation. An MRM
165 acquisition mode was used to monitor specific transitions of SDMT, TMP and internal
166 standards. The lower limit of quantification (LLOQ) of the method was 10 ng/ml or ng/g for
167 plasma, liver, kidney and muscle (except for SDMT in liver, for which it was 16.31 ng/g), and
168 20 ng/g for faeces. Validated concentration ranges and acceptance criteria for each matrix are
169 shown in Supplementary Table 2.

170 **2.4. Pharmacokinetic model development for SDMT**

171 **2.4.1. Modeling of plasma concentrations**

172 Monolix software (Lixoft, S. A. S. Monolix version 2021R2; Antony, France, 2021) was used
173 to develop the population PK model (or non-linear mixed effect model) based on the plasma
174 data for SDMT (not for TMP, see section 4.2). First, different structural models (one or two
175 compartments) were tested. The estimation of inter-individual variability (IIV) was tested for
176 each parameter. An additional inter-occasion variability (IOV) was also tested, where one
177 occasion corresponds to a new oral administration, assuming that individual parameters may
178 vary from occasion to occasion. Any potential effect of body weight as a continuous covariate
179 was then investigated for the apparent clearance and volume. The full model for each
180 structural parameter is described by Equation 1.

$$\log(X_i) = \log(X_{pop}) + \beta_X \times \log(BW_i/23.5) + \eta_i + \sigma_{ik} \quad \text{Eq. (1)}$$

181 Where X_{pop} is the population value of parameter X, β_X : the covariate coefficient linked to
182 body weight (only if significantly different from 0), BW_i : individual body weight (the median
183 weight of pigs at the beginning was 23.5 kg); η_i is the random effect for individual I (for IIV),
184 σ_{ik} is the variability at the occasion level for each individual (for IOV).

185 Different error models were evaluated (additive, proportional or a mixture of both).

186 The final PK model was selected based on the Bayesian information criterion (BIC) value and
187 the goodness-of-fit graphs for observed and predicted data. The IIV, IOV and covariate values
188 were kept only if the precision of estimation (based on the relative standard error (RSE)) was
189 <50% and associated with a decrease in BIC. Visual predictive checks (VPCs) were used to
190 validate the model, with 500 simulations and a 90% prediction interval.

191 **2.4.2. Prediction of edible tissue concentrations for SDMT**

192 One goal of this work was to use the PK model to predict SDMT tissue concentrations after
193 exposure to food contaminated at a population level with different SDMT concentrations. For
194 that purpose, the ratio of tissue concentration over plasma concentration (named partition
195 coefficient, K_p) at the time of slaughter (on average 18 h after the last meal) was calculated
196 for each edible tissue (kidneys, muscle, liver), assuming that the concentration steady-state
197 was achieved. Monte Carlo simulations were then performed using Simulix software (Lixoft,
198 S.A.S. Simulix version 2021R2 Antony, France, 2021). Briefly, a virtual pig population
199 ($n=1000$) was generated based on the final population PK model to get the predicted
200 distributions of SDMT plasma concentrations. For tissues, a log-normal distribution was
201 assumed for the calculated K_p s that was multiplied to the plasma concentrations at 18h after
202 the last administration (as in our experimental design) to get the predicted distributions of
203 SDMT concentration in each edible tissue. Three different scenarios of exposure to SDMT for
204 12 days were used: a contamination rate of 2% (identical to the experimental design), a

205 contamination rate of 1% (corresponding to the regulatory tolerated maximum) and the
206 maximal contamination rate ensuring to get tissue concentrations below the MRL. Then, the
207 maximum exposure scenario that would guarantee that tissue concentrations would not exceed
208 MRLs for 95% of pigs was determined.

209 **2.5. Seeking of SDMT metabolites by LC-HRMS**

210 Targeted and non-targeted LC-HRMS analyses were carried out in order to seek SDMT
211 metabolites in liver samples.

212 **2.5.1. Liver sample preparation**

213 In addition to the eight liver samples prepared for the pharmacokinetic and residue study, five
214 blank pig liver samples providing from animals of a pilot study were also extracted using the
215 same extraction as described previously. These non-treated animals were from the same
216 breeding as for the pharmacokinetic and residue study. These blank liver samples were used
217 as control samples for the non-targeted seeking of SDMT metabolites.

218 **2.5.2. LC-HRMS**

219 Both targeted and non-targeted approaches were performed on a system composed of an
220 Acquity H-class UPLC system coupled with a Vion IMS QTOF mass spectrometer
221 manufactured by Waters (Guyancourt, France). Resolution was higher than 50 000 for m/z
222 956, 44 000 for m/z 785 and 35 000 for m/z 152. Compounds were separated with a Kinetex
223 Biphenyl column (100 x 2.1 mm, 2.56 mm) by Phenomenex (Le Pecq, France) protected by a
224 C18 guard column (4 mm x 2 mm) heated to 35°C. Mobile phases were 0.1% formic acid in
225 water (A) and 0.1% formic acid in methanol (B) with a flow rate of 0.3 ml/min.

226 Electrospray ionisation (ESI) in positive mode was used and the acquisition was performed in
227 MS^E mode, with a low collision energy of 6 eV and a high collision energy ranging from 15
228 eV to 50 eV. The scan time was of 0.2 s and the mass range covered was from 50 m/z to 1200
229 m/z. Other source parameters were the desolvation temperature (550°C), the source

230 temperature (120°C), the capillary voltage (0.80 kV), the desolvation gas (800 L/h) and the
231 cone gas (50 L/h).

232 **2.5.3. Targeted approach acquisition**

233 The gradient for the targeted approach lasted 13 minutes. It started with 100% of A for 2
234 minutes, then 100% of B from minute 2 to minute 10, after which the composition was stable
235 until minute 11. Finally, at minute 11, the gradient returned to the initial conditions (100% of
236 A) and remained constant for two minutes.

237 There were two acquisitions in order to look for phase I and phase II metabolites. The targeted
238 compounds used to check acquisition quality are shown Table 1. In addition to these three
239 compounds, 20 phase 1 and 20 phase 2 metabolites of SDMT were investigated and are listed
240 in Supplementary Table 3.

<i>Component</i>	Adduct	Retention time (min)	m/z
<i>Sulphadimethoxine</i>	[M+H] ⁺	7.88	311.0736
<i>Sulphadimethoxine-d6</i>	[M+H] ⁺	7.88	317.1112
<i>Trimethoprim</i>	[M+H] ⁺	6.18	291.1379

241 **Table 1. Targeted analytes for the quality control in HRMS analysis**

242 **2.5.4. Non-targeted approach acquisition**

243 For the non-targeted approach, the elution gradient was longer. It started with one minute at
244 100% of A, then 100% of B for 24 min, where it remained stable for over two minutes.
245 Finally, at minute 27, the gradient returned to the initial conditions (100% of A) and was
246 constant until minute 30.

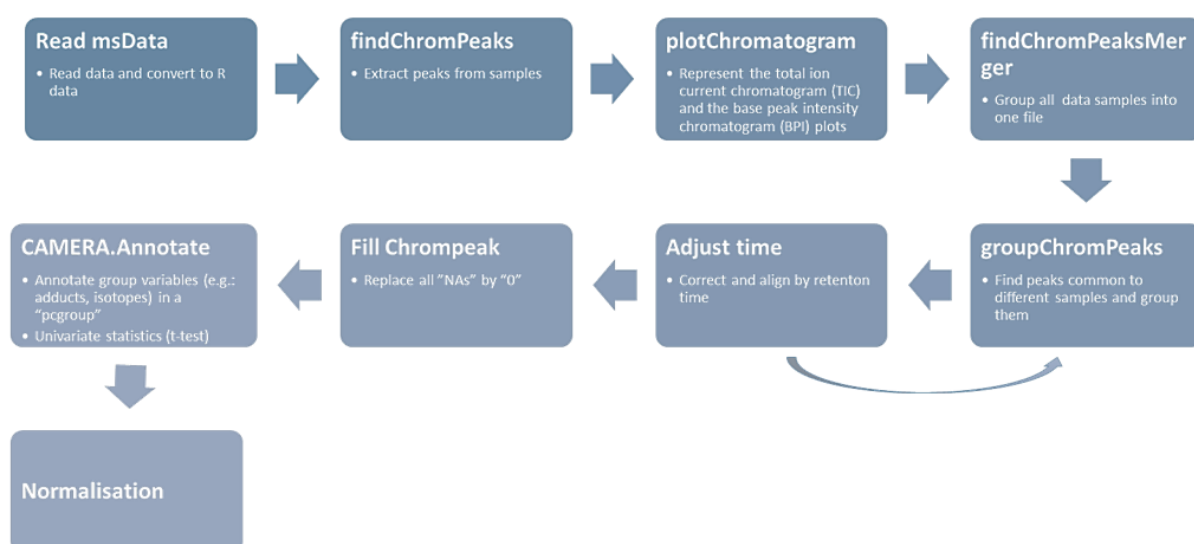
247 The targeted adducts used to check acquisition quality were the same as for the targeted
248 approach (Table 1). In this approach, the order of sample injections was randomised to avoid
249 any bias linked to the analytical instrument. Furthermore, a quality control (QC), which was a
250 mixture of all the samples, was injected every five injections.

251 **2.6. Data analysis for the non-targeted research of SDMT metabolites**

252 Data processing and statistical analysis were performed with Workflow4Metabolomics
253 (W4M) on the Galaxy platform.

254 2.6.1. Data processing workflow

255 First of all, raw acquisition files were transformed to mzML format with MS-Convert
256 software (version 3.0). At this step, only the precursor ions were kept, even when an MS^E
257 acquisition mode was used. After uploading mzML files to W4M, there was a preprocessing
258 step to first extract the peaks from all chromatograms, then group similar peaks from different
259 samples and finally apply a correction to the retention time if needed (Giacomoni et al., 2015;
260 Smith, Want, O'Maille, Abagyan & Siuzdak, 2006). The goal was to obtain a variable data
261 table in which each feature is represented with its m/z mass, time retention and presence in
262 each group (treated, control or QC) in addition to a data matrix table containing the intensities
263 of all the features in each sample. Before starting the statistical analysis, there was a
264 normalisation step. This entailed applying a linear correction taking into account the variation
265 in signal intensities of the features (m/z associated with a retention time) in the QC over time.
266 The detailed preprocessing and normalisation workflow is represented in Figure 2.



267

268 **Figure 2. Schematic workflow of data preprocessing and normalisation in**
269 **Workflow4Metabolomics on the Galaxy platform**

270 2.6.2. Statistical analysis

271 First, a univariate analysis (t-test with FDR correction for multiple tests) was carried out to
272 seek those variables with a significant ($P < 0.01$) difference in response between the control
273 and the treated samples.

274 Multivariate analyses were then performed (Guitton et al., 2017; Thevenot, Roux, Xu, Ezan &
275 Junot, 2015). A principal component analysis (PCA) was carried out after Pareto scaling of
276 the variable in order to see if there was a different metabolomic profile in the liver of SDMT-
277 treated pigs compared with control pigs. The goal was to see whether an unsupervised model
278 with projection of the whole dataset according to two components was sufficient to highlight
279 differences between the two groups at a first glance.

280 Secondly, a supervised partial least squares-discriminant analysis (PLS-DA) model was built
281 taking into account the groups to which each of the samples belonged (treated or control) to
282 create the orthogonal components. The PLS-DA was designed to reveal any differences in the
283 metabolomic profile of the two groups, as well as to identify which variables are more
284 important for group separation, necessary in the model's construction. Pareto scaling of the
285 data was used to provide the same scale for all variables. Model performance was determined
286 by R^2Y and Q^2Y , diagnostic statistics whose significance was then checked by permutation
287 tests.

288 **3. Results**

289 **3.1. Plasma analysis and PK model development**

290 **3.1.1. Plasma analysis**

291 Plasma concentrations of SDMT/TMP over time are shown in Supplementary Figure 1. After
292 the first two oral administrations of premix (D0-D1), the concentration of SDMT in plasma
293 increased to reach a maximum mean concentration of 2724.0 ± 561.2 ng/ml. For TMP, the
294 maximal concentrations were much lower (around 15 ng/mL, which is close to the LLOQ).

295 The premix feed intake was then replaced by antibiotic-free feed for two days to observe the
 296 profile for decreasing concentrations (useful for developing the PK model, see section 4.2).
 297 On D4, antibiotics were administered again and SDMT concentrations increased before
 298 remaining relatively stable (steady-state reached) until the animals' slaughter (between 2124
 299 and 4330 ng/ml).
 300 For TMP, 60% of the samples were below the LLOQ of the analytical method (10 ng/ml) (but
 301 above the LOD), which means that the measurements are subject to great uncertainty.

302 3.1.2. PK model development

303 For TMP, no PK model was developed due to the high proportion of samples (60%) being
 304 below the LLOQ.
 305 Regarding SDMT, a one-compartment model was finally selected based on a decrease in BIC
 306 value (the lower being the better). The corresponding structural parameters were apparent
 307 clearance (Cl/F), apparent volume (V/F) (apparent because only the oral route was used in this
 308 PK study), and absorption rate (ka). Parameters of the final model are presented in Table 2.
 309 IIV and IOV could be estimated for ka and apparent clearance. Moreover, a low but
 310 significant relationship was found between the covariate body weight and clearance (with
 311 $\text{beta_Cl}_{\text{WT}} = 0.18$).

312

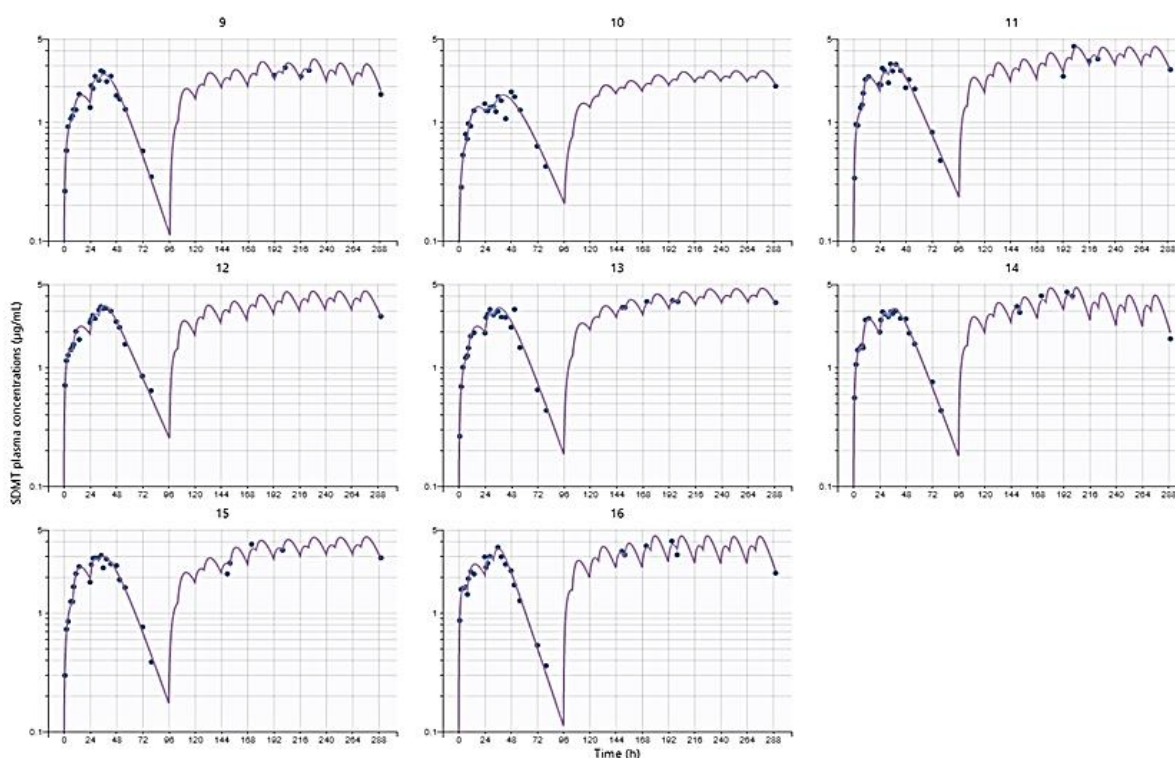
Parameters	Fixed Effects	
	VALUE	R.S.E. (%)
ka (h⁻¹)	0.18	20.4
V/F (L)	4.35	5.30
Cl/F (L/h)	0.24	5.01
beta_Cl_{WT} (no unit)	0.18	4.94
Parameters	<i>IIV : inter-individual variability</i>	
	Value as CV (%)	R.S.E. (%)
η_{ka}	46.6	34.0
η_{Cl}	12.8	31.8

Parameters	IOV: inter-occasion variability	
	Value as CV (%)	R.S.E. (%)
σ_{ka}	33.4	19.9
σ_{Cl}	11.4	24.0
Error Model Parameters		
a ($\mu\text{g/mL}$)	0.059	23.4
b (%)	0.11	7.76

313 **Table 2. Estimated parameter values from the final population PK model CI/F:**
314 **apparent clearance; V/F: apparent volume; ka: absorption rate; a: additive error**
315 **component; b: proportional error component, RSE: relative standard error**

316 The graph of individual fits (Figure 3) shows that the model was able to adequately describe
317 the observed data. The plasma steady-state was obtained from D8 (i.e. around 72 h after the
318 resumption of treatment).

319 The elimination half-life was calculated from the PK parameters to be equal to 12.5 +/- 1.3 h.



320
321 **Figure 3. Individual fits of SDMT plasma concentrations. The blue points are the**
322 **observed data and the purple line is the model's prediction**

323 The graphs of VPC (Supplementary Figure 2) showed some bias at the late time points but no
324 major bias, therefore the population pharmacokinetic (popPK) model was validated.

325 **3.2. Tissue and faecal samples analysis**

326 SDMT and TMP concentrations in tissues and the corresponding MRLs and Kp are shown in

327 Table 3.

328

<i>Matrix</i>	Antibiotic (MRL in µg/kg)	Mean concentration ± standard deviation (µg/kg)	Mean tissue-plasma partition coefficient (Kp) ± standard deviation
<i>Muscle (mean of loin and leg)</i>	SDMT (100)	187.36 ± 24.6	0.081 ± 0.018
	TMP (50)	<LLOQ*	NC
<i>Liver</i>	SDMT (100)	617.93 ± 95.6	0.27 ± 0.076
	TMP (50)	<LLOQ*	NC
<i>Kidney (mean of both left and right kidneys)</i>	SDMT (100)	509.85 ± 72.5	0.22 ± 0.076
	TMP (50)	18.9 ± 10.05	NC

329 *LLOQ: lower limit of quantification. NC: not calculated

330 **Table 3. Concentrations of sulphadimethoxine and trimethoprim in tissues of 8 pigs at**
331 **time of slaughter, along with the corresponding MRLs and Kp**

332 At an administration concentration corresponding to 2% of the therapeutic dose, SDMT
333 concentrations in all tissues after 12 days of exposure were higher than the MRLs allowed by
334 European legislation, especially in the liver and kidney. The individual results (SDMT tissue
335 and plasma concentrations at slaughter as per pig) are presented in Supplementary Table 4.

336 On the other hand, TMP concentrations were below the lower limit of quantification (LLOQ)
337 of the method in all matrices except kidney samples, where concentrations were quantifiable
338 but remained below the MRL.

339 The mean faecal concentrations are presented in Table 4. While TMP was not detected in
340 faeces, SDMT concentrations were constant after day 2 of the study, being between 600 and
341 750 µg/kg during the whole experiment.

342

<i>Day</i>	<i>Antibiotic</i>	<i>Mean concentration ± standard deviation (µg/kg)</i>
<i>Day 1</i>	SDMT	446.53 ± 107.2
<i>(n=8)</i>	TMP	ND
<i>Day 2</i>	SDMT	714.76 ± 105.1
<i>(n=8)</i>	TMP	ND
<i>Day 7</i>	SDMT	628.98 ± 25.9
<i>(n=4)</i>	TMP	ND
<i>Day 8</i>	SDMT	677.03 ± 71.5
<i>(n=8)</i>	TMP	ND
<i>Day 9</i>	SDMT	749.59 ± 117.8
<i>(n=8)</i>	TMP	ND

343 **Table 4. Concentrations of sulphadimethoxine and trimethoprim in pig faecal samples.**
344 **ND = Not Detected**

345 **3.3. Simulations based on PK model**

346 Monte Carlo simulations (n=1000) were then carried out based on the popPK model for
347 plasma and the calculated partition coefficients (Kp) in each tissue to predict the
348 concentrations of SDMT in edible tissue according to various exposure scenarios (see Table
349 5). For all the simulations, the tissue concentrations was predicted 18h after the last
350 contaminated feed as in our experimental design.

<i>Scenario</i>	<i>Liver concentrations (µg/kg)</i>	<i>Muscle concentrations (µg/kg)</i>	<i>Kidney concentrations (µg/kg)</i>
<i>2% contamination rate for 12 days</i>	577 [305-1085]	181 [102 - 317]	486 [254 - 917]
<i>1% contamination rate for 12 days</i>	295 [161 - 518]	92 [50 - 155]	244 [127- 484]
<i>0.2% contamination rate for 12 days</i>	59 [32-108]	1.8[1.0-3.2]	49 [24-96]

351 **Table 5. Simulated SDMT concentrations in edible tissues according to different**
352 **scenarios 18 h after the last administration. The median concentrations and [5th-95th]**
353 **percentiles are given.**

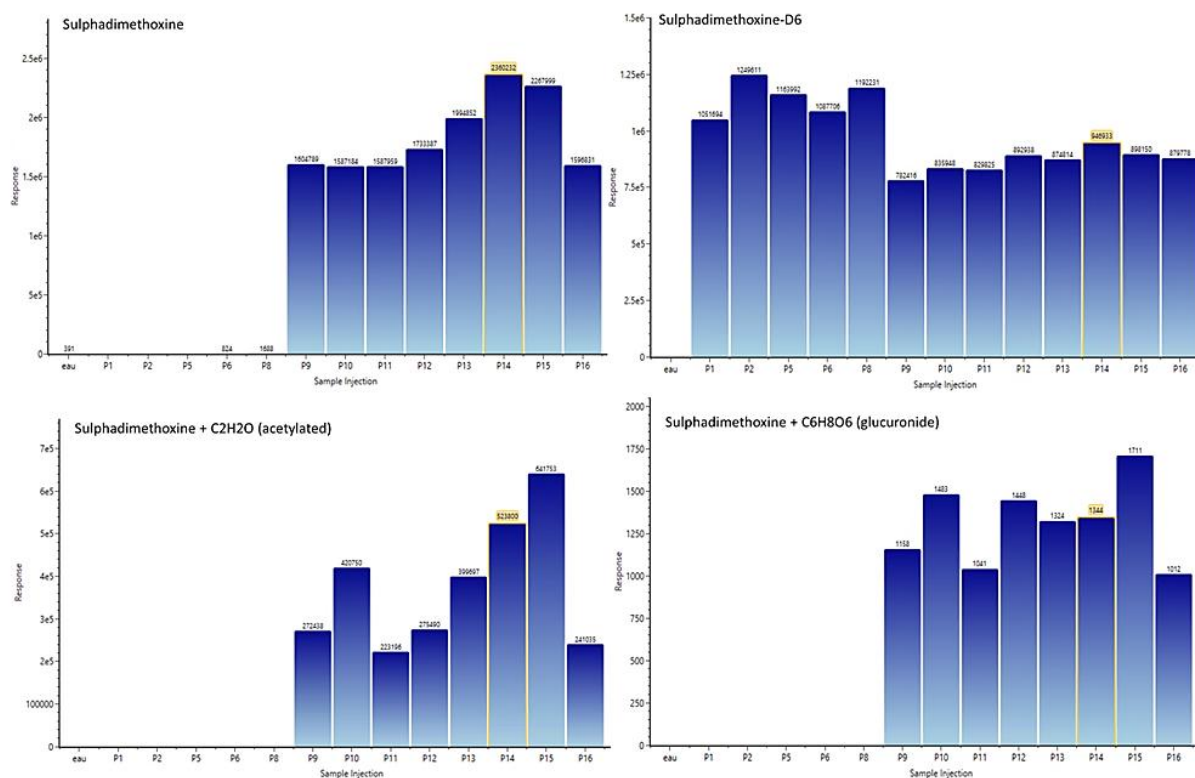
354 These simulations highlighted that with a scenario of a 2% contamination rate, 95% of the pig
355 population would have SDMT tissue concentrations above the MRL (100 µg/kg) in all edible
356 tissues. Similar results applied to the scenario with a 1% contamination rate, except for
357 muscles, for which at least 50% of pigs would have tissue levels under the MRL.

358 Based on the edible tissue with the highest SDMT concentrations in our experiment (liver)
359 and assuming that the kinetic disposition of SDMT is linear in liver, the maximal
360 contamination rate which ensures that 95% of pigs will have liver concentrations below the
361 MRL was calculated to be less than 0.2% of the therapeutic dosage.

362 **3.4. HRMS analysis and research of metabolites**

363 **3.4.1. Targeted approach**

364 Two phase II metabolites of SDMT were found after targeted HRMS analysis. First, the
365 potential acetylated metabolite was detected at a mean retention time of 8.15 min in all
366 samples from the treated pigs, but not from the control ones. In addition, the glucuronide
367 metabolite was also detected with a low-intensity signal. For this putative metabolite, a split
368 peak was observed (at 6.97 and 7.06 min), which could be two isomer forms of the
369 metabolite, as this type of isomerisation of glucuronide conjugates is described in the
370 literature (but not described in pigs) (Kong et al., 2018; Mitchell, Christopher, Behnaz, Peter
371 & Thomas, 1998). The results shown in this article are those corresponding to the first peak.
372 The responses of SDMT, SDMT-D6 and both putative metabolites across the samples are
373 shown in Figure 4.



374

375 **Figure 4. Sulphadimethoxine, sulphadimethoxine-d6,) sulphadimethoxine+C₆H₈O₆**
 376 **(glucuronide) and sulphadimethoxine+C₂H₂O (acetylated) responses in water (eau),**
 377 **control livers (P1, P2, P5, P6 and P8) and treated pig livers (P9-P16)**

378 Table 6 describes the observed m/z as well as the mean retention time for each of the
 379 metabolites.

<i>Putative metabolite</i>	<i>Observed m/z</i>	<i>Range of mass error (ppm)</i>	<i>Observed retention time (min, mean across the samples)</i>	<i>Adduct</i>
<i>Sulphadimethoxine + C₂H₂O (acetylated)</i>	353.092	-0.3 – 0.9	8.15	[M+H] ⁺
<i>Sulphadimethoxine + C₆H₈O₆ (glucuronide)</i>	487.115	0.3 – 14	6.97	[M+H] ⁺

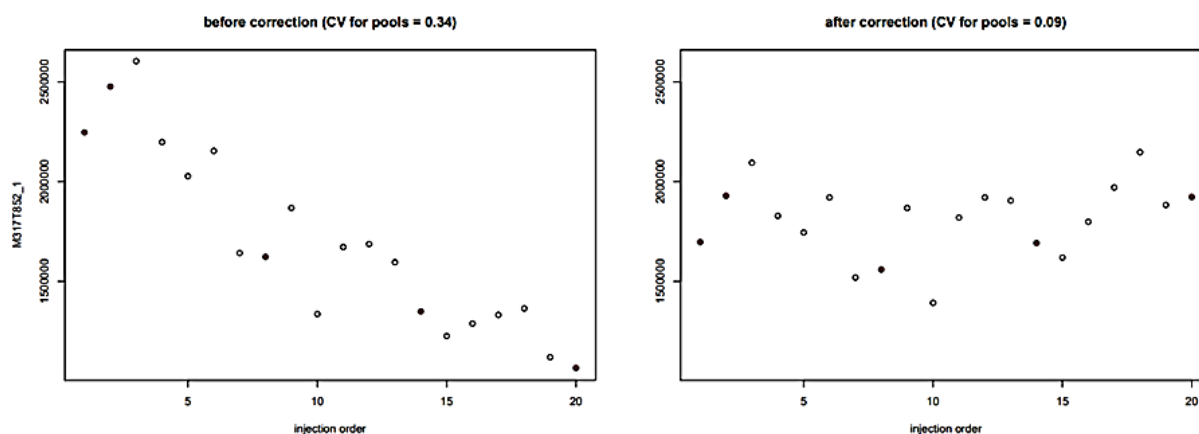
380 **Table 6. Observed mass and retention time for putative SDMT metabolites in liver**

381

382 **3.4.2. Non-targeted approach**

383 **Normalisation and quality of the data**

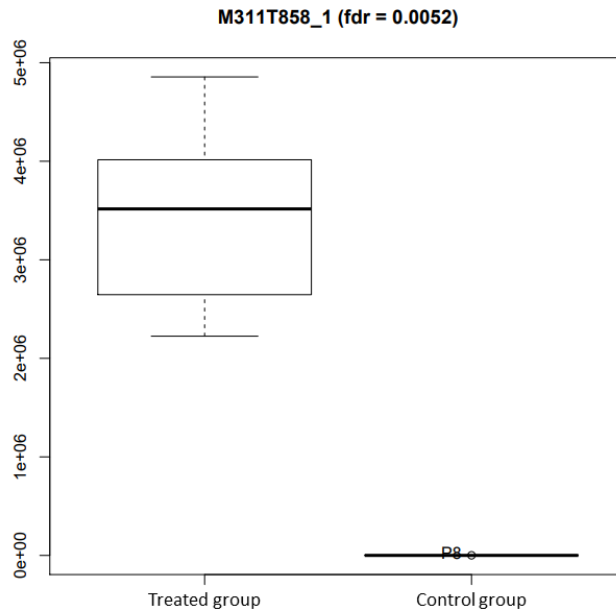
384 Some variables were subjected to linear normalisation because of a decrease in their signal
385 intensity in QCs over time. Whether or not this correction was applied depended on the
386 workflow developed by Van de Kloet (van der Kloet, Bobeldijk, Verheij & Jellema, 2009). A
387 filter was applied to eliminate variables with a high coefficient of variation (CV) in their
388 responses. This filter was set at 35% in order not to lose the SDMT, whose CV was of 33%.
389 Data quality was also checked using some of the parameters from the internal standard
390 (SDMT-D6), such as a constant retention time (14.19 ± 0.01 min) and the CV of the response
391 over the batch before and after normalisation (from 34% to 9%, see Figure 5).



392
393 **Figure 5. Coefficient of variation in the internal standard (sulphadimethoxine-d6)**
394 **response before and after normalisation**

395 **Univariate analysis**

396 The univariate analysis selected a total of 100 variables for which the response showed a
397 significant difference between the two groups ($p < 0.01$). Both SDMT and the potassium
398 adduct (SDMT+K) were found among these first 100 variables, but neither the acetylated nor
399 the glucuronide metabolites were.



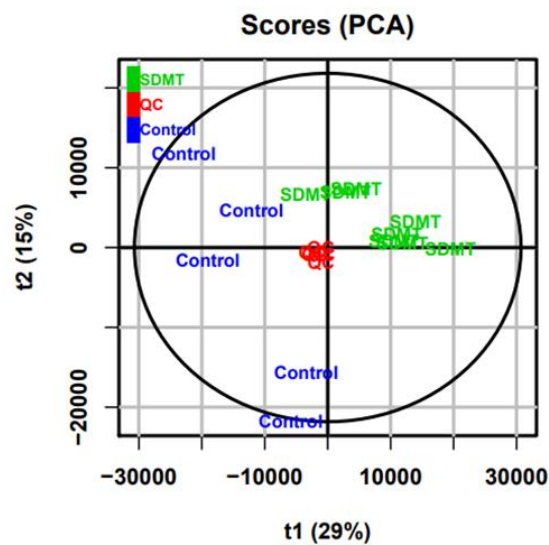
400

401

Figure 6. Univariate analysis result for sulphadimethoxine

402 **Multivariate analysis**

403 A first multivariate descriptive analysis was performed by doing a PCA. The goal was to see
 404 if patterns were observable in the projection of samples over the different components. As
 405 shown in Figure 7, clustering is clearly observed over the two main components and all the
 406 QCs are in the centre of the projection, demonstrating the contribution of normalisation. Up to
 407 44 % of the data set's variability is taken into account in this model.



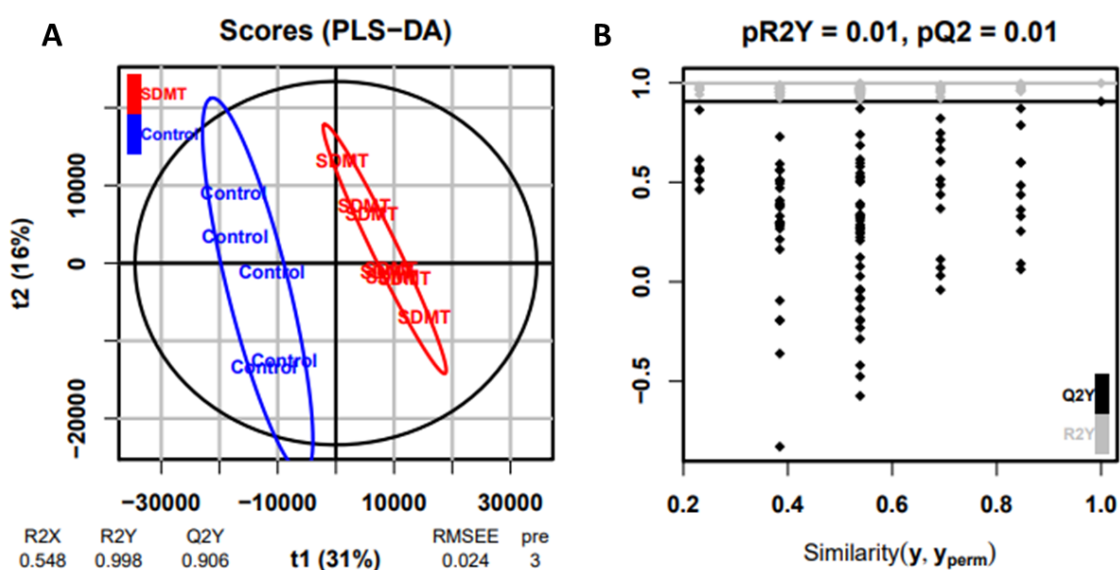
408

409

Figure 7. PCA scores for components 1 and 2 with identification of the samples according to group

410

411 A PLS-DA model was then constructed in order to better visualise any possible difference in
 412 the metabolomic profile between the two groups and to look for potential metabolites
 413 involved in this differentiation. The model's performance was assessed by evaluating
 414 different parameters. Firstly, R2Y —which is the percentage of variable Y (belonging to the
 415 treated or control group) explained by the model— was of 0.998. Next, Q2Y — which is the
 416 predictive capacity of the model —was of 0.906 (see Figure 8). Permutation tests were carried
 417 out to evaluate whether the model was overfitted or not. The proportion of times that the R2Y
 418 and Q2Y values obtained in the permutation tests were better than the original ones was
 419 calculated and p-values for both diagnostic parameters (pR2Y and pQ2Y) were of 0.01 (see
 420 Figure 8), which means that the diagnostic parameters are significant and that the model was
 421 valid. With this analysis, a difference between both groups is also observed over the first
 422 component of the PLS-DA model.



423

424 **Figure 8. PLS-DA score plot for components 1 and 2 (A) and results of the permutation**
 425 **tests (B)**

426 Furthermore, the PLS-DA analysis provided a features table where the variables were sorted
 427 according to their “variable importance projection” (VIP) score, which is a way of ranking the
 428 variables according to their contribution to group differentiation, thus used to create the

429 model. In this analysis, the SDMT was in ninth place but none of the putative SDMT
430 metabolites detected by the targeted approach was found by the non-targeted approach.

431 **4. Discussion**

432 The plasma PK profile was in good agreement with previous publications (Mengelers et al.,
433 2000; Mengelers et al., 2001). The development of a population PK model based on plasma
434 was successful for SDMT due to its rich sampling design. It was not possible for TMP due to
435 many data being below the LLOQ. Regarding SDMT, the popPK model was able to quantify
436 IIV and IOV for two parameters with very good confidence (Table 2): the oral absorption
437 constant (k_a) and apparent clearance (Cl/F). The variability for apparent clearance actually
438 represents both the variability of clearance and that of bioavailability (F), because F is
439 embedded in this parameter. Indeed, to compute the absolute bioavailability we would need
440 IV data.

441 A major result of this study is that even at low contamination rates tolerated by the current
442 French GMPs, residues of SDMT could be present in edible products at levels far above the
443 MRL (5 or 6 times higher, see Table 3). Furthermore, as this type of exposure of animals is
444 likely to be undetected on farms, there is no need for observing a withdrawal period as would
445 be the case with medicated feed and consequently consumers could be directly exposed to
446 antimicrobial residues. TMP concentrations were only quantifiable in the kidney, but
447 remained below the MRL.

448 The tissues/plasma ratios (K_p) for SDMT calculated at the end of the study were similar to
449 those found previously in the literature (Righter, Showalter & Teske, 1979), i.e. between 0.2
450 and 0.3 for kidney/liver and around 0.1 for muscles. This highlights a relatively poor affinity
451 of SDMT with these tissues. We used the popPK model with the calculated K_p values to
452 simulate and predict the concentrations of SDMT at a population level using various exposure
453 scenarios. Globally, our simulations showed that in order to avoid SDMT residues higher than

454 the MRL, the contamination rate should not exceed 0.2%. This value could be compared with
455 the contamination limits fixed in French GMPs that establish a 5% contamination rate for the
456 first non-medicated feed following a medicated one, and 1% for the following batches (
457 French Ministry of Agriculture and Fisheries, 2007). However, our simulations are based on
458 Kp ratios that were calculated for a specific time (i.e 18h) after the last antibiotic
459 administration, which may be a limit of the model. Indeed, because there are no kinetic
460 knowledge for tissues (i.e the elimination half-life), those simulations are subject to some
461 uncertainty. Furthermore, our animal study was carried out on piglets, which means that PK
462 parameters could be different for bigger and older animals. Therefore, the simulation results
463 should be considered carefully, as should extrapolation to pigs having reached the market
464 weight for slaughter.

465 The SDMT was a good choice for this study because of its relatively long half-life (around
466 13h), which increases the risk of accumulation in tissues when administered on a daily basis.
467 However, these results should not be extrapolated to other sulphonamides with different
468 pharmacokinetic parameters. Concentrations of SDMT were relatively low in faeces
469 (between 0.6 and 0.7 µg/g of faeces), which is in agreement with the available literature that
470 reported good oral absorption of this drug (Linkenheimer, 1965). Nevertheless, these low
471 levels of concentrations coupled with a chronic exposure (several weeks) could be a risk of
472 antibiotic resistance in gut commensal bacteria. Indeed, in our recent experimental study that
473 mimicked a cross-contaminated exposure with another antibiotic (oxytetracycline) in pigs, a
474 significant increase of the proportion of resistant *E.coli* was observed at the end of the study
475 (C. Santos-Santórum Suárez et al., 2023). Additionally, a recent EFSA work failed to
476 determine the Feed Antimicrobial Resistance Selection Concentration (FARSC) for
477 sulphonamides, due to a serious lack of data (EFSA, 2021). This work may help to overcome
478 these limitations.

479 The targeted approach of the HRMS analysis revealed two phase II metabolites. First, the
480 acetylated metabolite, which has already been described *in vitro* in pig hepatocytes
481 (Mengellers, 1997) and *in vivo* in pig plasma and urine (Mengelers et al., 2001; Shimoda et
482 al., 1990). Therefore, at the tolerated contamination rates, it is not only SDMT residues that
483 could be present in edible products but also its metabolites, which may be harmful for
484 consumers. For instance, the acetylated metabolite has been shown to be related to renal
485 toxicity because of its precipitation in the kidney (Kishida & Furusawa, 2004). Furthermore,
486 the glucuronide metabolite was potentially found but due to the low-intensity signal, this
487 needs to be investigated in a further study involving higher doses of SDMT. To our
488 knowledge, this metabolite has never been reported in pig livers, although it has been
489 described in the urine of humans (Vree, 1990; Vree, Beneken Kolmer & Peeters, 1991),
490 primates, rats, dogs, mice and guinea pigs (Adamson, Bridges, Kibby, Walker & Williams,
491 1970; Bridges, Kibby, Walker & Williams, 1968).

492 For the non-targeted approach, a PCA model was first developed that revealed a difference
493 between the treated and control groups in the metabolic profile of the liver. The PLS-DA
494 model then confirmed this clustering and allowed those features most involved in this
495 clustering to be selected. The results of the univariate and multivariate analysis showed the
496 most important variables involved in the differentiation of the groups. SDMT and one of its
497 adducts were found among these variables, but not the two putative SDMT metabolites
498 detected during the targeted approach. However, some potentially interesting features were
499 observed that could be further investigated in future studies. Nevertheless, the goal of this
500 study was to identify metabolites of SDMT. When seeking xenobiotic metabolites our study
501 clearly demonstrated that the targeted approach is more suitable than the non-targeted
502 approach, which makes the search very difficult and time-consuming due to the great number
503 of ions. Furthermore, some important metabolites may be lost due to the normalisation and

504 filtering. For example, the CV of the SDMT response was 33% (because the SDMT was not
505 normalised by the algorithm). This means that if a filter of 30% had been chosen, this
506 molecule would not have been found. What is more, the low SDMT concentrations, and thus
507 the low concentrations of its metabolites, makes it difficult to highlight them relative to the
508 control group.

509 In conclusion, this study aimed to evaluate level of antimicrobial residues in edible products
510 of animal origin after exposure to contaminated feed. The results showed that, at the tolerated
511 cross-contamination rates (in France), SDMT concentrations much higher than the authorised
512 MRL could be found in the muscle, liver and kidney. Furthermore, putative metabolites of
513 SDMT may be present in the liver, although further studies are needed to clarify this risk. In
514 the case of sulphonamides, MRLs were established for the whole class of veterinary
515 medicinal products as an extrapolation from studies performed with sulphadimidine alone
516 (EMA, 1995). Further studies should be carried out in order to improve our knowledge of
517 these drugs and specifically to check initial assumptions of pharmacokinetic similarities
518 between sulphonamides.

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527 **Declaration of Interest:**

528 The authors report there are no competing interests to declare

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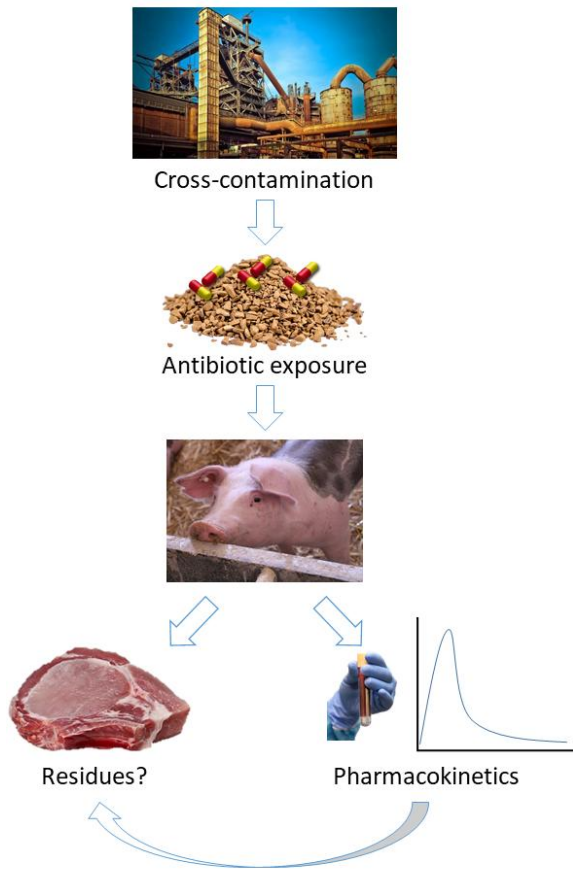
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674

675 **Graphic abstract**



676

677

678 **Supplementary material**

Feed composition
Raw protein
Raw fat
Raw cellulose
Raw ashes
Lysine
Methionine
Calcium
Phosphor sodium
Potassium

679 **Supplementary Table 1. Feed composition**

<i>Compound</i>	<i>Matrix</i>	<i>LLOQ-ULOQ (ng/mL or ng/g)*</i>	<i>Recovery (%)</i>	<i>Relative repeatability (VC, %)</i>	<i>Relative intermediate precision (VC, %)</i>	<i>Accuracy index</i>	<i>Relative expanded uncertainty (%)</i>
<i>SDMT</i>	Plasma	10-1000	97.67-100.6	2.39-3.42	2.93-3.42	0.872	5.52-7.51
	Muscle	10-1000	98.99-100.6	0.36-1.55	0.96-1.76	0.947	2.16-3.85
	Liver	16.31-1000	91.22-99.43	0.30-3.42	1.41-5.99	0.923	3.05-30.12
	Kidney	10-1000	97.95-102.2	0.58-3.6	0.58-3.82	0.886	1.26-8.32
	Faeces	20-1000	98.2-100.8	0.95-4.01	1.52-4.01	0.871	3.43-20.03
<i>TMP</i>	Plasma	10-1000	97.17-101.1	1.21-2.73	1.8-3.02	0.863	3.89-6.74
	Muscle	10-1000	97.64-102.2	0.81-2.65	0.81-2.80	0.948	1.76-6.10
	Liver	10-1000	97.3-102.2	0.85-13.87	1.76-13.87	0.911	3.79-29.95
	Kidney	10-1000	95.53-102.5	0.35-4.15	1.03-4.15	0.867	2.35-7.05
	Faeces	20-1000	96.97-99.83	1.04-3.65	1.82-8.00	0.866	4.13-18.37

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681 * LLOQ-ULOQ: Lower limit of quantification-upper limit of quantification

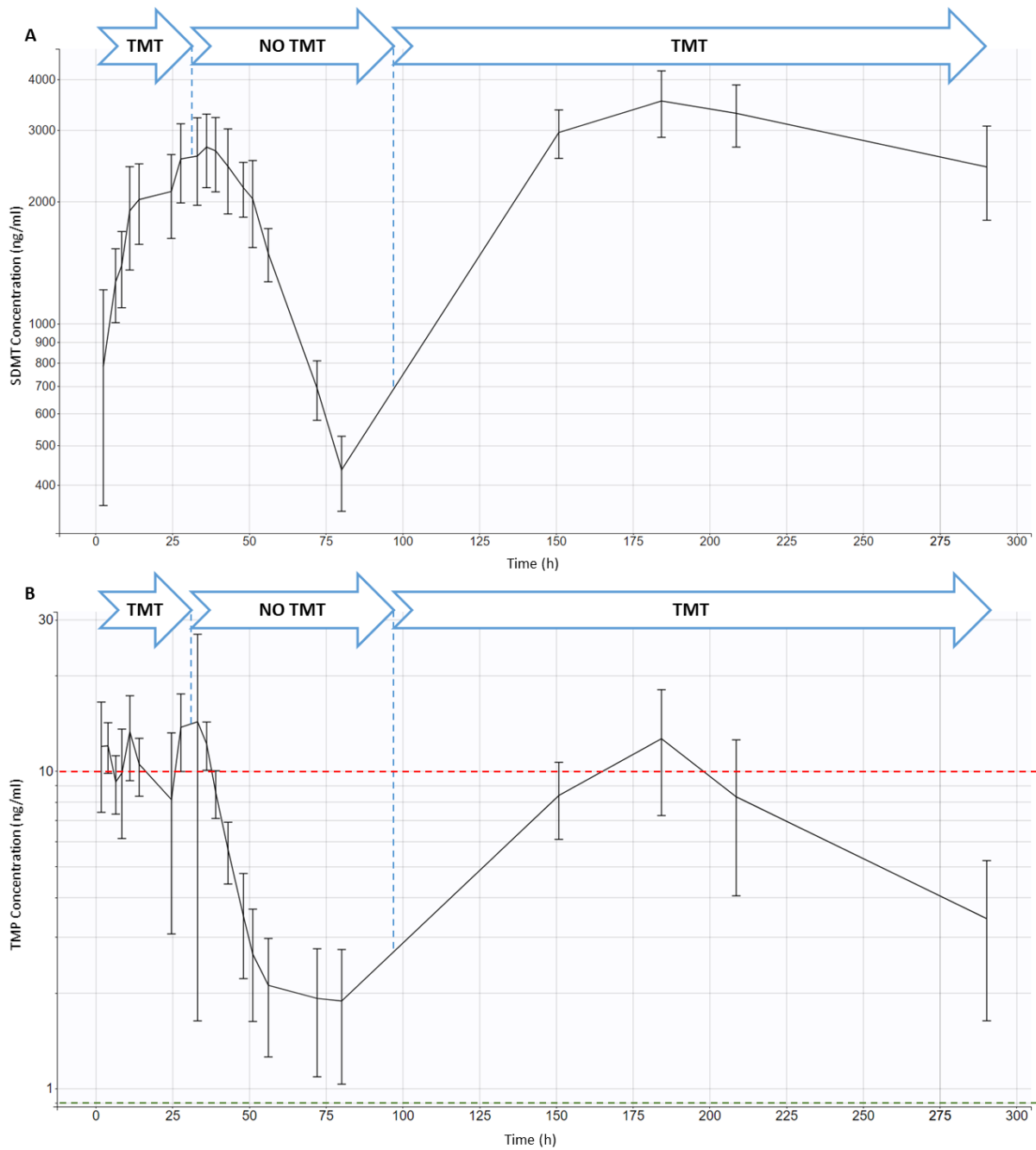
682 **Supplementary Table 2. LLOQ-ULOQ interval, recovery, relative repeatability, relative**
 683 **intermediate precision, recovery, accuracy index and relative expanded uncertainty for**
 684 **each analyte in each matrix**

685

	Transformation	Formula
<i>Phase I metabolites</i>	2 x Desaturation	-H4
	2 x Oxidation	+O2
	2 x Oxidation + Desaturation	-H2 +O2
	3 x Oxidation	+O3
	Decarbonylation	-CO
	Decarboxylation	-COO
	Deethylation	-C2H4
	Dehydration	-H2O
	Demethylation	-CH2
	Desaturation	-H2
	Dihydrodiol formation	+H2O2
	Ethyl ketone to acid	-C3H6+O
	Hidration	+H2O
	Isopropyl to alcohol	-C3H6+O
	Nitro reduction	-O2H2
	Oxidation	+O
	Oxidation + desaturation	+O-H2
	Reduction	+H2
	Propyl ketone to acid	-C4H8+O
	Tert-butyl to alcohol	-C4H8+O
<i>Phase II metabolites</i>	Acetyl cysteine conjugation	+C5H7NO3S
	Acetylation	+C2H2O
	Butyryl conjugation	+C4H6O
	Carnitine conjugation	+C7H14O2N
	CysGlys S adduction	+C5H8N2O3S
	Cysteine conjugation	+C3H5NOS
	Cysteine S-conjugation	+C3H5NO2S
	Formylation	+CO
	Glucuronidation	+C6H8O6
	Glucosylation	+C6H10O5
	Glucuronidation of carbamate	+C7H8O8
	Glutamine conjugation	+C5H7O3N
	Gluthatione conjugation	+C10H15N3O5S
	Gluthatione S-conjugation	+C10H15N3O6S
	Glycine conjugation	+C2H3NO
	Methylation	+CH2
	Phosphorylation	+HPO3
	Propionyl conjugation	+C3H4O
	Sulfation	+SO3
	Taurine conjugation	+C2H5NO2S

686 **Supplementary Table 3. Targeted transformation phase I and phase II metabolites**

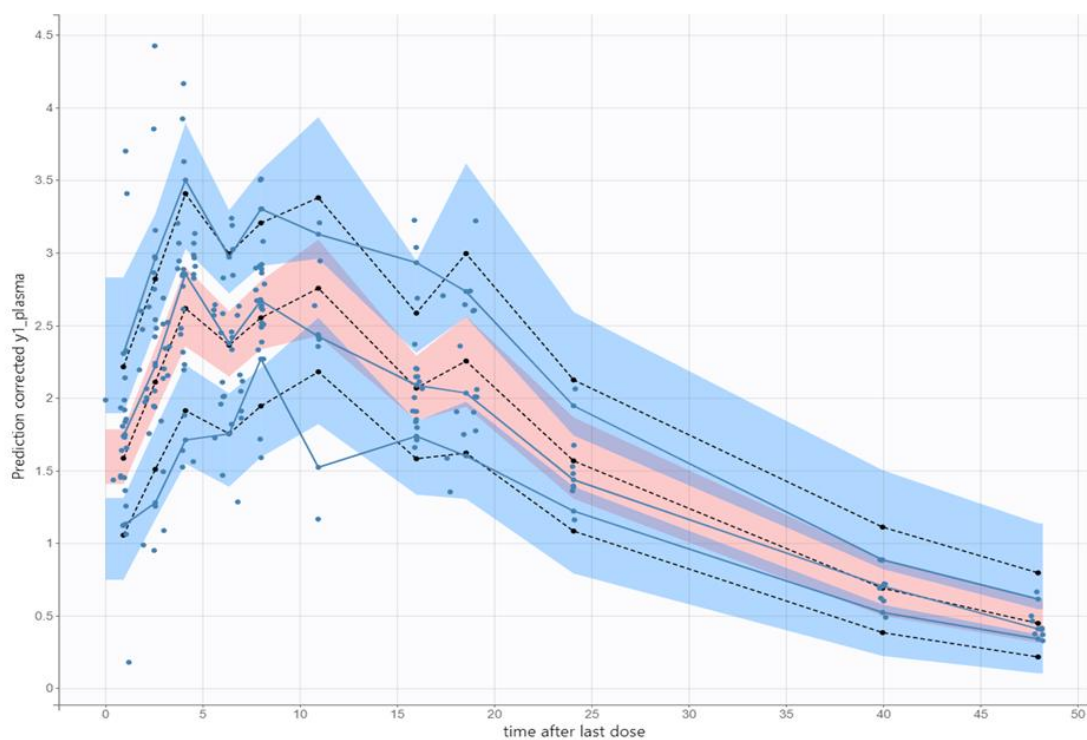
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689 **Supplementary figure 1. Mean SDMT (A) and TMP (B) concentrations in plasma**
 690 **(ng/ml, in log scale) and SD (error bar) for the 8 pigs. The red line represents the lower**
 691 **limit of quantification (LLOQ=10 ng/ml) and the green one represents the limit of**
 692 **detection (LOD=0.094 ng/ml) of the analytical method. TMT = treatment period**

693



694

695 **Supplementary figure 2. Visual predictive checks (VPCs) of the model with 500**
 696 **simulations and a 90% prediction interval**

<i>Pig</i>	Muscle concentration (mean of loin and leg) ($\mu\text{g}/\text{kg}$)	Liver concentration ($\mu\text{g}/\text{kg}$)	Kidney concentration ($\mu\text{g}/\text{kg}$)	Plasma concentration (ng/ml)
<i>Pig 1</i>	172.03	583.26	610.49	1704.78
<i>Pig 2</i>	158.26	550.87	437.46	2003.5
<i>Pig 3</i>	179.97	560.33	427.24	2759.68
<i>Pig 4</i>	198.68	576.75	508.43	2688.02
<i>Pig 5</i>	209.16	698.67	588.75	3510.47
<i>Pig 6</i>	203.27	721.70	565.60	1739.47
<i>Pig 7</i>	218.13	759.75	499.02	2902.07
<i>Pig 8</i>	161.39	492.13	441.83	2158.13

697 **Supplementary Table 4. Individual concentrations of sulphadimethoxine in pig tissues**
 698 **and plasma at time of slaughter**

699