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1 **Metabonomics-based analysis of *Brachyspira pilosicoli*'s response to**
2 **tiamulin reveals metabolic activity despite significant growth**
3 **inhibition.**

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14

15 **Key words: Metabonomics, *Brachyspira pilosicoli*, Tiamulin, Antibiotics.**

16

17 **Abstract**

18 Pathogenic anaerobes *Brachyspira spp.* are responsible for an increasing number of
19 Intestinal Spirochaetosis (IS) cases in livestock against which few approved treatments
20 are available. Tiamulin is used to treat swine dysentery caused by *Brachyspira spp.* and
21 recently has been used to handle avian intestinal spirochaetosis (AIS). The therapeutic
22 dose used in chickens requires further evaluation since cases of bacterial resistance to
23 tiamulin have been reported. In this study, we evaluated the impact of tiamulin at
24 varying concentrations on the metabolism of *B. pilosicoli* using a ¹H-NMR-based

25 metabonomics approach allowing the capture of the overall bacterial metabolic
26 response to antibiotic treatment. Based on growth curve studies, tiamulin impacted
27 bacterial growth even at very low concentration (0.008 µg/ml) although its metabolic
28 activity was barely affected 72 h post exposure to antibiotic treatment. Only the highest
29 dose of tiamulin tested (0.250 µg/ml) caused a major metabolic shift. Results showed
30 that below this concentration, bacteria could maintain a normal metabolic trajectory
31 despite significant growth inhibition by the antibiotic, which may contribute to disease
32 reemergence post antibiotic treatment. Indeed, we confirmed that *B. pilosicoli* remained
33 viable even after exposition to the highest antibiotic dose. This paper stresses the need
34 to ensure new evaluation of bacterial viability post bacteriostatic exposure such as
35 tiamulin to guarantee treatment efficacy and decrease antibiotic resistance
36 development.

37

38 **Highlight**

- 39 • *B. pilosicoli* metabolism was characterized using ¹H NMR-based metabonomics
- 40 • Tiamulin inhibited *B. pilosicoli* growth at very low dose (respectively < 0.016
41 µg/mL and > 0.032 µg/mL)
- 42 • *B. pilosicoli* metabolism is not inhibited for tiamulin concentration superior to
43 0.032 µg/mL
- 44 • *B. pilosicoli* metabolism is completely repressed at 0.250 µg/mL, but remain
45 viable

46 **1. Introduction**

47 *Brachyspira pilosicoli* is a gram-negative bacterium of the Spirochaetes family. It
48 colonizes the lower part of the gastrointestinal track of a large range of hosts including

49 pigs, birds, humans, monkeys, dogs and horses [1-4]. Once in the intestinal lumen, the
50 bacterium is attracted *via* chemotaxis to the mucin barrier [5,6] through which it swims
51 mediated by its unique “corkscrew” shape and rotation of its periplasmic flagella [7]
52 aided by the secretion of mucine degrading enzymes [5,8]. *B. pilosicoli* attaches to the
53 enterocytes in an end on fashion and may also infect these cells [9-12]. Colonization by
54 *B. pilosicoli* can lead to the development of intestinal spirochaetosis (IS), the signs of
55 which are diarrhea, poor overall condition, dehydration and decreased growth rate.
56 Mortality is often significant when the disease is left untreated [13-15], a consequence
57 that makes IS a serious economic and welfare problem in farming.

58 Tiamulin is effective in treating IS caused by *Brachyspira hyodystenteriae*, *B. hamptonii*
59 and *B. pilosicoli* in swine [16-18] and in poultry [19-21]. Tiamulin is a bacteriostatic
60 derived from a natural pleuromutilin that binds the 50S region of the ribosome to
61 inhibit protein synthesis [22]. The antibiotic blocks peptide bond formation by
62 interfering with substrate binding [22-25]. Tiamulin treatment in farms generally
63 results in clearance of infection and associated symptoms. However, reoccurrence of the
64 disease can be observed post treatment indicating incomplete clearance and possibly
65 decreased susceptibility [26,27] in response to treatment. The reason may be an
66 inappropriate dosing as there is currently there is a lack of an internationally
67 recognized standardized method to determine tiamulin minimum inhibitory
68 concentration (MIC) for this bacterium, which has impacts upon selection of an
69 appropriate treatment dose. Furthermore, recent studies have indicated that
70 *Brachyspira* may acquire resistance against tiamulin and, other than blocking protein
71 synthesis, nothing is yet known of the metabolic response of *B. pilosicoli* to tiamulin. We
72 argue that evaluating this using a metabonomics approach would allow a better

73 understanding of the bacterial response to tiamulin and give insights into improving
74 selection of effective dosing regimes.

75 Metabonomics allows non-targeted evaluation of the metabolic modifications occurring
76 in a biological system in response to a stress [28], which in this study is exposure to
77 tiamulin. By providing a general overview of the metabolic response, this technique
78 allows the generation of new hypotheses and to evaluate metabolic in response to
79 environmental stress or genetic modification. In this study, we used an NMR-based
80 metabonomics approach coupled with multivariate statistics to evaluate the metabolic
81 dose-response of *B. pilosicoli* to tiamulin. Bacteria were exposed to gradual antibiotic
82 doses and media were sampled over 120h in order to evaluate the evolution of its
83 metabolic composition during growth. This allowed to snapshot the metabolic response
84 of *B. pilosicoli* to tiamulin.

85

86 **2. Material and Methods**

87 2.1. Bacterial growth and antibiotic assay

88 *B. pilosicoli* B2904 isolated from chicken presenting clinical signs of AIS in the UK [29]
89 were grown from frozen stock on agar solidified BEB plate for four days under
90 anaerobic conditions (94% N₂ and 6% CO₂) at 37°C. Colonies were transferred into
91 *Brachyspira* enrichment broth media (BEB supplemented with heart infusion) for three
92 days under similar conditions. The bacterial concentration was then adjusted in BEB to
93 1 x 10⁶ CFU/ml and transferred into 24 well plates (2 ml per well) and incubated as
94 above for 120h. Every 24 h (with a first time point at 0 h growth), the entire well
95 content was taken and centrifuged for 2 min at 2400 g to separate growth medium from
96 bacteria. The supernatant was kept at - 80°C for further analysis. This process was

97 repeated at each time point in sextuplet to deliver the appropriate power for statistical
98 analysis.

99 The same method was used for the tiamulin assay. Bacterial cells were grown as above
100 and bacterial pellets were resuspended in BEB with antibiotic at six concentrations
101 (0.008, 0.016, 0.031, 0.062, 0.125 and 0.250 µg/ml plus control). Bacteria were then
102 inoculated into 24 well plates as previously described and incubated for 120h. For
103 metabolic analysis, each condition (tiamulin concentration) and time point (every 24 h
104 for 120 h) were also repeated in sextuplet. The medium was not changed for the
105 duration of the experiment so that antibiotic exposure was continuous.

106 To evaluate the viability of *B. pilosicoli* post- antibiotic exposure, the above experiment
107 was repeated in triplicate. Following 120 h incubation, *B. pilosicoli* was inoculated onto
108 fastidious anaerobic blood agar and incubated at 37°C, anaerobically for 48 h. Following
109 incubation all plates were visually inspected for bacterial growth.

110 *B. pilosicoli* growth was evaluated using the same experimental design as the one
111 previously described. Bacteria were grown in a 96 well plate (0.2 ml per well) and
112 bacterial growth was evaluated every 2 h for 120 h at an absorbance of 600 nm using a
113 Fluostar (Info). Water was used as blank and broth media without bacteria as a negative
114 control. Each condition (tiamulin concentration) was repeated in triplicate and results
115 are presented as an average of the log of the bacterial concentration calculated from the
116 absorbance observed at each tiamulin concentration per time point after correction
117 with standard curve.

118

119 2.2. NMR spectroscopy

120 For NMR spectroscopy, 0.4 ml of media was added to 0.2 ml of NMR phosphate buffer
121 (made in D₂O containing 10 % water and 0.05 % sodium 3-(tri-methylsilyl) propionate-

122 2,2,3,3-d₄ (TSP) as a ¹H NMR reference) and 0.5 ml of the solution was transferred into
123 5 mm of outer diameter NMR tubes. ¹H-NMR spectra were acquired on a Bruker Avance
124 DRX 700 MHz NMR Spectrometer (Bruker Biopsin, Rheinstetten, Germany) operating at
125 700.19 MHz and equipped with a cryogenic probe from the same manufacturer. A
126 standard 1-dimensional (1D) pulse sequence [recycle delay (RD)-90°-*t*₁-90°-*t*_m-90°-
127 acquire free induction decay (FID)] with water suppression applied during RD of 2 s
128 and a mixing time (*t*_m) of 100 ms and a 90° pulse set at 10 μs was applied. For each
129 spectrum 128 scans were recorded on a total of 32K data points. A broadening line
130 function of 0.3 Hz was used to multiply all FIDs. After acquisition, all spectra were
131 manually phased and baseline corrected using the software MestReNova® (version
132 2.1.8-11880, MestreLab, Spain). Finally, spectra were calibrated to the chemical shift of
133 TSP (δ 0.00). In order to facilitate metabolite identification based on literature, a series
134 of 2D spectra on selected samples were acquired using correlation spectroscopy (COSY)
135 NMR spectroscopy.

136

137 3.3. Statistical analysis

138 All spectra were scaled on unit variance and mean centered prior to analysis. To
139 evaluate metabolic variation between samples, principal component analysis (PCA) was
140 used. Orthogonal projection to latent structure discriminant analysis (O-PLS-DA) was
141 also performed, where ¹H-NMR spectra were used as a matrix of independent variables
142 (X) and time or antibiotic concentration were used as prediction vectors (Y) to capture
143 the metabolic variations linear to time and antibiotic concentration. O-PLS DA models
144 were generated between each tiamulin concentration at every time point
145 independently. A heat map was generated using each of this model strength in order to

146 visualize when tiamulin impacted bacterial metabolism in comparison to control and if
147 clusters related to dose could be observed.

148

149 **3. Results**

150 3.1. Modifications of *B. pilosicoli* metabolism during growth

151 The aim of this study was to characterize *B. pilosicoli* metabolism under optimum
152 growth conditions. Figure 1 presents *B. pilosicoli* growth and metabolic activity in broth
153 media. PC1, which captured 49% of the metabolic variation, indicated that a significant
154 metabolic shift was recorded after 96 h of incubation. Distinctions between 0 h, 24 h
155 and 48 h were observed on the 3rd component, representing only 9% of the total
156 variation, which suggests a modest effect on the composition of the culture medium
157 over the first 48 h. This metabolic trajectory indicates that bacterial metabolism might
158 change depending on the growth phase (Figure 1A and B). Scores from the same time
159 point were clustered together indicating good reproducibility of the experiment.

160 In the antibiotic free culture medium, the growth of *B. pilosicoli* was associated with a
161 decrease in glucose and an increase in amino acids (phenylalanine, alanine, tyrosine,
162 lysine, valine and methionine), fermentation products (lactate, acetate, butyrate and
163 isovalerate), as well as other compounds involved in the regulation of cell osmosis such
164 as *myo*-inositol and trimethylamine (TMA) as observed in the PCA results presented in
165 Figures 1A and C.

166 Glucose was the only readily identifiable substrate that showed a reduction over time
167 (Figure 1C). Decreased concentration of other substrates could not be detected and it is
168 possible that some may be below the detection limit of the NMR instrument.
169 Nevertheless, it is not unreasonable to assume that glucose was the only carbon source
170 used for bacterial anabolism and growth. *B. pilosicoli* and more especially the strain

171 used for this experiment (B2904) is able to use a wide range of carbohydrates and
172 hexoses as primary carbon sources [10] but it would seem in this study that glucose was
173 used preferentially.

174

175 3.2. Tiamulin impacts *B. pilosicoli* growth even at very low doses

176 Having characterized the metabolic footprint of *B. pilosicoli* when grown in optimal
177 conditions, we then challenged it with increasing tiamulin doses. Tiamulin impacted *B.*
178 *pilosicoli*'s growth at the lowest concentrations tested (0.008 and 0.016 µg/mL) as
179 displayed in Figure 2. For these two doses, the bacterial count observed at the
180 stationary phase was one log lower than for the control demonstrating the ability of
181 tiamulin to reduce the growth of *B. pilosicoli* at low concentrations. Up to 54 hours,
182 growth curves of the two lowest concentrations (0.008 and 0.016 µg/mL) were
183 identical to the control (T1 and T2 on the graph) but they stopped growing shortly after
184 and entered into the stationery phase. No bacterial growth was detected for higher
185 tiamulin concentrations (over 0.032 µg/mL) confirming its efficiency to stop bacterial
186 proliferation. Interestingly, no gradual tiamulin dose response of bacterial growth was
187 observed. Indeed, growth rates were similar for the two lowest concentrations (0.008
188 and 0.016 µg/mL) while higher doses induced a complete inhibition of *B. pilosicoli*'s
189 growth.

190

191 3.3. Metabolic response of *B. pilosicoli* to tiamulin

192 A clear metabolic response of *B. pilosicoli* to tiamulin could be observed when the
193 antibiotic dose exceeded to 0.032 µg/mL. At lower doses (0.008 and 0.016 µg/mL),
194 although *B. pilosicoli* growth was decreased by 1 log (Figure 2) compared with

195 untreated control, the metabolic trajectories remained unaffected (Figure 3A,
196 Supplemental Fig.1 and 2).

197 When bacteria were exposed to 0.032 $\mu\text{g}/\text{mL}$ of tiamulin (Supplemental Fig. 3), a
198 disruption of the metabolic trajectory was observed which was due to modifications of
199 amino acid concentration. A noticeable increase of tyrosine, methionine, valine,
200 phenylalanine and lysine into the medium from 0 to 96 h was observed. After that time,
201 their concentration reduced, indicating consumption of these amino acids until the end
202 of the experiment. After 120h of growth, the metabolic composition of the media was
203 comparable to control, indicating the full recovery of the metabolism following
204 antibiotic exposure.

205 Higher doses of tiamulin (0.062 and 0.125 $\mu\text{g}/\text{mL}$) induced similar responses to those
206 observed at 0.032 $\mu\text{g}/\text{mL}$ (Figure 3A and Supplemental Fig. 4. A and B). Amino acid
207 metabolism was affected in a greater extent.

208 At the maximum dose tested (0.250 $\mu\text{g}/\text{mL}$) the metabolic trajectory observed for the
209 media was drastically modified in comparison to those described previously (Figure 3
210 and Supplemental Fig. 5). The metabolic trajectory followed a circular shape where the
211 scores of the samples collected after 120h of bacterial growth were clustered with the
212 ones observed at T0, indicating metabolic similarities with the baseline time-point.
213 Once again, amino acids were released into the medium as well as butyrate and *myo*-
214 inositol.

215

216 *3.4. B. pilosicoli survives post-antibiotic exposure*

217 To evaluate the surviving potential of *B. pilosicoli* after tiamulin exposure, samples from
218 replication of the growth curve experiment were plated on agar plate at the end of the
219 antibiotic challenge (120 h). For all tiamulin doses applied it was possible to observe *B.*

220 *pilosicoli* colony formation on agar plates (Supplement Table 1). These results indicate
221 that *B. pilosicoli* is able to recover from tiamulin exposure (even at the highest dose of
222 0.250 µg/mL) once back in optimal growth conditions.

223

224 **4. Discussion**

225 The results obtained from *B. pilosicoli* growth in a control medium without tiamulin
226 provide new insights about its general metabolism. The bacteria were able to produce
227 lactate and acetate from glucose fermentation without secreting methanol, suggesting
228 the use of the bifidum pathway according to the following equation: glucose → 3 acetate
229 + 2 lactate [30]. However, lactate was generally found in very small quantity in
230 comparison to acetate, indicating its potential use in other metabolic reactions. Bacteria
231 were also able to secrete butyric acid but not propionic acid. Both of these short chain
232 fatty acids were found to be potential carbon sources for *B. pilosicoli* [10]. Bacteria also
233 released a large number of amino acids that could be caused either by synthesis and
234 active secretion of these amino acids, or more likely due to exogenous protein
235 degradation. This result is in accordance with the genetic results published by Mappley
236 et al. [10] that indicated a strong proteolytic capacity of the bacterium. This specific
237 strain of *B. pilosicoli* was also shown to be able to use amino acids as primary carbon
238 source [10]. However, as the bacterium favors glucose if available as primary carbon
239 source, amino acids may here only be used for protein synthesis and may therefore
240 become in excess in the culture medium where they accumulate. Finally, the bacteria
241 secreted TMA. Gut bacteria generally produce TMA from dietary L-carnitine, betaine or
242 choline. Yet, it was not possible to detect a decrease in concentration of these
243 compounds indicating that *B. pilosicoli* might not use these molecules as precursors or
244 that the technique used was not sensitive enough to detect such variations.

245 More importantly, this work confirmed tiamulin ability to significantly reduce at very
246 low doses (0.008 and 0.016 $\mu\text{g}/\text{mL}$) and inhibit at higher concentrations *B. pilosicoli*
247 growth. Decreased growth rate at such low antibiotic doses were unexpected, as
248 previous evaluation of minimum inhibitory concentration (MIC) values for this specific
249 strain were of 0.250 $\mu\text{g}/\text{mL}$ [31], furthermore, 10-15% of *B. pilosicoli* isolates presented
250 MICs > 4 $\mu\text{g}/\text{mL}$ [31]. Differences in the MIC values can be explained by experimental
251 conditions. Growth curves were acquired when *B. pilosicoli* was grown in BEB media
252 rather than on agar plates for MIC tests. The *B. pilosicoli* strain B2904 used in this study
253 is known to have an MIC of 0.250 $\mu\text{g}/\text{mL}$ [31,32] but showed clear inhibition of growth
254 with concentrations below this value. Thus, our findings confirm the previously
255 reported observation that lower tiamulin MIC values are generally found in broth
256 compared to agar for *B. hyodysenteriae* [33].

257 Despite these encouraging results regarding tiamulin efficiency to inhibit pathogen
258 growth, the evaluation of *B. pilosicoli* metabolic viability in response to antibiotic
259 treatment revealed that classic MIC calculations might not be sufficient to assess
260 antibiotic efficiency. Indeed, *B. pilosicoli* metabolic rate reduction did not mirror
261 previously commented reduced growth rate in response to tiamulin treatment. At the
262 two lowest doses used (0.008 and 0.016 $\mu\text{g}/\text{mL}$), *B. pilosicoli* growth was reduced by
263 one log in comparison to control. However, the metabolic trajectories observed by the
264 media were identical. Indicating that tiamulin was able to impact growth but that *B.*
265 *pilosicoli* basic metabolism remained unaffected. Higher doses were able to reduce but
266 not silent *B. pilosicoli* metabolism despite complete growth inhibition. Furthermore, the
267 metabolism of *B. pilosicoli* appears to slightly recover from all tiamulin concentrations
268 except from 0.250 $\mu\text{g}/\text{mL}$ after 120 h of growth. This might be due to the apparition of
269 resistance, which is known as being a slow bacterial development process [34,35].

270 In addition, it was demonstrated that *B. pilosicoli* was able to survive post-tiamulin
271 exposure even at the highest antibiotic dose when plated on agar (after 120 h drug
272 exposure). Indeed *B. pilosicoli* colonies were identified 48h after the end of the
273 antibiotic treatment for all doses tested (0.008-0.250 µg/mL). These results illustrate
274 the potential of *B. pilosicoli* to enter a dormancy state when exposed to tiamulin that is
275 reversible once the treatment period is over.

276 Besides, results demonstrated a slow response of the bacteria to antibiotic treatment as
277 modification of the metabolic footprint was only observed after more than 48 h of
278 growth in presence of tiamulin. From these results, it seems that metabolism was
279 stressed during the exponential phase, when bacterial division is compromised.
280 Metabolism modification was mainly associated with increased amino acid
281 consumption (that were produced in the control). However, as the provenance of these
282 amino acids remains unclear, two hypotheses can be formulated. Firstly, in response to
283 antibiotic stress bacteria could use amino acids as alternative energy substrates.
284 Secondly, *B. pilosicoli* might not be able to hydrolyse proteins present in the media
285 because new protein synthesis, such as secreted proteases, is blocked at the ribosome.
286 The specificity of the amino acids used indicates the first option is the most probable
287 and that catabolism repression could be overridden to secure energy from multiple
288 sources. This is an interesting hypothesis that needs confirmation by alternative
289 techniques such as transcriptomics.

290 The fact that *B. pilosicoli* remain viable and metabolically active without dividing despite
291 the antibiotic treatment and is able to recover after antibiotic exposure could partly
292 explain the IS relapse observed in farms after tiamulin intervention. Indeed, it seems to
293 arise from these results that bacteria remain viable but are not able to divide entering
294 therefore a dormancy stage. It is highly possible that such phenomenon occurs in the

295 intestinal lumen, where bacteria could suffer from inactivation of cell division but
296 remain viable. This bacterial state might be associated with a decrease in their
297 pathogenicity explaining the disappearance of associated symptoms. Nevertheless,
298 bacteria might remain viable but at a “dormancy” state in the intestinal lumen or animal
299 faeces until the environment becomes less hostile (end of antibiotic treatment) when
300 they can recover their pathogenic property. However, it is impotent to point out that
301 here *B. pilosicoli* was recovered on agar plates that represent optimal growth
302 conditions. This might not be the case in the intestinal lumen that is a more hostile
303 environment when the bacterium also needs to compete with other commensal bacteria
304 and is confronted to the host’s immune system.

305 As increasing antibiotic resistance mechanisms developed by pathogenic bacteria are
306 arising, many studies and reviews have stressed the concerns linked to inappropriate
307 antibiotic usage. Indeed, this is strongly linked to development of antibiotic resistance, a
308 burden for health and industry. There is therefore a surge for redefining appropriate
309 antibiotic use that would help minimizing the current concern linked to decreased
310 antibiotic efficiency. Thus, importance should be given to new methods development
311 aiming at better assessing antibiotic efficiency and to detect potential antibiotic
312 resistance factor development. This study indicates that metabonomics could be and
313 easy and practical way to evaluate bacterial metabolic activity and therefore assess
314 antibiotic efficiency to totally inactivate pathogens and therefore avoid infection
315 relapse.

316

317 **5. Conclusion**

318 This work gave a clearer understanding of *B. pilosicoli* metabolism in optimum growth
319 conditions, including indication regarding favored fermentation pathways and amino

320 acids metabolism. It supports the fact that tiamulin can inhibit efficiently bacterial
321 growth at low concentrations. However, it was surprising to observe that tiamulin could
322 impact *B. pilosicoli* growth without influencing its basic metabolism. It also reveals that
323 the bacterium try to maintain metabolic homeostasis despite an obvious stress visible
324 on the growth curve, demonstrating that in response to xenobiotic stress, bacterial
325 division is the first mechanism to be suspended. This indicates that tiamulin might
326 present a good solution against AIS outbreaks, as it is able to significantly reduce or stop
327 bacterial growth, provided that the efficient dose is achieved in the gut of every
328 individual. Even so, the treatment may not be sufficient to avoid relapse of the disease.
329 Such findings suggest that measurement of bacterial activity might be needed in order
330 to assess antibiotic efficiency against potential reoccurrence of the disease. In that
331 prospect, metabonomics appeared as a potential solution to evaluate if antibiotic
332 treatment can inactivate microbial metabolic activity.

333

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444

445 **Figures and tables**

446

447 Figure 1: *B. pilosicoli* consumed glucose and released amino acids and fermentation
448 products in its environment. (A) PCA scores plot. (B) *B. pilosicoli* growth curve in
449 *Brachyspira* enrichment broth media under anaerobic conditions made in triplicate. (C)
450 Associated loadings of the first component. The metabolic trajectories described by the
451 arrows were determined by the position of the centroids calculated at each time point
452 using the coordinate of the associated scores on the principal components.

453

454 Figure 2: Impact of tiamulin on *B. pilosicoli* growth in *Brachyospira* enrichment broth
455 media under anaerobic conditions. Growth was measured in triplicate every 2 h for 120
456 h.

457

458 Figure 3: (A) Metabolic trajectories derived from the PCA analysis performed using all
459 the sample population (N=252) of the study (i.e. control plus 6 tiamulin dilution) on PC1
460 and PC3 displaying the centroids for each time points of the control and three
461 concentrations of tiamulin. (B) Heat map representing the O-PLS DA model strength
462 existing between each tiamulin concentration at each time point, based on R²Y
463 (goodness of fit of the model) and Q²Y (goodness of prediction of the model) values

464 using the following formula: $Model\ strength = \frac{R^2Y \times Q^2Y}{R^2Y - Q^2Y}$.

465

466 **Supplemental material**

467

468 Supplement Fig. 1: Metabolic trajectories of *B. pilosicoli* footprints in broth media for
469 120 h at a tiamulin concentration of: 0.008 µg/mL (A) and 0.016 µg/mL (B). The arrows
470 indicate the metabolic trajectory. The metabolic trajectories described by the arrows
471 were determined by the position of the centroids calculated at each time point using the
472 coordinate of the associated scores on the PCs.

473

474 Supplement Fig. 2: Metabolic variation related to PCA scores plots presented in
475 Supplement Fig. 1. (A) loadings of the principal component 1 of the model presented in
476 Fig A.1.A. (B) loadings of the principal component 3 of the model presented in

477 Supplement Fig. 1.A. (C) loadings of the principal component 1 of the model presented
478 in Supplement Fig. 1.B. (D) loadings of the principal component 4 of the model
479 presented in Supplement Fig. 1.B.

480

481 Supplement Fig. 3: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120
482 h at a tiamulin concentration of 0.032 µg/ml on principal component 1 and 3. (A) PCA
483 score plot. (B) Associated loading plot for PC1. The metabolic trajectories described by
484 the arrows were determined by the position of the centroids calculated at each time
485 point using the coordinate of the associated scores on the PCs.

486

487 Supplement Fig. 4: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120
488 h at a tiamulin concentration of: 0.062 µg/ml (A) and 0.125 µg/ml (B). The arrows
489 indicate the metabolic trajectory. The metabolic trajectories described by the arrows
490 were determined by the position of the centroids calculated at each time point using the
491 coordinate of the associated scores on the PCs.

492

493 Supplement Fig. 5: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120
494 h at a tiamulin concentration of 0.250 µg/ml. (A) PCA score plot. (B) Associated loading
495 plot of PC1. The arrows indicate the metabolic trajectory. The metabolic trajectories
496 described by the arrows were determined by the position of the centroids calculated at
497 each time point using the coordinate on the associated scores on the PCs.

498

499 Supplement Tab. 1: Growth scores of *B. pilosicoli* on agar plate post-antibiotic exposure.
500 Detection of *B. pilosicoli*'s growth 48h after plating are indicated by a sign '+' and by '-' if
501 no growth was observed.