University of Reading

Metabonomics-based analysis of Brachyspira pilosicoli's response to tiamulin reveals metabolic activity despite significant growth inhibition

Article

Accepted Version

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

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

Caroline Ivanne Le Roy[a,b], Jade Louise Passey[c], Martin John Woodward[a], Roberto Marcello La Ragione[c], Sandrine Paule Claus[a]#.

[a] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, UK6 6AP, UK

Present address [b] Department of Twin Research & Genetic Epidemiology, King's College London, London SE1 7EH, UK

[c] Faculty of Health and Medical Sciences, School of Veterinary Medicine, University of Surrey, Guilford, Surrey GU2 7XH, UK

Corresponding author: s.p.claus@reading.ac.uk, Tel +44 118 378 8717

Abstract

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

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

Highlight

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

1. Introduction

Brachyspira pilosicoli is a gram-negative bacterium of the Spirochaetes family. It 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. hampsonii*

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% N_2 and 6% CO_2) 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 10^6 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 37^0C, 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 D_2O containing 10 % water and 0.05 % sodium 3-(tri-methylsilyl) propionate-

122   2,2,3,3-d_4 (TSP) as a ^{1}H NMR reference) and 0.5 ml of the solution was transferred into

123   5 mm of outer diameter NMR tubes. ^{1}H-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°-*t1*-90°-*tm*-90°-

127   acquire free induction decay (FID)] with water suppression applied during RD of 2 s

128   and a mixing time (*tm*) 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 ^{1}H-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 µg/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 µg/mL) induced similar responses to those

206   observed at 0.032 µg/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 µg/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 μg/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 μg/mL [31], furthermore, 10-15% of *B. pilosicoli* isolates presented

250   MICs > 4 μg/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 μg/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 μg/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 μg/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

References

335 [1] Stanton, T. B. & Hampson, D. J. Physiology of Ruminal and intestinal spirochaetes.
336   *CAB Int.* 7–45 (1997).

337 [2] Duhamel, G. E., Stryker, C. J., Lu, G., Wong, V. J. & Tarara, R. P. Colonic spirochetosis
338   of colony-raised rhesus macaques associated with *Brachyspira* and *Helicobacter*.
339   *Anaerobe.* **9,** 45–55 (2003).

340 [3] Hidalgo, A., Rubio, P., Osorio, J. & Carvajal, A. Prevalence of *Brachyspira pilosicoli*
341   and '*Brachyspira canis*' in dogs and their association with diarrhoea. *Vet.*
342   *Microbiol.* **146,** 356–60 (2010).

343 [4] Trott, D. J., Stanton, T. B., Jensen, N. S., Duhamel, G. E., Johnson, J. L., & Hampson, D.
344   J. *Serpulina pilosicoli* sp., the agent of porcine intestinal spirochetosis. *Int. J. Syst.*
345   *Bacteriol.* **46,** 206–15 (1996).

346 [5] Naresh, R. & Hampson, D. J. Attraction of *Brachyspira pilosicoli* to mucin.
347   *Microbiology.* **156,** 191–7 (2010).

348 [6] Hopwood, D., Pethick, D. & Hampson, D. Increasing the viscosity of the intestinal

349 contents stimulates proliferation of enterotoxigenic *Escherichia coli* and
350 *Brachyspira pilosicoli* in weaner pigs. *Br. J. Nurition.* **88,** 523–532 (2007).

351 [7] Prapasarakul, N., Lugsomya, K., Disatian, S., Lekdumrongsak, T., Banlunara, W.,
352 Chetanachan, P., & Hampson, D. J. Faecal excretion of intestinal spirochaetes by
353 urban dogs, and their pathogenicity in a chick model of intestinal spirochaetosis.
354 *Res. Vet. Sci.* **91,** e38–43 (2011).

355 [8] Li, C., Motaleb, A., Sal, M., Goldstein, S. F. & Charon, N. W. Spirochete periplasmic
356 flagella and motility. *J. Mol. Microbiol. Biotechnol.* **2,** 345–54 (2000).

357 [9] Dworkin, M. *The prokaryotes: Vol. 7: Proteobacteria: Delta and Epsilon Subclasses.*
358 S. Falkow, E. Rosenberg, K. H. Schleifer, & E. Stackebrandt (Eds.). Springer Science
359 & Business Media. 2006.

360 [10] Mappley, L. J., Black, M. L., AbuOun, M., Darby, A. C., Woodward, M. J., Parkhill, J.,
361 Turner, A. K., Bellgard, M. I., La, T., Philips, N. D., La Ragione, R. M. & Hampson, D.
362 J. Comparative genomics of *Brachyspira pilosicoli* strains: genome
363 rearrangements, reductions and correlation of genetic compliment with
364 phenotypic diversity. *BMC Genomics.* **13,** 454 (2012).

365 [11] Dassanayake, R. P. Biochemical properties of membrane-associated proteases of
366 *Brachyspira pilosicoli* isolated from humans with intestinal disorders. *J. Med.*
367 *Microbiol.* **53,** 319–323 (2004).

368 [12] Nakamura, S., Adachi, Y., Goto, T. & Magariyama, Y. Improvement in Motion
369 Efficiency of the Spirochete *Brachyspira pilosicoli*. *Biophys. J.* **90,** 3019–3029
370 (2006).

371 [13] Fellström, C. & Gunnarsson, A. Phenotypical characterisation of intestinal
372 spirochaetes isolated from pigs. *Res. Vet. Sci.* **59,** 1–4 (1995).

373 [14] Duhamel, G. E., Hunsaker, B. D., Mathiesen, M. R., & Moxley, R. A. Intestinal
374 spirochaetosis and giardasis in a beagle pup with diarrhoea. *Vet. Pathol.* **33**, 360-
375 362 (1996)

376 [15] Taylor, D. J., Simmons, J. R. & Laird, H. M. Production of dirrhoea and dysentery in
377 pigs by feeding pure cultures of spirochaete differing from *Treponemu*
378 *hyodysenteriae. Vet.* **106**, 326–332 (1980).

379 [16] Johnston, W. T., Dewey, C. E., Friendship, R. M., Smart, N., McEwen, B. J., Stalker,
380 M., & de Lange, C. F. An investigation of the etiology of a mild diarrhea observed in
381 a group of grower/finisher pigs. *Can. Vet. J.* **42,** 33–37 (2001).

382 [17] Wilberts, B. L., Arruda, P. H., Warneke, H. L., Erlandson, K. R., Hammer, J. M., &
383 Burrough, E. R. Cessation of clinical disease and spirochete shedding after
384 tiamulin treatment in pigs experimentally infected with 'Brachyspira hampsonii'.

385   *Res. Vet. Sci.* **97,** 341–347 (2014).

386   [18]   Burch, D. Tiamulin activity against *Brachyspira hyodysenteriae*. *Vet. Rec.* **163,** 698
387   (2008).

388   [19]   Burch, D. & Klein, U. Treatment of *Brachyspira* species with high MICs against
389   Tiamulin in layers. *6th Int. Conf. Avian Intest. Spyrochaetosis Infect. Anim. Humans.*
390   51 (2013).

391   [20]   Stephens, C. P. & Hampson, D. J. Evaluation of tiamulin and lincomycin for the
392   treatment of broiler breeders experimentally infected with the intestinal
393   spirochaete *Brachyspira pilosicoli*. *Avian Pathol.* **31,** 299–304 (2002).

394   [21]   Burch, D. G. S., Harding, C., Alvarez, R. & Valks, M. Treatment of a field case of
395   avian intestinal spirochaetosis caused by *Brachyspira pilosicoli* with tiamulin.
396   *Avian Pathol.* **35,** 211–6 (2006).

397   [22]   Schlünzen, F., Pyetan, E., Fucini, P., Yonath, A. & Harms, J. M. Inhibition of peptide
398   bond formation by pleuromutilins: the structure of the 50S ribosomal subunit
399   from *Deinococcus radiodurans* in complex with tiamulin. *Mol. Microbiol.* **54,** 1287–
400   1294 (2004).

401   [23]   Poulsen, S. M., Karlsson, M., Johansson, L. B., & Vester, B. The pleuromutilin drugs
402   tiamulin and valnemulin bind to the RNA at the peptidyl transferase centre on the
403   ribosome. *Mol. Microbiol.* **41,** 1091–1099 (2001).

404   [24]   Long, K. S., Hansen, L. H., Jakobsen, L. & Vester, B. Interaction of pleuromutilin
405   derivatives with the ribosomal peptidyl transferase center. *Antimicrob. Agents*
406   *Chemother.* **50,** 1458–1462 (2006).

407   [25]   Forschungsinstitut, S. The Mode of action of pleuromutilin derivatives effect on
408   cell-free polypeptide synthesis. *Europ. J. Biochem.* **533,** 527–533 (1974).

409   [26]   Stephens, C. P. & Hampson, D. J. Evaluation of tiamulin and lincomycin for the
410   treatment of broiler breeders experimentally infected with the intestinal
411   spirochaete *Brachyspira pilosicoli*. *Avian Pathol.* **31,** 299–304 (2002).

412   [27]   Burch, D. G. S., Harding, C., Alvarez, R. & Valks, M. Treatment of a field case of
413   avian intestinal spirochaetosis caused by *Brachyspira pilosicoli* with tiamulin.
414   *Avian Pathol.* **35,** 211–6 (2006).

415   [28]   Sperling, D., Smola, J. & Cízek, A. Characterisation of multiresistant *Brachyspira*
416   *hyodysenteriae* isolates from Czech pig farms. *Vet. Rec.* **168,** 215 (2011).

417   [29]   Karlsson, M., Aspan, A., Landén, A., & Franklin, A. Further characterization of
418   porcine *Brachyspira hyodysenteriae* isolates with decreased susceptibility to
419   tiamulin. *J. Med. Microbiol.* **53,** 281–285 (2004).

420   [30]   Nicholson, J. K., Lindon, J. C. & Holmes, E. 'Metabonomics': understanding the

metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica.* **29,** 1181–9 (1999).

[31]   Mappley, L. J., Tchórzewska, M. a, Cooley, W. a, Woodward, M. J. & La Ragione, R. M. Lactobacilli antagonize the growth, motility, and adherence of *Brachyspira pilosicoli*: a potential intervention against avian intestinal spirochetosis. *Appl. Environ. Microbiol.* **77,** 5402–5411 (2011).

[32]   Gottschalk, G. *Bacterial metabolism*. Springer Science & Business Media. (2012).

[33]   Pringle, M., Landén, A., Unnerstad, H. E., Molander, B. & Bengtsson, B. Antimicrobial susceptibility of porcine *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* isolated in Sweden between 1990 and 2010. *Acta Vet. Scand.* **54,** 54 (2012).

[34]   Woodward, M. J., Mappley, L., Le Roy, C., Claus, S. P., Davies, P., Thompson, G., & La Ragione, R. M. Drinking water application of Denagard® Tiamulin for control of *Brachyspira pilosicoli* infection of laying poultry. *Res. Vet. Sci.* **103,** 87–95 (2015).

[35]   Rohde, J., Kessler, M., Baums, C. G. & Amtsberg, G. Comparison of methods for antimicrobial susceptibility testing and MIC values for pleuromutilin drugs for *Brachyspira hyodysenteriae* isolated in Germany. *Vet. Microbiol.* **102,** 25–32 (2004).

[36]   Bock, A., Turnowsky, F., Hogenauer, G., Universitat, M. D. & Germany, W. Tiamulin resistance mutations in *Escherichia coli. J. Bacteriol.* **151,** 1253–1260 (1982).

[37]   Karlsson, M., Gunnarsson, A. & Franklin, A. Susceptibility to pleuromutilins in *Brachyspira* (*Serpulina*) *hyodysenteriae. Anim. Heal. Res. Rev.* **2,** 59–66 (2001).

Figures and tables

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

453

454   Figure 2: Impact of tiamulin on *B. pilosicoli* growth in *Brachyspira* 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 R^2Y

463   (goodness of fit of the model) and Q^2Y (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.