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

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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

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

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

2. Material and Methods

2.1. Bacterial growth and antibiotic assay

B. pilosicoli B2904 isolated from chicken presenting clinical signs of AIS in the UK [29] were grown from frozen stock on agar solidified BEB plate for four days under anaerobic conditions (94% N_2 and 6% CO_2) at 37°C. Colonies were transferred into *Brachyspira* enrichment broth media (BEB supplemented with heart infusion) for three days under similar conditions. The bacterial concentration was then adjusted in BEB to 1 x 10^6 CFU/ml and transferred into 24 well plates (2 ml per well) and incubated as above for 120h. Every 24 h (with a first time point at 0 h growth), the entire well content was taken and centrifuged for 2 min at 2400 g to separate growth medium from bacteria. The supernatant was kept at - 80°C for further analysis. This process was

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

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

To evaluate the viability of *B. pilosicoli* post- antibiotic exposure, the above experiment was repeated in triplicate. Following 120 h incubation, *B. pilosicoli* was inoculated onto fastidious anaerobic blood agar and incubated at 37^0C, anaerobically for 48 h. Following incubation all plates were visually inspected for bacterial growth.

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

2.2. NMR spectroscopy

For NMR spectroscopy, 0.4 ml of media was added to 0.2 ml of NMR phosphate buffer (made in D_2O containing 10 % water and 0.05 % sodium 3-(tri-methylsilyl) propionate-

2,2,3,3-d_4 (TSP) as a ^{1}H NMR reference) and 0.5 ml of the solution was transferred into 5 mm of outer diameter NMR tubes. ^{1}H-NMR spectra were acquired on a Bruker Avance DRX 700 MHz NMR Spectrometer (Bruker Biopsin, Rheinstetten, Germany) operating at 700.19 MHz and equipped with a cryogenic probe from the same manufacturer. A standard 1-dimensional (1D) pulse sequence [recycle delay (RD)-90°-*t1*-90°-*tm*-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s and a mixing time (*tm*) of 100 ms and a 90° pulse set at 10 μs was applied. For each spectrum 128 scans were recorded on a total of 32K data points. A broadening line function of 0.3 Hz was used to multiply all FIDs. After acquisition, all spectra were manually phased and baseline corrected using the software MestReNova® (version 2.1.8-11880, MestreLab, Spain). Finally, spectra were calibrated to the chemical shift of TSP (δ 0.00). In order to facilitate metabolite identification based on literature, a series of 2D spectra on selected samples were acquired using correlation spectroscopy (COSY) NMR spectroscopy.

3.3. Statistical analysis

All spectra were scaled on unit variance and mean centered prior to analysis. To evaluate metabolic variation between samples, principal component analysis (PCA) was used. Orthogonal projection to latent structure discriminant analysis (O-PLS-DA) was also performed, where ^{1}H-NMR spectra were used as a matrix of independent variables (X) and time or antibiotic concentration were used as prediction vectors (Y) to capture the metabolic variations linear to time and antibiotic concentration. O-PLS DA models were generated between each tiamulin concentration at every time point 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.

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

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

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

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

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

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

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

5. Conclusion

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

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

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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.

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

Figure 3: (A) Metabolic trajectories derived from the PCA analysis performed using all the sample population (N=252) of the study (i.e. control plus 6 tiamulin dilution) on PC1 and PC3 displaying the centroids for each time points of the control and three concentrations of tiamulin. (B) Heat map representing the O-PLS DA model strength existing between each tiamulin concentration at each time point, based on R^2Y (goodness of fit of the model) and Q^2Y (goodness of prediction of the model) values using the following formula: $Model\ strength = \frac{R^2Y \times Q^2Y}{R^2Y - Q^2Y}$.

Supplemental material

Supplement Fig. 1: Metabolic trajectories of *B. pilosicoli* footprints in broth media for 120 h at a tiamulin concentration of: 0.008 µg/mL (A) and 0.016 µg/mL (B). The arrows indicate the metabolic trajectory. 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 PCs.

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

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

Supplement Fig. 3: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120 h at a tiamulin concentration of 0.032 µg.ml on principal component 1 and 3. (A) PCA score plot. (B) Associated loading plot for PC1. 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 PCs.

Supplement Fig. 4: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120 h at a tiamulin concentration of: 0.062 µg/ml (A) and 0.125 µg/ml (B). The arrows indicate the metabolic trajectory. 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 PCs.

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

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