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Employing MIC data for mink pathogens to propose tentative epidemiological cut-off values: a step towards rationalizing antimicrobial use in mink

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17 **Keywords:** ECOFF, MIC, pharmacodynamics, mink, *E. coli*, *P. aeruginosa*, *S. canis*, *S. delphini*

18 **Abstract**

19 Optimizing antimicrobial dosage regimens and development of breakpoints for antimicrobial
20 susceptibility testing are important prerequisites for rational antimicrobial use. The objectives of the
21 study were 1) to produce MIC data for four mink pathogens and 2) to employ these MIC data to
22 support the development of tentative epidemiological cut-off values (TECOFFs), which may be used
23 for future development of mink-specific antimicrobial dosages and breakpoints.

24 Broth microdilution was used to establish MIC distributions for 322 mink bacterial isolates of clinical
25 origin from six European mink-producing countries. The included species were *E. coli* (n=162), *S.*
26 *delphini* (n=63), *S. canis* (n=42), and *P. aeruginosa* (n=55). Sixty-four *E. coli* isolates and 34 *S.*
27 *delphini* isolates were whole-genome sequenced and analyzed for antimicrobial resistance genes.

28 No EUCAST MIC data are available on *S. delphini* and *S. canis*, hence tentative ECOFFs were
 29 suggested for the majority of the tested antimicrobials. For *E. coli* and *P. aeruginosa*, the wildtype
 30 distributions were in accordance with EUCAST data. Overall, the genotypes of the sequenced isolates
 31 were in concordance with the phenotypes.

32 These data constitute an important piece in the puzzle of developing antimicrobial dosages and
 33 clinical breakpoints for mink. Until pharmacokinetic and clinical data become available, the
 34 (tentative) ECOFFs can be used for monitoring resistance development and as surrogates for clinical
 35 breakpoints.

36 **1 Introduction**

37 As in other species, mink become clinically ill due to various infectious agents, including a range of
 38 bacterial pathogens causing decreased animal welfare and affecting commercial fur production.
 39 Common bacterial pathogens in mink include *Escherichia coli*, which may cause diarrhea,
 40 *Pseudomonas aeruginosa*, which may cause hemorrhagic pneumonia, *Staphylococcus delphini*,
 41 which may cause urinary tract infections, and *Streptococcus canis*, which may cause skin infections
 42 (Pedersen et al., 2009). Bacterial infections in mink often require antimicrobial treatment. However,
 43 antimicrobial therapy in the mink industry is mostly based on empirical knowledge since clinical
 44 breakpoints and antimicrobial dosage regimens for mink are unavailable. Such non-evidence-based
 45 practice might lead to treatment failure, toxicity, and/or selection for antimicrobial resistance.
 46 Optimal treatment of bacterial infections relies on pharmacodynamic data pertaining to bacterial
 47 target pathogens and antimicrobial agents, respectively. Exploiting such data for development of
 48 clinical breakpoints and dosage regimens can help ensure a proper drug choice and an adequate
 49 antimicrobial concentration at the site of infection.

50 The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a scientific
 51 committee focusing on antimicrobial resistance and providing guidelines for procedures and
 52 interpretation of antimicrobial susceptibility testing. EUCAST defines the wildtype as isolates that
 53 have not acquired phenotypically detectable resistance mechanisms, and the epidemiological cut-off
 54 value (ECOFF) as the highest minimum inhibitory concentration (MIC) for the wildtype population
 55 (EUCAST 2019a). Thus, ECOFFs distinguish between isolates with and without phenotypically
 56 identifiable antimicrobial resistance, non-wildtype and wildtype respectively. Noteworthy, ECOFFs
 57 cannot necessarily be used to predict the outcome of therapy. Using the ECOFF as a biological
 58 phenomenon, *in vitro* resistance can be measured and the development of resistance can be monitored
 59 despite the lack of clinical breakpoints (Kahlmeter et al., 2003; Turnidge et al., 2006, Toutain et al.,
 60 2017, EUCAST 2019b).

61 Several requirements need to be met to suggest an ECOFF, e.g. the dataset needs to include at least
 62 five MIC distribution generated from separate laboratories. Furthermore, at least 15 isolates per MIC
 63 distribution must be represented in the putative wildtype population, and only a single peak (mode) in
 64 the MIC distribution of the putative wildtype distribution is allowed. One of the requirements for the
 65 aggregated distribution is that there must be at least 100 MIC values in the putative wildtype
 66 distribution (EUCAST 2019a). If some requirements are not met, a tentative ECOFF (TECOFF) can
 67 be proposed until more data become available (EUCAST 2019a).

68 Several antimicrobials can be used in veterinary practice. However, some are also applied in human
 69 medicine and for the treatment of infections involving multi-drug resistant bacteria. The World
 70 Health Organization (WHO) has published Model List of Essential Medicines 2019 (WHO 2019b).

71 One of the included antimicrobials is marked as reserved (colistin), five are marked as accessible
 72 (amoxicillin, doxycycline, spectinomycin, trimethoprim and sulfamethoxazole in combination with
 73 trimethoprim (SXT)), and one antimicrobial (lincomycin) as “watch”. Tylosin is only licensed for use
 74 in animals.

75 In this study, 322 bacterial isolates representing four bacterial species were tested against eight
 76 antimicrobials using an extended range of concentrations. Results of the relevant antimicrobials for
 77 each bacterial species are included (2-7 antimicrobials per species). The majority of the resulting
 78 MIC distributions allowed us to identify the wildtype and non-wildtype populations. This study
 79 provides valuable information on *in vitro* antimicrobial resistance in clinical bacteria from mink.
 80 Additionally, the MIC distributions data and (T)ECOFFs are important tools, together with
 81 pharmacokinetic and clinical data, for constructing dosage regimens and for suggesting relevant
 82 breakpoints.

83 2 Materials and methods

84 2.1 Bacterial isolates

85 Bacterial isolates were obtained from clinical material from mink submitted to diagnostic laboratories
 86 (The National Veterinary Institute DTU, Lyngby, Denmark; Institute for Experimental Pathology,
 87 Reykjavík, University of Iceland; veterinary clinic Pecon BV, Gemert, the Netherlands; INVESAGA
 88 Group, Department of Animal Pathology, University of Santiago de Compostela, Lugo, Spain;
 89 Finnish Food Authority, Seinäjoki, Finland) in the period 2006-2018. Each submission to the
 90 laboratory could consist of more than one animal. A maximum of one isolate of each of the four
 91 bacterial species was collected from each submission. A farm could be represented more than once if
 92 samples were submitted to the laboratory repeatedly for investigation. There was no limitation as to
 93 how many times each farm could be represented over the 12-year sampling period. Also, the
 94 antimicrobial treatment history for the farms was not a criterion for the inclusion of bacterial isolates.
 95 The mink industry follows the same seasonal pattern all over the world, and the animals have been
 96 submitted from the beginning of whelping (April) until pelting (November). The following species
 97 were included in the study: *E. coli* (n= 162), *S. delphini* (n= 63), *S. canis* (n= 42), and *P. aeruginosa*
 98 (n= 55). Isolates originated from Denmark, Finland, Iceland, Lithuania, the Netherlands, and Spain
 99 (Table 1). All isolates included in this study were identified by MALDI-TOF as described in
 100 Nikolaisen et al. (2017).

101 2.2 Antimicrobial susceptibility testing

102 All isolates were investigated using the broth microdilution semiautomated technique Sensititre
 103 (ThermoFisher Scientific, UK) according to methods described by the Clinical and Laboratory
 104 Standards Institute (CLSI 2018). For *E. coli*, *S. delphini*, and *P. aeruginosa*, cation-adjusted Mueller-
 105 Hinton broth (CAMHB) was used, and panels were incubated at 35 ±2 °C for 16-20 h, whereas for *S.*
 106 *canis* CAMHB with lyzed horse blood was used and panels were incubated at 35 ±2 °C for 20-24 h
 107 (CLSI 2018). Based on data from the national veterinary prescription database VetStat (Anonymous
 108 2017, Stege et al., 2003), some of the most frequently used antimicrobials in mink production in
 109 Denmark were chosen for designing a custom-made panel. This panel contained two-fold dilutions of
 110 amoxicillin (range 0.25 – 512 µg/mL), colistin (0.06 – 128 µg/mL), spectinomycin (0.25 – 512
 111 µg/mL), sulfamethoxazole-trimethoprim 19:1 (0.03 – 64 µg/mL), doxycycline (0.06 – 128 µg/mL),
 112 lincomycin (0.06 – 128 µg/mL), sulfamethoxazole (0.5 – 512 µg/mL), and tylosin (0.12 – 128
 113 µg/mL). Antimicrobial concentration ranges were based on MIC distributions in the EUCAST MIC

114 database (EUCAST 2020) and earlier reports on prevalence of antimicrobial resistance in bacterial
 115 pathogens from mink (Pedersen et al., 2009; Nikolaisen et al., 2017). A subset of isolates was further
 116 tested for susceptibility to trimethoprim (*E. coli*: n=53, *S. delphini*: n=38, *S. canis*: n=26) and
 117 penicillin (*S. delphini*: n=18) (Supplementary table 1 and 2) by broth microdilution (CLSI 2018).
 118 Trimethoprim test was performed to determine the added effect of the combinational drug
 119 sulfamethoxazole and trimethoprim. Susceptibility to penicillin was tested in isolates harboring the
 120 *blaZ* gene. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas*
 121 *aeruginosa* ATCC 27853 were used as quality control strains. Every 10th Sensititre panel was
 122 inspected and evaluated by a second investigator.

123 2.3 Epidemiological cut-off values

124 The protocol for data collection was performed according to the EUCAST SOP 10.1 for ECOFF
 125 setting (EUCAST 2019a). The MICs were determined in three different laboratories by different
 126 investigators at 1) the National Food Institute at the Technical University of Denmark, 2) the
 127 Department of Veterinary and Animal Sciences at the University of Copenhagen, and 3) the Institute
 128 for Experimental Pathology at the University of Iceland.

129 Firstly, the MIC distributions were visually inspected (e.g. the Gaussian wildtype MIC distributions
 130 were identified) to ascertain that the “ECOFFinder” version 2.0 software could be applied (nonlinear
 131 regression, at 99 %) (Turnidge et al., 2006). Additionally, the MIC distributions for each
 132 antimicrobial agent and species were compared to the modes and ECOFFs already established and
 133 available in the EUCAST database (EUCAST 2020).

134 Prior to analyzing results for SXT, MIC distributions for sulfamethoxazole and trimethoprim were
 135 created separately. A “true” SXT wildtype MIC distribution was solely defined on organisms, which
 136 were independently wildtype to both agents. Isolates in the SXT wildtype population with MICs >
 137 ECOFF for sulfamethoxazole alone were omitted, as the effect of the combinational drug, SXT, must
 138 be attributed by the addition of trimethoprim.

139 2.4 Identification of antimicrobial resistance genes

140 Resistance genes were deduced from whole-genome sequencing of randomly selected 64 *E. coli*
 141 (Danish) and 34 *S. delphini* isolates originating from Denmark, Spain, Iceland, the Netherlands, and
 142 Finland. Briefly, DNA was isolated from culture material using a Maxwell®16 equipment and the 16
 143 LEV Blood DNA Kit according to the manufacturer’s instructions (Promega Corporation, USA). The
 144 *S. delphini* isolates were treated with lysostaphin before extraction as described in Strube et al.
 145 (2018). DNA purity and concentration were assessed using NanoDrop ND-1000 (NanoDrop
 146 Technologies, USA) and Qubit® (Life Technologies, USA). Library preparation (NextEra XT DNA
 147 sample preparation kit, Illumina, USA) and sequencing (Illumina NextSEQ-based technologies in a
 148 150 base pair paired-end configuration with an expected coverage of 50) was outsourced to Statens
 149 Serum Institut, Denmark.

150 Sequences were quality-checked by fastx_quality_stats from the FASTX-Toolkit (FASTX-Toolkit,
 151 RRID:SCR_005534) (http://hannonlab.cshl.edu/fastx_toolkit/). Using Centrifuge (Centrifuge
 152 Classifier, RRID:SCR_016665), the reads were classified and checked for contamination (Kim et al.,
 153 2016). Contigs were assembled using SPAdes (SPAdes, RRID:SCR_000131) with the setting:
 154 settings ‘-k 21,33,55,77 --careful’ (Bankevich et al., 2013). The assemblies were checked using Quast
 155 (QUAST, RRID:SCR_001228) and annotated using Prokka (Prokka, RRID:SCR_014732) (Seemann,

156 2014, Gurevich et al., 2013). Subsequently, antimicrobial resistance genes were identified by running
 157 sequences through the ResFinder pipeline (Zankari et al., 2012).

158 3 Results

159 3.1 *Escherichia coli*

160 For *E. coli*, MIC distributions for six antimicrobial agents are presented (Figures 1-6). Data and
 161 derived TECOFFs were in accordance with the EUCAST ECOFFs (Table 2). Antimicrobial
 162 resistance genes were not detected in 18 of the 64 sequenced isolates. With only few exceptions,
 163 these isolates were found in the wildtype populations (Figures 1-6). With the exception of colistin
 164 and spectinomycin, a high number of isolates were part of the non-wildtype populations (Table 6).
 165 For three of the agents, the ECOFFfinder suggested a value one dilution lower than the EUCAST
 166 ECOFF. However, there were no indications other than that the range and mode of colistin,
 167 spectinomycin and SXT were in accordance with the EUCAST ECOFFs (Figure 2, 4 and 6). Hence,
 168 these TECOFFs were visually determined (Table 2).

169 For amoxicillin, a bimodal distribution was identified. The MIC range, TECOFF and mode for
 170 amoxicillin are presented in Figure 1 and Table 2, respectively. Beta-lactam resistance genes were
 171 not detected in 23 of the 64 sequenced isolates. All of these were in the wildtype population. Forty-
 172 one of the sequenced isolates harbored a β -lactam resistance gene. None of these isolates were in the
 173 wildtype population (Figure 1). Genes belonging to the *bla*_{TEM-1} family were most prevalent, while
 174 one isolate carried the *bla*_{CTX-M-1} gene encoding an extended-spectrum beta-lactamase (ESBL).

175 For colistin, the distribution was mono-modal exhibiting a Gaussian distribution in the range 0.25-2
 176 μ g/mL (Figure 2). The mode and TECOFF of the colistin MIC values are presented in Table 2. No
 177 colistin resistance genes were detected in any of the sequenced *E. coli* isolates (Figure 2).

178 Two apparently overlapping populations were detected for doxycycline. The range, TECOFF and
 179 mode of doxycycline MIC distribution are presented in Figure 3 and Table 2, respectively. The
 180 finding of two overlapping populations was supported by the results and distribution of the
 181 sequencing data (Figure 3). Three isolates had an MIC > 128 μ g/mL and might represent a third
 182 population. Thirty-six of the sequenced isolates had no tetracycline resistance genes and were part of
 183 the wildtype population. One isolate had no known tetracycline resistance genes despite having an
 184 MIC >128 μ g/mL. Twenty-seven of the isolates harbored a tetracycline resistance gene (*tet*(A) or
 185 *tet*(B)). None of these isolates were in the wildtype population (Figure 3).

186 For spectinomycin, the MIC distribution, TECOFF and mode is presented in Figure 4 and Table 2,
 187 respectively. Forty-two of the sequenced isolates had no spectinomycin resistance genes and were
 188 part of the wildtype population. Twenty-two of the sequenced isolates harbored a spectinomycin
 189 resistance gene (*aadA5* or *aadA1*). Seven of these had an MIC < ECOFF, and 15 of these had an MIC
 190 > ECOFF (Figure 4).

191 For sulfamethoxazole, a bimodal distribution was identified. The range, TECOFF and mode of
 192 sulfamethoxazole MIC values are presented in Figure 5 and Table 2, respectively. Resistance genes
 193 were not detected in 32 of the sequenced isolates. All of these were part of the wildtype population.
 194 Thirty-two of the sequenced isolates harbored a sulfonamide resistance gene with *sul2* being the most
 195 prevalent. None of these isolates were in the wildtype population (Figure 5).

196 For sulfamethoxazole in combination with trimethoprim (SXT), three populations were apparent, and
 197 the wildtype population displayed a Gaussian distribution in the range 0.03-0.25 $\mu\text{g/mL}$ (Figure 6). A
 198 “true” SXT wildtype MIC distribution was solely defined on organisms, which were independently
 199 wildtype to both agents. Therefore, 26 sulfamethoxazole non-wildtype and concomitantly SXT
 200 wildtype were omitted. One isolate in the SXT wildtype population was omitted due to high
 201 trimethoprim MIC (64 $\mu\text{g/mL}$) (Supplementary table 1A). All included isolates with SXT MICs of
 202 0.12 $\mu\text{g/mL}$ and 0.25 $\mu\text{g/mL}$ were sensitive to trimethoprim alone (MIC ≤ 1 $\mu\text{g/mL}$) (Supplementary
 203 table 1A). Thirty-nine of the isolates with an SXT MIC of 0.06 $\mu\text{g/mL}$ were tested and proved
 204 sensitive to trimethoprim alone (MIC ≤ 1 $\mu\text{g/mL}$) (Supplementary table 1A). The mode and range of
 205 the SXT MIC values are presented in Table 2. There was no indication other than that the MIC
 206 distribution from the current study was in accordance with the EUCAST database. All isolates
 207 without detected sulfonamide nor trimethoprim resistance genes were in the wildtype population.
 208 Three non-wildtype isolates (MIC of 0.5 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$) harbored only a sulfonamide
 209 resistance gene (Figure 6b). Eighteen of the sequenced isolates harbored both sulfonamide (*sul1*,
 210 *sul2*, *sul3*) and trimethoprim resistance genes (*dfrA1*, *dfrA5*, *dfrA8*, *dfrA14*). Of these 18 isolates, 14
 211 had an MIC > 64 $\mu\text{g/mL}$, two had an MIC = 64 $\mu\text{g/mL}$, and two had an MIC = 4 $\mu\text{g/mL}$ (Figure 6).

212 3.2 *Staphylococcus delphini*

213 For *S. delphini*, the results of the seven tested antimicrobials are presented in Figures 7-13. Tentative
 214 ECOFFs were suggested for six of the antimicrobials (Table 3). In seven of the 34 sequenced
 215 isolates, no resistance genes were detected, and these isolates were mostly located in the wildtype
 216 population (Figures 7-11, 13). For doxycycline and lincomycin, high fractions of isolates were in the
 217 non-wildtype populations (Table 6).

218 All isolates had an MIC ≤ 0.25 $\mu\text{g/mL}$ to amoxicillin (Figure 7), truncating the dataset to the left.
 219 Since the test range did not cover the MIC distribution, it was not possible to suggest a TECOFF.
 220 Beta-lactam resistance genes were not detected in 16 of the sequenced isolates. Eighteen of the
 221 sequenced isolates harbored the β -lactam resistance gene *blaZ*; these isolates were tested against
 222 penicillin. Five of those were non-wildtype against penicillin (MIC ≥ 0.25 $\mu\text{g/mL}$, Supplementary
 223 table 2) when using the EUCAST ECOFF for *S. aureus* (EUCAST 2020).

224 A bimodal distribution was apparent for doxycycline; the wildtype population was truncated to the
 225 left in the range ≤ 0.06 -0.25 $\mu\text{g/mL}$. A TECOFF of 0.12 $\mu\text{g/mL}$ was suggested (Table 3, Figure 8).
 226 Nineteen of the sequenced isolates had no tetracycline resistance genes and were part of the wildtype
 227 population. Fifteen of the sequenced isolates harbored the tetracycline resistance gene *tet(M)*, none of
 228 these isolates were in the wildtype population (Figure 8).

229 For spectinomycin, the apparent wildtype population was in the range 16-64 $\mu\text{g/mL}$, but due to the
 230 lack of a Gaussian distribution it was not possible to apply the ECOFFinder 2.0 software (Turnidge et
 231 al., 2006, Figure 9). A TECOFF of 128 $\mu\text{g/mL}$ was suggested by visual inspection (Table 3). Thirty-
 232 two of the sequenced isolates had no spectinomycin resistance genes and were part of the wildtype
 233 population. Two of the sequenced isolates harbored the spectinomycin resistance gene *spc*, and both
 234 had MICs above the test range > 512 $\mu\text{g/mL}$ (Figure 9).

235 For tylosin, three populations could be identified. The wildtype population displayed a Gaussian
 236 distribution in the range 0.25-2 $\mu\text{g/mL}$. A TECOFF of 2 $\mu\text{g/mL}$ was suggested (Table 3, Figure 10).
 237 Twenty-nine of the sequenced isolates had no macrolide resistance genes and were part of the
 238 wildtype population. Five of the sequenced isolates harbored macrolide resistance genes, none of

239 these isolates were in the wildtype population (Figure 10). Four different macrolide resistance genes
 240 were identified, all belonging to the *erm* gene family encoding macrolide, lincosamide and
 241 streptogramin B resistance (MLS_B).

242 At least two populations were apparent for lincomycin with the wildtype population displaying a
 243 Gaussian distribution in the range 0.12-2 µg/mL. A TECOFF of 2 µg/mL was suggested (Table 3,
 244 Figure 11). Twenty of the sequenced isolates had no macrolide nor lincomycin resistance genes, all
 245 but three were part of the wildtype population. Ten of the sequenced isolates harbored the lincomycin
 246 resistance gene, *lnu*(A); none of these isolates were in the wildtype population. Additionally, five of
 247 the sequenced isolates harbored *erm* genes, all had a lincomycin MIC above the test range (> 128
 248 µg/mL, Figure 11).

249 There was only one apparent population for sulfamethoxazole, and a TECOFF of 128 µg/mL was
 250 suggested (Table 3, Figure 12).

251 For SXT, the wildtype population displayed a Gaussian distribution in the range ≤ 0.03-0.5 µg/mL.
 252 Two isolates were omitted, so that all isolates within the SXT wildtype population (Figure 13) were
 253 sensitive to sulfamethoxazole alone (MIC ≤ 128 µg/mL) (Figure 12). Thirty-eight randomly selected
 254 isolates in the SXT wildtype population were tested against trimethoprim alone, and all were
 255 sensitive (MIC ≤ 8 µg/mL) (Supplementary table 1b). A TECOFF of 0.25 µg/mL was suggested for
 256 SXT (Table 2, Figure 13). Two isolates harbored two different trimethoprim resistance genes, *dfrK*
 257 and *dfrG*, and these displayed MICs of 2 and 8 µg/mL, respectively (Figure 13).

258 3.3 *Streptococcus canis*

259 The MIC distributions of *S. canis* are presented in Supplementary figure 1. Tentative ECOFFs were
 260 suggested for five of the seven antimicrobials tested (Table 4). With the exception of SXT, a high
 261 number of isolates were found in the non-wildtype populations (Table 6).

262 For amoxicillin, all isolates had an MIC ≤ 0.25 µg/mL (Supplementary figure 1A), truncating the
 263 dataset to the left. Since the test range did not cover the MIC distribution, it was not possible to
 264 suggest a TECOFF.

265 The majority of the isolates displayed a Gaussian distribution for doxycycline in the range 8-32
 266 µg/mL (Supplementary figure 1B). However, this distribution was most likely not the wildtype
 267 distribution, since two isolates had MIC values of 0.25 µg/mL and 2 µg/mL, respectively, and since
 268 the ECOFF for the closely related species *S. pyogenes* (Table 4) and *S. pneumoniae* is 0.5 µg/mL
 269 (EUCAST 2020). Consequently, a TECOFF was not proposed.

270 Two main distributions were apparent for spectinomycin; the wildtype population displayed a
 271 Gaussian distribution in the range 8-32 µg/mL (Supplementary figure 1C). A TECOFF of 32 µg/mL
 272 was suggested (Table 4).

273 Two distributions were apparent for tylosin, and the wildtype population was truncated in the range ≤
 274 0.125-0.25 µg/mL (Supplementary figure 1D). Visual inspection of the truncated data indicated a
 275 tylosin TECOFF of 0.25 µg/mL (Table 4).

276 Similarly, for lincomycin, two populations were apparent with the wildtype population truncated in
 277 the range ≤ 0.06-0.25 µg/mL (Supplementary figure 1E). Visual inspection of the truncated data
 278 indicated a TECOFF of 0.5 µg/mL (Table 4).

279 For sulfamethoxazole, probably two overlapping populations were apparent in the range 8- >512
 280 µg/mL. The wildtype distribution was most likely in the range 8-128 µg/mL (Supplementary figure
 281 1F). A TECOFF of 128 µg/mL was suggested (Table 4).

282 For SXT, the wildtype population displayed a Gaussian distribution in the range ≤ 0.03 -0.12 µg/mL
 283 (Supplementary figure 1G). Eight isolates were omitted, so that all isolates within the SXT wildtype
 284 population were sensitive to sulfamethoxazole alone ($MIC \leq 128$ µg/mL, Table 4). Further, 26
 285 randomly selected isolates in the SXT wildtype population were tested against trimethoprim alone,
 286 and one isolate with $MIC \geq 4$ µg/mL was omitted (Supplementary table 1C). A TECOFF of 0.12
 287 µg/mL was suggested (Table 4).

288 3.4 *Pseudomonas aeruginosa*

289 For *P. aeruginosa*, MIC distributions and results of the tested antimicrobials are presented in
 290 Supplementary figure 2 and Table 5.

291 For colistin, only one population was apparent (Supplementary figure 2A). The MIC range and mode
 292 for colistin were similar to the EUCAST MIC distribution and the ECOFF of 4 µg/mL (Table 5). All
 293 isolates were in the wildtype population (Table 6).

294 For SXT, the wildtype population displayed a Gaussian distribution in the range 2-64 µg/mL
 295 (Supplementary figure 2B). A tentative ECOFF of 32 µg/mL was suggested (Table 5). The TECOFF
 296 places 24 % of the isolates in the non-wildtype population (Table 6).

297 4 Discussion

298 An ECOFF indicates the cut-off for the sensitive wildtype population, whereas a clinical breakpoint
 299 indicates the lowest concentration for which treatment is likely to be successful. Often an ECOFF
 300 corresponds to a clinical breakpoint, or the ECOFF is a lower concentration than the clinical
 301 breakpoint. In the absence of a clinical breakpoint, the ECOFF may be used to infer susceptibility of
 302 a pathogen (Toutain et al., 2017). In that regard, it is worth noticing the high proportion of isolates
 303 above the ECOFF in some occasions (Table 6); e.g. for *E. coli*, 56 % of the isolates were above the
 304 amoxicillin ECOFF, while 40 % and 46 % were above the ECOFF for doxycycline and
 305 sulfamethoxazole, respectively. These findings are in accordance with the clinical resistance results
 306 found by Nikolaisen et al. (2017), who applied clinical breakpoint adapted from other host species
 307 and closely related bacterial species. Further, these authors recorded marked differences in resistance
 308 between hemolytic and non-hemolytic *E. coli* isolates, i.e. the proportion of resistant isolates was
 309 significantly higher for the hemolytic isolates compared to non-hemolytic ones. For *S. delphini*, 52 %
 310 and 19 % were above the TECOFF for doxycycline and tylosin, respectively, which is almost
 311 identical to the proportion of resistant isolates found by Nikolaisen et al. (2017) for tetracycline (51
 312 %), and erythromycin (20 %). Likewise, a similarity was seen for *S. canis* where 57 % of the isolates
 313 were above the tylosin TECOFF (Table 6), while Nikolaisen et al. (2017) found 53 % resistant to
 314 erythromycin using the adapted clinical breakpoints. Thus, there seems to be a good congruence
 315 between the number of isolates above the (T)ECOFFs found in this study compared to our
 316 knowledge about clinical resistance for these bacterial species (Nikolaisen et al. 2017). High
 317 percentages of isolates above the (T)ECOFF may indicate that the chance of clinical cure is low and
 318 the risk of selecting for antimicrobial resistance is high. Accordingly, we recommend susceptibility
 319 testing for these antimicrobial/pathogen combinations and using the established (T)ECOFFs as
 320 surrogate clinical breakpoints.

321 The ECOFFs are based on phenotypic antimicrobial resistance patterns. In this study, genotypic data
 322 on the presence of antimicrobial resistance genes were included for *E. coli* and *S. delphini* to confirm
 323 the phenotypic antimicrobial resistance patterns. Overall, the distributions of genotypes support the
 324 interpretation of the distributions and evaluation of the ECOFFs. For example, in most cases
 325 antimicrobial resistance genes were detected only in isolates with MICs above the (tentative) ECOFF
 326 (*E. coli* 96 % (154/161), *S. delphini* 100 %, Figure 1-13).

327 All *S. delphini* and *S. canis* isolates had amoxicillin MICs ≤ 0.25 $\mu\text{g/mL}$ (Figure 7, Supplementary
 328 figure 1A). However, 18 of the 34 sequenced *S. delphini* isolates harbored *blaZ*. The *blaZ* gene
 329 encodes a β -lactamase conferring resistance to certain β -lactam antimicrobials such as penicillins and
 330 aminopenicillins but not cephalosporins. Five of these 18 isolates were phenotypically resistant to
 331 penicillin with MICs of 0.25 $\mu\text{g/mL}$ (Supplementary table 2). Other studies have reported isolates
 332 being phenotypically sensitive to β -lactam antimicrobials despite harboring *blaZ* (Haveri et al., 2005;
 333 Ruegg et al., 2015; Ferreira et al., 2017; Turchi et al., 2020). This can be explained by failure to
 334 induce the *blaZ* gene (Lowy, 2003) or the use of incorrect penicillin breakpoints (Haveri et al., 2005;
 335 Ruegg et al., 2015; Ferreira et al., 2017; Turchi et al., 2020). In that regard, it should be noted that the
 336 available penicillin ECOFF for *S. aureus* was applied (ECOFF 2020).

337 The majority of the *S. delphini* isolates were wildtype to tylosin, and all isolates harboring macrolide
 338 resistance *erm* genes were above the TECOFF (Figure 10). Some lincosamide and macrolide
 339 resistance genes confer cross resistance (MLS_B) (Leclercq, 2002). Such cross resistance is visualized
 340 in the lincomycin MIC distribution, as the isolates harboring *erm* genes all have lincomycin MICs
 341 above the test range (> 128 $\mu\text{g/mL}$, Figure 11). In contrast, *S. delphini* isolates without *erm* genes,
 342 but harboring the lincosamide resistance gene *lnu(A)*, were only resistant to lincomycin.

343 The tetracycline resistance genes *tet(A)* and *tet(B)* were identified in all sequenced *E. coli* isolates
 344 representing the doxycycline non-wildtype population. However, the two genes allocated differently
 345 in the MIC distribution of the non-wildtype population, as *tet(A)* was present in isolates with
 346 doxycycline MICs of 8 - 32 $\mu\text{g/mL}$, whereas *tet(B)* was found in isolates with slightly higher MICs of
 347 16 - 64 $\mu\text{g/mL}$ (Figure 3). This difference in doxycycline MIC related to presence of different *tet*
 348 genes has been described previously (Alexander et al., 2013). In the doxycycline distribution, three
 349 isolates had an MIC that exceeded the test range, > 128 $\mu\text{g/mL}$ (Figure 3). In the EUCAST database,
 350 very few *E. coli* with MIC > 64 $\mu\text{g/mL}$ are reported representing only 0.1 % of the isolates
 351 (EUCAST 2020). This proportional difference might indicate that mink have been exposed to a high
 352 selection pressure for this drug. One of these mink isolates was sequenced, but interestingly no
 353 known tetracycline resistance genes were detected. The mechanism behind the resistance of this
 354 isolate is therefore currently unknown.

355 For the combinational drug SXT, all isolates in the wildtype population were cross-referenced with
 356 the results for sulfamethoxazole alone. Isolates with sulfamethoxazole non-wildtype MICs could not
 357 truly belong to the wildtype population for the combinational drug and were therefore omitted from
 358 the dataset for the combinational drug (*E. coli* $n=26$, *S. delphini* $n=2$, *S. canis* $n=8$). The low MIC
 359 values for SXT in these omitted isolates (0.03 - 0.5 $\mu\text{g/mL}$) likely reflect an effect of trimethoprim.
 360 The majority of the SXT wildtype population was further tested using trimethoprim alone and all
 361 except one *S. canis* and one *E. coli* isolate were found to be wildtype with respect to trimethoprim.
 362 These two isolates were therefore also omitted from the distribution for the combinatorial drug
 363 (Supplementary table 1A and 1C). Hence, the isolates in the SXT wildtype population were all
 364 wildtype to sulfamethoxazole alone. Furthermore, all the randomly chosen isolates from the SXT
 365 wildtype population that were trimethoprim tested were also wildtype to trimethoprim alone

366 (Supplementary table 1). The ECOFFs for the individual antimicrobials are of more biological interest
 367 than those of the combinational drug, the latter is however more widely applied in veterinary
 368 medicine.

369 *Pseudomonas aeruginosa* displays intrinsic resistance against the majority of the antimicrobials
 370 included in this study, except colistin. None of the isolates had a colistin MIC higher than the
 371 EUCAST ECOFF (4 µg/mL), so all isolates were wildtype. Colistin is administered orally to mink,
 372 but the absorption of colistin from the intestinal tract is known to be minimal (Guyonnet et al., 2010;
 373 Rhouma et al., 2015). Consequently, colistin treatment of the often severe lower respiratory *P.*
 374 *aeruginosa* infection in mink are not feasible. In addition, colistin is categorized as a reserved group
 375 of antimicrobials in the WHO's List of Essential medicines (WHO/AGISAR 2019a, WHO 2019b).
 376 Other agents to consider are aminoglycosides and fluoroquinolones, for which intrinsic resistance is
 377 not recorded in *P. aeruginosa*. However, aminoglycosides (e.g. neomycin and gentamicin) are also
 378 poorly absorbed from the intestinal tract. A systemic effect with high antimicrobial concentration in
 379 the lungs would therefore demand each animal to be treated individually by injection, something that
 380 is not feasible in modern mink farming. Fluoroquinolones, such as enrofloxacin, can be used orally
 381 for systemic infections but are listed as "Highest priority" among critically important antimicrobials
 382 (WHO/AGISAR 2019a). These drugs should therefore not be used for treatment of mink, except in
 383 particular situations where there are no other alternatives (Panzuti et al., 2020). Sulfonamides in
 384 combination with trimethoprim are used empirically to treat *P. aeruginosa* mink pneumonia, even
 385 though this pathogen is intrinsically resistant to these combinational drugs. Due to the widespread
 386 use and allegedly good clinical effect (Tina Struve, Personal communication, February 10, 2020), we
 387 have included data for SXT against *P. aeruginosa* (Supplementary figure 2B). Based on the MIC
 388 distribution and the TECOFF, most (76 %) mink *P. aeruginosa* isolates are wildtype, but the
 389 TECOFF of 32 µg/mL is high (Supplementary figure 2B). Furthermore, pharmacokinetic studies
 390 conducted by our group (Ronaghinia et al., 2020) indicate that a clinical effect of sulfonamide and
 391 trimethoprim against *P. aeruginosa* cannot be expected in mink, even for wildtype isolates.

392 A careful selection of antimicrobial test ranges was done to confirm concordance with a EUCAST
 393 ECOFF or to suggest a TECOFF. Despite the wide test ranges, some challenges occurred when
 394 interpreting the MIC distribution results; 1) the wildtype population was truncated resulting in the
 395 absence of a mode and the ECOFF being impossible to infer, 2) only one distribution was present, in
 396 which case, it was most likely the wildtype population, or, 3) the distribution was not truly Gaussian.
 397 These problems could be addressed in future studies by increasing the test range further and/or
 398 including more isolates.

399 **5 Conclusion**

400 With the MIC Sensititre panels, it was possible to verify ECOFFs and determine new TECOFFs for
 401 the majority of the tested mink-specific combinations of microorganism and antimicrobial agents.
 402 These TECOFFs may serve as surrogate clinical breakpoints when there is reasonable clinical
 403 experience with the antimicrobial in mink. Additionally, it can serve as pharmacodynamic data for
 404 future determination of dosage regimens and clinical breakpoints. Further MIC and pharmacokinetic
 405 studies are needed for most compounds to establish clinical breakpoints for common mink
 406 pathogenic bacteria. Results of this study can help as one step to promote prudent use of
 407 antimicrobials in mink and decrease the risk of selecting for antimicrobial resistance.

408 **6 Tables**

409 **Table 1: The 322 isolates included in the study, divided into species and country of origin.**

| | Denmark | Iceland | The Netherlands | Finland | Spain | Lithuania | Total |
|--------------------------------|---------|---------|-----------------|---------|-------|-----------|-------|
| <i>Escherichia coli</i> | 103 | 23 | 4 | 26 | 5 | 1 | 162 |
| <i>Staphylococcus delphini</i> | 24 | 14 | 1 | 20 | 4 | | 63 |
| <i>Pseudomonas aeruginosa</i> | 24 | 13 | 18 | | | | 55 |
| <i>Streptococcus canis</i> | 35 | 1 | 5 | | 1 | | 42 |

410

411

412 **Table 2: *Escherichia coli* isolated from mink – tentative ECOFFs and modes of MIC wildtype**
 413 **distributions and the official ECOFFs from EUCAST.**

| | Current study (mink) | | EUCAST (mixed origins) | |
|------------------|-------------------------|-------------------|---------------------------|-------|
| | MODE | TECOFF | MODE | ECOFF |
| Amoxicillin | 4 | 8 | 4 | 8 |
| Colistin | 0.5 | 2 ^v | 0.5 | 2 |
| Doxycycline | 2 | 4 | 2 | 4 |
| Spectinomycin | 16 | 64 ^v | 16 | 64 |
| Sulfamethoxazole | 16 | 64 | 16 | 64 |
| Sulfa. + TMP | 0.06 | 0.25 ^v | 0.06 | 0.25 |

414 All values are given as µg/mL. The MIC wildtype distributions were visually inspected and the
 415 ECOFFs were tested by nonlinear regression analysis using the ECOFFinder 2.0 software (Turnidge
 416 et al., 2006). Compared with data retrieved from EUCAST (EUCAST 2020). ^v: visually determined,
 417 as the MIC distribution was very similar to the EUCAST distribution. Sulfa. + TMP:
 418 sulfamethoxazole in combination with trimethoprim (19:1).

419

420

421 **Table 3: *Staphylococcus delphini* isolated from mink – tentative ECOFFs and modes of MIC**
 422 **wildtype distributions, compared with modes and ECOFFs for *S. aureus* from EUCAST.**

| | Current study (mink) | | EUCAST, <i>S. aureus</i> (mixed origins) | |
|------------------|-------------------------|---------------------|---|-------|
| | MODE | TECOFF | MODE | ECOFF |
| Amoxicillin | - | - | - | - |
| Doxycycline | 0.06 ^t | 0.12 ^{t v} | 0.12 | 0.5 |
| Spectinomycin | 64 | 128 ^v | - | - |
| Tylosin | 0.5 | 2 | - | - |
| Lincomycin | 0.5 | 2 | 1 | 2 |
| Sulfamethoxazole | 16 | 128 | 16 | 128 |
| Sulfa. + TMP | 0.12 | 0.25 | 0.06 | 0.25 |

423 All values are given as µg/mL. The MIC wildtype distributions were visually inspected and the
 424 tentative ECOFFs (TECOFFs) were suggested by nonlinear regression analysis using the
 425 ECOFFinder 2.0 software (Turnidge et al., 2006). Compared with data for *S. aureus* retrieved from
 426 EUCAST (EUCAST 2020). ^t: truncated data, ^v: visually determined, Sulfa. + TMP: sulfamethoxazole
 427 in combination with trimethoprim (19:1).

428

429

430 **Table 4: *Streptococcus canis* isolated from mink – tentative ECOFFs and modes of MIC**
 431 **wildtype distribution, compared with modes and ECOFFs for *S. pyogenes* from EUCAST.**

| | Current study (mink) | | EUCAST, <i>S. pyogenes</i> (mixed origins) | |
|------------------|-------------------------|---------------------|---|-------|
| | MODE | TECOFF | MODE | ECOFF |
| Amoxicillin | - ^t | - ^t | 0.016 | 0.06 |
| Doxycycline | - | - | 0.12 | 0.5 |
| Spectinomycin | 16 | 32 | - | - |
| Tylosin | 0.12 ^t | 0.25 ^{t v} | - | - |
| Lincomycin | 0.25 | 0.5 ^v | - | - |
| Sulfamethoxazole | 32 | 128 | - | - |
| Sulfa. + TMP | 0.06 | 0.12 | 0.12 | 0.5 |

432 All values are given as µg/mL. The MIC wildtype distributions were visually inspected and the
 433 tentative ECOFFs (TECOFFs) were suggested by nonlinear regression analysis using the
 434 ECOFFfinder 2.0 software (Turnidge et al., 2006). Compared with data for *S. pyogenes* retrieved from
 435 EUCAST (EUCAST 2020). ^t: truncated data, ^v: visually determined, Sulfa. + TMP: sulfamethoxazole
 436 in combination with trimethoprim (19:1).
 437

438 **Table 5: *Pseudomonas aeruginosa* isolated from mink – tentative ECOFFs and modes of MIC**
 439 **wildtype distributions and the official ECOFF from EUCAST.**

| | Current study (mink) | | EUCAST (mixed origins) | |
|--------------|-------------------------|--------|---------------------------|-------|
| | MODE | TECOFF | MODE | ECOFF |
| Colistin | 2 | 4 | 1 | 4 |
| Sulfa. + TMP | 8 | 32 | - | - |

440 All values are given as µg/mL. The MIC wildtype distributions were visually inspected and the
 441 (T)ECOFFs were tested by nonlinear regression analysis using the ECOFFfinder 2.0 software
 442 (Turnidge et al., 2006). Compared with data retrieved from EUCAST (EUCAST 2020). Sulfa. +
 443 TMP: sulfamethoxazole in combination with trimethoprim (19:1).
 444

445 **Table 6: Percentages of isolates in non-wildtype population.**

| | <i>E.coli</i> | <i>S. delphini</i> | <i>S. canis</i> | <i>P. aeruginosa</i> |
|------------------|---------------|--------------------|-----------------|----------------------|
| Amoxicillin | 56 | - | - | - |
| Colistin | 0 | - | - | 0 |
| Doxycycline | 40 | 52 | - | - |
| Spectinomycin | 13 | 3 | 31 | - |
| Tylosin | - | 19 | 57 | - |
| Lincomycin | - | 54 | 67 | - |
| Sulfamethoxazole | 46 | 3 | 19 | - |
| Sulfa. + TMP | 30 | 6 | 0 | 24 |

446 Tentative epidemiological cut-off values (TECOFFs) from this study were applied (Table 2-5). Sulfa.
 447 + TMP: sulfamethoxazole in combination with trimethoprim (19:1).

448 **7 Conflict of Interest**

449 The authors declare that the research was conducted in the absence of any commercial or financial
 450 relationships that could be construed as a potential conflict of interest.

451 **8 Author Contributions**

452 NKN drafted the manuscript. AAR, DCKL, CNC and NKN provided raw data. GK, KP, PD and
 453 NKN conducted the analysis of the MIC distribution data. ML conducted the analysis of sequence
 454 data. MC, TS, LBJ and KP supervised the project. All authors critically reviewed the manuscript.

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 457 00061B), Pelsdyrafgiftsfonden, and Dansk Pelsdyravlerforeningens Forskningsfond.

458 **10 Abbreviations**

459 ECOFF: Epidemiological cut-off value

460 TECOFF: Tentative epidemiological cut-off value

461 SXT: Sulfamethoxazole in combination with trimethoprim (19:1)

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569 **13 Supplementary Material**

570 In separate file (Supplementary figure 1-2, Supplementary table 1 and 2)

571 **14 Data Availability Statement**

572 The raw data supporting the conclusions of this manuscript will be made available by the authors,
 573 without undue reservation, to any qualified researcher.

574 **15 Figure legends**

575 **Figure 1:** MIC distribution of *E. coli* (n=162) against amoxicillin in the test range of 0.25-512
 576 µg/mL. The arrow indicates the epidemiological cut-off value (ECOFF, EUCAST). Colors indicate if
 577 the isolates have been sequenced and whether they harbor known relevant resistance genes. WGS:
 578 whole-genome sequencing, res: resistance.

579 **Figure 2:** MIC distribution for *E. coli* (n=162) against colistin in the test range 0.06-128 µg/mL. The
 580 arrow indicates the epidemiological cut-off value (ECOFF, EUCAST). Colors indicate if the isolates
 581 have been sequenced and whether they harbor known relevant resistance genes. WGS: whole-genome
 582 sequencing, res: resistance

583 **Figure 3:** MIC distribution of *E. coli* (n=162) against doxycycline in the test range of 0.06-128
 584 µg/mL. The arrow indicates the epidemiological cut-off value (ECOFF, EUCAST). Colors indicate if
 585 the isolates have been sequenced and whether they harbor known relevant resistance genes. WGS:
 586 whole-genome sequencing, res: resistance.

587 **Figure 4:** MIC distribution of *E. coli* (n=162) against spectinomycin in the test range of 0.25-512
 588 µg/mL. The arrow indicates the epidemiological cut-off value (ECOFF, EUCAST). Colors indicate if
 589 the isolates have been sequenced and whether they harbor known relevant resistance genes. WGS:
 590 whole-genome sequencing, res: resistance.

591 **Figure 5:** MIC distribution of *E. coli* (n=162) against sulfamethoxazole in the test range of 0.5-512
 592 µg/mL. The arrow indicates the epidemiological cut-off value (ECOFF, EUCAST). Colors indicate
 593 if the isolates have been sequenced and whether they harbor known relevant resistance genes. WGS:
 594 whole-genome sequencing, res: resistance.

595 **Figure 6:** MIC distribution of *E. coli* (n=135) against sulfamethoxazole in combination with
 596 trimethoprim (19:1) in the test range of 0.03-64 µg/mL. The arrow indicates the epidemiological cut-

597 off value (ECOFF, EUCAST). Colors indicate if the isolates have been sequenced (n=51) and
 598 whether they harbor known A) trimethoprim resistance gene and/or B) sulfonamide resistance genes.
 599 WGS: whole-genome sequencing, res: resistance.

600 **Figure 7:** MIC distribution of *Staphylococcus delphini* (n=63) against amoxicillin in the test range of
 601 0.25-512 µg/mL. Colors indicate if the isolates have been sequenced and whether they harbor known
 602 relevant resistance genes. WGS: whole-genome sequencing, res: resistance.

603 **Figure 8:** MIC distribution of *Staphylococcus delphini* (n=63) against doxycycline in the test range
 604 of 0.06-128 µg/mL. The broken arrow indicates the tentative epidemiological cut-off value
 605 (TECOFF). Colors indicate if the isolates have been sequenced and whether they harbor known
 606 relevant resistance genes. WGS: whole-genome sequencing, res: resistance.

607 **Figure 9:** MIC distribution *Staphylococcus delphini* (n=63) against spectinomycin in the test range
 608 0.25-512 µg/mL. The broken arrow indicates the tentative epidemiological cut-off value (TECOFF).
 609 Colors indicate if the isolates have been sequenced and whether they harbor known relevant resistance
 610 genes. WGS: whole-genome sequencing, res: resistance.

611 **Figure 10:** MIC distribution of *Staphylococcus delphini* (n=63) against tylosin in the test range of
 612 0.12-128 µg/mL. The broken arrow indicates the tentative epidemiological cut-off value (TECOFF).
 613 Colors indicate if the isolates have been sequenced and whether they harbor known relevant
 614 resistance genes. WGS: whole-genome sequencing, res: resistance.

615 **Figure 11:** MIC distribution of *Staphylococcus delphini* (n=63) against lincomycin in the test range
 616 of 0.06-128 µg/mL. The broken arrow indicates the tentative epidemiological cut-off value
 617 (TECOFF). Colors indicate if the isolates have been sequenced and whether they harbor known
 618 relevant resistance genes. WGS: whole-genome sequencing, res: resistance.

619 **Figure 12:** MIC distribution of *Staphylococcus delphini* (n=63) against sulfamethoxazole in the test
 620 range of 0.5-512 µg/mL. The broken arrow indicates the tentative epidemiological cut-off value
 621 (TECOFF).

622 **Figure 13:** MIC distribution of *Staphylococcus delphini* (n=61) against sulfamethoxazole in
 623 combination with trimethoprim (19:1) in the test range of 0.03-64 µg/mL. The broken arrow indicates
 624 the tentative epidemiological cut-off value (TECOFF). Colors indicate if the isolates have been
 625 sequenced and whether they harbor known relevant resistance genes. WGS: whole-genome
 626 sequencing, res: resistance.