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## Effects of antimicrobial exposure on the antimicrobial resistance of *Escherichia coli* in the digestive flora of dairy calves

Nathalie Jarrige, G. Cazeau, G. Bosquet, J. Bastien, Fabienne Benoit, E. Gay

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1 **Title**

2 Effects of antimicrobial exposure on the antimicrobial resistance of *Escherichia coli* in the digestive flora of  
3 dairy calves

4

5 **Author names and affiliations**

6 N. Jarrige<sup>a</sup>, G. Cazeau<sup>a</sup>, G. Bosquet<sup>b</sup>, J. Bastien<sup>b</sup>, F. Benoit<sup>c</sup>, E. Gay<sup>a</sup>

7 <sup>a</sup> Université de Lyon, Anses, Laboratoire de Lyon, Unité Epidémiologie et appui à la surveillance, 31 avenue  
8 Tony Garnier, 69364 Lyon Cedex 07, France.

9 <sup>b</sup> SNGTV - Société nationale des groupements techniques vétérinaires, 5 rue Moufle, 75011 Paris, France.

10 <sup>c</sup> Laboratoire Labéo-Manche, 1352 avenue de Paris CS 33608, 50008 Saint-Lô Cedex, France.

11

12 **Corresponding author:** Nathalie Jarrige. Electronic address: [nathalie.jarrige@anses.fr](mailto:nathalie.jarrige@anses.fr)

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14

15 **Summary**

16 Veal calves are often identified as reservoirs for antimicrobial resistant *Escherichia coli* (*E. coli*). This production  
17 is closely linked with dairy production, as young calves — mostly males — are collected from dairy farms to  
18 enter the fattening process. The aim of this prospective study was to explore the factors on dairy farms that  
19 favour the selection of antimicrobial resistance (AMR) in the digestive *E. coli* strains of young calves and to  
20 assess whether the resistance levels and selection pressure were the same for males and females. The  
21 exposure of calves to antimicrobials was investigated through three factors: antimicrobial treatment of calves;  
22 feeding of calves with milk from cows treated with antimicrobials; and the consumption of colostrum from  
23 cows treated with antimicrobials at dry-off.

24 The study design involved 100 dairy farms. A calf of each sex was selected from birth on each farm. Information  
25 on the calves' exposure to antimicrobials was collected daily and calves were sampled (rectal swab) two weeks  
26 after birth, then seven weeks after birth for females only. Laboratory analyses included culture on two distinct  
27 media: a non-selective medium (identifying dominant flora) and a medium containing ceftiofur to select the  
28 extended-spectrum beta-lactamase (ESBL) phenotype. Susceptibility testing was performed on an *E. coli* strain  
29 from each medium. Generalised linear models were used to assess associations between the resistance of *E.*  
30 *coli* strains and antimicrobial exposure. A set of 280 swabs from healthy calves were analysed. In dominant

31 flora, high levels of resistance (>60%) were identified for streptomycin, tetracycline and amoxicillin but AMR  
32 levels were low (3%) for critically important antimicrobials (3<sup>rd</sup>- and 4<sup>th</sup>-generation cephalosporins and  
33 fluoroquinolones). For females staying in dairy farms, a marked decrease in resistance was observed for almost  
34 all antimicrobials between the age of 15 days and 7 weeks. A selective medium revealed an ESBL phenotype for  
35 20.7% of the calves. Whether for AMR or antimicrobial exposure, no significant difference was found between  
36 male and female calves.

37 The antimicrobial treatment of calves was associated with an increased resistance of *E. coli* from dominant  
38 flora for amoxicillin (OR=2.9), gentamicin (OR=4.6), florfenicol (OR=5.0) and trimethoprim-sulfonamide  
39 (OR=5.6). The consumption by calves of milk from cows treated with antimicrobials was also associated with an  
40 increased resistance to amoxicillin (OR=2.6), gentamicin (OR=4.0), tetracycline (2.6) and trimethoprim-  
41 sulfonamide (OR=2.2). In contrast, the models did not reveal any association between AMR and consumption  
42 of colostrum from cows treated with antimicrobials at dry-off.

43

44

## 45 INTRODUCTION

46 The commensal and pathogenic nature of *Escherichia coli* associated with its great genomic plasticity makes it a  
47 central species in the emergence and spread of antimicrobial resistance (AMR). The epidemic dissemination of  
48 extended-spectrum beta-lactamase (ESBL) enterobacteriaceae in humans, animals and the environment is  
49 particularly worrying.

50 Among food production animal species, calves are considered to be reservoirs of AMR strains. Data from the  
51 French monitoring network for antimicrobial resistance in bacteria from diseased animals (Anses, 2019)  
52 indicate that *E. coli* strains isolated from neonatal calf diarrhoea carry most of the resistances observed in the  
53 beef sector. On dairy farms where calves are born, some of the females are kept in the farm for herd renewal,  
54 while the other females and most of the males are sold for fattening around the age of 15 days. Calves from  
55 different farms are grouped together in batches and fattened on specialised farms to be slaughtered at the age  
56 of 5 to 6 months. Antimicrobial treatments are numerous during the fattening process (Jarrige et al., 2017) and  
57 AMR monitoring programmes and epidemiological studies have measured the high prevalence of *E. coli*  
58 harbouring resistances and ESBL in calves at slaughterhouses (Haenni et al., 2014). These veal calves are  
59 directly introduced into the food chain and could expose human health through meat consumption. However,

60 it has also been evidenced that by the time they arrive at fattening farms, these calves are already carrying  
61 many resistant *E. coli* in their digestive tract. Major resistances concern amoxicillin, tetracycline and  
62 streptomycin. ESBL *E. coli* from subdominant flora is also frequent (Gay et al., 2019). Calves therefore appear to  
63 acquire AMR very early in their life, even on their farm of birth (Berge et al., 2005).

64 The European Food Safety Authority (EFSA) has investigated this issue and identified two putative sources of  
65 calves' exposure to antimicrobials or their residues on dairy farms. First, the colostrum from cows treated with  
66 antimicrobials at the beginning of their dry period can be administrated to calves. Second, the milk from cows  
67 treated with antimicrobials during their lactation (waste milk, prohibited for sale) that can also be used to feed  
68 calves during the withdrawal period in order to limit economic losses (EFSA Panel on Biological Hazards  
69 (BIOHAZ) et al., 2017). The EFSA report finally recommended avoiding feeding calves colostrum and milk  
70 containing residues of antimicrobials that could select for antimicrobial-resistant bacteria, particularly those  
71 selecting for resistance to high-priority critically important antimicrobials (CIAs).

72 The aim of the present longitudinal study was to explore on dairy farms the factors associated with the  
73 selection of antimicrobial resistance among *E. coli* strains in the digestive flora of calves of two weeks of age,  
74 and to verify whether the resistance and selection pressure were the same in males and females. The  
75 assumption was that males and females could be treated differently on farms according to their future  
76 destination (fattening or herd renewal). The hypotheses included examining the influence of three potential  
77 modes of exposure of calves to antimicrobials: (i) antimicrobial treatment given directly, (ii) consumption of  
78 milk from cows treated with antimicrobials, (iii) consumption of colostrum from cows having received  
79 antimicrobial treatment at dry-off.

80

## 81 **MATERIALS AND METHODS**

82 The study design involved a cohort of 100 dairy farms. We selected ten voluntary veterinary practitioners  
83 spread over the main cattle production areas in France. Each veterinarian was responsible for choosing ten  
84 farms: six farms that usually feed their calves with milk from cows treated with antimicrobials and four farms  
85 never doing so. All the farms selected had to meet the following inclusion criteria: being dairy farms with at  
86 least 40 cows, having at least five cows close to calving, usually selling their males for veal calf production and  
87 keeping their females for herd renewal.

88

89 At each farm, two calves — one male and one female — were studied from birth to two weeks of age for males  
90 (corresponding to their departure to a fattening farm) and from birth to seven weeks for females (before  
91 completing the weaning). Farmers completed questionnaires on farm characteristics (herd size, main breed,  
92 average milk production per cow, geographical area), calf feeding (type of milk, distribution system) and the  
93 selected calves' characteristics (breed, estimated birth weight, conditions of birth). They also completed daily  
94 questionnaires on the antimicrobial exposure of selected calves: *i*) consumption of colostrum during the first 48  
95 hours of life and the antimicrobial treatments used at dry-off for the cow that supplied the colostrum, *ii*)  
96 consumption of milk from cows treated with antimicrobials, whether during the cow's treatment or the  
97 withdrawal period (date, antimicrobial used and route of administration), *iii*) the antimicrobial treatments  
98 given to the calf (date of treatment, antimicrobial used).

99

100 All these calves were sampled at the age of 15 days (rectal swab) and females were sampled again seven weeks  
101 after birth. The sample were undertaken by veterinarians. Sick calves at sampling time were excluded from the  
102 study. The digestive flora of diseased calves can be considerably modified and it was estimated that their  
103 inclusion in that study, focused on commensal *E. coli*, could bias the results. If the calf was sick at sampled time,  
104 another calf was selected at birth in the farm to restart the protocol. Rectal swabs were sent to the veterinary  
105 laboratory selected for the study within 24 hours of sampling and processed upon arrival. They were directly  
106 plated in parallel: *i*) onto MacConkey agar (bioMérieux, Marcy l'Etoile, France) for the culture of the dominant  
107 flora and *ii*) onto selective ChromID ESBL agar containing ceftiofur (bioMérieux) for the selection of ESBL from  
108 the subdominant flora. After incubation at 37°C for 24 hours, one presumptive *E. coli* colony was arbitrary  
109 selected from each plate and isolates were identified through matrix-assisted laser desorption ionization using  
110 a time-of-flight (MALDI-TOF) mass spectrometer (van Veen et al. 2010). If the isolate was not identified as *E.*  
111 *coli*, another colony was selected and identified. The process was repeated twice if needed.

112

113 Antimicrobial susceptibility was tested using the disc diffusion method on Mueller-Hinton agar and results were  
114 interpreted according to the breakpoints recommended by the veterinary section of the Antibiogram  
115 Committee of the French Society of Microbiology (CA-SFM) (<http://www.sfm-microbiologie.org/>). The  
116 antimicrobials used were seven beta-lactams (amoxicillin, amoxicillin/clavulanic acid, cefalotin, ceftiofur,  
117 cefquinome, ceftiofur and ertapenem) and nine non-beta-lactam antimicrobials (tetracycline, streptomycin,  
118 gentamicin, kanamycin, florfenicol, colistin, trimethoprim-sulfamethoxazole, nalidixic acid and enrofloxacin).

119 Florfenicol was tested for epidemiological purposes using the breakpoints assigned for *Pasteurella spp.*  
120 Ertapenem is not authorised in veterinary medicine, but the molecule was tested considering its importance for  
121 human health. Synergy between the amoxicillin/clavulanic acid and ceftiofur discs was used to detect the  
122 presence of an ESBL phenotype, according to CA-SFM recommendations. Cefoxitin was used to highlight the  
123 AmpC phenotype. Multidrug resistance (MDR) was defined as resistance to at least three antimicrobial  
124 molecules of distinct categories, using the following seven molecules as markers of the antimicrobial  
125 categories: amoxicillin, ceftiofur, gentamicin, tetracycline, trimethoprim-sulfamethoxazole, enrofloxacin and  
126 florfenicol.

127

128 The resistance ratio for each antimicrobial tested was calculated as the number of animals harbouring resistant  
129 *E. coli* divided by the total number of animals tested. Comparisons of resistance ratios between males and  
130 females at the age of 15 days and comparisons of resistance ratios for females at different stages (15 days  
131 versus seven weeks after birth) were made using a simple Chi-squared test. The significance level was set to  $p \leq$   
132 0.05.

133

134 Logistic models were used to evaluate associations between antimicrobial resistance and exposure factors. The  
135 models have been implemented for samples taken from 15 days calves whose males are directly sent to  
136 fattening workshops and slaughtered around 5-6 months. For *E. coli* isolated from dominant flora, resistance  
137 was the dependent variable and was taken into account in various forms in different models: *i*) resistance to  
138 each antimicrobial tested (antimicrobials for which resistance ratios were less than 1% were not tested in the  
139 modelling process), *ii*) multidrug resistance, *iii*) the ESBL phenotype. For *E. coli* isolated from sub-dominant  
140 flora, only the ESBL phenotype was tested.

141 In order to select the variables to include in the models, univariate analyses were first performed. Variables  
142 concerning calves, their birth, and their feeding conditions were tested: sex of the calves, birth weight, calf  
143 breed (dairy, cross breed, mixed), farms geographical areas (4 classes), lactation rank, birth area (separate and  
144 clean or not), type of milk given to the calves (dairy milk or powder), milk distribution system used (individual,  
145 collective) and contact between calves. Three antimicrobial exposure variables were also tested: feeding with  
146 colostrum from cows treated with antimicrobials at dry-off, feeding with milk from cows treated with  
147 antimicrobials, and antimicrobial treatment of the calf.

148 To take into account that two calves were studied per farm and that each veterinarian had included ten farms  
149 in the sample, mixed models were used with two variables introduced as random effects (the farm and the  
150 veterinarian).

151 The significant variables at the univariate steps were introduced in multivariate models.

152 Exposures were considered globally, all antimicrobials taken together. The odds ratio (OR) and 95% confidence  
153 intervals were calculated. The associations were considered significant at  $p \leq 0.05$ .

154 In this work, several antimicrobials were studied jointly each having its own prevalence of resistance.  
155 Antimicrobial exposures practices had also specific frequencies. It was then difficult to determine the optimal  
156 sample size. However, by including 182 calves in the study, it was possible to identify, with a power of 80%, an  
157 OR of 2.2, out of a proportion of 40% of resistance in the unexposed, with as many exposed as unexposed  
158 units. Statistical analyses were performed with the R software (R Core Team, 2017, R Foundation for  
159 Statistical Computing, version 3.4.0).

160

161

## 162 **RESULTS**

163 One hundred farms located in the main French dairy areas were monitored from April to September 2017. The  
164 average herd size was 84 cows, with an average milk production of 8,303 L per lactation. The first male and  
165 female calves born on the farm and meeting the inclusion criteria were included in the cohort. These calves  
166 were mainly Prim'holstein (61%), Montbéliarde (19%) or crossbred (15%). The calves with diarrhoea at the time  
167 of sampling were removed from the statistical analyses, which finally focused on 185 calves: 90 males and 95  
168 females.

169

### 170 **Exposure of calves to antimicrobials**

171 All the calves received colostrum within 48 hours of their birth. For 65% of calves, the colostrum came from a  
172 cow treated with antimicrobials at dry-off. For 71% of them, the time between the cow's treatment and the  
173 distribution of colostrum (mean of 64 days) was compliant with the time defined in the summary of product  
174 characteristics (SPC) for the antimicrobial used. Cow treatments at dry-off mostly included 1<sup>st</sup>-generation  
175 cephalosporins (35% of the calves concerned), penicillins (29%), aminoglycosides (11%), macrolides (2%) and  
176 4<sup>th</sup>-generation cephalosporins (<1%).

177 The exact antimicrobial treatments of calves were recorded daily. By two weeks after birth, 22% of the calves  
178 had already received at least one antimicrobial treatment. These treatments mostly contained aminoglycosides  
179 (11% of the total calves), penicillins (8%), polypeptides, i.e. colistin (6%), amoxicillin with clavulanic acid (4%) or  
180 trimethoprim with sulfonamides (3%). Other antimicrobial classes (macrolides, tetracyclines and phenicols)  
181 were rarely used (less than 1% of the calves). By seven weeks old, 31% of the female calves had been treated at  
182 least once by antimicrobials.

183 By two weeks after birth, one-third of the calves (31%) had consumed, at least once, milk from cows treated  
184 with antimicrobials, whether during the treatment or withdrawal periods. The milk came from cows treated by  
185 the intramammary route (50% of the calves concerned), by the parenteral route (14%) or both of these routes  
186 (36%). Cow treatments contained aminoglycosides (21% of the calves consumed milk from cows treated with  
187 this antimicrobial class), tetracycline (16%), polypeptides, i.e. colistin (13%) or penicillins (11%). By seven weeks  
188 old, nearly 40% of the females had consumed milk from cows treated with antimicrobials.

189 For these three antimicrobial exposures (colostrum from cow treated with an antimicrobial at dry-off;  
190 antimicrobial treatment of the calf; consumption of milk from cows treated with antimicrobials), no significant  
191 difference was found according to the sex of the calves.

192

### 193 **Resistance of *E. coli* strains in dominant flora**

194 A strain of *E. coli* was isolated from the dominant flora for each of the 280 samples (185 samples at 15 days and  
195 95 samples at 7 weeks). For 15-day-old calves, the proportion of *E. coli* resistance in dominant flora was high  
196 for streptomycin (70%), tetracycline (68%), amoxicillin (64%) and kanamycin (55%) (Table 1). Resistance  
197 proportions for CIAs were low: 3% for ceftiofur, 2% for cefquinome and 3% for enrofloxacin. There was no  
198 significant difference between the resistance proportions observed for males and females. For females,  
199 resistance was lower at 7 weeks old than at 15 days old for almost all the antimicrobials and significant for the  
200 following ones: amoxicillin (23.2% vs. 64.2% at 15 days old), tetracycline (29.5% vs. 66.3%), nalidixic acid (1.1%  
201 vs. 14.7%), trimethoprim-sulfamethoxazole (4.2% vs. 27.4%) and the three aminoglycosides (streptomycin  
202 26.3% vs. 66.3%, kanamycin 17.9% vs. 52.6% and gentamicin 1.1% vs. 9.5%) (Table 1).

203 Almost a third (32%) of isolates from 15-day-old calves had an MDR profile. The most frequent MDR  
204 associations (75% of multidrug resistant strains) combined amoxicillin, tetracycline and trimethoprim-  
205 sulfamethoxazole resistances. For 7-week-old females, MDR was less common (8%).



206 The ESBL phenotype was identified for 2% of *E. coli* strains isolated from calves' dominant flora. There was no  
207 ESBL phenotype among samples from 7-week-old females.

208

#### 209 **Resistance of *E. coli* strains in sub-dominant flora**

210 An *E. coli* strain was isolated on selective medium for 21% of the samples (n=58/280). Most of them (93.5%)  
211 were confirmed as having an ESBL profile after phenotypic analysis. One isolate was confirmed as AmpC.

212 By two weeks after birth, 22% of the calves were carrying ESBL *E. coli* in their digestive subdominant flora, with  
213 equivalent levels for males and females. The proportions of co-resistance were very high for tetracycline  
214 (82.5%), streptomycin (>85.0%) and kanamycin (72.5%), and lower for gentamicin (10.0%) and enrofloxacin  
215 (15.0%) (Table 2). The proportion of ESBL *E. coli* detected on selective medium decreased slightly over time  
216 from 22% at 15 days to 19% for 7-week-old females. Almost half of females with ESBL *E. coli* at 15 days old  
217 were still positive at 7 weeks old.

218

#### 219 **Association between antimicrobial resistance and exposure to antimicrobials**

220 The univariate analyses carried out on the variables concerning calves and their conditions of birth and feeding,  
221 did not highlight specific factors having impact on the resistance of antimicrobials studied. Only sparse  
222 associations were observed. Considering these findings, the very low levels of resistance of some antimicrobials  
223 (not allowing many explanatory variables to be tested), it was decided to favor a model restricted to the main  
224 exposure variables in order to use similar models for all the antimicrobials tested. Consequently, four bimodal  
225 explanatory variables were kept in the final models: sex of the calf (whose impact assessment was a study  
226 objective) and three antimicrobial exposure variables (feeding with colostrum from cows treated with  
227 antimicrobials at dry-off; feeding with milk from cows treated with antimicrobials; and antimicrobial treatment  
228 of the calf). The implementation of mixed models to take into account the presence of two animals from the  
229 same farm or the involvement of the same veterinarian for ten farms was tested but the models showed little  
230 or no effect for random variables and prevented from reaching convergence for some of the models. It was  
231 verified that removing the random effects had no impact on the results for the other exposure indicators  
232 studied, they were therefore removed from the final models to help convergence and to keep the same model  
233 for all the antimicrobials.

234 No statistical association was identified between calves' consumption of colostrum from cows treated with  
235 antimicrobials at dry-off and proportions of resistant *E. coli* strains, whatever the resistance indicator used.

236 Similarly, no models revealed a link between the ESBL phenotype and calves' antimicrobial exposure on farms  
237 for either dominant or subdominant flora.

238 Feeding calves with milk from cows treated with antimicrobials was associated with significantly increased  
239 resistance in the dominant flora from 15-day-old calves for the following antibiotics: amoxicillin (OR=2.6[1.3;  
240 5.6]), streptomycin (OR=2.7[1.3; 6.2]), gentamicin (OR=4.0[1.2; 14.5]), kanamycin (OR=3.0[1.5; 6.0]),  
241 tetracycline (OR=2.6[2.3; 5.8]) and trimethoprim-sulfonamides (OR=2.2 [1.1; 4.4]) (Table 3). The presence of  
242 MDR *E. coli* strains was also significantly associated with consumption of milk from treated cows (OR=2.3 [1.2;  
243 4.7]).

244 Treating calves with antimicrobials was also associated with increased resistance to amoxicillin (OR=2.9[1.3;  
245 7.2]), gentamicin (OR=4.6[1.3; 16.2]), florfenicol (OR=5.0[2.0; 12.6]) and trimethoprim-sulfonamides  
246 (OR=5.5[2.6; 12.2]) (Table 3). The presence of MDR *E. coli* at 15 days old was greatly increased by treatment of  
247 the calf (OR=6.0 [2.8; 13.3]).

248

## 249 **DISCUSSION**

250 Study results highlight that feeding calves with milk from cows treated with antimicrobials and treating calves  
251 themselves with antimicrobials increase faecal *E. coli* resistance to many antimicrobials and increase *E. coli*  
252 MDR phenotypes. The results also show time trend on resistance since an overall decrease in resistance burden  
253 was observed in female calves between the age of 15 days and seven weeks after birth.

254 From a methodological point of view, one of the strengths of the study lies in the daily data collection, which  
255 guarantees the high precision of data collected. The location of the farms reflected the main dairy production  
256 areas in France, and the farm characteristics were consistent with national data in terms of breed and milk  
257 production. The mean herd size of study farms was higher than the national mean, but this does not impact the  
258 observed relationships between resistance and antimicrobial exposure.

259 On the other hand, it must also be considered that in a field study context, the number of calves to which the  
260 study relates is still limited. It should be taken into account in the interpretation of the model results that  
261 certain relationships between the exposures studied and the AMR may not have been identified due to a lack  
262 of power. In the statistical modelling process, it was not possible to integrate random effects to avoid  
263 convergence issues. That could lead to an overestimation of significance. However, for risk factors and

264 antimicrobials for which there was a significant effect, the p-values resulting from the models are clearly lower  
265 than 0.05, which allows being confident about the significance of the results obtained.

266

267 The study was designed to highlight associations between exposure and resistance, and did not estimate the  
268 prevalence of resistance or antimicrobial exposure since the farms were not randomly selected but chosen  
269 according to their practice concerning the feeding of calves with milk from treated cows. Nevertheless, the  
270 ratio between the six farms that usually fed their calves with milk from cows treated with antimicrobials and  
271 the four farms that never did seems to be realistic according to field veterinarians. The highest levels of  
272 resistance in the *E. coli* of dominant flora were for aminoglycosides, tetracyclines and penicillins. These results  
273 are consistent with those of a previous study using quite different methods of analysis, i.e. by determination of  
274 minimal inhibitor concentrations (CLSI 2013) and EUCAST epidemiological cut-offs (Duse et al., 2015).

275 The results of our study largely suggest a correlation of resistance for the four antimicrobials with higher levels  
276 of resistance and common patterns over time. This correlation is also supported by the modelling results,  
277 which highlighted very similar odds ratios for these antimicrobials. This situation could be linked to different  
278 phenomena: *i)* these antimicrobials are the most used in veterinary medicine causing significant selection  
279 pressure, *ii)* some are frequently used jointly for their synergistic effect (aminoglycosides and penicillins), *iii)*  
280 common resistance mechanisms with the acquisition by *E. coli* of one or more resistance genes carried by  
281 transferable mobile structures (Poirel et al., 2018).

282 The correlation between resistances was also highlighted through the MDR results in the models. The choice  
283 was made to consider gentamicin, among aminoglycosides in the MDR definition, whose impact on human  
284 health is significant. This choice may have led to an underestimation of the MDR rates compared to those  
285 which would have been obtained by choosing streptomycin, widely used in veterinary medicine.

286 Our study also showed a sharp decrease in resistance during the first weeks of life for females. For some  
287 antimicrobials, this decrease was not statistically significant but it could be linked to an insufficient statistical  
288 power, the prevalence for these antimicrobials being low (<10%) and most below 5% for calves at 15 days.  
289 Some publications (Berge et al., 2003; Pereira et al., 2014; Pereira et al., 2018) have also stressed a peak of  
290 resistance towards the second week of life and then a decrease, probably resulting from the natural evolution  
291 of the intestinal microbiota of calves in connection with the maturation of their digestive system until weaning.

292 The present study did not reveal any difference in antimicrobial exposure according to the sex or destination of  
293 the calves (fattening for males and herd renewal on the same farm for most of the females). The resistance

294 rates for antimicrobials tested were equivalent for males and females and very similar to those found on  
295 fattening farms (Gay et al., 2019).

296 Strains with an ESBL phenotype were rare (2%) among dominant flora but frequently identified (22%) using  
297 selective medium. However, the proportions of resistance found in our study were far below those observed in  
298 calves on arrival at fattening farms after departure from dairy farms (respectively 3% and 68%) (Gay et al.,  
299 2019). Two hypotheses may explain such a disparity: *i*) practices changed between the two studies (a new  
300 French decree in 2016 led in particular to a reduction in the use of 3<sup>rd</sup>- and 4<sup>th</sup>-generation cephalosporins), *ii*)  
301 the other production steps between dairy farms and fattening farms (i.e. batching centres) have an influence  
302 on resistance selection.

303

304 Until it develops its own ability to resist disease, the calf is entirely dependent on the immunity acquired by  
305 colostrum consumption (Barrington and Parish, 2001; McGuirk and Collins, 2004). This contains high  
306 concentrations of antibodies that provide temporary and passive immunity against infections. Our study  
307 reported a widespread distribution of colostrum on the farms surveyed, reflecting the farmers' good  
308 knowledge of the importance of this practice. Most of calves received colostrum from cows for which the  
309 drying period after treatment was in accordance with the SPCs. Finally, no link was reported between  
310 consumption of colostrum from cows treated with antimicrobials at dry-off and the resistance levels of faecal  
311 *E. coli* from calves. This corroborates and supplements the findings of a previous study that focused on  
312 penicillin and aminoglycoside treatments because they were the only antimicrobials used in dry cow therapy in  
313 Sweden (Duse et al., 2015). Maximum Residue Limit (MRL) assessments and the scientific literature have also  
314 shown that levels of antimicrobial residues in colostrum are low and decrease in keeping with the duration of  
315 the dry period (Johnson et al., 1977; Oliver et al., 1984; Rangel-Lugo et al., 1998; Hausler et al., 2013). However,  
316 EFSA has suggested that further studies are needed on antimicrobial residues in colostrum and the thresholds  
317 at which selection for AMR occurs in calves (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2017).

318

319 In our study, many calves were treated with antimicrobials during their first two weeks of life. The  
320 antimicrobials used corresponded globally to the French recommendations of good practices for the use of  
321 antimicrobials, particularly the use of colistin, penicillins and aminoglycosides as first-line treatments. No  
322 critically important antimicrobials were reported to have been used to treat calves. This is not surprising  
323 because since 2016 in France, the use of these molecules is subject to the result of a prior susceptibility test.

324 The results showed that the *E. coli* strains from treated calves had higher levels of resistance for amoxicillin,  
325 gentamicin and trimethoprim-sulfonamide, but also for florfenicol which was rarely used on these farms. The  
326 use of one antimicrobial selects resistance to this antimicrobial but also frequently participates in the co-  
327 selection of other resistances. This is because some resistance genes are genetically linked, being carried by  
328 mobile genetic supports such as plasmids (Carattoli, 2009; Meunier et al., 2010).

329 The impact of antimicrobial treatment on resistance has previously been demonstrated on a macroscopic scale  
330 by linking antimicrobial consumption and resistance levels in different countries and animal species  
331 (Chantziaras et al., 2013). Available studies on calves have either been experimentation-based (Berge et al.,  
332 2006; Bosman et al., 2014) or have focused on calves on fattening farms (Catry et al., 2016), making  
333 comparisons with our study limited. However, they have generally shown that an increase in resistance—  
334 though sometimes transient—was associated with the use of antimicrobials in calves.

335 Nevertheless, although neonatal diarrhoea is a common disease, the routine use of antimicrobials cannot be  
336 recommended in calves without systemic illness (normal appetite for milk, no fever) (Constable, 2004; Berge et  
337 al., 2009). Providing better care and healthier conditions for calves or using oral rehydration therapy, originally  
338 developed in human medicine, may also be helpful (Victoria et al., 2000).

339

340 The most striking result of this study is the negative impact of the distribution of milk from cows treated with  
341 antimicrobials on the AMR of calves' commensal digestive flora. There is no regulation prohibiting the  
342 distribution of this milk to calves in France. This practice is most often carried out for economic reasons, to limit  
343 the losses linked to this non-marketable milk (Brunton et al., 2012; Rollin et al., 2015). Our study did not  
344 identify the frequency of this practice, the sample being selected on this criterion, but veterinarians  
345 encountered no difficulty in finding such farms even though they had to include six farms giving this milk to  
346 calves, which shows that this practice is quite common.

347 In our study, the consumption of milk from treated cows increased the resistance of *E. coli* to a broad range of  
348 antimicrobials (amoxicillin, aminoglycosides, tetracycline, trimethoprim-sulfonamide association). It would  
349 have been beneficial to distinguish cow treatments according to whether they were intramammary or  
350 parenteral. This was not possible in the modelling process considering the very small number of parenteral  
351 route treatments.

352 The effect of milk consumption from treated cows on streptomycin resistance has already been evidenced in  
353 Sweden and Spain (Duse et al., 2015; Maynou et al., 2017). These studies also found effects for quinolones and

354 fluoroquinolones. Other experimental protocols have also demonstrated an impact on C3G/4G (Brunton et al.,  
355 2014; Pereira et al., 2014). This was not the case in our study conducted under field conditions, where these  
356 antimicrobials were not used (in the case of fluoroquinolones) or rarely used (in the case of cephalosporins) to  
357 treat cows on the selected farms.

358

## 359 **CONCLUSION**

360 This study highlights the impact of feeding calves with milk from cows treated with antimicrobials and the  
361 impact of calves' antimicrobial treatments on the AMR of commensal *E. coli* in their digestive flora. It therefore  
362 appears important to consider the impact of these resistant bacteria in calves and their potential dissemination  
363 to other bacteria or other species — including humans — via their environment, contact with animals or  
364 consumption of their meat. Conversely, feeding calves with colostrum from cows treated with antimicrobials at  
365 dry-off does not appear to induce an increase in resistance levels of the commensal digestive flora. The impact  
366 of this practice also depends on the period of time between the date of the cow's antimicrobial treatment at  
367 dry-off and consumption by the calf of the treated cow's colostrum, which in our study often complied with the  
368 recommended periods.

369

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373

## 374 **Declarations of interest**

375 None to declare.

376

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462 Table 1: Proportion of resistant *E. coli* strains isolated from the dominant flora (rectal swab) of healthy calves sampled at 15 days and 7 weeks after birth on dairy farms.

Antimicrobials	Breakpoints (mm: S≥/R<)	15 days			p of comparison, male 15 days versus female 15 days	7 weeks Female n=95 (%)	p of comparison, female 15 days versus female 7 weeks
		Male n=90 (%)	Female n=95 (%)	Total n=185 (%)			
Amoxicillin	21/14	63.3	64.2	63.8	1.00	23.2	<0.01
Amoxicillin/clavulanic acid	21/14	6.7	6.3	6.5	0.76	2.1	0.28
Cefalotin	18/12	6.7	4.2	5.4	0.52	1.1	0.39
Cefoxitin	22/15	3.3	2.1	2.7	0.67	0.0	0.50
Ceftiofur	21/18	2.2	3.2	2.7	1.00	1.1	0.62
Cefquinome	22/19	2.2	2.1	2.2	1.00	1.1	1.00
Ertapenem	28/26*	0.0	0.0	0.0	1.00	1.1	1.00
Streptomycin	15/13	73.3	66.3	69.7	0.26	26.3	<0.01
Gentamicin	18/16	4.4	9.5	7.0	0.25	1.1	0.02
Kanamycin	17/15	57.8	52.6	55.1	0.55	17.9	<0.01
Tetracycline	19/17	70.0	66.3	68.1	0.53	29.5	<0.01
Nalidixic acid	20/15	10.0	14.7	12.4	0.38	1.1	<0.01
Enrofloxacin	19/19	4.4	2.1	3.2	0.43	0.0	0.50
Florfenicol	19/15†	12.2	14.7	13.5	0.67	7.4	0.16
Trimethoprim-sulfamethoxazole	16/10	28.9	27.4	28.1	0.87	4.2	<0.01
Colistin	18/15	0.0	0.0	0.0	1.00	0.0	1.00

463 \* Human breakpoints. † Breakpoints for *Pasteurella spp.*

464 Table 2: Proportion of co-resistant ESBL *E. coli* strains isolated from the subdominant flora (selective culture medium containing ceftiofur) of healthy calves sampled at 15  
 465 days and 7 weeks after birth on dairy farms (rectal swab).

Antimicrobials	Breakpoints (mm: S $\geq$ /R<)	15 days			7 weeks
		Male n=20 (%)	Female n=20 (%)	Total n=40 (%)	Female n=18 (%)
Streptomycin	15/13	85.0	85.0	85.0	66.7
Gentamicin	18/16	15.0	5.0	10.0	16.7
Kanamycin	17/15	70.0	75.0	72.5	50.0
Tetracycline	19/17	85.0	80.0	82.5	88.9
Nalidixic acid	20/15	40.0	40.0	40.0	22.2
Enrofloxacin	19/19	15.0	15.0	15.0	11.1
Florfenicol	19/15 <sup>†</sup>	40.0	35.0	37.5	22.2
Trimethoprim-sulfamethoxazole	16/10	50.0	55.0	52.5	33.3
Colistin	18/15	0.0	0.0	0.0	0.0

466 <sup>†</sup> Breakpoints for *Pasteurella* spp.

467 Table 3: Results from generalised linear models relating significant associations between antimicrobial resistance of commensal *E. coli* isolates from the dominant flora of  
 468 dairy calves (sampled at 15 days after birth by rectal swab) and antimicrobial exposure of calves on farms, all antimicrobial classes combined.

469

Resistance modelled Antimicrobial	Explanatory variables Modality (number of calves)									
	Sex of the calf		Colostrum <sup>1</sup>		Milk <sup>2</sup>			Calf treatment <sup>3</sup>		
	Male*/Female (90/95)		No*/Yes (65/120)		No*/Yes (127/58)		Resistant strain (%)	No*/Yes (144/41)		Resistance strain (%)
	OR [95% CI]	p	OR [95% CI]	p	OR [95% CI]	p		OR [95% CI]	p	
Amoxicillin	1.0 [0.5; 1.9]	(0.93)	0.9 [0.4; 1.7]	(0.68)	<b>2.6 [1.3; 5.6]</b>	<b>(0.01)</b>	57.5/77.6	<b>2.9 [1.3; 7.2]</b>	<b>(0.02)</b>	59.0/80.5
Streptomycin	0.7 [0.4; 1.3]	(0.28)	1.0 [0.5; 2.1]	(0.92)	<b>2.7 [1.3; 6.2]</b>	<b>(0.01)</b>	63.8/82.8	2.1 [0.9; 5.4]	(0.09)	66.7/80.5
Gentamicin	1.8 [0.5; 7.2]	(0.37)	1.3 [0.4; 5.3]	(0.70)	<b>4.0 [1.2; 14.5]</b>	<b>(0.02)</b>	3.9/13.8	<b>4.6 [1.3; 16.2]</b>	<b>(0.01)</b>	4.2/17.1
Kanamycin	0.8 [0.4; 1.5]	(0.47)	0.9 [0.5; 1.7]	(0.73)	<b>3.0 [1.5; 6.0]</b>	<b>(&lt;0.01)</b>	47.2/72.4	1.8 [0.9; 3.9]	(0.12)	-
Tetracycline	0.8 [0.4; 1.6]	(0.55)	1.6 [0.8; 3.1]	(0.19)	<b>2.6 [2.3; 5.8]</b>	<b>(0.01)</b>	62.2/81.0	2.0 [0.9; 4.8]	(0.10)	-
Florfenicol	1.1 [0.4; 2.6]	(0.90)	0.7 [0.3; 1.7]	(0.39)	1.3 [0.5; 3.2]	(0.59)	-	<b>5.0 [2.0; 12.6]</b>	<b>(&lt;0.01)</b>	8.3/31.7
Trimethoprim-sulfamethoxazole	0.8 [0.4; 1.6]	(0.50)	1.3 [0.6; 2.7]	(0.52)	<b>2.2 [1.1; 4.4]</b>	<b>(0.04)</b>	23.6/37.9	<b>5.5 [2.6; 12.2]</b>	<b>(&lt;0.01)</b>	20.1/56.1
Multidrug resistance <sup>4</sup>	1.2 [0.6; 2.3]	(0.68)	0.8 [0.4; 1.6]	(0.55)	<b>2.3 [1.2; 4.7]</b>	<b>(0.02)</b>	29.1/46.6	<b>6.0 [2.8; 13.3]</b>	<b>(&lt;0.01)</b>	25.7/65.9

470 Significant associations are in bold; \*Reference class; \*\*OR [95% CI] (p): odd ratio with 95% confidence interval and p value.

471 <sup>1</sup>Consumption by calf of colostrum from cow treated with antimicrobial at dry-off; <sup>2</sup>Consumption by calf of milk from cow treated with antimicrobial; <sup>3</sup>Calf treated with at least one  
 472 antimicrobial treatment. <sup>4</sup>Multidrug resistance: resistance to at least three antimicrobial molecules among seven tested (amoxicillin, ceftiofur, gentamicin, tetracycline, trimethoprim-  
 473 sulfamethoxazole, enrofloxacin and florfenicol).