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

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Title

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Summary

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

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

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

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

INTRODUCTION

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

Among food production animal species, calves are considered to be reservoirs of AMR strains. Data from the French monitoring network for antimicrobial resistance in bacteria from diseased animals (Anses, 2019) indicate that *E. coli* strains isolated from neonatal calf diarrhoea carry most of the resistances observed in the beef sector. On dairy farms where calves are born, some of the females are kept in the farm for herd renewal, while the other females and most of the males are sold for fattening around the age of 15 days. Calves from different farms are grouped together in batches and fattened on specialised farms to be slaughtered at the age of 5 to 6 months. Antimicrobial treatments are numerous during the fattening process (Jarrige et al., 2017) and AMR monitoring programmes and epidemiological studies have measured the high prevalence of *E. coli* harbouring resistances and ESBL in calves at slaughterhouses (Haenni et al., 2014). These veal calves are directly introduced into the food chain and could expose human health through meat consumption. However, it has also been evidenced that by the time they arrive at fattening farms, these calves are already carrying many resistant *E. coli* in their digestive tract. Major resistances concern amoxicillin, tetracycline and streptomycin. ESBL *E. coli* from subdominant flora is also frequent (Gay et al., 2019). Calves therefore appear to acquire AMR very early in their life, even on their farm of birth (Berge et al., 2005).

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

Exposure of calves to antimicrobials

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

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

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

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

Resistance of *E. coli* strains in dominant flora

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

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

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

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

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

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

Association between antimicrobial resistance and exposure to antimicrobials

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

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

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

Feeding calves with milk from cows treated with antimicrobials was associated with significantly increased resistance in the dominant flora from 15-day-old calves for the following antibiotics: amoxicillin (OR=2.6[1.3; 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]), tetracycline (OR=2.6[2.3; 5.8]) and trimethoprim-sulfonamides (OR=2.2 [1.1; 4.4]) (Table 3). The presence of MDR *E. coli* strains was also significantly associated with consumption of milk from treated cows (OR=2.3 [1.2; 4.7]).

Treating calves with antimicrobials was also associated with increased resistance to amoxicillin (OR=2.9[1.3; 7.2]), gentamicin (OR=4.6[1.3; 16.2]), florfenicol (OR=5.0[2.0; 12.6]) and trimethoprim-sulfonamides (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 the calf (OR=6.0 [2.8; 13.3]).

DISCUSSION

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

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

On the other hand, it must also be considered that in a field study context, the number of calves to which the study relates is still limited. It should be taken into account in the interpretation of the model results that certain relationships between the exposures studied and the AMR may not have been identified due to a lack of power. In the statistical modelling process, it was not possible to integrate random effects to avoid convergence issues. That could lead to an overestimation of significance. However, for risk factors and antimicrobials for which there was a significant effect, the p-values resulting from the models are clearly lower than 0.05, which allows being confident about the significance of the results obtained.

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

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

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

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

The present study did not reveal any difference in antimicrobial exposure according to the sex or destination of the calves (fattening for males and herd renewal on the same farm for most of the females). The resistance rates for antimicrobials tested were equivalent for males and females and very similar to those found on fattening farms (Gay et al., 2019).

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

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

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

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

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

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

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

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

The effect of milk consumption from treated cows on streptomycin resistance has already been evidenced in Sweden and Spain (Duse et al., 2015; Maynou et al., 2017). These studies also found effects for quinolones and fluoroquinolones. Other experimental protocols have also demonstrated an impact on C3G/4G (Brunton et al., 2014; Pereira et al., 2014). This was not the case in our study conducted under field conditions, where these antimicrobials were not used (in the case of fluoroquinolones) or rarely used (in the case of cephalosporins) to treat cows on the selected farms.

CONCLUSION

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

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Declarations of interest

None to declare.

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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	p of comparison, female 15 days versus female 7 weeks
		Male n=90 (%)	Female n=95 (%)	Total n=185 (%)		Female n=95 (%)	
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

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

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 days and 7 weeks after birth on dairy farms (rectal swab).

		15 days			7 weeks
Antimicrobials	Breakpoints (mm: S≥/R<)	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[†]	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

[†] Breakpoints for *Pasteurella spp*.

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

Resistance modelled	Explanatory variables Modality (number of calves)									
	Sex of the calf Male*/Female (90/95)		Colostrum[1] No*/Yes (65/120)		Milk[2] No*/Yes (127/58)			Calf treatment[3] No*/Yes (144/41)		
Antimicrobial	OR [95% CI]	p	OR [95% CI]	p	OR [95% CI]	p	Resistant strain (%)	OR [95% CI]	p	Resistance strain (%)
Amoxicillin	1.0 [0.5; 1.9]	(0.93)	0.9 [0.4; 1.7]	(0.68)	**2.6 [1.3; 5.6]**	**(0.01)**	57.5/77.6	**2.9 [1.3; 7.2]**	**(0.02)**	59.0/80.5
Streptomycin	0.7 [0.4; 1.3]	(0.28)	1.0 [0.5; 2.1]	(0.92)	**2.7 [1.3; 6.2]**	**(0.01)**	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)	**4.0 [1.2; 14.5]**	**(0.02)**	3.9/13.8	**4.6 [1.3; 16.2]**	**(0.01)**	4.2/17.1
Kanamycin	0.8 [0.4; 1.5]	(0.47)	0.9 [0.5; 1.7]	(0.73)	**3.0 [1.5; 6.0]**	**(<0.01)**	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)	**2.6 [2.3; 5.8]**	**(0.01)**	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)	-	**5.0 [2.0; 12.6]**	**(<0.01)**	8.3/31.7
Trimethoprim-sulfamethoxazole	0.8 [0.4; 1.6]	(0.50)	1.3 [0.6; 2.7]	(0.52)	**2.2 [1.1; 4.4]**	**(0.04)**	23.6/37.9	**5.5 [2.6; 12.2]**	**(<0.01)**	20.1/56.1
Multidrug resistance[4]	1.2 [0.6; 2.3]	(0.68)	0.8 [0.4; 1.6]	(0.55)	**2.3 [1.2; 4.7]**	**(0.02)**	29.1/46.6	**6.0 [2.8; 13.3]**	**(<0.01)**	25.7/65.9

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

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