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Effects of feeding pasteurized waste milk or saleable milk to calves on weight, health and fecal *Escherichia coli* antimicrobial resistance

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Research Article

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Abstract

The aim of this study was to compare the effects of feeding pasteurized waste milk or saleable milk to calves on weight, health and emergence of antimicrobial resistance in *Escherichia coli* strains isolated from those calves. An experimental study under field conditions on a commercial pasture-based Argentinian dairy farm was carried out. Forty Holstein calves were assigned randomly to either pasteurized waste milk (PWM) or non-pasteurized saleable milk (SM). The antimicrobial agents (AM) used on the farm, both to treat or prevent diseases, were recorded. The passive immunity level, calf live weight, AM presence in milk, clinical examination of calves, and *E. coli* isolation and identification, were performed. A total of 258 *E. coli* strains were isolated from fecal samples (132 isolates from SM calves and 126 from PWM calves at six sampling times). All *E. coli* isolated were used to perform AM susceptibility tests (disc diffusion and agar dilution). No differences were observed between groups in health parameters, average daily gain or prevalence of resistant *E. coli* strains to any AM evaluated throughout the study. Peaks of trimethoprim, sulfamethoxazole and enrofloxacin minimum inhibitory concentration (MIC) were observed at 30 d in *E. coli* from both groups of calves, whilst additional peaks to tetracyclin and ampicillin were observed only in SM calves. All MIC apart from gentamicin decreased at 75 and 90 d of age (during the weaning period). Gentamicin MIC behaved differently, having no peaks and increasing at 90 d only in PWM group. In conclusion, we found no evidence that emergence of antibiotic resistance is related to the consumption of pasteurized waste milk.

There are several liquid feeds available for calves after the initial colostrum, which include stored colostrum, saleable milk, waste or discard milk and milk replacer. Among them, feeding saleable milk is ideal but not always economically viable (Margerison *et al.*, 2013; Ricci *et al.*, 2017). An alternative is to feed calves with waste milk (WM) which consists of excess colostrum and transition milk, milk from cows with clinical mastitis and from cows treated with veterinary drugs for mastitis or other diseases (Maynou *et al.*, 2017). This is a management practice with two basic objectives: maintaining the nutritional requirements of calves and giving a destiny to non-saleable milk (Aust *et al.*, 2013; Kertz *et al.*, 2017).

However, one of the main concerns related to feeding WM to calves is the antimicrobial (AM) content and the potential risk of development of antimicrobial resistance (AMR) in the gastrointestinal tract microbiota of calves (Berge *et al.*, 2005; Aust *et al.*, 2013; Pereira *et al.*, 2014; Maynou *et al.*, 2017; Ricci *et al.*, 2017). Exposure to subtherapeutic levels of AM favors the emergence of resistant bacterial strains, which can cause diseases in calves and/or transfer resistance genes horizontally to other bacteria (Pereira *et al.*, 2014; Maynou *et al.*, 2017).

Resistance level in commensal bacteria, such as *E. coli,* is considered a good indicator of expected resistance problems in pathogens since this organism rapidly acquires AMR to commonly used drugs (Duse *et al.*, 2015). The effect of feeding WM to dairy calves on the emergence of AMR bacteria is controversial. Maynou *et al.* (2017) concluded that the AM pressure *via* WM is not sufficient for the emergence of resistance in gut bacterial population. However, other studies (Langford *et al.*, 2003; Aust *et al.*, 2013; Duse *et al.*, 2015; Jarrige *et al.*, 2020) reported that although AMR *E. coli* are commonly isolated from feces of preweaned dairy calves offered saleable milk, the use of WM to feed them increased the probability of shedding resistant *E. coli*. However, in none of these studies was *E. coli* AMR assessed in dairy calves during both pre-weaning and post- weaning periods under field conditions. This is particularly relevant since it is unclear whether the increased prevalence of AMR *E. coli* isolates in the calf gastrointestinal tract is a transient or long-lasting effect (Berge *et al.*, 2005; Aust *et al.*, 2013). The aim of this study was to assess the effects of feeding pasteurized waste milk and whole

saleable milk to calves on live weight gain, health and emergence of AMR in *E. coli* strains isolated during pre- and post-weaning period from calves managed in a pastured-based dairy production system in Argentina.

Materials and methods

Experimental design

This study was approved by the Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina with the protocol number: Expte 12345/001. Protocolo 064/10. An experimental study under field conditions on a commercial dairy farm was carried out. The farm was located in Grütly (31°15′16.5″S 61°07′47.0″W, Santa Fe, Argentina). Holstein calves were selected according to the following inclusion criteria: calves born in normal labor (with no human-assistance), clinically healthy and that had received good quality colostrum (4 l) through an esophageal tube within 4–6 h after birth. Fifty calves were assigned randomly to one of two treatment groups following birth and offered pasteurized waste milk (PWM) and non-pasteurized saleable milk (SM). Male and female calves were assigned proportionally to both groups. All calves assigned to the study were followed for 14 weeks. Further information available in the online Supplementary File.

Variables measured

Passive immunity acquisition was assessed by the whole-blood glutaraldehyde coagulation test (Sandholm, 1974). This test was performed on calves to detect hypogammaglobulinemia between 2 and 5 d after birth. Only calves that received an adequate intake of colostrum and acquired passive immunity with IgG levels in sera >18 g/l were included in the study (Lombard *et al.*, 2020). The live weight (LW) of calves was measured using calibrated livestock scale (Hook, AT-100, precision = 0.1 kg) weekly from 1 to 8 weeks of age, followed by an assessment at 10 weeks of age. Global average daily gain (GADG) was estimated for each individual calf by calculating the difference in final weight minus baseline weight and dividing this result over the 70 d (10 weeks) of the study duration. Weaning average daily gain (WADG) was estimated for each individual calf by calculating the difference between weight at week 6 minus baseline weight and dividing this result over the 56 d (6 weeks) of age. Post-weaning average daily gain (PWADG) was estimated for each individual calf by calculating the difference between weight at week 10 minus weight at week 6 and dividing this result over the 28 d (4 weeks) evaluated post-weaning.

The antimicrobial presence in milk was evaluated three times per week during the preweaning period. Presence of AM residues on the milk fed to calves (PWM and SM) was determined with a commercial microbiological test (ResScreen®, Argentina). Ninety-one samples of milk were analyzed by both Rescreen® methods (BT and BS). Forty-four of the samples were taken from PWM and forty-seven from SM. Samples of milk offered in the morning and afternoon were analyzed. More information about the tests used is available in the online Supplementary File.

A clinical examination was performed daily on the calves to observe signs of illness (general condition, diarrhea, posture, attitude, respiratory symptoms, fever and dehydration). Calves that presented clinical signs of illness and received AM or any kind of treatment were excluded from the study. All calves that got sick, either those that have received AM treatment or not, ultimately died and were removed from the study. Nevertheless, there were no differences in mortality rate between groups.

For the isolation and identification of *E. coli* an individual fecal sample was taken directly from the rectum every 15 d, four times from each calf during pre-weaning period. After weaning, an individual fecal sample was taken every 15 d, two times, giving a total of six fecal samples per calf (at 15, 30, 45, 60, 75 and 90 d of age). Samples were taken aseptically in sterile bags directly from the rectum and refrigerated until they were cultured in selective media (MacConkey Agar, Britania®, Argentina) at 37°C for 24 h. at the Microbiology laboratory (Facultad de Ciencias Veterinarias, UNL). *E. coli* presumptive colonies were further identified by biochemical tests according to standardized procedures (Koneman *et al.*, 1999) and preserved in cryoprotective medium at −80°C.

Antimicrobial susceptibility was evaluated by disc diffusion test (DDT) and agar dilution test (minimal inhibitory concentration, MIC) in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2018). Amoxicillin/clavulanic acid (AMC), ampicillin (AMP), tetracycline (TET), trimethoprim sulfamethoxazole (TMS), gentamicin (GEN) and enrofloxacin (ENRO) were used. Isolates were categorized as susceptible, intermediate and resistant based on the CLSI (2018) interpretive criteria. Further descriptions about these tests are available in the online Supplementary File.

Statistical analysis

All data were unified in an Excel spreadsheet. This database was reviewed to ensure that there were no errors in the transfer of the information from paper to the spreadsheet. If, as a result of DDT or MIC, an isolate was categorized as intermediate, it was considered as resistant for the purposes of the analyses. A descriptive analysis of data was performed, including graphics of the distribution and normality analysis using Shapiro–Wilk test of the scale variables (LW and ADGs) to select the better statistical approach for the subsequent analysis. As data from dead calves were excluded from future analysis on this study, differences in mortality rates between treatment groups were evaluated by means of χ^2 test to ensure there was no bias.

To assess if there were differences in LW between treatment groups a generalized linear model (GLM) with treatment group as fixed factor, sex as a covariable, and LW as dependent variables (using gamma distribution and probit link function) was carried out. Another GLM with treatment group as fixed factor and LW as dependent variables (using gamma distribution and probit link function) was carried out to assess if there were differences in LW between male and female calves. In order to assess if there were weekly differences in LW between treatments, one *T*-Student test per week was carried out. The effect of treatment on ADGs (GADG, WAGD and PWAGD) was evaluated by means of Student *t* tests. The effect of treatment on presence of AMR was evaluated by means of a generalized estimating equation with treatment group (PWM/SM) as fixed factor and AMR (resistant/susceptible) to a specific drug as dependent variable for each AM analyzed in the study (using binomial distribution and probit link function). That is, one equation for AMR to AMC, one for AMR to AMP, one for AMR to TET, etc. The effect of treatment by sampling time was evaluated by means of a GLM with treatment group as fixed factor and AMR to a specific drug as dependent variables for each AM analyzed in the study and each sampling time (using binomial distribution and probit link function). Concordance between DDT and MIC results was assessed by means of Cohen concordance test and kappa coefficient

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calculation. All statistical analyses were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

Results

Mortality rate was 12% (3/25) and 16% (4/25) in SM and PWM group, respectively and did not differ significantly. The data from 22 calves in SM group and 21 calves from the PWM group were included in the final analyses. In SM group were 11 bull and 11 heifer calves and in PWM group 10 bull and 11 heifer calves.

Live weight and average daily weight gain

There were no LW differences between treatment groups throughout the study ($P > 0.05$). The average LW of calves from SM group was 49.13 ± 2.59 kg and of calves from PWM group was 47.71 ± 2.29 Kg. There were no LW weekly differences between treatment groups (online Supplementary Fig. S1). There were no differences in LW between calves' gender along the study ($P > 0.05$). Male calves weighted 47.8 ± 1.4 kg and female calves weighed 49.0 ± 1.0 kg at the end of the pre-weaning period.

There were no differences between treatment groups in any of the ADG measures (GADG, WADG and PWADG). Values for GADG were (PWM/SM) 0.43 ± 0.11 and 0.43 ± 0.10 kg, for WADG they were 0.26 ± 0.11 and 0.29 ± 0.10 kg and for PWADG they were 0.44 ± 0.09 and 0.41 ± 0.12 kg.

Antimicrobial presence in milk

Thirteen percent of SM samples were positive to beta lactams and tetracyclines and to beta lactams and sulfonamides. The positive samples were from weeks 4 (three samples), 5 (one sample) and 7 (two samples). Four of these samples; those from weeks 4 and 5 were simultaneously positive for both tests. More than 86% of PWM samples were positive to beta lactams and tetracyclines and 77.28% were positive to beta lactams and sulfonamides. All the samples positive to BS test were also positive to BT. The negative PWM samples were detected on weeks 3 (two samples), 4 (two samples) and 5 (two samples).

Antimicrobial susceptibility tests

A total of 258 *E. coli* strains were isolated from fecal samples (132 isolates from SM calves and 126 from PWM calves at six sampling times). Antimicrobial susceptibility tests (MIC and DDT) were performed on every isolate and regardless of feeding treatment, prevalence of AMR between *E. coli* strains during the study was variable, according to the AM evaluated (Table 1). The highest prevalence of AMR was observed for TET (87.2% and 64%, according to MIC and DDT, respectively), followed by AMP, ENRO and TMS. GEN resistant strains were not detected.

No differences were found between resistant/susceptible *E. coli* strains throughout the study between treatment groups for any AM evaluated or by any technique (online Supplementary Table S1). When performing the analysis by days of age, some AM presented differences in their resistance prevalence between treatment groups (Table 2). At the first three sampling times, differences in the prevalence of resistant strains for AMP between treatment groups were observed. At 15 d of age, the prevalence of resistant strains was higher in SM group but at 30 and 45 d, it was higher in PWM group. Antimicrobial resistance prevalence differences were also observed for TMS, being higher in PWM group at 30 d and higher in SM group at 60 d. Differences in AMR prevalence for ENRO was observed at 60 d, being higher in SM group (Table 2).

The MIC evolution through time was different according to the AM evaluated (Fig. 1). A peak in the MIC of TET, AMP, trimethoprim, sulfamethoxazole and ENRO (all $P < 0.05$ or better) was observed at 30 d in strains from calves of PWM group. Another peak in the MIC of trimethoprim, sulfametoxazol (both $P < 0.05$) and ENRO ($P = 0.05$) was observed at 30 d in strains from calves of SM group. The MIC of all the AM evaluated (except for GEN) decreased at 75 and 90 d of age (during the weaning period) in strains from calves from both treatment groups, while GEN MIC behaved differentially, showing no peaks, and increasing at 90 d in strains from calves of PWM group ($P = 0.05$; Fig. 1).

Concordance and correlation between antimicrobial susceptibility tests

The concordance between DDT and MIC was statistically significant for TET, AMP, ENRO, and TMS ($P < 0.001$, online Supplementary Table S2). More than 63% of isolates classified as susceptible for TET by MIC were also classified susceptible by DDT (concordance = 63.6%). For AMP, ENRO, and TMS the concordance between techniques for susceptible isolates was 87.6, 94.1 and 96.6%, respectively.

Table 1. Antimicrobial resistance prevalence of *E. coli* strains isolated from feces of Holstein calves during the pre-weaning period (approximately eight weeks) and two weeks post-weaning period

	ADT (MIC)		DDT	
Antimicrobial	S*n* (%)	R*n* (%)	S*n* (%)	R*n* (%)
Tetracycline	33 (12.8)	225 (87.2)	93 (36)	165 (64)
Ampicillin	177 (68.6)	81 (31.4)	173 (67.1)	85 (32.9)
Enrofloxacin	204 (79.1)	54 (20.9)	206 (79.8)	52 (20.2)
Gentamicin	258 (100)		258 (100)	
Trimethoprim sulfamethoxazole	208 (80.6)	50 (19.4)	211 (81.8)	47 (18.2)
Amoxicillin – clavulanic acid			250 (96.9)	8 (3.1)

ADT, agar dilution test; MIC, minimal inhibitory concentration; DDT, disc diffusion test; S, susceptible; R, resistant.
Isolates were categorized as susceptible, intermediate and resistant based on the CLSI (2018) criteria.

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Table 2. Antimicrobial resistance prevalence (AMR) between *Escherichia coli* isolated from feces of Holstein calves offered pasteurized waste milk (PWM, $n = 21$) or saleable milk (SM, $n = 22$) in an Argentinian commercial dairy farm ($n = 43$)

		Antimicrobial susceptibility test					
		MIC % Resistance (*n*)		*P*	DDT % Resistance (*n*)		*P*
Antimicrobial	Days of age	PWM	SM		PWM	SM	
TET	15	81 (17)	95.5 (21)	0.147	81 (21)	95.5 (22)	0.147
	30	100 (21)	86.4 (19)	–	100 (21)	81.8 (18)	–
	45	90.5 (19)	72.7 (16)	0.240	71.4 (15)	81.8 (18)	0.488
	60	81 (17)	86.4 (19)	0.698	66.7 (14)	77.3 (17)	0.438
	75	85.7 (18)	72.7 (16)	0.457	42.9 (9)	27.3 (6)	0.284
	90	95.2 (20)	100 (22)	–	19 (4)	22.7 (5)	0.767
Ampicillin	15	23.8 (5)	63.6 (14)	0.009	38.1 (8)	68.2 (15)	0.049
	30	61.9 (13)	22.7 (5)	0.009	71.4 (15)	31.8 (7)	0.009
	45	66.7 (14)	40.9 (9)	0.091	71.4 (15)	36.4 (8)	0.021
	60	33.3 (7)	27.3 (6)	0.665	28.6 (6)	31.8 (7)	0.817
	75	19 (4)	13.6 (3)	0.698	0	14.3 (3)	–
	90	0	4.5 (1)	–	0	4.5 (1)	–
TMS	15	28.6 (6)	45.5 (10)	0.252	19 (4)	45.5 (10)	0.065
	30	42.9 (9)	13.6 (3)	0.033	52.4 (11)	13.6 (3)	0.007
	45	33.3 (7)	27.3 (6)	0.665	23.8 (5)	27.3 (6)	0.795
	60	4.8 (1)	31.8 (7)	0.023	4.8 (1)	27.3 (6)	0.095
	75	4.8 (1)	0	–	4.8 (1)	0	–
	90	0	0	–	0	0	–
ENRO	15	33.3 (7)	31.8 (7)	0.916	42.9 (21)	54.5 (22)	0.444
	30	57.1 (12)	36.4 (8)	0.172	47.6 (10)	45.5 (10)	0.887
	45	33.3 (7)	13.6 (3)	0.162	14.3 (3)	9.1 (2)	0.664
	60	4.8 (1)	31.8 (7)	0.046	4.8 (1)	18.2 (4)	0.345
	75	4.8 (1)	0	–	4.8 (1)	0	–
	90	0	4.5 (1)	–	0	0	–
AMC	15				4.8 (1)	13.6 (3)	0.327
	30				4.8 (1)	0 (0)	0.488
	45				0	0	–
	60				0	0	–
	75				9.5 (2)	4.5 (1)	0.607
	90				0	0	–

PWM, pasteurized waste milk; SM, saleable milk; MIC, minimal inhibitory concentration; DDT, disc diffusion test; TET, tetracycline; TMS, trimethoprim-sulfamethoxazole; ENRO, enrofloxacin; AMC, amoxicillin-clavulamic acid; bold numbers represent statistical significance; – Fisher's exact test cannot be performed since zero is present in the crosstab; empty spaces represent that susceptibility test was not performed for that antimicrobial.
Results from the generalized linear models performed per days of age of calves with treatment group (PWM/SM) as fixed factor and AMR to each specific drug by disc diffusion test (DDT) or agar dilution test (MIC) as dependent variables (binomial distribution and probit link function).

Discussion

Dairy producers have several options for pre-weaning calf feeding to reduce production costs while raising healthy calves. Among these options, the use of non-saleable milk to feed calves is a common practice in Argentina (González Pereyra *et al.*, 2015) as well as in other countries (Duse *et al.*, 2015; Kertz *et al.*, 2017; Maynou *et al.*, 2017). This study aimed to assess the effect of feeding calves SM or PWM on calf performance (ADG) and AMR in *E. coli* isolated from feces during pre and post-weaning, under management conditions of a commercial dairy farm.

No differences in weekly LW throughout the observation period were detected, neither between treatment groups nor between calves' gender. We also found no differences in GADG, WADG or PWADG between treatment groups. These findings agree with previous reports that did not find detrimental effects of PWM on growth (Aust *et al.*, 2013; Vieira *et al.*, 2021). The ADG

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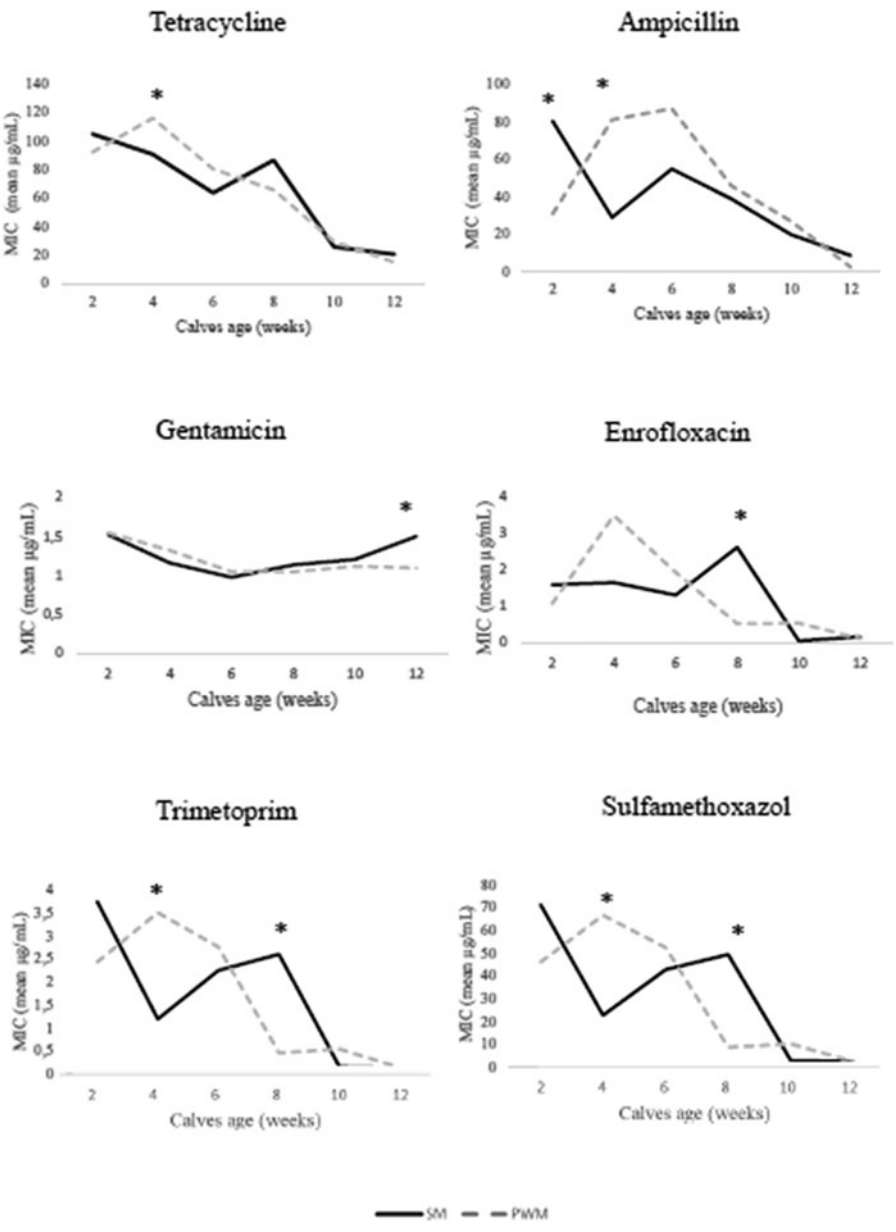

Figure 1. Minimal inhibitory concentration (MIC) evolution of selected antimicrobials for *Escherichia coli* isolated from feces of calves throughout the study. Black line represents MIC from isolates of the saleable milk treatment group (SM) and gray line represents MIC from isolates of the pasteurized waste milk group (PWM).

observed in this study during the first 15 d of life was similar to that reported in other studies in which PWM was offered, however, the WADG was lower than that recorded in those studies (Aust *et al.*, 2013; Vieira *et al.*, 2021). Differences between studies may be due the experimental conditions used (housing, breed, volume of milk fed, among other factors). Outdoor housing and feeding 2 L of milk twice daily were the most frequently used practices on local dairy farms at the time this study was carried out (Carbonero *et al.*, 2007) and the WADG observed was the expected for the management conditions used (Juliano *et al.*, 2016).

The largest expected difference between feeding with SM or PWM was the presence of AM residue. Antimicrobial residues in six (13%) SM samples were detected, which can be attributed to lack of identification of AM treated cows, inadequate record of treated animals and/or presence of residues below the MRL after the withdrawal period in some animals, taking into account that the AM screening methods used in this study detect AM below the MRL (Nagel *et al.*, 2011). This implies that calves in the SM group were exposed to AM residues in weeks 4, 5 and 7 of age, which could be associated with the increased AM resistance of fecal *E. coli* observed for ampicillin, tetracyclin, trimethoprim and sulfametoxazol between weeks 6 and 8. The presence of AM residues in some SM samples was unexpected and not simultaneously detected by the processing dairy industry. However, as this trial was conducted in a commercial dairy farm evaluating the actual feedings offered to calves, these results may reflect the potential management errors expressed above.

Globally, prevalence of resistant *E. coli* strains was not different between treatment groups as was observed in other studies (Aust *et al.*, 2013; Maynou *et al.*, 2017). Nevertheless, some antimicrobials presented differences between treatment groups at some days of age of the calves. Resistant *E. coli* were more frequently isolated from fecal samples of PWM group at 30 and 45 d of age for AMP and TMS. This could have been associated with AM usage since both AMP and TMS were frequently used at the farm, so resistant

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strains could be present in the environment, with the possibility of colonizing the intestinal microbiota of untreated calves (Sayah *et al.*, 2005). Other authors found that farm-type and antibiotic treatment effects were associated with specific antibiotic-resistance phenotypes in fecal *E. coli* from pre-weaned calves. However, the age of the calf was the variable most strongly associated with phenotypic AMR (2–4 weeks of age), suggesting that host-specific factors not directly related to AM usage are associated with emergence of resistance (Berge *et al.*, 2005). In humans, the influx of antimicrobial-resistant bacteria into the enteric microbiota is contingent upon their capacity to outcompete the indigenous microbiota (Jernberg *et al.*, 2010). Neonatal calves exhibit an underdeveloped and less diverse intestinal microbiota, diminishing the protective barrier against the colonization of bacteria incurring a higher fitness cost, such as antimicrobial-resistant and pathogenic enteric bacteria (Oikonomou *et al.*, 2013; Song *et al.*, 2018; Hang *et al.*, 2021).

Resistant *E. coli* strains to TMS and ENRO were isolated more frequently from the fecal samples of calves offered SM at 60 d of age, however, this was only detected by means of MIC. This could be due to the presence of TMS AM residues in SM, favoring selection of resistance strains in this group of calves. In contrast, ENRO was not used in lactating cows on the dairy farm while this study was carried out, suggesting in this case a lack of direct association. Rather, AMR strains from the environment may have colonized the intestinal microbiota of calves (Sayah *et al.*, 2005).

At 30 d of age fecal *E. coli* isolated from calves offered PWM showed a peak in the MIC values for the majority of the antimicrobial drugs tested. Other authors found that the proportion of fecal *E. coli* resistant to AM in calves fed with milk containing AM residues was higher between 14 and 28 d of age (Aust *et al.*, 2013; Pereira *et al.*, 2014). However, the former authors did not detect a systematic shift in resistance during the experimental period and the latter authors worked with artificially spiked milk, which makes comparisons difficult. At 60 d of age *E. coli* isolated from calves offered SM presented a peak in the MIC, while MIC of isolates from both groups decreased at 75 d of age. The prevalence of AMR strains in the intestine depends on their capability to effectively compete with the existing gastrointestinal microbiota. There was no difference in the present study in the prevalence of AMR *E. coli* to the AM evaluated between calves offered SM or PWM. This may be because calves acquire the bacteria from the environment and the selection pressure for residues in milk was not sufficient to increase the prevalence or because the amount of these AMs present in milk was too low to generate enough selection pressure, as described by Langford *et al.* (2003).

The kappa statistic, calculated to estimate the overall precision of the DDT when compared to MIC results was high for almost all the AM, showing that DDT and MIC were similar in determining susceptibility/resistance of strains tested (Klement *et al.*, 2005).

In conclusion, the emergence of antibiotic resistance observed cannot be justified only by the PWM offered to calves but involves a complex interaction in an ecosystem including microbial communities and antimicrobials.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000219

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