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

Cellone, Ivana, Russi, Norma, Calvinho, Luis F., Signorini, Marcelo, Molineri, Ana

MATERIALS AND METHODS

Experimental design

An experimental study under field conditions on a commercial dairy farm was performed. Holstein calves from a dairy farm located in Grütly (31°15'16.5"S 61°07'47.0"W, Santa Fe, Argentina) were used. Fifty calves were initially selected according to the inclusion criteria and assigned randomly to two treatment groups directly after birth: pasteurized waste milk (PWM) and non-pasteurized saleable milk (SM). Male and female calves were assigned proportionally to both groups. Each calf was identified with a number and color ear tag according to sex and date of birth. Sample size was determined to detect a significant difference of 45% between proportions of resistance in each treatment group with 95% of confidence and 80% power. The calculated n was 38 calves, 19 per group of treatment. More calves were enrolled to guarantee the estimated n at the end of the study (22 calves in SM group and 21 in PWM group reached the end of the study).

The inclusion criteria were 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 (which was further evaluated by whole-blood glutaraldehyde coagulation test (Sandholm, 1974)). All calves assigned to the study were followed for 14 weeks.

Calves were reared outdoors, tethered by a chain to a stake that held the feeding bucket (stake system). The experimental groups of calves (PWM and SM) were 20 meters apart from each other and were fed and sampled by a different operator to avoid horizontal bacteria transmission. Within each group, calves were separated by a distance that did not allow contact between them. All calves received four liters of milk at 37°C ± 1ºC daily (PWM or SM) divided into two equal volumes until weaning time (approximately eight weeks), according to the usual management practice used at the farm. Group PWM received waste milk collected daily into a specific tank and pasteurized at 73-75 ºC per 30 seconds (pasteurizer Alfa Laval®, Argentina). Waste milk consisted of transition milk, milk from cows with clinical mastitis and milk from cows treated with AM due to mastitis or other diseases. Group SM received non-pasteurized milk suitable for sale collected daily from the milking line. Additionally, from day seven of life, calves received water *ad libitum* and a commercial calf starter twice per day (20.89% crude protein, 2.5% crude fat, 6.15% crude fiber; AB Iniciador® Guillermo Lehmann Cooperativa Agrícola Ltda., Santa Fe, Argentina). The commercial calf starter was gradually increased according to the intake up to a maximum of 2 Kg/day. Experimental conditions were similar for both groups; being the only difference between treatment groups the milk they were fed.

Each group was composed of male and female calves that were kept together until weaning time. Then, male calves and female calves were allocated to separate feed-lot pens, with differential diets. Male calves’ diet included corn, soy expeller and vitamins, while female calves’ diet included corn, soy expeller and alfalfa hay.

All procedures used in this study were consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 2010). 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.

The AM agents used on the farm, both to treat or prevent diseases, were recorded. Cows with clinical mastitis were treated with intramammary amoxicillin-clavulanic acid (Clavamox®, Zoetis). All cows received blanket dry cow therapy with cloxacillin (Orbenin Extra®, Zoetis) at the end of lactation. The AM used to treat calves’ infectious diseases were tilmicosin, danofloxacin, penicillin-streptomycin, trimethoprim sulfamethoxazole (TMS) and gentamicin, administered parenterally at recommended doses. All calves that received AM treatment were excluded from the study.

Variables measured

-Passive inmunity: before entering the study whole-blood glutaraldehyde coagulation test (Sandholm, 1974) was performed to calves to detect hypogammaglobulinemia on day 2 – 5 of life. Only calves that received an adequate intake of colostrum and acquired passive immunity properly (serum IgG levels >18 g/l) were included in the study (Lombard et al., 2020).

-Weight: all calves were weighed weekly during the pre-weaning period (first eight weeks of life, approximately) and once at the end of their tenth week of age using a calibrated livestock scale (Hook, AT-100, precision= 0.1 kg). Average daily gain was estimated for each individual calf by dividing weekly weight by seven.

-Antimicrobial presence in milk: three times per week during the preweaning period AM residues presence on the milk feed to calves (PWM and SM) was determined with a commercial microbiological test (ResScreen®, Argentina). Two kinds of ReScreen® tests were used: BT and BS (Nagel et al., 2011). The BT test detected beta lactams (amoxicillin, ampicillin, cloxacillin, oxacillin, penicillin G, cefalexin, cefoperazone, cefadroxil, cefuroxime, and ceftiofur) and tetracyclines (chlortetracycline, oxytetracycline, and tetracycline). The BS test detected the same beta lactams and sulfonamides (sulfadiazine, sulfadimetoxin, sulfamerazine, sulfametazine, sulfamethoxazole, and sulfathiazole). These tests provide a qualitative detection of the AM in milk at levels below their maximum residue limits (MRLs) according to Codex Alimentarius (Argentina).

-Clinical examination: calves were examined daily for clinical signs of illness (general condition, diarrhea, posture, attitude, respiratory symptoms, fever, and dehydration). Diarrhea was defined as augmentation of frequency of deposition, with a decreased consistency of feces (Windeyer et al., 2014). Calves that presented clinical signs of illness were excluded from the study and treated.

-Isolation and identification of *E. coli*: an individual fecal sample was taken every 15 days, four times from each calf during pre-weaning period (first 60 days of life). After weaning, an individual fecal sample was taken every 15 days, two times, giving a total of six fecal samples per calf (15/30/45/60/75/90 days of age). Samples were taken directly from the rectum and placed into sterile plastic bags. All samples were immediately refrigerated, transported to the laboratory, and cultured within 6 h. Samples were cultured in selective media (MacConkey Agar, Britania®, Argentina) at 37°C for 24 h. *E. coli* presumptive colonies were identified by biochemical tests according to standardized procedures (Koneman et al., 1999) and preserved in cryoprotective medium at -80°C.

-Antimicrobial susceptibility tests: 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, 2013).

Disc diffusion test: briefly, the isolates kept at -80° C were reactivated on MacConkey agar, four to five colonies were suspended in tryptic soy broth (Laboratorio Britania®, Buenos Aires, Argentina) and adjusted to a turbidity equivalent to a 0.5 McFarland standard. A swab with the inoculum was streaked over a Mueller-Hinton agar plate (Laboratorio Britania®, Buenos Aires, Argentina). The following disks were used: amoxicillin/clavulanic acid (AMC) 20/10 µg; ampicillin (AMP) 10 µg; tetracycline (TET) 30 µg; trimethoprim sulfamethoxazole (TMS) 25 µg; gentamicin (GEN) 10 µg (Britania®); enrofloxacin 5 µg (ENRO) (Neo- Sensitabs ®, Rosco, Denmark). *E. coli* ATCC 35218 was used as control strain. Isolates were categorized as susceptible, intermediate and resistant based on CLSI interpretive criteria (CLSI, 2013). For ENRO, interpretive criteria developed by the laboratory producer of the discs were used (Rosco, Denmark).

Agar dilution test (CLSI, 2013): briefly, isolates were suspended in tryptic soy broth and adjusted to a turbidity equivalent to a 0.5 McFarland standard. This inoculum was then diluted in tryptic-soy broth 1:10 to obtain an inoculum of 107 UFC/ml that was inoculated in Mueller-Hinton agar plates containing serial 2-fold dilutions from 126.56 to 0.025 µg (or units)/ml for each AM, using a multipoint inoculum replicator that delivered 1 µl. Plates were incubated at 37°C for 20 h. *E. coli* ATCC 35218 was used as control strain. Mueller-Hinton plates without AM drugs were incubated each time as controls. The MIC was defined as the lowest concentration at which no bacterial growth was detected. The AM agents tested were AMP, TET, TMS, GEN, and ENRO (Sigma-Aldrich, Germany). Isolates were categorized as susceptible, intermediate, and resistant based on the CLSI (2013) criteria.

Statistical analysis

First, a descriptive analysis of data was performed. Differences in mortality rates between treatment groups were evaluated by means of chi-squared test to ensure that there were no differences between treatment groups in this regard.

To evaluate weight differences between treatment groups, a Generalized Estimating Equation with treatment group (PWM/SM) as fixed factor, sex as a covariable, and weight as dependent variable was performed (using gamma distribution and log link function). A gamma distribution was used because weight was not normally distributed.

A Generalized Estimating Equation with treatment group (PWM/SM) as fixed factor and AMR (resistant/susceptible) to a specific drug as dependent variable was performed for each antibiotic analyzed in the study (using binomial distribution and probit link function). If, as a result of DDT or MIC, an isolate was categorized as intermediate, it was considered as resistant for analyses purposes. In addition, a generalized linear model (GLM) per moment (15/30/45/60/75/90 days of age) with treatment group (PWM/SM) as fixed factor and AMR (resistant/susceptible) to a specific drug as dependent variables were performed for each antibiotic analyzed in the study (using binomial distribution and probit link function).

Concordance between DDT and agar dilution test (MIC) results was assessed by means of Cohen concordance test and kappa coefficient calculation. If, as a result of DDT or MIC an isolate was categorized as intermediate, it was considered as resistant for analyses purposes. All statistical analyses were carried out using InfoStat software (Universidad Nacional de Córdoba, Argentina).

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Table 1 supplementary file. Results of Generalized Estimating Equation with treatment group (pasteurized waste milk -PWM- or saleable milk -SM) as fixed factor and antimicrobial resistance of *E. coli* isolated from Holstein calves to each specific drug by disc diffusion test (DDT) or agar dilution test (MIC) as dependent variables (using binomial distribution and probit link function).

|  |  |  |  |
| --- | --- | --- | --- |
| MIC | | | |
| Antimicrobial | Group | OR (CI 95%) | *P* |
| Tetracycline | PWM | 0.854 (0.575-1.268) | 0.434 |
| SM (Ref.) |
| Ampicillin | PWM | 0.860 (0.624-1.186) | 0.358 |
| SM (Ref.) |
| TMS | PWM | 1.024 (0.715-1.467) | 0.897 |
| SM (Ref.) |
| Enrofloxacin | PWM | 0.916 (0.634-1.323) | 0.640 |
| SM (Ref.) |
| DDT | | | |
| Antimicrobial | Group | OR (CI 95%) | *P* |
| AMC | PWM | 0.980 (0.563-1.705) | 0.942 |
| SM (Ref.) |
| Tetracycline | PWM | 1.024 (0.777-1.351) | 0.864 |
| SM (Ref.) |
| Ampicillin | PWM | 0.790 (0.603-1.036) | 0.088 |
| SM (Ref.) |
| TMS | PWM | 1.058 (0.728-1.537) | 0.769 |
| SM (Ref.) |
| Enrofloxacin | PWM | 1.080 (0.823-1.417) | 0.578 |
| SM (Ref.) |

References: PWM: pasteurized waste milk; SM: saleable milk; TMS: Trimethoprin sulfamethoxazole; AMC: Amoxicillin/clavulanic acid; OR: odds ratio; CI95%: confidence interval 95%

Table 2 supplementary file. Concordance analysis between minimal inhibitory concentration (MIC) and disc diffusion test (DDT) results for *E. coli* strains isolated from Holstein calves’ feces.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tetracycline | | DDT | | Total |
| S n (%) | R n (%) |
| MIC | S | 21 (63.6%) | 12 (36.4%) | 33 (100%) |
|  | R | 72 (32%) | 153 (68%) | 225 (100%) |
| Total |  | 93 (36%) | 165 (64%) | 258 (100%) |
| *P* | <0.001 |  |  |  |
| *Kappa* | 0.178 |  |  |  |
| Ampicillin | | DDT | | Total |
| S n (%) | R n (%) |
| MIC | S | 155 (87.6%) | 22 (12.4%) | 177 (100%) |
|  | R | 18 (22.2%) | 63 (77.8%) | 81 (100%) |
| Total |  | 173 (67.1%) | 85 (32.9%) | 258 (100%) |
| *P* | <0.001 |  |  |  |
| *Kappa* | 0.645 |  |  |  |
| Trimethoprim sulfamethoxazole (TMS) | | DDT | | Total |
| S n (%) | R n (%) |
| CIM | S | 201 (96.6%) | 7 (3.4%) | 208 (100%) |
|  | R | 10 (20%) | 40 (80%) | 50 (100%) |
| Total |  | 211 (81.8%) | 47 (18.2%) | 258 (100%) |
| *P* | <0.001 |  |  |  |
| *Kappa* | 0.784 |  |  |  |
| Enrofloxacin | | DDT | | Total |
| S n (%) | R n (%) |
| MIC | S | 192 (94.1%) | 12 (5.9%) | 204 (100%) |
|  | R | 14 (25.9%) | 40 (74.1%) | 54 (100%) |
| Total |  | 206 (79.8%) | 52 (20.2%) | 258 (100%) |
| *P* | <0.001 |  |  |  |
| *Kappa* | 0.691 |  |  |  |

Reference: S= Susceptible, R= Resistant

Figure 1 supplementary file. Holstein calves weight in SM (saleable milk) and PWM (pasteurized waste milk) treatment groups throughout the study.

