Objectives:

1. Document the exposure of calves to antimicrobial residues in WM on Swiss dairy farms.
2. Evaluate at the herd level the effect of feeding WM with residues to dairy calves on AMR in calves' fecal *E. coli* compared to no WM.
3. Evaluate at the herd level the effect of feeding WM with residues to dairy calves on the reservoir of AMR in calves' environment.

Pilot study: Estimate prevalence and farm-level clustering of multidrug resistance (MDR) in calves' environment on farms where WM is fed to calves and on farms where no WM is fed to calves.

Study design: Cross-sectional study, 1 visit per farm

Inclusion criteria: farm size: 15-60 cows (based on quantiles 10-90 from 1st questionnaire)  
Exclusion criteria: organic production/rarely treating lactating cows, seasonal production, shared pasture (with other farms), only feeding WM to calves in the fattening period

Sample size calculation to estimate a single prevalence (for each category of herd): using same data as in calves' fecal samples: expected MDR prevalence of 0.5 in herds feeding WM, 0.2 in herds feeding no WM, and ICC 0.193; 4 isolates/farm  
Herds feeding WM: 30 farms  
Herds feeding no WM: 30 farms

Total number of farms: 60  
Total number of cultures: 120  
Total number of isolates to characterize (MIC): 240

Recruitment:   
Stratification by average cow milk yield; random recruitment of herds of both categories among email list. The project will be briefly described (objectives, timeline, inclusion criteria) and compensation will be mentioned.

Timeline: Invitations for recruitment will be sent in the week of May 17th. Farm visits will be scheduled between June 21st to approximately September 1st. The visits will take place on Mondays through Wednesdays, at 2-4 farms/day.

Sample collection: 2 gauzes, each wiped over a strip of approximately 30 cm x 150 cm, on the wall (or railings if no wall available) of calves' pen or hutches. In case of individual housing, 2 different hutches/pens will be sampled. Each gauze will be put in a 50-ml plastic tube, to which will be added 30 ml of Mueller-Hinton broth. The tube will be vortexed, then incubated for 18-24h at 37 °C. Following enrichment, 10 μl of broth will be inoculated onto a BROLAC plate, and incubated for 18-24h at 37 °C. Two *E. coli* colonies per plate will be selected (morphologically different, if possible), grown in pure culture, species identification will be confirmed by MALDI-TOF, and MIC will be determined using EUVSEC3 plates (15 antimicrobials).

Data collection: AMU (currently possible from records?); general farm characteristics, housing type, number of calves in each pen, cleaning frequency (see annex)

Main study

Study design: Repeated cross-sectional

Target population: Swiss dairy farms producing waste milk containing antimicrobial residues  
Source population: producers who participated in the 1st questionnaire study and volunteered for further studies

Study unit: Farm. Level of exposure: farm; level of measurement of outcome: *E. coli* isolates from pre-weaned calves  
Inclusion criteria: farm size: 15-60 cows  
Exclusion criteria: organic production/no treating lactating cows, seasonal production, only feeding WM to calves in the fattening period, only feeding WM after 1d of withdrawal

1. Control herds: Herds not feeding waste milk with antimicrobial residues to calves
2. Exposed herds: Herds feeding waste milk with antimicrobial residues to all calves

Sample size calculation:   
Expected prevalence of MDR in calves on farms feeding WM: 0.5  
Expected prevalence of MDR in calves on farms feeding no WM: 0.2  
Expected intra-class clustering: 0.193  
Sample size per group: 45 isolates  
Design effect due to clustering with 3 isolates/farm: 1.386  
Design effect due to repeated sampling + management factors: 1.25  
n = 45 samples / 3 isolates/farm x 1.386 x 1.25 = 26; round up for drop-off: 30 farms/group

Total number of farms: 60  
Total number of fecal cultures: 240  
Total number of fecal isolates to characterize (MIC): 720  
Total number of environmental cultures: 480  
Total number of environmental isolates to characterize (MIC): 960

Recruitment: Same participants as pilot study

The study will be conducted into 1 cohort, of which recruitment and initial farm visits will span over 3 months. Approximately equal numbers of herds from both groups will be visited per month. A total of approximately 20 visits per month (5 visits/week) will take place.



Data collection   
Questionnaire at initial visit: see Annex   
Questionnaire at last visit: repeat 1st questionnaire  
Data collection by participants between visits:   
- Group A: AMU data, volume of WM and antibiotic used  
- Group B: AMU data, records of contents of WM and ID of calves that receive it, period of exposure of calves, WM samples

Sample collection during visits:

1. Fecal samples from 1 to 3 pre-weaned calves 30-60 d of age, that would have received WM if there was any (if belonging to group B), and that have not received any antimicrobial treatment. If there are more than 3 calves corresponding to the selection criteria, 3 will be selected by decreasing age. Age (in days) and prior treatments of the sampled calves will be recorded.
2. Environmental samples: Samples will be collected from hutches/pens walls using dry gauzes (see pilot study above). Number of isolates per visit to be selected for MIC will be determined based on pilot study.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | visit 1 | visit 2 | visit 3 | visit 4 |
| questionnaire | x |  |  | x |
| fecal samples | x | x | x | x |
| envir. Samples | x | x | x | x |
| WM samples |  | x | x | x |

Laboratory analyses:

1. Fecal samples: After each farm visit, fecal samples will be pooled prior to submission to a commercial laboratory. Fecal culture on selective medium will be performed, and identification of suspected *E. coli* isolates will be confirmed by MALDI-TOF mass spectrometry. Three *E. coli* isolates per fecal pool will be selected. Minimum inhibitory concentrations of 15 different antimicrobials will be determined by broth microdilution method using commercial Sensititre test plates. Results will be interpreted according to EUCAST epidemiological cut-off values.
2. Environmental samples:
3. WM samples: Concentration of antimicrobial residues in thawed WM samples will be determined using high-performance liquid chromatography (HPLC).

Exposure: documentation + measurement of concentrations of antimicrobial residues fed to calves

Outcomes: AMR patterns of *E. coli* isolates from pooled samples from calves and their environment (MDR, specific clusters of resistance TBD based on preliminary analysis)

Blinding: participants and research staff visiting the farms not blinded; laboratory staff measuring outcome blinded to group assignment

Summary of potential confounders and ways to control them:

* Variables affecting both AMU and the feeding of WM to calves:
  + SCC: -
  + Cow-level milk production: stratification
  + Canton/region: data analysis
  + Organic production: exclusion
* AMU in cows (potential confounder on the relationship between feeding WM and AMR in calves, through volume and contents of WM produced, and AMR in the farm environment): control in data analysis
* AMU in calves (covariate)

Data analyses

1. AMU: AMU will be quantified as treatment incidence based on defined daily doses (DDDvet) (European Medicines Agency, 2016), on a quarterly basis and evaluated at the herd level and for the groups of lactating cows (intramammary treatments; systemic treatments) and pre-weaned calves.
2. Antimicrobial exposure: Descriptive statistics of antimicrobial residues in WM samples will be performed. Herd-level exposure to residues will be quantified as treatment incidence and UDD/calf/60 days.
3. AMR: Prevalence of multidrug resistance (MDR) will be calculated, and clustering of isolates into AMR patterns will be evaluated. The association between WM feeding and AMR will be analyzed using multivariable mixed logistic regression models, taking into account season, confounding by AMU in cows, and clustering of isolates within herd. Models will be built for each tested antimicrobial and for MDR.