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Associations between antimicrobial treatment modalities and antimicrobial susceptibility in *Pasteurellaceae* and *E. coli* isolated from veal calves under field conditions

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ABSTRACT

Antimicrobial consumption, with bovine respiratory disease as main indication, is higher in the veal calf industry compared to other livestock production branches. The aim of the present study was to investigate possible associations between antimicrobial drug use and resistance in *Pasteurellaceae* and indicator *Escherichia* (*E.*) *coli* from veal calves under field conditions in a prospective trial.

Over a period of one year, nasopharyngeal and rectal swabs were collected from 2587 animals on 12 and 43 farms, respectively. Antimicrobial susceptibility testing was performed on 346 *Mannheimia* (*M.*) *haemolytica*, 1162 *Pasteurella* (*P.*) *multocida* and 2138 *E. coli*. Drug use was quantified as treatment incidence for each farm based on the used daily dose methodology (TI_{UDD}), separately for group and individual treatments, and for antimicrobial classes.

In multivariable mixed logistic regression analyses, risk factors could be identified for reduced susceptibility to certain antimicrobial classes. Group treatment was generally associated with higher rates of not susceptible (NS) *M. haemolytica* and *P. multocida* and non-wildtype (non-WT) *E. coli*. Individual treatment was associated with less NS and non-WT isolates. Age and entry protocol were important confounders with younger animals showing higher rates of NS and non-WT strains.

The present findings suggest that, under field conditions, targeted individual treatment of calves can reduce the development of antimicrobial resistance compared to oral group treatment. For the different microorganisms, risk factors for resistance were partially different. This demonstrates that indicator organisms like *E. coli* do not necessarily reflect the associations observed in respiratory pathogens.

1. Introduction

Veal production is a widely discussed topic in the scientific community as well as in the broader public, due to concerns regarding animal welfare and also antibiotic use. Antimicrobial consumption has been reported to be higher in the veal calf fattening sector than in other production branches and includes an alarming rate of antimicrobial classes of critical importance (Pardon et al., 2012; Bos et al., 2013; Lava et al., 2016b). Traditional veal calf farming comprises the transporting of veal calves at a young age and continuous arrival and commingling of calves from multiple birth farms, all of which affect animal health negatively and are classic factors fostering preventive use of antimicrobials (Caruso, 2018). Preventive antimicrobial treatment has been restricted in the European Union by guidelines and regulations (commission notice 2015/C 299/04 and regulation (EU) 2019/6) as well as in Switzerland (Swiss Ordinance on Veterinary Medicinal Products, Art. 11). However, arrival of sick animals at the fattening units is common and antimicrobial group treatment of healthy but potentially infected animals alongside sick calves (metaphylaxis) is largely practised (Pardon et al., 2012; Baptiste and Kyvsgaard, 2017; Renaud et al., 2018).

The main indication for antimicrobial drug use on veal calf farms is bovine respiratory disease (BRD). It is also the main reason for calf mortality in veal calf fattening units alongside gastrointestinal disease (Pardon et al., 2013; Lava et al., 2016b; Schnyder et al., 2019). A number of opportunistic bacterial pathogens including *Mannheimia* (*M.*) *haemolytica*, *Pasteurella* (*P.*) *multocida* and *Histophilus somni*, as well as *Mycoplasma bovis* regarded as a primary pathogen are involved in the

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BRD-complex (Nicholas and Ayling, 2003; Dedonder and Apley, 2015).

Resistance to antimicrobial classes of very high and high importance for human health, including cephalosporins, fluoroquinolones, aminoglycosides and macrolides used in veterinary medicine, has been reported for *M. haemolytica* and *P. multocida* isolated from the bovine respiratory tract (Anholt et al., 2017). Moreover, the first ESBL gene in a *M. haemolytica* strain from a case of BRD in the USA was recently described, highlighting the global threat of emerging antimicrobial resistance to both veterinary and human medicine (Kadlec et al., 2019).

Reduced susceptibility in bacteria isolated from cattle has been associated with antimicrobial drug use (Pereira et al., 2014; Catry et al., 2016). Treatment modalities, differentiating group from individual or oral from parenteral therapy, are usually not included in such analyses. Total antimicrobial drug use has been quantified in several studies for veal calf farms in particular (Pardon et al., 2012; Lava et al., 2016b; Jarrige et al., 2017). Likewise, European reports on antibiotic use and antimicrobial resistance include data from both cattle younger than one year and veal calves in their calculations, granting relevance to calves as a livestock category of their own (EFSA and ECDC, 2019). Studies on associations between antimicrobial resistance and drug use often focus on faecal *Escherichia* (*E.*) *coli*. These are considered suitable microorganisms for determining an effect of antimicrobial use on the prevalence of resistance (Bosman et al., 2014; Pereira et al., 2014; EFSA and ECDC, 2019). However, detailed studies also considering potential pathogens are sparse and no such study has prospectively focused on veal calves under field conditions exclusively.

The aim of the present study was therefore to investigate a possible association between antimicrobial drug use and resistance of opportunistic pathogens involved in BRD, one of the most important disease complexes in young cattle. The same association was investigated in faecal indicator *E. coli*. Hence, prospective data on antimicrobial resistance, treatment incidences and confounding farm factors in intensively reared veal calf populations of different sizes were compared.

2. Materials and methods

2.1. Study design and farm characteristics

Forty-three farms participated in this prospective longitudinal study on veal calf health, antimicrobial drug use and antimicrobial susceptibility, which was advertised for farm recruitment as described by Schnyder et al. (2019). The main inclusion criterion was a minimum of 21 fattened calves per year. The study farms were classified into small- or large-scale operations prior to the observation period. Based on the information from the previous year received from the farm managers, this categorisation resulted in 31 small operators fattening between 21 and 99 animals per year and 12 large farms with 100 or more fattened calves per year. Each of the 43 farms was monitored for a period of 12 months between July 2016 and November 2017. Forty-one farms were visited six times, one farm was visited only four times due to a break during the summer months and another farm was visited eight times because of its shorter fattening periods compared to the other participating farms. Twelve farms with an all-in/all-out system were visited for three consecutive fattening periods shortly after the calves' arrival on the farm and again shortly before they left the farm for slaughter. Farms with continuous arrival of animals ($n = 31$) were visited every two to two and a half months. An extensive treatment record journal was handed out to the farmers to gather detailed information about the calves' medications during the observation period. In addition, information on farm characteristics were recorded in a detailed questionnaire completed with the farmer, as described by Schnyder et al. (2019).

2.2. Sample collection

Half of the samples were collected from the youngest and half from the oldest calves present on the farm at each visit. Sampling was performed on treated and untreated as well as clinically healthy and diseased animals alike. Every sampled animal's individual ear tag number was registered. Dates of birth of the sampled calves were retrieved from the Swiss national animal movement database (Tierverkehrsdatenbank) based on their identification number. Their age at sampling was then calculated.

For isolation of *M. haemolytica* and *P. multocida*, deep nasopharyngeal swabs were collected from 50% of all calves present on large farms at the time of sampling. Only large operations were included to gather representative numbers of *Pasteurellaceae* isolates per sampled farm. Prior to swabbing, the calves' nostrils were wiped and disinfected with gauze swabs (Provet AG/ Henry Schein Animal Health, Lyssach, Switzerland) soaked in 70% propylalcohol (F25-A Feinsprit 2% MEK, Alcosuisse AG, Bern, Switzerland). The nasopharynx was reached through the nasal cavity with a sterile swab (COPAN Italia SpA, Brescia, Italy) which was transferred into liquid Amies transportation medium (Axonlab SwabAX, liquid Amies, Axon Lab AG, Baden, Switzerland) immediately after sampling.

For isolation of *E. coli* faecal swabs were collected on all study farms. On large farms, 25% of all calves present that day were swabbed, and on small farms six animals were chosen at random for sampling. These numbers were based on two factors: firstly, an anticipated detection rate for faecal *E. coli* of up to 100% and secondly, the assumption that at least six calves would be present at any time on every small farm. Faecal samples were collected from the rectum using sterile swabs (BD BBL CultureSwab, Becton Dickinson AG, Basel, Switzerland) which were immediately transferred into a liquid transportation medium for faecal samples (DeltaSwab Cary Blair, deltalab, Barcelona, Spain). All samples were transported to the laboratory within 48 h.

All procedures had been approved by the competent Committee for Animal Welfare and Protection (authorisation number BE 63/16).

2.3. Laboratory analyses

Nasopharyngeal samples were either cultivated for *M. haemolytica* and *P. multocida* within 72 h of sampling or cryopreserved at −80 °C for cultivation at a later time. Ten microliter (µl) of liquid Amies-media were spread on Pasteurella Selective Medium (Thermo Fisher Scientific, Pratteln, Switzerland) and incubated at 37 ± 1 °C for 48 ± 6 h in an atmosphere containing 5% CO_2. Phenotypically suspicious colonies were cultured on BD Trypticase Soy Agar with 5% Sheep Blood (TSA SB, Becton Dickinson AG) for a further 48 ± 6 h under the same conditions. Confirmation of bacterial species was performed by Matrix Assisted Laser Desorption Ionisation Time Of Flight Mass Spectrometry (MALDI TOF MS) using the direct transfer protocol according to the manufacturer's instructions (Biotyper 3.0, Bruker Daltonics GmbH, Bremen, Germany). Isolates were cryopreserved for further analyses in tryptone soy bouillon containing 30% glycerol at −80 °C.

Faecal samples were cultivated for *E. coli* within 72 h of sampling. Ten µl of each sample were spread on MacConkey Agar No. 3 (Thermo Fisher Scientific) and incubated at 37 ± 1 °C for 24 ± 3 h under aerobic conditions. Morphologically typical, lactose-positive cultures were subcultured onto TSA SB and incubated at 37 ± 1 °C for another 24 ± 3 h under aerobic conditions. Strains were confirmed to be *E. coli* by an indole positive reaction (DMACA Indole, Becton Dickinson AG) and cryopreserved for further analyses as described above.

Isolates were re-cultivated from cryopreservation on TSA SB and identification was confirmed with MALDI TOF MS prior to antimicrobial susceptibility testing. All isolated *M. haemolytica* and *P. multocida* could be regrown for susceptibility testing. From 2145 cryopreserved *E. coli* isolates, 2138 could be regrown and were tested for antimicrobial susceptibility.

Minimal inhibitory concentrations (MICs) were determined for *M. haemolytica*, *P. multocida* and *E. coli* with the broth microdilution method using commercially available Sensititre test plates (BOPO6F,

Thermo Fisher Scientific (TREK Diagnostic Systems)). The procedures followed the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI, 2013). In brief, an approximate inoculum of 5×10^5cfu/ml in cation adjusted Mueller-Hinton broth (CAMBH) (Thermo Fisher Scientific) supplemented with 5% lysed horse blood (Thermo Fisher Scientific) for optimal growth of primary cryopreserved isolates was achieved for *M. haemolytica* and *P. multocida*. For susceptibility testing of *E. coli*, the inoculum was adjusted to 1×10^5cfu/ml in CAMBH. Inoculated Sensititre plates were incubated aerobically for 18–24 hours at 36 ± 1 °C in a humidified chamber. Quality control was run using *E. coli* ATTC 25922, which showed MIC results within the acceptable range.

Based on the MICs, *M. haemolytica* and *P. multocida* isolates were classified as clinically susceptible (S), intermediate (I) or resistant (R) using clinical breakpoints (CB) issued by the CLSI (2018). Intermediate and resistant isolates were combined into a single category of not susceptible isolates (NS). For *E. coli*, epidemiological cut-off values (ECOFF) provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) were applied. Based on their MICs, they were categorised as either wildtype (WT) or non-wildtype (non-WT). Antimicrobials tested and applied interpretive criteria are shown in Table 1.

As a summary measure of susceptibility, we defined "incomplete susceptibility/WT": For *M. haemolytica*, this included isolates NS to more than one of the evaluated antimicrobial drugs, for *P. multocida* isolates NS to more than two of the evaluated antimicrobials and for *E. coli* all isolates non-WT to at least one of the evaluated antimicrobials. Complete susceptibility alone was not meaningful as a summary measure for *M. haemolytica* and *P. multocida*, because only 15% and 5% of the isolates, respectively, were susceptible to all tested antimicrobials.

2.4. Data analysis

The following seven antimicrobial classes (and corresponding antimicrobial drugs tested) were taken into account for *Pasteurellaceae*: cephalosporins (ceftiofur), fluoroquinolones (danofloxacin and enrofloxacin), macrolides (tulathromycin and tilmicosin for *M. haemolytica*, tulathromycin for *P. multocida*), aminoglycosides (spectinomycin), penicillins (penicillin), tetracyclines (oxytetracycline), and phenicols (florfenicol).

For commensal *E. coli* isolates, five antimicrobial classes (and corresponding antimicrobials tested) were taken into account for statistical modelling: cephalosporins (ceftiofur), fluoroquinolones (enrofloxacin), aminoglycosides (gentamicin, neomycin and spectinomycin), penicillins (ampicillin), and tetracyclines (oxytetracycline).

Non-susceptibility to one agent in the corresponding antimicrobial class resulted in classification of an isolate as NS/non-WT to the entire corresponding class.

Treatment records from the farm-specific treatment journals were entered into a spreadsheet (Excel 2010, Microsoft, Redmont, WA, USA). Antimicrobial drug use was quantified based on the used daily dose (UDD) (Timmerman et al., 2006). Treatment incidences (TI) were calculated for each farm individually as a total, including all antimicrobials applied on the respective farm, as well as on antimicrobial class level separately for group and individual treatments. Group treatment was defined as administration of antimicrobial drugs to all animals of a respective pen, individual treatment as antibiotic use on one or a subgroup of animals in a respective pen. Regarding β-lactam antibiotics, TI_{UDD}-values were calculated for cephalosporins as one class, and penicillins as a second class. The class of penicillins included all other β-lactam antibiotics used on the study farms (benzylpenicillin, aminopenicillins and combinations with clavulanic acid) in this study.

Table 1
Test range and interpretive criteria for the antimicrobials tested and prevalence [%] of not susceptible (NS)* *P. multocida* and *M. haemolytica* isolated from veal calves in 12 farms and non-wildtype (non-WT) *E. coli* isolated from veal calves in 43 farms.

	Antimicrobial class	Antimicrobial drug	Test range	CB[1] (cattle, respiratory tract, according to CLSI) and ECOFF[2] (according to EUCAST)	Overall prevalence† of NS and non-WT
M. haemolytica	**Cephalosporins**	**Ceftiofur**	0.25-8	S ≤2, I = 4, R ≥8	0
Number of isolates:	**Fluoroquinolones**	**Danofloxacin**	0.12-1	S ≤0.25, I = 0.5, R ≥1	14
346		**Enrofloxacin**	0.12-2	S ≤0.25, I = 0.5-1, R ≥2	18
	Macrolides	**Tilmicosin**	4-64	S ≤8, I = 16, R ≥32	53
		Tulathromycin	1-64	S ≤16, I = 32, R ≥64	0
	Aminoglycosides	**Spectinomycin**	8-64	S ≤32, I = 64, R ≥128[3]	0.3
	Penicillins	**Penicillin**	0.12-8	S ≤0.25, I = 0.5, R ≥1	52
	Tetracyclines	**Oxytetracycline**	0.5-8	S ≤2, I = 4, R ≥8	27
	Phenicols	**Florfenicol**	0.25-8	S ≤2, I = 4, R ≥8	0
P. multocida	**Cephalosporins**	**Ceftiofur**	0.25-8	S ≤2, I = 4, R ≥8	2
Number of isolates:	**Fluoroquinolones**	**Danofloxacin**	0.12-1	S ≤0.25, I = 0.5, R ≥1	36
1162		**Enrofloxacin**	0.12-2	S ≤0.25, I = 0.5-1, R ≥2	26
	Macrolides	**Tulathromycin**	1-64	S ≤16, I = 32, R ≥64	30
	Aminoglycosides	**Spectinomycin**	8-64	S ≤32, I = 64, R ≥128[3]	81
	Penicillins	**Penicillin**	0.12-8	S ≤0.25, I = 0.5, R ≥1	42
	Tetracyclines	**Oxytetracycline**	0.5-8	S ≤2, I = 4, R ≥8	94
	Phenicols	**Florfenicol**	0.25-8	S ≤2, I = 4, R ≥8	2
E. coli	**Cephalosporins**	**Ceftiofur**	0.25-8	WT ≤1	2
Number of isolates:	**Fluoroquinolones**	**Enrofloxacin**	0.12-2	WT ≤0.125	14
2138	**Aminoglycosides**	**Gentamicin**	1-16	WT ≤2	15
		Neomycin	4-32	WT ≤8	26
		Spectinomycin	8-64	WT ≤64	25
	Penicillins	**Ampicillin**	0.25-16	WT ≤8	54
	Tetracyclines	**Oxytetracycline**	0.5-8	WT ≤8[4]	66

* NS: intermediate and resistant isolates combined.
† Overall prevalence: total rates [%] of NS/non-WT isolates for each antimicrobial agent including all isolates of the respective bacterial species.
[1] CB = clinical breakpoints (*M. haemolytica* and *P. multocida*): CLSI Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 4th ed. CLSI supplement VET08. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
[2] ECOFF = epidemiological cut-off values (*E. coli*): European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website, last accessed 22.02.2019. http://www.eucast.org.
[3] Applied as R > 64.
[4] Value for tetracycline.

Calculations of TI_{UDD} were performed as described by Schnyder et al. (2019). Farm-level $TI_{UDD\ Total}$ described the number of used daily doses of antimicrobial drug per animal and year on the corresponding farm without consideration of the different antimicrobial classes or application routes. On the antimicrobial class level, TI_{UDD} described the number of used daily doses of the respective antimicrobial class in group or individual treatments per animal and year on the corresponding farm.

Every isolate's susceptibility data were combined with the respective farm TIs in separate spreadsheets for *M. haemolytica, P. multocida* and *E. coli*. Those spreadsheets were imported into SAS 9.4 (SAS Institute Inc., Cary, USA) for further analyses. Descriptive statistics included calculation of overall prevalence for resistance data as well as mean, standard deviation, median and range for TIs.

The association between antimicrobial use and resistance was analysed with multivariable mixed logistic regression models. Statistics were performed separately for each bacterial species and every evaluated antimicrobial class. The unit of analysis in each model was the individual bacteria isolate. Every isolate of the same species originated from one specific calf at each of the sampling times. The models corrected for clustering of isolates on the farm level by including the farm as a random effect. In the models for *Pasteurellaceae*, 12 clusters were included in the models, representing the 12 large farms on which the respective swabs had been collected. In the models for *E. coli* that had been isolated from swabs taken on all participating study farms, 43 clusters were included.

The isolates' susceptibility data to the respective antimicrobials tested (S versus NS for *M. haemolytica* and *P. multocida*, WT versus non-WT for *E. coli*) was used as binary outcome to a specific model for each antimicrobial class. Additionally, to analyse the contribution of the various antimicrobial drugs used in the participating farms on resistance selection, risk factor analyses were performed for the outcome incomplete susceptibility/WT in *M. haemolytica*, *P. multocida* and *E. coli*. For the models on class specific resistance, the two respective TI_{UDD} (group and individual) specific for each antimicrobial class were used as numeric predictor variables. For the models on incomplete susceptibility/WT, group and individual TI_{UDD} for all antimicrobial classes were included in the analysis. Only two potential confounders could be included in the models for the *Pasteureallceae* because of the comparatively small number (12) of large farms where deep nasopharyngeal swabs had been collected: "entry protocol" (continuous arrival of animals or all-in/all-out protocol) and "age at sampling" (young (up to 90 days) or old (> 90 days)). Additionally, for *E. coli* isolated from rectal swabs from 43 farms, "treatment regime" (farms using only individual treatment versus all others using group and individual treatments) and "farm size" (small versus large farms) were included. Treatment regime could not be analysed in the *Pasteurellaceae* models because none of the 12 farms used individual treatment only.

The screening of all potential risk factors was performed with univariable mixed regression analysis. Correlation between all group and individual antimicrobial class level TI_{UDD} as potential risk factors was tested with the Spearman correlation coefficient for continuous variables. If correlation coefficients were > 0.7, only the TI_{UDD} of the antimicrobial drug with a higher use on the 43 farms was used for further analysis. An antimicrobial class had to be used in the respective application route in a minimum of five farms, otherwise it was excluded from the analysis. Only variables with a p-value < 0.2 in the univariable screening were entered into the respective multivariable model. The variable selection was stepwise backward selection. Non-significant variables ($p > 0.05$) were excluded from a model, unless they changed the coefficient of another predictor by more than 20%. Model fit was assessed by QIC and QICu and by visual assessment of residuals.

3. Results

A total of 2506 deep nasopharyngeal swabs and 2260 rectal swabs were collected from 2587 animals between July 2016 and November 2017. The calves' mean age at the beginning of the fattening period (age at purchase) was 33 (± 4.5) days. Mean duration of the fattening period was 116.4 (± 14.2) days. Overall, 346 *M. haemolytica* and 1162 *P. multocida* as well as 2145 *E. coli* were isolated, respectively.

Approximately the same number of nasopharyngeal swabs were taken from younger and older calves. *M. haemolytica* strains were isolated from animals with a median age of 116 days (interquartile range (IQR) = 84–135) and *P. multocida* from animals with a median age of 113 days (IQR = 66–134). The sampling strategy and the almost 100% isolation rate resulted in a bimodal distribution of the *E. coli* isolates, with a median age of 89 days dividing the population into two groups. The younger group had a median age of 47 days (IQR = 34–61), the older group had a median age of 129 days (IQR = 115–144).

The overall proportions of NS isolates for *M. haemolytica* and *P. multocida* as well as non-WT isolates for *E. coli* are presented in Table 1. Of all *M. haemolytica* isolates, 15% (52/346) were susceptible to all antimicrobials tested, 35% (120/346) were susceptible to all but one, and 27% (92/346) to all but two antimicrobials. *P. multocida* was susceptible to all antimicrobials tested in 5% (60/1162) of the cases, susceptible to all but one in 7% (84/1162) of the cases, and susceptible to all but two antimicrobials in 41% (475/1162) of the cases. The proportion of WT *E. coli* for all antimicrobials tested was 28% (597/2138), WT for all but one antimicrobial 14% (303/2138), and WT for all but two antimicrobials 17% (354/2138). In addition to the overall proportions, the prevalence of non-WT *E. coli* was calculated separately for 13 farms using individual treatment only (total number of *E. coli* $n = 357$) and 30 farms also applying group therapy (total number of *E. coli* $n = 1781$). Rates of non-WT *E. coli* were lower in farms applying individual treatment only for all antimicrobial drugs evaluated in this study (ceftiofur: 1% vs. 2%, enrofloxacin: 8% vs. 16%, gentamicin: 9% vs. 16%, neomycin: 22% vs. 27%, spectinomycin: 7% vs. 29%, ampicillin: 34% vs. 58%, oxytetracycline: 47% vs. 70%).

An overview of the study farms' TIs is given in Table 2. The overall antimicrobial drug use on the study farms ranged from 0 to 50 antimicrobial treatment days per animal and year. This corresponds to a range of 0 to 16 antimicrobial treatments per animal per fattening

Table 2
Treatment incidence in used daily doses per calf and year (TI_{UDD}) for all antimicrobial classes applied on 43 calf fattening farms calculated as a total and separately for each antimicrobial class and for group and individual treatments.

TI_{UDD}	Range		Median	Mean ± SD
	Minimum	Maximum		
Total	0	50.2	8.1	11.8 ± 12.2
Cephalosporins group	0	0	0	0 ± 0
Cephalosporins individual	0	0.3	0	0.02 ± 0.05
Fluoroquinolones group	0	0.4	0	0.02 ± 0.09
Fluoroquinolones individual	0	0.6	0.05	0.1 ± 0.2
Macrolides group	0	10.4	0	1.9 ± 3.2
Macrolides individual	0	1.7	0	0.1 ± 0.4
Aminoglycosides group	0	0.5	0	0.01 ± 0.08
Aminoglycosides individual	0	4.2	0	0.3 ± 0.8
Penicillins group	0	25.2	1.6	3.0 ± 4.7
Penicillins individual	0	5.2	0.4	0.7 ± 1.1
Tetracyclines group	0	11.3	0	2.4 ± 3.6
Tetracyclines individual	0	3.1	0.09	0.3 ± 0.6
Phenicols group	0	0.6	0	0.02 ± 0.1
Phenicols individual	0	0.9	0	0.08 ± 0.2
Sulfonamides group	0	18.8	0	2.1 ± 4.0
Sulfonamides individual	0	3.1	0	0.2 ± 0.5
Trimethoprim group	0	9.4	0	0.4 ± 1.6
Trimethoprim individual	0	3.1	0	0.09 ± 0.5

Group: TI_{UDD} calculated from those treatments administered to all animals of a respective pen.
Individual: TI_{UDD} calculated from those treatments administered to one or a subgroup of animals of a respective pen.

period using the participating farms' mean fattening period of 116.4 days as a reference. The mean TI_{UDD} for one fattening period was 3.8 treatments. Penicillins, accounting for 32% of the overall TI_{UDD} in all participating farms, represented the most frequently used antimicrobial class followed by tetracyclines (23%), sulfonamides (19%) and macrolides (17%). Trimethoprim (4%), aminoglycosides (2%) and fluoroquinolones (1%) were used distinctly less frequently. Phenicols and cephalosporins accounted for less than 1% of the overall antimicrobial drug use in the participating farms. The most common application of antimicrobial drugs was as oral group therapy, representing more than 80% of $TI_{UDD\ Total}$. Orally administered antimicrobials represented 87% of the overall use, 13% of $TI_{UDD\ Total}$ were administered parenterally. Group treatments represented 83% of $TI_{UDD\ Total}$, individual treatments 17% of $TI_{UDD\ Total}$. Individually administered antimicrobial drugs, although accounting for a limited percentage of the overall drug use, were used on numerous farms (fluoroquinolones in 27 farms, aminoglycosides 21, florfenicol 14, 3rd and 4th generation cephalosporins eight).

Generally, group treatment was associated with more NS/non-WT isolates and incomplete susceptibility. Individual treatment was associated with less NS/non-WT and less incomplete susceptibility/WT. For *M. haemolytica*, a higher $TI_{UDD\ Macrolides\ individual}$ was associated with less NS to macrolides. A higher $TI_{UDD\ Tetracyclines\ group}$ was associated with more NS to oxytetracycline (Table 3). For *P. multocida*, a higher $TI_{UDD\ Macrolides\ group}$ was associated with more NS to macrolides, a higher $TI_{UDD\ Pencicillins\ individual}$ was associated with less NS to penicillin (Table 4). For *E. coli*, farms using group treatments had a higher prevalence of non-WT isolates for aminoglycosides, ampicillin and oxytetracycline. For aminoglycosides, a higher $TI_{UDD\ Aminoglycosides\ individual}$ was associated with less non-WT (Table 5).

Younger age was associated with more NS/non-WT and incomplete susceptibility/WT, except for the macrolides model in *M. haemolytica*. An all-in/all-out protocol was associated with less NS isolates for several antimicrobials in *M. haemolytica* (Table 3) and *P. multocida* (Table 4). For *E. coli*, on the other hand, an all-in/all-out protocol was associated with more non-WT isolates (Table 5). Detailed results from the mixed logistic regression models are shown in Tables 3–6.

4. Discussion

Bacterial opportunists from the upper respiratory tract with potential involvement in BRD as well as faecal *E. coli* from veal calves were tested for their antimicrobial susceptibility in this study. Binary outcome variables were obtained from the interpretation of MIC values for the investigation of possible associations between antimicrobial drug use and reduced bacterial susceptibility. Following the guidelines published by Schwarz et al. (2010), interpretive criteria were kept uniform for the different microorganisms tested. The MICs of opportunistic pathogenic *M. haemolytica* and *P. multocida* were interpreted using CBs specific for the respiratory tract of cattle. Faecal commensal *E. coli* isolates were investigated as indicator organisms, hence ECOFFs were applied. Unlike CBs, these threshold values are not meant to guide individual therapy but can be used as measures of emerging reduced susceptibility (Kahlmeter et al., 2003; Simjee et al., 2008). For statistical analyses, non-susceptibility to at least one agent of a certain class resulted in classification of a respective isolate as NS/non-WT to the entire corresponding antimicrobial class. This approach is in accordance with definitions applied when assessing multidrug resistance (Sweeney et al., 2018).

Susceptibility rates themselves were not the focus of this investigation. However, data for *Pasteurellaceae* found in this study were comparable to most numbers from other recent studies focusing on BRD (Catry et al., 2016; Anholt et al., 2017). The prevalence of non-WT *E. coli* for comparable drugs was higher in the present study than the calculated average from a study including data from 2010/11 from seven European countries (Chantziaras et al., 2014) as well as values from the Swiss Antibiotic Resistance Report 2018 (FOPH and FSVO, 2018). In those studies, *E. coli* had been isolated from apparently healthy animals mostly at slaughter. In contrast, *E. coli* in our study originated from veal calves of different ages and health statuses, who may have been under antimicrobial treatment.

The most frequently used antimicrobials in this study belonged to the classes of penicillins, tetracyclines, sulfonamides and macrolides, which is in accordance with other European studies (Pardon et al., 2012; Lava et al., 2016b; Jarrige et al., 2017). The mean TI of 3.8 days

Table 3
Results from the multivariable mixed logistic regression analyses of the associations between reduced antimicrobial susceptibility, antimicrobial treatment and potential confounders in 346 *M. haemolytica* isolates from 12 large veal calf farms.

Outcome = classification as not susceptible	Variable	Categories	Odds Ratio	95% CI		*p*-value
				lower	upper	
Ceftiofur	N/A[3]					
Fluoroquinolones	Age[1]	old	Reference			
		young	3.02	0.89	10.30	0.0773
	Entry protocol[2]	continuous	Reference			
		all-in/all-out	0.16	0.03	0.80	0.0252
Macrolides	$TI_{UDD\ Macrolides\ individual}$		0.42*	0.19	0.91	0.0286
	Age[1]	old	Reference			
		young	0.23	0.14	0.38	< 0.0001
Spectinomycin	N/A[4]					
Penicillin	N/A[5]					
Oxytetracycline	$TI_{UDD\ Tetracyclines\ group}$		1.55*	1.11	2.18	0.0102
	Age[1]	old	Reference			
		young	4.85	1.08	21.87	0.0398
Florfenicol	N/A[3]					

N/A: not applicable.
[1] Age: old group = calves > 90 days, young group = calves ≤ 90 days.
[2] Entry protocol: continuous arrival of animals or all-in/all-out.
[3] All isolates susceptible.
[4] All isolates but one susceptible.
[5] All variables not significant.
* Odds ratios for TI specify the odds of an isolate being classified as not susceptible per increase in TI_{UDD} of 1 used daily dose per animal and year.

Table 4
Results from the multivariable mixed logistic regression analyses of the associations between reduced antimicrobial susceptibility, antimicrobial treatment and potential confounders in 1162 *P. multocida* isolates from 12 large veal calf farms.

Outcome = classification as not susceptible	Variable	Categories	Odds Ratio	95% CI		*p*-value
				lower	upper	
Ceftiofur	N/A[3]					
Fluoroquinolones	N/A[4]					
Tulathromycin	$TI_{UDD\ Macrolides\ group}$		1.63*	1.17	2.28	0.0041
	Entry protocol[1]	continuous	Reference			
		all-in/all-out	0.004	0.0001	0.10	0.0009
Spectinomycin	N/A[5]					
Penicillin	$TI_{UDD\ Penicillins\ individual}$		0.18*	0.04	0.77	0.021
	Age[2]	old	Reference			
		young	1.33	1.01	1.75	0.0442
	Entry protocol[1]	continuous	Reference			
		all-in/all-out	0.16	0.03	0.86	0.0325
Oxytetracycline	Entry protocol[1]	continuous	Reference			
		all-in/all-out	0.29	0.09	0.97	0.0436
Florfenicol	N/A[6]					

N/A: not applicable.
[1] Entry protocol: continuous arrival of animals or all-in/all-out.
[2] Age: old group = calves > 90 days, young group = calves ≤ 90 days.
[3] No cephalosporin group treatment on the 12 large farms, cephalosporin individual treatment in less than 5 farms and other variables not significant.
[4] No fluoroquinolone group treatment on the 12 large farms, fluoroquinolone individual treatment and other variables not significant.
[5] Aminoglycoside group treatment in less than 5 farms, aminoglycoside individual treatment and other variables not significant.
[6] Phenicol group and individual treatment in less than 5 farms and other variables not significant.
* Odds ratios for TI specify the odds of an isolate being classified as not susceptible per increase in TI_{UDD} of 1 used daily dose per animal and year.

Table 5
Results from the multivariable mixed logistic regression analyses of the associations between reduced antimicrobial susceptibility, antimicrobial treatment and potential confounders in 2138 *E. coli* isolates from 43 veal calf farms.

Outcome = classification as non-wildtype	Variable	Categories	Odds Ratio	95% CI		*p*-value
				lower	upper	
Ceftiofur	Age[1]	old	Reference			
		young	2.95	1.14	7.61	0.0254
	Farm size[2]	small	Reference			
		large	5.96	1.22	29.10	0.0274
Enrofloxacin	Age[1]	old	Reference			
		young	2.45	1.20	4.98	0.0133
	Farm size[2]	small	Reference			
		large	2.42	1.27	4.61	0.0073
Aminoglycosides	$TI_{UDD\ Aminoglycosides\ individual}$		0.85*	0.74	0.98	0.021
	Treatment regime[3]	individual	Reference			
		group	2.15	1.28	3.61	0.0039
	Age[1]	old	Reference			
		young	1.97	1.28	3.02	0.002
	Entry protocol[4]	continuous	Reference			
		all-in/all-out	1.60	1.10	2.33	0.0137
Ampicillin	Treatment regime[3]	individual	Reference			
		group	2.20	1.12	4.32	0.0218
	Age[1]	old	Reference			
		young	2.41	1.46	4.01	0.0006
	Entry protocol[4]	continuous	Reference			
		all-in/all-out	1.97	1.24	3.12	0.0041
Oxytetracycline	Treatment regime[3]	individual	Reference			
		group	2.02	1.36	2.98	0.0004
	Age[1]	old	Reference			
		young	1.65	1.02	2.68	0.0431
	Entry protocol[4]	continuous	Reference			
		all-in/all-out	1.91	1.13	3.23	0.0157

[1] Age: old group = calves > 90 days, young group = calves ≤ 90 days.
[2] Farm size: large farms ≥ 100 fattened calves per year, small farms < 100 fattened calves per year.
[3] Treatment regime: individual = farms using only individual treatment, group = all other farms (using group and individual treatment).
[4] Entry protocol: continuous arrival of animals or all-in/all-out.
* Odds ratios for TI specify the odds of an isolate being classified as not susceptible per increase in TI_{UDD} of 1 used daily dose per animal and year.

Table 6
Results from the multivariable mixed logistic regression analyses of the associations between "incomplete susceptibility/WT", antimicrobial treatment and potential confounders in 346 *M. haemolytica*, 1162 *P. multocida* and 2138 *E. coli* isolates from 12 and 43 veal calf farms, respectively.

Outcome = classification as "incompletely susceptible/WT"	Variable	Categories	Odds Ratio	95% CI		*p*-value
				lower	upper	
M. haemolytica[1]	$TI_{UDD\ Fluoroquinolones\ individual}$		0.001*	6.79E-05	0.03	< 0.0001
	$TI_{UDD\ Macrolides\ individual}$		0.003*	0.0003	0.03	< 0.0001
P. multocida[2]	$TI_{UDD\ Penicillins\ group}$		1.10*	1.07	1.13	< 0.0001
	$TI_{UDD\ Penicillins\ individual}$		0.07*	0.01	0.49	0.0074
	Entry protocol[4]	continuous	Reference			
		all-in/all-out	0.17	0.06	0.49	0.001
E. coli[3]	$TI_{UDD\ Penicillins\ group}$		0.96*	0.94	0.99	0.0036
	Treatment regime[5]	individual	Reference			
		group	2.27	1.50	3.43	0.0001
	Age[6]	old	Reference			
		young	1.71	1.10	2.67	0.0175
	Entry protocol[4]	continuous	Reference			
		all-in/all-out	2.58	1.81	3.69	< 0.0001

[1] "Incompletely susceptible" *M. haemolytica*: isolates not susceptible to more than one of the evaluated antimicrobials (n = 172/346).
[2] "Incompletely susceptible" *P. multocida*: isolates not susceptible to more than two of the evaluated antimicrobials (n = 619/1162).
[3] "Incompletely WT" *E. coli*: isolates of the non-wildtype to at least one of the evaluated antimicrobials (n = 597/2138).
[4] Entry protocol: continuous arrival of animals or all-in/all-out.
[5] Treatment regime: individual = farms using only individual treatment, group = all other farms (using group and individual treatment).
[6] Age: old group = calves > 90 days, young group = calves ≤ 90 days.
* Odds ratios for TI specify the odds of an isolate being classified as not susceptible per increase in TI_{UDD} of 1 used daily dose per animal and year.

under treatment per calf for one fattening period was lower than values from previous studies in Switzerland (7 treatment days per calf and fattening period on average; Lava et al., 2016b) and France (mean TI of 8.55; Jarrige et al., 2017). These values should be compared with caution, as different methods were applied to quantify antimicrobial drug use in these studies. However, the lower TI correlates with the sales data of antimicrobials in Europe and the USA where sales numbers have decreased continuously in past years (FOPH and FSVO, 2018; FDA, 2018; MARAN, 2018; Bokma et al., 2019).

The most common application of antimicrobial drugs in this and other studies was via oral route (Catry et al., 2016; Lava et al., 2016b). Oral group treatment can be easily administered via automatic milk feeder without causing stress to the animals, whereas individual treatment is more difficult to apply. However, a clear disadvantage of oral medication may be the potential insufficient suckling because of depression caused by sickness. Further problematic factors might be insufficient uptake because of taste alterations or due to limited access to the feeder for animals of low social rank. In the present study, TIs were calculated separately for group and individual treatments rather than for oral vs. parenteral treatment. Although long-acting macrolides are administered parenterally, they were taken into account in the category of group treatments if they were applied to all animals in a pen similar to oral group treatments. The overall TI-values for group treatments were close to those for oral treatments in this study. For other animal species and bacteria, oral administration of antimicrobials has been shown to select for more resistance compared to the parenteral route (Wiuff et al., 2003; Chantziaras et al., 2017). The findings of our statistical analyses suggest that a similar effect is also present in veal calves. In those models for *Pasteurellaceae* and *E. coli* where antimicrobial class level TI_{UDD} remained as significant variables, group treatment was associated with increased odds of NS/non-WT isolates. Individual treatment, on the other hand, was associated with decreased odds for NS/non-WT. Furthermore, treatment regime as categorical variable in the models for *E. coli* (farms where exclusively individual treatments were applied vs. farms with group and individual treatments) was an important factor, with group treatment representing a significant risk factor for reduced susceptibility in *E. coli*. A significant association between antimicrobial drug use and increased resistance rates in bacteria is well established (Pereira et al., 2014; Catry et al., 2016). For this reason, it was surprising that higher individual TI_{UDD} was associated with a decreased risk of reduced susceptibility in five models, including all three bacterial species under observation. The reason for this result is likely that less group treatments were applied in farms with higher individual TI. Indeed, no group treatment at all was applied in 13 of the 43 participating farms. Thus, these results suggest treatment modalities rather than treatment intensity to be of major importance in the association between antimicrobial drug use and resistance.

Other risk factors for reduced susceptibility identified in our analyses included age at sampling, entry protocol and farm size, confirming that antimicrobial resistance, especially under field conditions, is indirectly influenced by many factors (Murphy et al., 2018).

Younger age (≤ 90 days) was associated with higher rates of NS/non-WT isolates. One exception was the macrolides model for *M. haemolytica* where young age was associated with less NS isolates. This latter result must be interpreted with caution because the proportion of *M. haemolytica* isolated from younger calves was low (97/346). The association between age and NS *M. haemolytica* was not significant for fluoroquinolones (mostly used for individual treatments). However, the risk for NS *M. haemolytica* was significantly higher in younger than in older calves for tetracyclines (mostly applied as group treatments). In contrast, Catry et al. (2016) did not find age to be a factor significantly associated with resistance rates. However, they did observe an age related decline in multidrug resistant *E. coli* from veal calves. The mean age of veal calves receiving antimicrobial treatment has been reported to be 51 days (Lava et al., 2016b) which further supports our findings of an association between antimicrobial group treatment, younger age and reduced susceptibility.

An all-in/all-out entry protocol was associated with a decreased risk of NS *Pasteurellaceae* but with an increased risk of non-WT *E. coli*. All-in/all-out farms generally purchase calves and often treat new groups of animals metaphylactically upon arrival. At that time, *Pasteurellaceae* are not highly prevalent in veal calves as confirmed by our isolation. Farms with continuous arrival of animals, fattening their own (and purchased) animals, rarely apply metaphylaxis upon arrival (Lava et al., 2016a), but those calves are more likely to become ill and to be treated during the fattening period because of new entries into the fattening unit at any time. This in turn leads to the exposition of pathogens such as *Pasteurellaceae* to antimicrobial selection pressure in farms with continuous stocking. In contrast, *E. coli* is always present in the calf's gut at

any time antimicrobial treatment might occur, which is a possible explanation to why all-in/all-out protocols with a more prevalent application of group treatment is associated with increased risk of reduced susceptibility in indicator *E. coli*.

The definitions of "incomplete susceptibility" differed slightly for the three bacterial species under observation to create outcomes with sufficient sample sizes. For *E. coli* the number of isolates susceptible (WT) to all antimicrobials was sufficient for statistical modelling with completely susceptible isolates as one category. For *M. haemolytica* on the other hand, isolates susceptible to all but one of the evaluated antimicrobials had to be added to the group of "overall susceptible" isolates to obtain a sample size allowing for further statistical evaluation. Isolates not susceptible to one or two antimicrobials tested were added to the group of "overall susceptible" *P. multocida* due to the small numbers of isolates susceptible to all and all but one antimicrobial drug. No antimicrobial class was identified as predominant contributor to reduced susceptibility in all three bacterial species. Treatment regime was once more a significant factor in the analysis for *E. coli* with group treatment increasing the odds for non-WT in comparison to individual treatment only. In contrast, group treatment with penicillins was associated with less non-WT in the same model. This was the only occurrence of group treatment being associated with less NS/non-WT. However, the odds ratio for this finding was weak (OR = 0.96, 95% CI = 0.94-0.99), whereas all other ORs for significant associations between TI_{UDD} and NS/non-WT were distinctly stronger. Alternatively, this observation may be due to a random effect (type I error).

Interested farmers had to reach out to the study team for participation. This represents a potential selection bias toward particularly motivated farmers. However, a partly positive and partly negative association between farm performance parameters and voluntary participation in a herd health programme has been demonstrated (Derks et al., 2014). Supported by the fact that some of the farmers in this study reported to have herd health or farm management problems motivating them to participate in the project, this minimizes such a potential selection bias. Indeed, the study population eventually consisted of a representative spectrum of farms, not only geographically across Switzerland but also regarding management standards.

5. Conclusions

In this study focusing exclusively on veal calves under field conditions, associations were found between antimicrobial drug use and the occurrence of reduced antimicrobial susceptibility in bacteria. These findings suggest that treatment modalities (group vs. individual treatment) rather than treatment intensity represent a key factor, with group treatment associated with increased odds for the presence of NS/non-WT isolates. Moreover, several management factors were associated with reduced susceptibility of the bacteria under study. Faecal indicator *E. coli* was confirmed as suitable organism to study risk factors for antimicrobial resistance. Partially differing associations were observed for the *Pasteurellaceae* species under observation, highlighting the importance of a differentiated assessment regarding resistance rates.

Declaration of Competing Interest

The authors declare no conflict of interest.

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