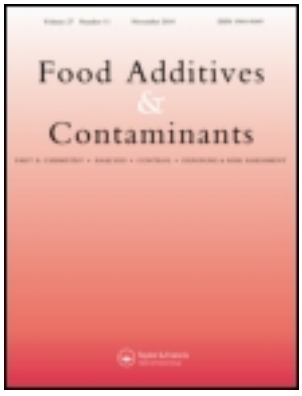

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Developing a microbiological growth inhibition screening assay for the detection of 27 veterinary drugs from 13 different classes in animal feedingstuffs

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Developing a microbiological growth inhibition screening assay for the detection of 27 veterinary drugs from 13 different classes in animal feedingstuffs

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Many regulations prohibit using veterinary drugs in feedingstuffs to protect consumers and animals alike. Within this investigation we developed a simple, cost-efficient primary screening method for detecting antibiotics and coccidiostats in animal feeds. Thirty-two veterinary drugs were originally considered. Following matrix-free testing to optimise detection, an assay based on matrix extraction with methanol/acetonitrile/phosphate buffer followed by inoculation and diffusion in agar plates was developed. Final validation was performed with 14 representative drugs (one per drug class) and four bacteria (*Escherichia coli* ATCC11303 and ATCC27166, *Staphylococcus aureus* ATCC6538P, *Micrococcus luteus* ATCC9341) in bovine, lamb and swine fodder, measuring growth inhibition zones. Of the original drugs tested, 27 remained detectable in feed matrices at or below 20 mg kg^{-1}. Of the 14 validated representatives, two had estimated minimum detectable concentrations of 10–11 mg kg^{-1}, others of 5 mg kg^{-1} or lower, an earlier minimum European Union inclusion rate for many veterinary drugs. No significant matrix effect on inhibition zones was detected. Per cent wrong negative deviations ranged from 0% (nine of 14 compounds) to 20–27% (two of 14), while inter-day precision based on inhibition zones had relative standard deviations (RSDs) of 6–109% (mean of 40%). When setting a 1 mm inhibition zone, the maximum observed for negative controls, as a cut-off level, no false-positives were found. While not all targeted antibiotics were detectable in complex matrices, the majority of veterinary drugs were detected with reasonable sensitivity, indicating that this method could be suitable for screening feedingstuffs prior to further confirmatory investigation of positive findings such as by LC-MS/MS.

Keywords: antibiotics; coccidiostats; animal feeds; growth inhibition; matrix; multi-method

Introduction

Veterinary drugs in feedingstuffs have in the past been used to enhance animal growth intended for meat production, and also to avoid diseases (Dibner & Richards 2005). All feed additives classified as growth promoters were banned by the European Union in 2006, except for some coccidiostats and histomonostats (EU Commission 2003) where phasing out is still under discussion, and their control is regulated by Regulation 882/2004/EC (EU Commission 2004). While in the European Union only few veterinary drugs are still in use for restricted medical conditions, in the United States, regulated by the US-FDA, many of these compounds are still allowed, with few exceptions such as cephalosporins (Food and Drug Administration 2012). The main concern that has led to their banning is the risk of developing bacterial resistances, loosing valuable medication to combat life-threatening bacterial infections in both animals and humans (Lathers 2002); albeit there has been some dispute on this topic (Phillips 2007). In addition, wrong applications and dosing of some antibiotics have been related to toxic effects in animals (Higgins et al. 1999). Finally, the potential risk of allergic reactions following consumption of food containing antibiotics, even though apparently small, has been discussed (Dewdney et al. 1991).

In addition to the still permitted ones, a variety of banned veterinary drugs are occasionally detected in feedingstuffs, and the overall sales for veterinary drugs have remained more or less stable in recent years (Goodyear 2010). For example, in a study with 12 samples of feather meals for broilers, eight were found to be positive for banned antibiotics, i.e. fluoroquinolones (Love et al. 2012), indicating that veterinary drugs are still fraudulently or carelessly employed in animal production facilities. When used earlier or still not prohibited in animal feed, minimum inclusion rates (MIRs) had been suggested by the European Union, varying between 5 and 1875 mg kg^{-1} (Higgins & McEvoy 2002), depending on drug, animal and purpose of application. Higher concentrations may be used for medicated feedingstuffs according to the individual European Union member states' regulations. As in fact complete absence of veterinary drugs in final food products may not be possible, i.e. due to cross-contamination of animal fodder, or therapeutic use of drugs, MRLs of veterinary drugs in foodstuffs have been set in many countries, including the European Union (European Commission 1990, 2010b), typically

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ranging between 25 and 300 μg kg^{-1}. Coccidiostats were incompletely addressed in these regulations, but were included in later ones, at least for certain food items (EU Commission 2010a, 2013b), and MRLs were also published for other countries (reviewed by Renshaw 2013).

Detection of veterinary drugs can be carried out by several means, differentiable into confirmatory methods, typically based on LC-MS/MS (Stolker et al. 2007; Boscher et al. 2010), and screening methods. While confirmatory techniques such as LC-MS/MS are more sensitive and thus allow for detecting simultaneously a number of antibiotics, lengthy sample preparation is usually required, and instrumentation use and cost of sample analysis are intensive. Thus, screening methods that are simple, rapid, low cost and do not require sophisticated instrumentation are desired prior to confirmatory analysis. For screening purposes, immunoassays such as ELISA (Williams et al. 1994; Gaudin et al. 2003), biosensors (Chafer-Pericas et al. 2010), LC techniques (Benito-Pena et al. 2009; Gonzalez de la Huebra et al. 2012), and microbiological methods employing mostly bacteria growth inhibition (Higgins & McEvoy 2002; Pikkemaat et al. 2009) are among the most common. However, especially for secondary screening, combinations of separatory techniques such as TLC coupled to microbiological methods (Gafner 1999; Vincent et al. 2007) have also been developed. While the development of biosensors has only recently accelerated, ELISA methods typically target only one antibiotic at a time, and may be prone to matrix interferences and cross-reactivities (Barrett 2004; Toldra & Reig 2012), with a risk of producing a higher rate of wrong positive/wrong negative findings.

Thus, microbiological methods based on bacterial growth inhibition are still the most common means to detect veterinary drugs. While many such methods have been developed for detecting antibiotics in foodstuffs, such as the five-plate screening test for antibiotic residues (STAR) in milk (Gaudin et al. 2004) or meat (Gaudin et al. 2010), or the five-plate screening test of Nouws, van Egmond, Loeffen, et al. (1999) with additional post-screening tests, only very few methods have targeted veterinary drug screening in feedingstuffs. Among the latter are: a single-plate inhibitory substance test (IST) with *Bacillus stearothermophilus* for detecting up to 15 (Lynne & Lott 2012) antibiotics (Higgins et al. 1999); a two-plate system with *Bacillus subtilis* and *Micrococcus luteus* (Higgins & McEvoy 2002) for detecting five veterinary drugs; a test system based on three plates with three bacteria for five growth promoters (Pol-Hofstad et al. 2008); and a system based on eight plates with four bacteria at two different pHs (Przenioslo-Siwczynska & Kwiatek 2007).

The present study presents the development of a microbiological multi-screening method allowing for the detection of the presence of 27 antibiotics from 13 drug classes from several types of feedingstuffs, i.e. lamb, swine and bovine feed.

Materials and methods

Test matrices, chemicals and standards

Test matrices (Table 1), i.e. feedingstuffs, included piglet (Vormast), bovine (K11) and lamb feed (Lammstar 2), and were obtained from Versis (Mersch, Luxembourg); their pellets were ground prior to usage. Storage was within packed sacks at RT.

The six coccidiostats: lasalocid A sodium salt solution (100 μg ml^{-1} in acetonitrile), monensin sodium salt, maduramycin ammonium, narasin, salinomycin monosodium salt hydrate and robenidine HCl; and penicillin G procaine, amoxicillin trihydrate; nalidixic acid sodium salt, oxolinic acid, flumequine, ciprofloxacin, enrofloxacin, florfenicol, chloramphenicol, trimethoprim, tilmicosin, spiramycin, tylosin tartrate; virginiamycin M1, valnemulin, bacitracin zinc salt, carbadox, olaquindox tetracycline hydrochloride, oxytetracycline hydrochloride, doxycycline hyclate, sulfadiazine, chlortetracycline hydrochloride, colistin sulfate, and lincomycin hydrochloride monohydrate were purchased from Sigma-Aldrich (Seelze, Germany), as were methanol and ethylenediamine tetra-acetic acid (EDTA). Flavophospholipol (Flavomycin) was obtained from Biovet/Huvepharma (Anvers, Belgium). All antibiotics had a certified purity of 90% or higher, except for bacitracin (61%), monensin (84%), tylosin (85%) and tilmicosin (87%).

Unless otherwise mentioned, all other chemicals were of analytical grade or superior; and only 18 MΩ water was used throughout. Acetonitrile was purchased from Biosolve (Valkenswaard, the Netherlands). Citric acid and disodiumhydrogen phosphate (Na_2HPO_4) were purchased from VWR (Leuven, Belgium). DST agar was from BD Biosciences (Erembodegem, Belgium), as were antibiotic media (Table 2). Dipotassium hydrogenphosphate (K_2HPO_4, 100%) and potassium dihydrogen phosphate (KH_2PO_4, 99.5%) were purchased from UCB (Leuven, Belgium).

McIlvaine buffer (pH 4.6) was prepared by mixing 53.3 ml citric acid (0.1 M) with 46.8 ml Na_2HPO_4 (0.2 M). Phosphate buffer (pH 7.0) was constituted by mixing 13.6 g K_2HPO_4 with 4 g KH_2PO_4 in 1 L of water and adjusting the pH, if needed, with 1 M NaOH or 1 M HCl. All buffer composites and the final buffer were stored at 4°C.

Bacteria

A total of 22 bacterial strains were tested originally. The following Gram-positive bacilli were originally considered: *Bacillus stearothermophilus* (spores) Merck[5] and

Table 1. Composition of animal feedingstuffs employed in the study as test matrices.

Matrix component	Vormast DSM flour (kg/100 kg)	Lammstar 2 pellets (kg/100 kg)	K11 special pellets (kg/100 kg)
Wheat	31.60	–	18.56
Winter barley	25.00	35.00	3.00
Triticale	15.00	–	5.00
Soy shred	6.95	9.70	3.90
Rapeseed, shred	5.00	5.00	13.99
Beans	4.00	–	–
Wheat bran	4.00	4.40	12.00
Soyax[a]	2.00	–	–
Malt germs	1.90	–	8.38
VEME Vormast LMT 1.5%	1.50	–	–
Soy oil	1.00	–	0.50
Calcium carbonate[c]	0.90	0.69	1.00
Lysine 25%	0.70	–	–
Cattle salt	0.35	0.21	0.67
Monocalcium phosphate	0.10	–	–
Alfalfa granulate	–	20.00	–
Sugar beet pulp	–	18.00	–
Sheep minerals[b]	–	2.00	–
Molasses, liquid	–	5.00	3.00
Sack of sheep	–	0.75	–
Palm kernel expeller	–	–	11.00
Corn (maize)	–	–	8.00
Maize, gluten feed	–	–	10.00
VM, Rinder Mersch 1%	–	–	1.00

Notes: [a]Velthof Export B.V., Borne, Netherlands.
[b]Salvana, Ahlhorn, Germany.
[c]Fodder chalk, < 500 μm.

Table 2. Preparation of bacterial suspensions and media for veterinary drug testing.[a]

Bacteria strain	Growth media	Temperature (°C)	Preparation of bacterial suspension (absorption), dilution	pH
Staphylococcus aureus ATCC 6538P	AM 2 (#226340)[b]	30 or 37	0.09–0.11, then 1/200	6.55
S. aureus ATCC 29213	AM 2	37	0.09–0.11, then 1/200	6.55
Escherichia coli ATCC 11303	AM 1 (#227020)	37	0.09–0.11, then 1/100	6.55
E. coli ATCC 27166	AM 1	37	0.09–0.11, then 1/100	6.55
Micrococcus luteus ATCC 9341	AM 5 (#227710), including K_2HPO_4 (1 g l^{-1}) and tylosine (5 μg l^{-1})	37	0.09–0.11, then 1/100	7.90
M. luteus ATCC 9341	AM 2, including K_2HPO_4 (1 g l^{-1})	37	0.09–0.11, then 1/100	6.55
M. luteus ATCC 9341 A	AM 5 + tylosine (5 μg l^{-1})	37	0.09–0.11, then 1/100	7.90
Yersinia ruckeri NCIMB 13282	DST agar, micro-aerophilic atmosphere	27–28	0.09–0.11, then 1/100[c]	7.00–7.20
Corynebacterium xerosis NCTC 9755	DST + K_2HPO_4 (2 g l^{-1})	37	0.09–0.13, then 1/100[d]	6.55
Bordetella bronchisepta ATCC 4617	AM 2 + K_2HPO_4 (1 g l^{-1}), trimethoprime (10 μg l^{-1})	37	0.09–0.10, then 1/100	7.00–7.40

Notes: [a]All incubation times were 24 h.
[b]AM, antibiotic medium (DIFCO, BD Biosciences); DST, diagnostic sensitive test agar (OXOID).
[c]Pre-trials were also conducted at acidic conditions (pH 6.55) with the same media including nalidixic acid (100 μg l^{-1}); however, the bacterial growth was too irregular to produce an interpretable inhibition zone.
[d]Pre-trials were also conducted at basic conditions (pH 7.9) with the same media including K_2HPO_4 (2 g l^{-1}) and tylosin (5 μg l^{-1}); however, the bacterial growth was too irregular to produce an interpretable inhibition zone.

ATCC 10149[1]; *Bacillus subtilis* BGA (spores) Merck[5] and ATCC 6633[1]; *Bacillus cereus* (spores) ATCC 11778[1]; *Bacillus pumilis* (spores) CN 607 and ATCC 14884[1]; *Bacillus megaterium* (spores) ATCC 10778[1]; and *Corynebacterium xerosis* NCTC 9755[6]. With respect to Gram-negative bacilli, the following were purchased:

Escherichia coli ATCC 11303, 29998 and 27166[1]; *Yersinia ruckeri* NCIMB 13282[3]; and *Bordetella bronchiseptica* ATCC 4617[0]. As for Gram-positive cocci, the following bacteria were obtained: *Micrococcus luteus* ATCC 9341, 9341a, 10240 and 15957[1]; *Staphylococcus aureus* ATCC 29213 and 6538P[0]; *Staphylococcus epidermidis* ATCC 12228[0]; and *Staphylococcus warneri*/spp. ATCC 12715[0]. Bacteria were obtained from either BCCM (University Gent, Belgium)[0], LGCstandards (Wesel, Germany)[1], the Centre de Ressources Biologiques de l'Institut Pasteur (Paris, France)[2], NCIMB (Aberdeen, UK)[3], DSMZ (Braunschweig, Germany)[4], Merck (Darmstadt, Germany)[5], or NCTC (Salisbury, UK)[6].

Extraction of antibiotics from feedingstuffs

In brief, about 50 g of crude fodder were ground using a mortar and pestle, and an aliquot of this (about 20 g) was further fine grounded. From this powder, 4 g of material were weighed into a 15 ml falcon tube (Fisher Scientific, Erembodegem, Belgium). For spiking experiments, 10–100 µl antibiotic standard solutions were mixed with 4 g of fodder, shaken by vortex and hand, and left to equilibrate for about 15 min without light. Then, 12 ml of extraction buffer were added, consisting of methanol, acetonitrile and McIlvaine buffer containing 0.3% EDTA (37.5/37.5/25%, v/v). The mixture was vortexed for 30 s, ultrasonicated (UP 200S, Hielscher, Teltow, Germany) for 15 min at 37 kHz and 300 W, further soaked for 2–5 min, and finally centrifuged for 15 min at 4500*g* at 15°C (Multifuge×3R, VWR, Darmstadt, Germany). From the resulting supernatant, 4 ml were collected in a 10 ml glass vial and evaporated to about 0.8 ml using a Turbo-Vap evaporator (Biotage, Hengoed, UK) at 40°C. The final volume was brought to 4 ml with phosphate buffer (pH 7), and again ultrasonicated for 5–10 min at 37 kHz and 300 W. From this mixture, 100 µl were further used for microbiological testing.

Microbiological testing

For vegetative bacterial strains, the bacteria stored at –80°C were revitalised in 2 ml tryptic soy broth (#211824, BD Biosciences) for approximately 2–3 h at bacterial growth temperature (28–37°C). Tryptone soy agar plates (#236940, BD Biosciences) were inoculated with 10 µl of this suspension and incubated overnight at bacterial growth temperature (28–37°C).

Afterwards, isolated colonies were taken and placed into sterile physiological saline (0.9%) to reach a turbidity of about 0.1 (0.9–1.1) in a Genesys 10 ultraviolet (UV) spectrophotometer (Thermo Scientific, Erembodegem, Belgium) at 620 nm. For antibiotic testing, 0.25 ml (*S. aureus* and *B. bronchiseptica*) or 0.5 ml (all other bacteria) of this bacterial suspension was brought up to 50 ml with liquid culture medium (Table 2) at 55°C. For the *Bacillus* spp., an adequate volume of spore suspension (depending on the spore concentration) was brought up to 50 ml with liquid culture medium. The final targeted bacteria concentrations were around 1–5 × 10^5 cfu ml^{-1} medium.

To these media, additional reagents were added where necessary, i.e. tylosine (5 µg l^{-1} final concentration) and trimethoprime (10 µg l^{-1} final concentration) to prevent growth of undesired bacteria, and 0.5 or 1 ml dipotassium hydrogen phosphate (1 or 2 g l^{-1}) as a buffering agent. Finally, 30 ml of these mixtures were poured into a screening plate (120 × 120 mm diameter, Greiner Bioone, Frickenhausen, Germany) and left to cool at RT for 15 min. The plates were then placed in a fridge for 1 h at 4°C until solidification of the media. Subsequently, 11 holes with 10 mm diameter per screening plate were punched into the solidified media, about 3 cm apart from one another. Into these holes, 100 µl of the samples (or blanks) were pipetted, and the plate was equilibrated for 1 h in the dark at RT prior to incubation (incubator type 9010–0078, Binder, Tuttlingen, Germany) for 24 h. Following incubation, inhibition zones were measured with a calliper.

Condition optimisation without matrix

The first aim was to optimise conditions for the detection of veterinary drugs without matrix. Pre-tests (data not shown) suggested the use of 10 bacterial conditions to be retained (Table 3), while bacterial strains resulting in less repeatable or interpretable results were not further considered. The remaining 10 bacterial conditions were tested for 32 veterinary drugs in solution. Concentrations of veterinary drugs varied according to pre-test results (data not shown), but in general were in between 0.01 and 10 mg l^{-1}. Each condition was tested in triplicate.

Studying the impact of matrix extracts without added antibiotics

Following identification of the most suitable bacterial incubation conditions, the effect on feed matrix on the detection of veterinary drugs was investigated, especially with respect to producing wrong positive results. For this purpose, three different antibiotic-free animal feeds (Lammstar, K11 and Vormast) were extracted 10 times each, and the supernatants were tested to observe effects such as overgrowth or growth inhibition against the various bacteria. Samples (4 g) were weighed, extracted and tested against seven bacterial conditions remaining after optimisation without matrix (Table 4), which yielded a sufficient inhibition zone (generally > 3 mm) and allowed a clear interpretation without a high rate of wrong positives or further complications such as overgrowth.

Table 3. Lowest detectable concentrations (μg l^{-1}) of veterinary drugs tested in solution (without matrix), with 10 various bacterial growth conditions (n = 3 per condition).

Antibiotic	*E. coli* ATCC 11303	*E. coli* ATCC 27166	*Y. ruckeri* micro-aerophilic	*B. bronchi-septica* ATCC 4617	*C. xerosis* NCTC 9755	*M. luteus* ATCC 9341 basic	*M. luteus* ATCC 9341 acidic	*M. luteus* ATCC 9341A	*S. aureus* ATCC 29213	*S. aureus* ATCC 6538P
Amoxicillin					100	50	**50**	**50**		
Bacitracin					**1000**	1000	**500**	1000		
Carbadox	**5000**									
Chloramphenicol	5000				**5000**	5000		5000		
Chlortetracycline	500	100			**500**	500	1000		**100**	**100**
Cirpofloxacin	**10**	10	50							
Colistin		**500**		**500**						
Doxycycline		500		**500**	500		500		**100**	100
Enrofloxacin	**50**	**50**								
Flavomycin									500	**100**
Florfenicol			5000		5000					
Flumequine	**500**	500	500							
Lasolacid A					**5000**				**1000**	**1000**
Lincomycin						**500**	1000	**500**		
Maduramycin					**5000**				5000	5000
Monensin					**5000**				5000	5000
Nalidixic acid	**5000**	5000								
Narasin					500					500
Olaquindox	5000									
Oxolinic acid	500	100	500							
Oxytetracyline	500	500			1000		1000		**500**	**500**
Penicillin G					100	**100**	50	**100**		**50**
Robenidine		**5000**			**10,000**	**10,000**	**10,000**	**10,000**		
Salinomycin					2000				1000	1000
Spiramycin						**100**	**500**	**100**		
Sulfadiazine				5000						5000
Tetracycline	500	500			1000		500		**500**	**500**
Tilmycosin					**500**	**100**	**500**	**100**		
Trimethoprim			1000							
Tylosin					**100**	**100**	**500**	**100**		
Valnemulin		500			**100**	**100**	100	**100**		500
Virginiamycin					**500**	500	500	1000		

Note: Values show the lowest detectable concentrations (μg l^{-1}) clearly giving a signal, i.e. an inhibition zone of at least 2 mm. Numbers in bold indicate that all three out of three replicates were clearly interpretable; and non-bold figures indicate a slight halo observed in some replicates, or the result was otherwise not always clearly interpretable.

Table 4. Effect of extracted blank matrices on bacterial growth against seven bacterial growth conditions selected for antibiotic detection.[a]

	Vormast		K11		Lammstar	
	Bacterial growth, quality of inhibition zone	Inhibition diameter (mm)	Bacterial growth, quality of inhibition zone	Inhibition diameter (mm)	Bacterial growth, quality of inhibition zone	Inhibition diameter (mm)
S. aureus ATCC 6538P (acidic)	Good, yellow colour. Small – no overgrowth	About 1, sz, dm[b]	Very good, yellow colour. Overgrowth around holes	1 (3/10)[c], < 1 (7/10), sz, dm[a]	Very good, yellow colour. Overgrowth around holes	< 1
S. aureus ATCC 29213 (acidic)	Very good, yellow colour. No overgrowth	0	Very good, yellow colour. Overgrowth around holes	0, st (3/10)[a]	Very good, yellow colour. Overgrowth around holes	0, st (3/10)[a]
E. coli ATCC 11303 (acidic)	Very good. No overgrowth	0	Good. Small overgrowth	0	Good. Small overgrowth	0
E. coli ATCC 27166 (acidic)	Very good, yellow colour. No overgrowth	0	Very good. No overgrowth	0	Good. Small overgrowth	0
Y. ruckeri (micro-aerophilic, neutral pH)	Good. No overgrowth	0	Good. No overgrowth	0	Very good. No overgrowth	0
C. xerosis (acidic)	Very good, yellow colour. Visible overgrowth	3–4, mean = 3.5	Very good, yellow colour. Pronounced overgrowth around holes	2–4, mean = 3.1	Very good. Pronounced overgrowth	2–3, mean = 12.5, sz, dm[a]
M. luteus ATCC 9341 (basic)	Very good. No overgrowth	0	Very good. Slight overgrowth	0	Very good. Slight overgrowth	0

Notes: [a]n = 10 extractions per condition.
[b]sz, sharp zone; dm, difficult to measure; and st, small turbidity.
[c]Should read, for example, three out of 10 replicates.

Pre-tests with a single matrix

Following the optimisation of the bacterial conditions without matrix, the finally selected bacteria conditions were tested in a first matrix. For this purpose, the lamb feed (Lammstar) was chosen. Veterinary drugs (n = 32) were tested and spiked into the matrix at two concentrations (Table 5). For this purpose, parental solutions of 10–100 µl were added and extractions carried out. As a blank, the matrix was processed without the addition of any veterinary drugs.

Seven bacterial conditions were tested: *E. coli* ATCC 11303 (acidic medium) and ATCC 227166 (acidic medium); *Y. ruckeri* NCIMB 13282 (neutral medium and micro-aerophilic condition); *M. luteus* ATCC 9341 (basic medium) and ATCC 9341 (acidic medium); and *S. aureus* ATCC 29213 (acidic medium) and ATCC 6538P (acidic medium). Concentrations of veterinary drugs were between 0.25 and 20 mg kg^{-1}.

Pre-test with several matrices

In the final step of pre-tests, it was verified whether there were differences in the veterinary drug detection in dependence of different test matrices (Table 6). Thus, two different concentrations per antibiotic were tested for lamb feed (Lammstar), piglet feed (Vormast) and bovine feed (K11). Bacterial conditions were further reduced to four and included the following: *E. coli* ATCC 11303 (acidic medium) and ATCC 227166 (acidic medium); *M. luteus* ATCC 9341 (antibiotic medium 5, basic); and *S. aureus* ATCC 6538P (acidic medium). Concentrations of antibiotics tested were between 0.25 and 20 mg kg^{-1}.

Final validation

The aim of the final validation was to obtain more thorough insights of the performance of the method, measuring parameters such as reproducibility, false-positive and false-negative rates, to estimate minimum detectable concentrations and to detect differences between matrices. For this purpose, all three matrices (Lammstar, Vormast and K11), the remaining four bacterial conditions, 14 veterinary drugs (Table 7), and at least one representative veterinary drug from each class were chosen, a procedure not uncommon (Community Reference Laboratories Residues 2010; Gaudin et al. 2010). In addition, 20 blank measurements (unspiked matrices) were conducted per bacterial condition (i.e. 80 blanks in total). Tests were conducted on different days in order to determine between-day (inter-day) precision (i.e. five replicates per antibiotic and bacteria condition were run on different days), defined as the RSD (%) of these five results. In addition, results were combined with pre-tests (Tables 5 and 6) (to obtain several sets of concentrations and inhibition zones per veterinary drug) in order to estimate minimum detectable concentrations (MDC) as reported by Higgins et al. (1999). For this purpose, plots for each veterinary drug were done of the $\log_{10}$ values of the two to three concentrations (mg kg^{-1}), tested against the square of the resulting inhibition zone (mm diameter) and linear regression curves estimated. The MDC was then defined as the concentration corresponding to an inhibition zone of 2 mm (the size of inhibition never observed for any blank) and was calculated based on six to nine replicates per drug.

Statistics

Unless otherwise stated, all values represent means ± SD. For statistical evaluation, SPSS v.19 (IBM, Chicago, IL, USA) was used. For the final validation, a linear mixed model was employed in order to study the effect of the matrix on the detection of veterinary drugs, i.e. to verify if the matrix had a significant effect on the inhibition zone (mm). Thus, the inhibition area (mm) was set as the observed (dependent variable), and veterinary drugs, matrix and bacteria condition as the fixed (independent) factors. Following significant Fisher F-tests, Bonferroni post-hoc tests for further pairwise comparisons for the matrices (n = 3) and bacterial conditions (n = 4) were conducted. Assumptions such as homogeneity of variance and normality of distribution were tested with box plots and normality plots, respectively. A p-value < 0.05 (two-sided) was assumed to indicate significant differences.

Results

Optimisation of detection without matrix

Detectable concentrations of veterinary drugs in solution varied between 0.01 mg l^{-1} for ciprofloxacin to 10 mg l^{-1} for robenidine (Table 3). Thus, all 32 antibiotics could be successfully detected. It was found that some of the bacterial conditions tested (a total of 10 remaining of the original 22) were either not suitable (e.g. producing wrong negative results) or redundant. These included *B. bronchiseptica*, *M. luteus* ATCC 9341 A (basic medium), and *C. xerosis*. These were therefore excluded from the further optimisation experiments with fodder matrix.

Impact of matrix extracts without added antibiotics

Of the remaining seven bacterial conditions tested, *C. xerosis* NCTC 9755 appeared to produce a large overgrowth, and the inhibition size was seen as problematic as it was quite large (Table 4). Consequently, *C. xerosis* was replaced by *M. luteus* ATCC 9341 (acidic pH, with K_2HPO_4 at 2 g l^{-1}). *E. coli* ATCC 11303, *E. coli* ATCC 27166, *Y. ruckeri* NCIMB 13282, and *M. luteus* appeared to constitute suitable species. With *S. aureus* ATCC

Table 5. Inhibition area produced by extraction supernatant of matrix (Lammstar) spiked with 32 antibiotics against 7 bacterial growth conditions selected for antibiotic detection. Values show inhibition zones at detectable concentrations.[a]

Antibiotic	Concentration (mg kg^{-1})	*S. aureus* ATCC 6538P	*S. aureus* ATCC 29213	*Y. ruckeri*	*E. coli* ATCC 11303	*E. coli* ATCC 27166	*M. luteus* ATCC 9341 acidic	*M. luteus* ATCC 9341 basic + tylosin
		Net inhibition (mm)[a]						
Control[b]	0	1	0	6	0	0	0	0
Control	0	1	1	5	0	0	0	0
Control	0	1	0	11	0	0	0	0
Amoxicillin	0.25	1	0.5	0	0	0	**12**[c]	**9**
Amoxicillin	0.5	2	0.5	0	0	0	**17**	**13**
Bacitracin	2	0	0	0	0	0	0	0
Bacitracin	4	0	0	0	0	0	0	0
Carbadox	5	1	0	1.5	0	**7**	0	0
Carbadox	15	1	0.5	**3.5**	**9**	**10**	0	0
Chloramphenicol	10	0	0	**4.5**	0	0	2	**4**
Chloramphenicol	20	1	0	**8.5**	0	0	**3**	**6**
Chlortetracycline	0.5	0	0	0.5	0	2	0	0
Chlortetracycline	1.5	**5**	0	0	0	**6**	0	0
Ciprofloxacine	0.25	0	0	**8**	**15**	**12**	0	0
Ciprofloxacine	0.5	0	0	**14**	**19**	**17**	0	0
Colistin	2	0	0	0	0	0	0	0
Colistin	4	0	0	0	0	0	0	0
Doxycycline	0.5	0	0	0	0	0	0	0
Doxycycline	1.5	0	**4**	0	0	**4**	0	0
Enrofloxacin	0.25	0	1	**15**	**14**	**15**	0	0
Enrofloxacin	0.5	0	1	**15**	**18**	**17**	0	0
Flavomycin	0.5	0	0	**9**	0	0	0	0
Flavomycin	1.5	0	0	**7**	0	0	0	0
Florfenicol	10	0	0	0	0	**5**	0	0
Florfenicol	20	1	**4**	**3**	0	**12**	0	0
Flumequine	2	0	0.5	1.5	**5**	**8**	0	0
Flumequine	4	1	0.5	**14.5**	**9**	**14**	0	0
Lasolacid A	5	0	0	0	0	0	0	0
Lasolacid A	15	**5**	**4**	0	0	0	0	0
Lincomycin	2	1	0.5	0	0	0	2	**5**
Lincomycin	4	**3**	1.5	0.5	0	0	**4**	**10**
Maduramycin	10	0	0	0	0	0	0	0
Maduramycin	20	1	0	1	0	0	0	0
Monesin	5	2	1.5	0	0	0	0	0
Monesin	15	**4**	**4.5**	**2.5**	0	0	0	0
Nalidixic acid	5	0	0.5	1.5	0	**5**	0	0
Nalidixic acid	15	1	0	**14.5**	**7**	**10**	0	0
Narasin	2	**3**	0.5	0	0	0	0	0
Narasin	4	**4**	1.5	0	0	0	0	0
Olaquindox	10	0	0	0	0	0	0	0

(*continued*)

Table 5. (*Continued*).

Olaquindox	20	0	0	0	**7**	0	0	0
Oxolinic acid	0.5	0	0	1	0	**4**	0	0
Oxolinic acid	1.5	0	0	1	7	**14**	0	0
Oxytetracycline	2	**3**	0.5	0	0	**6**	1	0
Oxytetracycline	4	**9**	**8.5**	0.5	**4**	**10**	1	**3**
Penicillin G	0.25	0	0	**9**	0	0	**8**	**6**
Penicillin G	0.5	**14**	2	**4**	0	0	**13**	**12**
Robenidine	10	0	0	1	0	0	0	0
Robenidine	20	0	0	0	0	0	0	0
Salinomycin	2	1	0	0	0	0	0	0
Salinomycin	4	**3**	0	0	0	0	0	0
Spiramycin	0.5	0	0	0	0	0	0	1
Spiramycin	1.5	1	0.5	0	0	0	0	**10**
Sulfadiazin	10	1	0	0	0	0	0	0
Sulfadiazin	20	0	0	0	0	0	0	0
Tetracycline	2	**3**	**3**	0	0	2	0	0
Tetracycline	4	**6**	**5**	0	0	**7**	0	0
Tilmicosin	0.5	1	0	0	0	0	0	**3**
Tilmicosin	1.5	0	0	2	0	0	0	**12**
Trimethoprim	2	1	0	**4.5**	0	0	0	0
Trimethoprim	4	0	0	**14.5**	0	0	0	0
Tylosin	0.5	0	0	0	0	0	0	**5**
Tylosin	1.5	1	1	0	0	0	**8**	**12**
Valnemulin	0.5	0	0	0	0	0	0	0
Valnemulin	1.5	0	0	2	0	0	0	**3**
Virginiamycin	2	0	0	0	0	0	2	**3**
Virginiamycin	4	0	0	0.5	0	0	**6**	**7**

Notes: [a]Net inhibition is the diameter of the inhibition zone minus the diameter of the control sample (blank), except for controls, where net diameter is the diameter of the inhibition zone minus the diameter of the whole (10 mm).
[b]Controls without addition of veterinary drugs were done in triplicate; all other values are individual measurements.
[c]Figures in bold indicate the largest inhibitory zones of the 7 bacterial conditions.

Table 6. Inhibition area produced by extraction supernatant of three matrices spiked with 32 antibiotics against four bacterial growth conditions selected for antibiotic detection.

Bacteria		SA 6538P[a]	SA 6538P	SA 6538P	EC 11303	EC 11303	EC 11303	EC 27166	EC 27166	EC 27166	MI 9341 basic	MI 9341 basic	MI 9341 basic
Matrix	Concentration (mg kg^{-1})	Lammstar	K11	Vormast	Lammstar	K11	Vormast	Lammstar	K11	Vormast	Lammstar	K11	Vormast
Controls[b]	0	1	0	0	0	0	0	0	0	0	1	0	0
Amoxicillin	0.25	1	1	2	0	0	6	0	0	0	9	0	7
Amoxicillin	0.5	2	1	0	0	0	0	0	0	0	13	10	11
Bacitracin	10	0	0	0	0	0	0	0	0	0	0	0	0
Bacitracin	20	2	0	0	0	0	0	0	0	0	0	0	0
Carbadox	5	1	0	0	0	0	1	7	0	0	0	0	0
Carbadox	15	1	0	0	9	7	8	10	8	3	0	0	0
Chloramphenicol	10	0	0	1	0	0	8	0	0	0	4	15	0
Chloramphenicol	20	1	2	1	0	5	0	0	0	0	6	0	1
Chlortetracycline	0.5	0	0	0	0	0	0	2	0	0	0	1	0
Chlortetracycline	1.5	5	1	1	0	0	3	6	0	0	0	4	0
Ciprofloxacin	0.25	0	5	7	15	27	20	12	23	23	0	0	0
Ciprofloxacin	0.5	0	9	11	19	27	21	17	25	28	0	0	0
Colistin	10	0	0	0	0	0	0	0	0	0	0	0	0
Colistin	20	1	0	0	0	0	0	0	0	0	0	0	0
Doxycycline	0.5	0	6	13	0	0	0	0	14	10	0	1	6
Doxycycline	1.5	0	11	16	0	0	6	4	19	17	0	9	11
Enrofloxacin	0.25	0	9	12	14	23	18	15	24	29	0	0	0
Enrofloxacin	0.5	0	13	16	18	27	21	17	26	26	0	0	0
Flavomycin	1.5	0	4	1	0	0	0	0	0	0	0	0	0
Flavomycin	4	5	9.5	10	0	0	0	0	0	0	0	0	0
Florfenicol	10	0	0	0	0	0	0	5	6	3	0	0	0
Florfenicol	20	1	0	0	0	0	0	12	13	13	0	0	0
Flumequine	2	0	0	0	5	4	5	8	10	9	0	0	0
Flumequine	4	1	2	0	9	10	10	14	14	15	0	0	0
Lasolacid A	5	0	0	0	0	0	0	0	0	0	0	0	0
Lasolacid A	15	5	3	3	0	0	0	0	0	0	0	0	0
Lincomycin	2	1	4	5	0	0	0	0	0	0	5	18	19
Lincomycin	4	3	7	8	0	0	0	0	0	0	10	27	26
Maduramycin	10	0	1	0	0	0	5	0	0	0	0	4	0
Maduramycin	20	1	1	1	0	0	10	0	0	5	0	0	0
Monesin	5	2	0	0	0	0	0	0	0	0	0	0	0
Monesin	15	4	2	0	0	0	0	0	0	0	0	0	0
Nalidixic acid	5	0	0	0	0	0	0	5	0	0	0	0	0
Nalidixic acid	15	1	0	0	7	7	8	10	10	9	0	0	0
Narasin	2	3	0	0	0	0	0	0	0	0	0	0	0
Narasin	4	4	1	4	0	0	0	0	0	0	0	0	0
Olaquindox	10	0	2	0	0	0	0	0	0	0	0	0	0
Olaquindox	20	0	1	0	7	5	0	0	0	0	0	0	0
Oxolinic acid	0.5	0	0	n.d.[c]	0	25	n.d.	4	29	n.d.	0	0	n.d.
Oxolinic acid	1.5	0	0	n.d.	7	33	n.d.	14	35	n.d.	0	0	n.d.

(*continued*)

Table 6. (*Continued*).

Oxytetracycline	2	3	11	15	0	0	6	6	16	12	0	5	6
Oxytetracycline	4	9	14	18	4	0	12	10	19	16	3	9	11
Penicillin G	0.25	0	27	25	0	0	0	0	0	0	6	27	28
Penicillin G	0.5	14	30	29	0	0	0	0	8	7	12	30	30
Robenidine	10	0	0	0	0	0	0	0	0	0	0	0	0
Robenidine	20	0	0	0	0	0	0	0	0	0	0	0	0
Salinomycin	2	1	1	1	0	0	0	0	0	0	0	0	0
Salinomycin	4	3	1	1	0	0	0	0	0	0	0	0	0
Spiramycin	0.5	0	0	0	0	0	0	0	0	0	1	0	0
Spiramycin	1.5	1	1	0	0	0	0	0	0	10	10	3	6
Sulfadiazin	10	1	0	0	0	0	0	0	0	0	0	0	0
Sulfadiazin	20	0	0	0	0	0	0	0	0	0	0	0	0
Tetracycline	2	3	5	3	0	0	0	2	6	9	0	0	0
Tetracycline	4	6	6	9	0	3	2	7	11	9	0	3	4
Tilmicosin	0.5	1	1	0	0	0	0	0	0	0	3	14	15
Tilmicosin	1.5	0	0	1	0	0	0	0	0	0	12	19	21
Trimethoprim	4	0	0	0	0	0	8	0	0	0	0	0	0
Trimethoprim	10	0	0	0	15	13	15	0	0	0	0	0	0
Tylosin	0.5	0	1	3	0	0	0	0	0	0	5	19	18
Tylosin	1.5	1	6	8	0	0	0	0	3	4	12	23	26
Valnemulin	0.5	0	0	0	0	0	0	0	0	0	0	0	0
Valnemulin	1.5	0	0	0	0	0	0	0	0	0	3	2	1
Virginiamycin	2	0	4	5	0	0	0	0	7	5	3	14	15
Virginiamycin	4	0	7	8	0	0	0	0	8	8	7	19	19

Notes: Values are net inhibition zones (mm), i.e. blank values (controls) were already subtracted.
[a]SA, *Staphylococcus aureus*; EC, *Escherichia coli*; Ml, *Micrococcus luteus*.
[b]Mean of $n = 4$; zone = measured minus whole (10 mm).
[c]n.d., No data (trial not conducted).

6538P, a very sharp inhibition barely extending beyond the hole size (10 mm diameter) was noticed, never exceeding 1 mm.

Pre-tests with single matrix

Detectable concentrations of veterinary drugs within the Lammstar matrix varied between 0.25 mg kg^{-1} (ciprofloxacin) and 20 mg kg^{-1} (maduramycin), being already considerably higher compared with the matrix-free conditions (Table 5). Some antibiotics remained completely undetectable up to 20 mg kg^{-1}, including bacitracin, colistin, robenidine and sulfadiazine. Maduramycin produced only very small inhibition zones (1 mm). Furthermore, of the seven bacterial conditions tested, it was found that *Y. ruckeri* yielded highly wrong positive results in the presence of feedingstuff matrix; thus, this bacterium was excluded in further experiments. Other bacteria were found to be redundant, including *M. luteus* ATCC 9341 (acidic condition) and *S. aureus* ATCC 29213 (acidic condition). Thus, the number of test bacteria retained for further investigations could be reduced to four.

Pre-tests with several matrices

A slight fluctuation of results between the matrices could be seen (Table 6). Lowest detectable concentrations were 0.25 mg kg^{-1} (e.g. ciprofloxacin); highest were 20 mg kg^{-1} (maduramycin). As seen before with the lamb matrix, some veterinary drugs could not be detected even at levels of 20 mg kg^{-1}, including bacitracin, robenidine, colistin and sulfodiazine. Consequently, it appeared not meaningful to include them further in the following investigation; thus, 28 detectable veterinary drugs remained. For all other veterinary drugs, at least one bacterial condition of the four remaining existed that allowed for a satisfactory detection (i.e. inhibition zone equally or larger than 2 mm).

Final validation

For six out of 14 veterinary drugs tested, two or even three bacterial conditions employed were able to detect the antibiotic (Tables 7 and 8). No strong effect of the matrix was seen. In fact, the *p*-value for matrix (0.297) indicated no significant impact of the matrix on the results, suggesting that there were similar inhibition results no matter what was the matrix. However, a significant interaction of matrix with bacteria was found ($p = 0.044$), indicating that bacterial growth differed to some extent, depending on the matrix, i.e. that the matrix had an effect on the growth of some, but not all, bacteria. The interaction of matrix and antibiotics was likewise significant ($p = 0.018$), indicating that some antibiotics produced a somewhat different inhibition size zone, depending on the matrix. Nevertheless, the mean inhibition zones (all results pooled including negative results) of the matrices were almost identical, with 5.3, 5.6 and 5.4 mm for K11, Lammstar and Vormast, respectively, being in line with limited general differences of measured inhibition zones between various matrices.

All chosen veterinary drugs were found to have MDCs between 0.25 mg kg^{-1} (ciprofloxacin) and 20 mg kg^{-1} (maduramycin); however, the detection of maduramycin at this concentration was not reliable, as the inhibition area observed was low, only 1–3 mm above the inhibition area of the blank, and about 27% of the tests were wrong negatives (Table 7). One problem contributing to this less reliable detection was the fact that maduramycin could only be detected by *S. aureus*, and that the inhibition area this bacterium produced was somewhat impacted by the matrix alone, usually producing about a 1 mm inhibition zone. None of the other bacteria employed reacted negatively to the matrix, i.e. no false-positives were observed. Olaquindox generally produced sufficiently large inhibition zones of 4–7 mm, but still had a false-negative rate of 20% at 20 mg kg^{-1}. Chloramphenicol produced only in some cases (one to two out of five trials) a low inhibition zone of 1–2 mm, and thus its detection up to 20 mg kg^{-1} could not be termed reliable. For all other antibiotics, the estimated MDC varied between 0.03 mg kg^{-1} (ciprofloxacin) and 10.4 mg kg^{-1} (maduramycin) (Table 7).

For the majority of veterinary drugs (nine out of 14) the false-negative rate, at the respective concentration tested, was zero. All other veterinary drugs had a false-negative rate below 27% (Table 7). Thus, the recovery rate (expressed as 100 – false-negative rate) was always above 73% and in most cases 100%. Concerning the false-positives (as defined as inhibition zone > 1 mm), as calculated from the blanks (no added antibiotic), only one out of 80 samples was positive (1.25%), which was regarded as an outlier.

The inter-day (between-day) precision of the inhibition zone (mm), estimated based on five replicas of the same antibiotic, detected by the same bacteria, with experiments conducted on different days, varied between 6% (lincomycin) and 109% (maduramycin) RSD, being higher for the drugs with a lower inhibition zone and higher detection limits.

Discussion

Within this present investigation, a microbiological method was developed allowing for the detection of 27 veterinary drugs from 13 different chemical classes in various feedingstuffs, at estimated MDCs between 0.03 and 11 mg kg^{-1}, with a rate of 0% false-positives (matrices without antibiotics) and 7–27% false-negatives (matrix spiked with antibiotics) for some veterinary drugs

Table 7. Summary table for veterinary drugs[c] tested in the final validation stage. Results were pooled for all three matrices tested, as the matrix effect appeared to be non-significant in the linear mixed model.[g]

Veterinary drug	Class of veterinary drug	Concentration tested (mg kg^{-1})	Mean inhibition zone (mm)[b]	SD of inhibition zone (mm)[e]	Interday precision (%)	Best bacterial condition	Wrong negative cases (%)[d]	Minimum detectable concentration (mg kg^{-1})
Amoxycillin, *Penicillin G*[a]	Penicillins/ β-lactams	0.25	19.0	0.8	<28	MI 9341[f]	0	0.21
Chloramphenicol, *Florfenicol*	Phenicols	20	<1	n.a.	n.a.	MI 9341, EC 27166	n.a.	n.a.
Ciprofloxacin *Enrofloxacin*	Quinolones	0.25	22.4	1.5	<19	EC 27166	0	0.03
Doxycycline *Oxytetracyline*, *Chlorotetracycline, Tetracycline*	Tetracyclines	1.5	16.4	1.0	<20	EC 27166	0	0.36
Flavomycin	Glycolipids	1.5	12.6	1.0	<27	SA 6538P	0	0.51
Lincomycin	Lincosamides	4.0	13.5	1.0	<6	SA 6538P	0	1.16
Maduramycin, *Lasolacid*, *Monensin*	Ionophores (polyether)	20	1.1	0.3	<109	SA 6538P	26.7	10.35
Nalidicic acid, *Flumequine*, *Oxolinic acid*	Quinolones	15	9.0	2.1	<61	EC 27166	6.7	5.07
Narasin, *Salinomycin*	Ionophores, salinomycins	4.0	5.7	5.6	<20	SA 6538P	0	2.14
Olaquindox, *Carbadox*	Quinoxalines	20	5.2	1.0	<95	EC 11303	20.0	11.06
Spiramycin, *Tilmicosin*, *Tylosine A*	Macrolides	1.5	20.0	0.4	<43	MI 9341 basic	0	0.51
Trimethoprim	Diaminopyrimidines	4.0	19.0	0.8	<9	EC 11303	0	2.02
Valnemulin	Pleurotumilins	1.5	18.2	0.6	<31	MI 9341 basic	0	0.51
Virginiamycin	Streptogramins	2.0	12.0	0.6	<57	MI 9341 basic	13.3	1.05

Notes: [a]Italic entries are antibiotics from the same family for which detection is equally well assumed, based on the authors' results (Table 6) and due to structural similarity.
[b]Blank (negative controls) already subtracted.
[c]Bacitracin, robenidine and sulfadiazine: could not be detected by this method.
[d]No wrong negatives were observed, as defined by a diameter causing an inhibition area more than or equal to 2 mm.
[e]SD, standard deviation; RSD, relative standard deviation (%).
[f]Basic condition (pH 7.9); all others acidic (pH 6.55).
[g]Each veterinary drug was tested on 5 different days measured in duplicate, resulting in a total of $n = 5 \times 2 \times 3 = 30$ samples per veterinary drug when matrices were combined and $n = 120$ when considering tested bacteria conditions.

Table 8. Activity overview of the veterinary drugs investigated, indicating positive results of the various bacterial conditions/plates involved, considering both results with and without matrix.

					Bacterial strains/plates			
Veterinary drug class	Veterinary drug	Minimum – maximum inclusion rates (mg kg^{-1})	Allowed in the European Union until and for use	Concentration tested (mg kg^{-1})	*S. aureus* ATCC 6538P	*E. coli* ATCC 11303	*E. coli* ATCC 27166	*M. luteus* ATCC 9341[m]
Diaminopyrimidines	Trimethoprim	250–250[c]	Banned gp	4.0	(+)	+	(+)	
Glycolipids	Flavomycin	1–25[a]	n.d.	1.5	+			
Ionophores	Maduramycin	5–5[a]	5/2021[d]	20	+			
(polyether)	Lasolacid	75–125[a]	9/2021[d]	15	+			
Ionophores	Monensin	10–125[a]	3/2022[d]	15	+			
(salomycin derivatives)	Narasin	60–100[a]	10/2020[d]	4.0	+			
	Salinomycin	15–70[a]	2/2018[d]	4.0	+			
Lincosamides	Lincomycin	2–10[e]	Banned gp	4.0	+			+
Macrolides	Spiramycin	5–80[b]	All banned gp	1.5	(+)			+
	Tilmicosin	as tylosine A		0.5	(+)			+
	Tylosin A	5–40[e]		0.5	(+)			+
Penicillins	Amoxycillin	250–500[l]	All banned gp[c]	0.25	+	+	(+)	+
	Penicillin G	5–80[b]		0.25	+		(+)	+
Phenicols	Chloramphenicol	as florfenicol	All banned gp	20	(+)			
	Florfenicol	<6000[f]		10			+	
Pleurotumilins	Valnemulin	25–200[g]	All banned gp	1.5	+		(+)	+
Polypeptides/	Bacitracin	5–80[b]	All banned gp	20				(+)
polymyxins	Colistin	as bacitracin		20			(+)	
Quinolones	Ciprofloxacin	as enrofloxacin	All banned gp	0.25	+	+	+	(+)
	Enrofloxacin	<200[i]		0.25	(+)	+	+	
	Nalidixic acid	as enrofloxacin		15		+	+	
	Flumequine	as enrofloxacin		2.0		+	+	
	Oxolinic acid	500–2000[j]		1.5		(+)	+	
Quinoxalines	Olaquindox	<50[k,b]	All banned gp	20		+	(+)	
	Carbadox	as olaquindox		15		+	+	
Streptogramins	Virginiamycin	0.5–80[b]	All banned gp	2.0	(+)		+	+
Tetracylines	Doxycycline	5–80[b]	All banned gp	1.5	+	+	+	+
	Oxytetracyline	5–80[b]		2.0	+	+	+	+
	Chlortetracycline	5–80[b]		1.5	+	+	+	+
	Tetracycline	5–80[b]		2.0	+	+	+	+
Sulphonamides	Sulfadiazine	188–1875[c]	Banned gp	20	(+)			
Others	Robenidine	30–66[a]	6/2021[d]	20			(+)	
Number of optimal detections/plate					15	12	12	12

Notes: Final concentration in animal feeds: [a]2004/C50/01 (EU Commission 2004); [b]70/524/EEC (EU Commission 1970); [c]Higgins and McEvoy (2002); [d]European Union Register of Feed Additives (EU Commission 2013a); [e]Directive 74/180/EEC (EU Commission 1974); [f]for medical purposes (fish) (Hayes 2013); [g]for medical purposes (see http://www.ah.novartis.com/products/en/econor_pig.shtml); [i]for medical purposes (Sarközy 2001); [j]for medical purposes (Higgins et al. 1999); [k]for gp (Barber et al. 1979); and [l]for medical purposes (De Baere & De Backer 2007).

+, Very strong response or strong response; (+), slight response, does not work reliably in the presence of feedingstuff matrix; [m]basic condition (pH 7.9), all others acidic (pH 6.55); n.d., not determined; gp, growth promotion, since 2006 (EU Commission 2003).

(four) and 0% for the remaining ones of the 14 validated compounds. Non-detectable, up to 20 mg kg^{-1} remained sulfadiazine, zinc-bacitracin and robenidine, while the detection of chloramphenicol appeared non-reliable and that of maduramycin with 27% false-negatives unsatisfactory. Inhibition zone differences observed between matrices were overall non-significant, and four bacterial conditions were sufficient to detect the antibiotics and coccidiostats in spiked feedingstuffs. Globally, detection of tetracyclines, penicillins and macrolides appeared very sensitive, with estimated MDCs at or below 0.5 mg kg^{-1} in animal feeds, while detection of some quinolones, some ionophores and quinoxalines was less sensitive, around 5, 10 and 11 mg kg^{-1}, respectively.

To date, no regulation exists clearly defining the analysis parameters of antibiotics in feedingstuffs, and no MRLs have been defined. Instead, veterinary drug residues should normally not be present in feedingstuffs, impeding setting up useful limits of detection for these compounds in animal feeds. In the present investigation, most veterinary drugs were detectable at concentrations at 5 mg kg^{-1}, except for olaquindox and maduramycin, which were detectable above 10–20 mg kg^{-1}, concentrations that are comparable with limits of detection in feedingstuffs as reported in earlier studies (van Egmond 2005). Furthermore, earlier MIR recommended by the European Union for many now banned antibiotics and the partly still allowed coccidiostats were often around 5 mg kg^{-1} (Table 8), thus the range of detectable concentrations appears meaningful. Despite the estimated MDCs obtained in this study, it should be mentioned that the relation between concentration and inhibition zones is not always reliable and can be impacted by the matrix, as shown for meat (Pikkemaat et al. 2009). Thus, unlike studies focusing on a single matrix (Althaus et al. 2009), care should be taken not to overstress the quantitative interpretation when multiple matrices are investigated.

In this study, concentrations detectable in the matrices were relatively high compared with those in pure solutions (range of 0.01–10 mg l^{-1}), and four antibiotics, i.e. robenidine, sulfadiazine, colistin and batricacin, could not be detected at all until 20 mg kg^{-1}. The general difficulty to apply growth inhibition tests to complex matrices such as for foods and feedingstuffs has been observed in several studies for various matrices such as for meat (Pikkemaat et al. 2007), but also in animal feeds (Higgins et al. 1999). This is possibly attributable to several factors, mainly the incomplete extraction of the antibiotic from the matrix, extractable matrix compounds that could either likewise inhibit bacterial growth (causing wrong positive results), such as the presence of polyphenols (Hafidh et al. 2011) or metals and vitamin C (McCarrell et al. 2008), or which could interact with the veterinary drug, such as high concentrations of magnesium, reducing the activity of especially tetracyclines and quinolones (Ferrini et al. 2006).

We do not know the reasons for the inability to detect sulfadiazine, robenidine, bacitracin and colistin and the problems observed for chloramphenicol. Earlier reports have likewise noted that the detection of chloramphenicol by microbiological tests lacks in sensitivity (Bogarts & Wolf 1980; Gaudin et al. 2003), and low sensitivity for bacitracin, colistin and chloramphenicol was also reported in the STAR protocol for milk and muscle (Gaudin et al. 2004, 2010). In another study on feedingstuffs, bacitracin was detectable by *B. stearothermophilus* in solution (0.7 mg l^{-1}), but not following matrix extraction, up to 100 mg kg^{-1} (Higgins et al. 1999). In the same study, detection of sulfadiazine was possible neither in solution nor in matrix up to 2000 mg kg^{-1}, and a low detection capability for sulphonamides by microbiological methods has been reported (Gaudin et al. 2010). Przenioslo-Siwczynska and Kwiatek (2007) also stated that sulphonamides in feedingstuffs were undetectable below 100 mg kg^{-1}, and also colistin showed poor detectability below 20 mg kg^{-1}. In addition to suboptimal bacterial conditions, limited diffusion of comparatively apolar (sulfadiazine) or of large compounds (colistin) in the polar agar medium may have limited their detection to some extent.

As in this study we aimed to develop a simple method for detecting a large array of antibiotics from various matrices, we did not strive to purify the extract further and to minimise matrix effects, which was deemed too time-consuming and problematic given the large variety of compounds and matrix interactions involved. Solid–liquid extraction (SLE), as carried out in the present study, is, due to its rapidness and simplicity, the most common treatment for extracting veterinary drugs in screening tests (Chafer-Pericas et al. 2010). SLE was compared with other feed extraction techniques such as pressurised liquid extraction (Higgins & McEvoy 2002), which is equally effective for extracting veterinary drugs, as shown by Boscher et al. (2010). Extraction at acidic pH with a mixture of phosphate buffer and organic solvents, i.e. acetonitrile and methanol, usually allows for a maximum number of veterinary drugs to be extracted, including the more polar tetracyclines, polypeptides and quinolones, and the relatively more apolar veterinary drugs such as penicillins, macrolides and sulphonamides. Recovery with this solvent mixture produced reasonable recoveries (>70%) for most veterinary drugs from feedingstuffs (Boscher et al. 2010), except for penicillins (a mean recovery of about 55%) and tetracyclines (a mean recovery of about 35%), as determined by LC-MS/MS. Thus, it appears unlikely that the inability to detect sulfadiazine, robenidine, bacitracin and colistin resulted from poor extraction. Therefore, a two-step procedure for extraction, e.g. carried out with trichloracetic acid followed by acetonitrile (Gaudin et al. 2009) or acetone/water/hydrochloric acid followed by phosphate buffer

(van Egmond 2005), did not appear to be required, but it may be superior for complete extraction of varying classes of veterinary drugs. Earlier extractions from feedingstuffs were likewise based on mixtures of methanol, acetone and water (Higgins et al. 1999); however, in the present study, it was preferred to further evaporate organic solvents post-extraction to minimise their potential negative effects on bacterial growth.

Following extraction, the minimum number of bacterial conditions to be finally employed were four (*E. coli* ATCC 11303, *E. coli* ATCC 27166, *S. aureus* ATCC 6538P, and *M. luteus* ATCC 9341), all of which appeared sufficiently able to grow in the presence of matrix extracts, with only a small inhibition caused by the matrix, as observed for *S. aureus* ATCC 6538P, of typically 1 mm, which was therefore chosen as a cut-off level to differentiate wrong positives. The fact that these bacteria were not considerably impacted by the matrix may also be reflected in the fact that independent of the matrix, similar inhibition zones were produced by these four bacteria. The final choice of four bacteria is in line with earlier reports, showing that strains of *E. coli* including the one used (ATCC 11303) were able to detect quinolones (Gaudin et al. 2004; van Egmond 2005), and *M. luteus* macrolides and streptogramins (van Egmond 2005). *S. aureus* has not to our knowledge been used for veterinary drug detection in feedingstuffs, but other staphylococci, e.g. *S. epidermis*, have been employed for the detection of, for example, aminoglycosides in meat and poultry (USDA 2011). Some studies have used a higher number of bacteria, up to six or seven, to detect veterinary drugs in milk (Nouws, van Egmond, Smulders, et al. 1999; Althaus et al. 2009), and many studies accept a somewhat more arguable cut-off level of an inhibition zone of several millimetres for separating wrong positives from true positives, which was avoided in the present investigation. On the other hand, the detection of sulphonamides such as sulfadiazine may require additional types of bacteria such as *Bacillus pumilus* (Bushby & Hitchings 1968). In the present study, this strain was shown to react strongly with matrix extracts alone. For the detection of bacitracin at 3–5 mg kg^{-1}, *M. luteus* ATCC 10240 may have been superior (van Egmond 2005; Pol-Hofstad et al. 2008); however, this strain produced extremely variable results in pre-tests and was therefore not further considered.

In addition to choosing bacterial conditions, detection of veterinary drugs with microbiological tests is further dependent on optimal pH. For example, as noted above, macrolides are better detected and are more active at higher pH, as are the majority of quinolones (except for oxolinic acid and flumequine) (Dang et al. 2010), while beta-lactams are preferably detected at acidic pH (Yamada et al. 1981), similar to tetracyclines (Kavanagh 1963). This was similar to observations in the present study, e.g. macrolide analysis in the present study was superior at pH 7.9 with *M. luteus*, while the majority of classes were better detected at acidic pH, except for phenicols, steptogramins and pleurotumulins. With respect to the detection of most quinolones in the present study, e.g. ciprofloxacine and enrofloxacine, detection was sufficiently sensitive at low pH. However, detection of nalidixic acid with *E. coli* ATCC 11303 may have been more sensitive at higher pH, but would have meant introducing another bacterial condition.

The highest number of veterinary drugs were detected by *S. aureus* ATCC 6538P, followed by an equal number of detections by the other bacterial conditions (Table 8). Although, especially in the final validation procedure, only a reasonable number of antibiotics were tested, many constitute representatives of their respective class in terms of activity profile and properties. For example, ciprofloxacin has been reported to constitute a good representative for marbofloxacin and danofloxacin, and lincomycin for pirlimycin, three compounds that were not included in the present investigation (Gaudin et al. 2010).

Despite some deductions on the type of the veterinary drugs that are possible using the present four-plate test (Table 8), one of the limitations of using only four bacteria was that the exact nature of the veterinary drug could not be finally deduced, and that secondary screening tests or confirmatory methods would be needed for compound identification. Furthermore, as only three types of animal feeds were tested, it cannot be completely excluded that other matrix compounds not contained in the feedingstuffs, or strong deviations in composition, could have a negative impact on the four bacterial strains, resulting in false-positives which were not observed in the present investigation. Thus, only a confirmatory method producing four points of identification for each veterinary drug, such as LC-MS/MS methods generating retention times, parent mass and two transition masses, as demanded for antibiotics in foodstuffs, can be reliably used to identify veterinary drugs (Boscher et al. 2010).

In conclusion, the present study suggested a comparably simple microbiological method for the detection of a number of antibiotics and coccidiostats from various chemical classes, allowing for a large number of sample processing in parallel. Compared with several earlier published methods, the method included extraction and detection in real feeds, and was tested against several matrices, which is often neglected. Furthermore, no arbitrary cut-off levels of inhibition zones were needed, and the assay produced reasonable detectable concentrations with a limited number of bacterial strains. Further improvements in the future would aim at identifying antibiotics that remained undetectable in the present investigation, and to detect aminoglycosides that were not included in the present investigation, and to strive to lowering limits of detection further by altering, for example, extraction

techniques, such as by further concentration or simple additional clean-ups.

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