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Contents

Samenvatting

De hedendaagse intensieve vleeskuikenhouderij is sterk afhankelijk van het gebruik van coccidiostatica. Deze antiparasitaire middelen worden op grote schaal als diervoederadditief toegepast. De belangrijkste groep coccidiostatica, de ionoforen, heeft naast een antiparasitaire werking ook een antimicrobiële werking tegen grampositieve bacteriën. Ionoforen worden niet ingezet bij humane infecties en daarom werd tot nu toe aangenomen dat het grootschalig gebruik van ionoforen bij pluimvee geen consequenties heeft voor de humane gezondheidszorg. Uit onderzoek uitgevoerd in Noorwegen en Zweden is onlangs echter gebleken dat antimicrobiële resistentie tegen vancomycine, een voor humane behandeling belangrijk antibioticum, in enterococcen samen voorkomt met resistentie tegen ionoforen (*narAB* genen). Dit heeft als consequentie dat er gekoppelde uitwisseling tussen bacteriën plaats kan vinden, waardoor het gebruik van ionoforen kan leiden tot de verspreiding van vancomycine resistentie.

Resistentie tegen ionoforen, in het bijzonder salinomycine (SAL), wordt vanwege de beperkte humane relevantie al enige jaren niet meer gemonitord. Uit historische gegevens kan echter worden opgemaakt dat het percentage SAL resistente *Enterococcus faecium* en *Enterococcus faecalis* in isolaten uit pluimvee(producten) aanzienlijk is. De Scandinavische resultaten vormden aanleiding om te onderzoeken in hoeverre de aanwezigheid van ionoforenresistentie in in Nederland geïsoleerde enterococcen is gekoppeld aan resistentie tegen andere antibiotica. In dit onderzoek is daartoe het resistentieprofiel bepaald van 137 isolaten *E. faecium* en *E. faecalis* uit pluimveeproducten, waarvan een deel uit de biologische pluimveehouderij afkomstig was. Deze resistentieprofielen zijn statistisch geanalyseerd op co-resistenties, waarna van een subselectie van de enterococcen het DNA met behulp van sequentieanalyse is onderzocht op de aanwezigheid van resistentiegenen.

Resistentie tegen SAL blijkt aanwezig in bijna 40% van de isolaten. De resultaten laten een statistisch significant verband zien tussen de aanwezigheid van SAL resistentie en erythromycine (ERY) resistentie in *E. faecium* uit reguliere houderij. Ditzelfde verband is ook aanwezig bij de *E. faecalis* isolaten van zowel reguliere als biologische herkomst. Daarnaast is in zowel *E. faecium* als *E. faecalis* een statistisch significant verband aangetoond tussen de aanwezigheid van SAL en tetracycline (TET) en SAL en ampicilline (AMP) resistentie.

De DNA analyseresultaten laten een match zien tussen de waargenomen (fenotypische) resistentie en aanwezige resistentiegenen. In alle onderzochte isolaten met een fenotypische SAL resistentie zijn de uit eerder genoemd onderzoek geïdentificeerde *narAB* genen aangetroffen. Daarnaast kon worden aangetoond dat *narAB* zich in vrijwel alle gevallen bevond op een plasmide met daarop tevens genen coderend voor resistentie tegen andere typen antibiotica, met name tegen TET en ERY. Dit is een verontrustende constatering. Het betekent dat het gebruik van ionoforen kan leiden tot de verspreiding en het in stand houden van deze andere typen resistentie, zonder dat het betreffende antibioticum zelf toegepast wordt.

Deze resultaten vormen aanleiding tot een heroverweging van het gebruik van ionoforen als diervoeder additief in de pluimveehouderij. Meer onderzoek is nodig om de omvang en de impact van de in dit rapport gepresenteerde bevindingen te bepalen. Het is onbekend of ionoforenresistentie ook bij andere grampositieve bacteriën, zoals bijvoorbeeld *Staphylococcus aureus,* voorkomt. Het is aannemelijk dat er naast de nu bekende *narAB* genen nog andere ionoforenresistentiegenen in pluimveegeassocieerde bacteriën voorkomen. *E. faecium* en *E. faecalis* zijn belangrijke veroorzakers van infecties bij ziekenhuispatiënten. Uit analyse van humane enterococcen isolaten zal moeten blijken of het gebruik van ionoforen in pluimvee consequenties heeft voor de humane gezondheidszorg.

Summary

Today's intensive broiler production is highly dependent on ionophore coccidiostats. These antiparasitic agents are applied as feed additives. The most important group, the ionophores, not only exhibit antiparasitic activity but are also antimicrobial against Gram-positive bacteria. Because these ionophores are not used in humans, it is widely assumed their use does not impact human health. However, recent Swedish and Norwegian research shows that ionophores can cause the co-selection of vancomycin resistance in enterococci. Vancomycin is a critically important antimicrobial for human medicine.

Because of its limited relevance for human medicine, monitoring of antimicrobial resistance against ionophores, in particular salinomycin (SAL), was discontinued several years ago. From historical data, however, it can be retrieved that a fair percentage of the *Enterococcus faecium* and *Enterococcus faecalis* isolates from poultry origin show SAL resistance. The current research was initiated to determine whether ionophore resistance in enterococci from poultry co-occurs with other clinically relevant types of antimicrobial resistance.

A set of 137 *E. faecium* and *E. faecalis* isolates from poultry products from conventional and organic origin was phenotypically characterized. The resistance profiles were statistically analysed and subsequently a selection of 20 isolates was DNA sequenced and analysed for the presence of resistance genes.

Almost 40% of the isolates appeared to have a SAL resistant phenotype. Results of the statistical analysis showed a significant correlation between the presence of SAL resistance and erythromycin (ERY) resistance in *E. faecium* from conventional farming. The same correlation was also observed in *E. faecalis* from both conventional and organic origin. Besides this, both *E. faecium* and *E. faecalis* show significant correlation between the presence of SAL and tetracycline (TET) resistance and SAL and ampicillin (AMP) resistance.

DNA sequencing results show good agreement between the observed phenotypic resistance and the identified resistance genes. In all of the isolates showing phenotypic SAL resistance the *narAB* genes could be identified. Moreover, in most isolates *narAB* is found to be located on a plasmid carrying additional genes for other types of antibiotic resistance. This is an alarming observation, since it implies that the use of ionophores may drive the transfer and dissemination of other, clinically relevant types of antimicrobial resistance by co-selection.

These results question the sustainability of the prophylactic use of ionophores in broiler production. More research is needed to determine the extent and the impact of this issue. It is unknown whether ionophore resistance also occurs in other Gram-positive bacteria, like *Staphylococcus aureus*. It is plausible that ionophore resistance genes other than *narAB* will be present in poultry associated bacteria. *E. faecium* and *E. faecalis* are an important cause of hospital-acquired infections. Analysis of human enterococcal isolates should reveal whether transmission from the poultry reservoir is occurring and thus whether the use of ionophores in poultry impacts human healthcare.

1 Introduction

Antimicrobial resistance (AMR) is increasing worldwide and considered to be a major threat to human welfare and modern medicine. Excessive or imprudent use of antibiotics in animal production provides a breeding ground for bacteria expressing AMR, which can be transmitted to humans through consumption of contaminated food, occupational contact between humans and livestock, and environmental dispersion. It is generally recognized that fighting AMR can only be successful if it is approached from a One Health perspective, combining efforts within the human, animal, and environmental domain.

Coccidiosis, an intestinal tract infection caused by protozoa belonging to the genus *Eimeria*, is a significant health problem in poultry. It has an adverse impact on animal welfare and impairs growth, causing losses in production. To treat and prevent coccidiosis, coccidiostats are applied. The majority of these coccidiostats belong to the polyether ionophores. According to EU legislation these coccidiostats are considered feed additives (EC 1831/2003), which allows for (at a specified dietary concentration) a more or less unrestricted use in broiler production. Consumption figures in the Netherlands as well as on EU scale are lacking, but given that the inclusion of coccidiostats in the diet is considered an unavoidable health management measure in conventional broiler production systems, usage is expected to be significant.

Besides the anticoccidial activity of the polyether ionophores (narasin, monensin, salinomycin, lasalocid, semduramicin and maduramicin), these compounds also possess antibacterial activity, in particular against Gram-positive bacteria. Concerns about the systematic use of ionophores with respect to AMR development have always been countered by arguing that the ionophores are not medically important (they are not used in human medicine because of their toxicity) and that there are no indications that their use in poultry has any effect on therapeutical application of other antibiotics, neither in animals nor in humans. Because of their insignificance for human medicine, ionophore resistance is currently not included in the harmonized EU monitoring of AMR in zoonotic and commensal bacteria (EC 2020/1729). However, historical data show that in 2013 salinomycin (SAL) resistance in *Enterococcus faecalis* isolates from poultry was >40% in the Netherlands (NethMap/MARAN, 2014). Even higher incidence of SAL resistance (64%) was reported from Denmark (DANMAP, 2014), while at that time Norway and Sweden also reported similar figures for narasin resistance (NORM-VET, 2014; SVA, 2014).

Although recent figures are lacking, it is evident that ionophore resistance is highly prevalent among (at least) the enterococci in poultry. It is important to note that bacterial genes encoding AMR are often located on plasmids, small circular pieces of DNA that can easily be transmitted between bacteria ("horizontal gene transfer"). Despite ionophore coccidiostats being used since the 1970s, there were no indications of transferable ionophore resistance genes until 2012, when researchers from the Swedish National Veterinary institute found evidence for plasmid-mediated narasin resistance. More importantly, they showed co-transfer of narasin and vancomycin resistance, a clinically important glycopeptide antimicrobial (Nilsson et al., 2012). In 2020 the genes conferring the narasin resistance were cloned and named *narAB* (Naemi et al., 2020). The operon was shown to confer reduced susceptibility not only to narasin, but also to salinomycin and maduramicin.

The fact that the *narAB* operon is plasmid-located and the observation of co-transfer with a clinically important AMR is very alarming. If such a plasmid is present in poultry-associated bacteria, exposure of the poultry to ionophores will pose selective pressure promoting not only the spread of narasin resistance but also of the secondary AMR gene(s) (Figure 1).

Since AMR to clinical antibiotics in bacterial isolates from poultry is much more frequently observed in the Netherlands (NethMap/MARAN, 2021) compared to the Scandinavian countries, where the *narAB* operon was first discovered, it is to be expected that enterococci carrying plasmids co-harboring ionophore and clinical AMR might be prevalent in Dutch poultry as well.

The aim of the research presented in this report was to determine the prevalence of these *narAB* genes in enterococcal isolates from Dutch poultry and the association with other, therapeutically relevant types of AMR. Samples originating from both conventional and organic production were included. In organic broiler production the preventative application of coccidiostats is not allowed and subsequently the prevalence of ionophore resistance was expected to be lower.

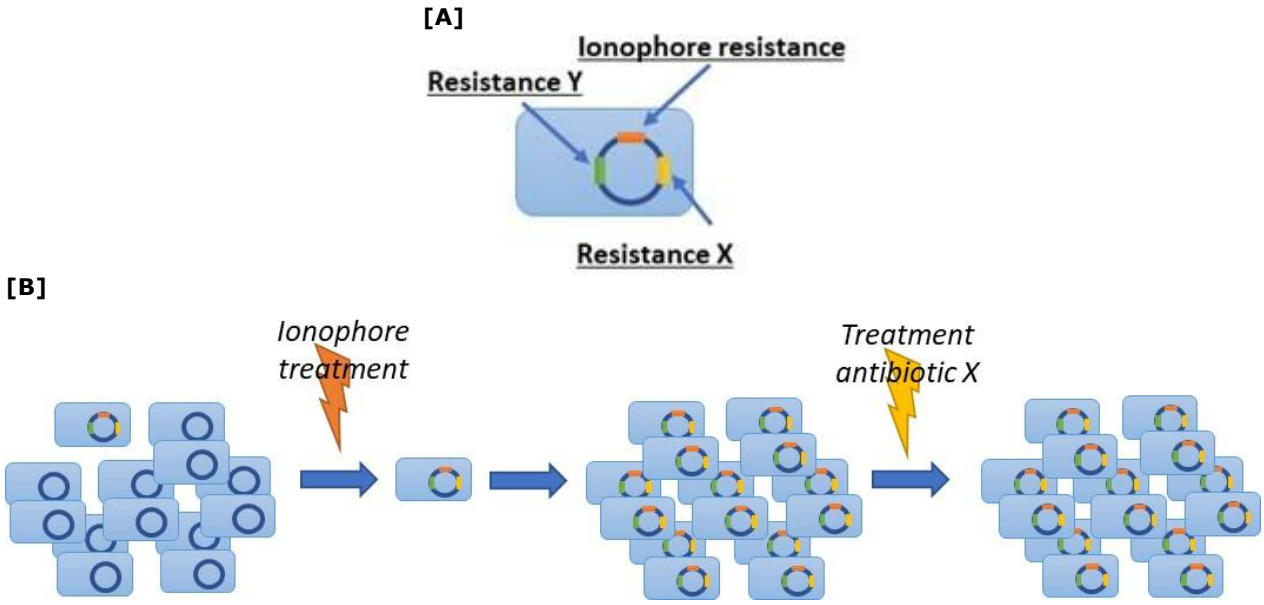

Figure 1 *Explanation of the principle of co-selection. [A] Bacterium carrying a plasmid with genes conferring resistance to ionophores and antibiotics X and Y. [B] When ionophores are applied (orange lightning) this yields selective pressure in favor of the bacteria carrying iononophore resistance genes. If these bacteria cause an infection, either in poultry or humans, they are no longer susceptible to antibiotics X and Y, and cannot be treated with these antibiotics (yellow lightning).*

2 Experimental setup and procedures

2.1 Collection of isolates

A total of 137 enterococcal isolates from poultry origin were included in the study. Of these, forty-one (*E. faecalis* n= 28 and *E. faecium* n=13) originated from organic poultry production. Part of the enterococci *(E. faecalis* n= 27 and *E. faecium* n=15) were isolated from fresh poultry meat retail samples obtained from local supermarkets during April and May 2020. The remainder (*E. faecalis* n= 62 and *E. faecium* n=33) was obtained from an existing in house culture collection. These strains were originally isolated between 2013 and 2018.

2.2 Isolation, Identification, MIC determination

Isolation of enterococci was performed according to an in house procedure. A sample of 25 g of poultry meat was homogenized in 225 mL of buffered peptone water using a stomacher, and incubated at 37 °C for 16-22 hrs. Subsequently 10 ml of the enrichment broth was transferred to 90 ml Azide dextrose broth and incubated at 44°C for 18-22 hrs. After incubation, 10 µl of broth was streaked on Slanetz-Bartley agar and incubated at 44°C for 44-52 hrs. From this plate a single typical colony was transferred to a Trypton Soya Agar plate (TSA) and incubated at 37°C for 22-26 hrs. These pure cultures were subjected to MALDI-TOF for confirmation and species identification. All *E. faecalis* and *E. faecium* isolates were stored at -80°C.

Minimum Inhibitory Concentrations (MIC) were determined with a microbroth dilution method described in NEN-EN-ISO 20776-1:2020, using the Sensititre™ EU Surveillance Enterococcus EUVENC Plate. This plate contains the following antibiotics: vancomycin (VAN), teicoplanin (TEI), quinupristin/dalfopristin (SYN), tetracycline (TET), daptomycin (DAP), ciprofloxacin (CIP), erythromycin (ERY), tigecycline (TGC), linezolid (LZD), gentamicin (GEN), ampicillin (AMP), chloramphenicol (CHL). The Sensititre™ Vizion™ MIC viewing system was employed for MIC read out of the EUVENC plates. The MIC for salinomycin (SAL) was determined using a similar approach, using an in house prepared microbroth dilution series, ranging 0.25-0.5-1-2-4-8-16-32 µg/ml salinomycin.

Epidemiological cut-off values (ECOFFs) established by EUCAST were applied (EUCAST, 2020). For salinomycin currently no ECOFF is defined. Historically, a cut-off of 4 mg/L salinomycin was used (NethMap/MARAN, 2014). Other published data, however, suggest that a lower cut-off might be applicable (Butaye, 2000), so for our initial analysis a cut-off of 2 mg/L was applied. Sequencing results obtained in this project, however, substantiated a cut-off of 1 mg/L.

2.3 Statistical analysis

Statistical analysis of co-occurrence patterns was based on calculating Jaccard's index of similarity (Mainali, 2017). Jaccard's similarity index for each pair of antibiotics is calculated as

$$J = \frac{a}{(a + b + c)}$$

where *a* is the number of isolates with resistance to both antibiotics, and *b* and *c* are the number of isolates with resistance only to either of these two antibiotics. To calculate these values, MIC have been thresholded into binary results: resistant or not. "Resistance" was defined as the state that the MIC was larger than the ECOFF for that subspecies. For SAL resistance was defined at an MIC >1 mg/L. The dataset was split into four subsets based on species (*faecalis* and *faecium*) and production method (organic or conventional),

resulting in 13 (*faecium*/org), 35 (*faecium*/conv), 28 (*faecalis*/org), and 61 (*faecalis*/conv) enterococcal AMR profiles. Observed Jaccard's indices were calculated for each of these four subsets.

To test whether the observed co-occurrence values are significant, i.e. if co-occurrence is significantly higher than expected by random co-occurrence from the occurrence rate of two individual antibiotics, resampling techniques have been used. Resampling was done using a custom script in R (R 3.6.1, R Core Team, 2019). The AMR profiles of the four subsets were resampled, with replacement, to a sample size of 10x the actual size for resolution issues. All possible Jaccard's indices were calculated and stored. This sampling was repeated 10000x, which appeared sufficient to yield stable results between runs. Confidence intervals for Jaccard's indices (pairwise AMR) and individual occurrence AMR were defined as the lower and upper 5% quantiles for the pool of 10000 results.

2.4 Genome Sequencing and analysis

2.4.1 Short-read sequencing

Single colonies were selected from a tryptone soya agar plate and grown in Brain Heart Infusion broth for max. 24 hrs. Of the resulting culture, 1 ml was spun down (10 min, 5000 *g*) to obtain a pellet from which DNA was extracted using Qiagen's DNeasy Blood & Tissue kit according to manufacturer's instructions including their protocol 'Pretreatment for Gram--Positive Bacteria'. Sequencing library preparation and short-read sequencing (PE 150 bp) was outsourced and performed using the NexteraXT library preparation kit and Illumina NovaSeq sequencer.

2.4.2 Long-read sequencing

A separate aliquot of the DNA extracted for short-read sequencing was used as starting point for barcoded library preparation using Nanopore EXP-NBD104, EXP-NBD114 and SQK-LSK109 kits according to manufacturer's instructions. Samples were sequenced on a FLO-MIN106 flow cell on a Nanopore MinION device.

2.4.3 Sequence pre-processing and assembly

Using Trimmomatic (v 0.36; Bolger, 2014) Illumina reads were adapter-trimmed (ILLUMINACLIP:NexteraPE-PE.fa:2:20:12:1:true) and quality trimmed (SLIDINGWINDOW:3:22), retaining only reads longer than 36 bases. Nanopore fast5 files were based called using ONT's guppy (version 5.0.11; chunk_size 3500, min_qscore 7), and de-multiplexed and adapter trimmed using guppy_basecaller. A hybrid assembly using Illumina and Nanopore data was performed using Unicycler (version 0.4.9; Wick, 2017). Internally, Unicycler uses SPAdes to produce an initial assembly from Illumina data. It attempts to pick an optimal k-mer size to use based on a trialling different k-mer sizes. Whilst this approach yielded decent results when considering the bacterial chromosome, this optimisation had poor effect on the quality of potential plasmid sequences and subsequent recall of AMR genes. A manual assessment of sequence size, circularity, and recall of AMR genes was performed to select a k-mer size of 71 as a reasonable parameter for all data sets in this study. Coverage profiles of Nanopore reads across the assembled genomes were checked for evidence of incorrect assembly resulting from, for example, the rare presence of chimeric reads. Minimap2 (2.17-r941; Li, 2018) was used to map reads to the assembly, alignments were further processed using samtools (1.11; Danecek, 2021) to keep only primary alignments, sambamba (0.8.0; Tarasov, 2015) for sorting and bedtools (2.28.0; Quinlan, 2010) to summarise the alignment into genome coverage. A rolling mean of the coverage profiles was calculated and visualised in R (R 4.0.2; R core Team, 2020), using rollmean from package zoo (1.8-9; Zeileis, 2005), tidyverse packages (1.3.1; Wickham, 2019), gggenes (0.4.1; Wilkins, 2020) and ggpubr (0.4.0; Kassambara, 2020).

2.4.4 Detection of AMR genes and sequence annotation

An initial inventory of potentially present AMR genes was determined from trimmed Illumina reads using Resfinder (4.1.3; Bortolaia, 2020) using a customised database version to which *narA* and *narB* gene sequences were added. To this end, a collection of non-redundant *narA* and *narB* genes was built from sequences submitted by Naemi et al. (Naemi, 2020) to NCBI Genbank (MN590304–MN590310). The same version of Resfinder and custom database were used to determine the presence of AMR genes in assembled data. Genes were predicted and annotated using Prokka (version 0.1; Seemann, 2014) using default settings. Plasmid replicons were determined using PlasmidFinder (version 2.1.1; Carattoli, 2014).

3 Results

3.1 MIC profiles of the isolates

Table 1 shows an overview of the results of the susceptibility testing. Only antibiotics for which resistance was observed, and vancomycin, are included. Results for individual strains can be found in Annex 1.

Table 1 *Overview of the results of the susceptibility testing.*

Species		n	Res	AMP	CIP	ERY	SYN	TET	TGC	VAN	SAL >1	SAL >2
E. faecalis	conv	61	abs	0	1	33	Intr	46	0	0	29	14
			%	0,0	1,6	**54,1**	nd	**75,4**	0,0	0,0	**47,5**	23,0
	org	28	abs	1	0	11	Intr	21	0	0	5	1
			%	3,6	0,0	**39,3**	nd	**75,0**	0,0	0,0	**17,9**	3,6
E. faecium	conv	35	abs	2	6	20	0	12	1	0	17	11
			%	5,7	17,1	**57,1**	0,0	**34,3**	2,9	0,0	**48,6**	31,4
	org	13	abs	0	2	3	0	4	0	0	1	1
			%	0,0	15,4	**23,1**	0,0	**30,8**	0,0	0,0	**7,7**	7,7

Antibiotics for which results are included: ampicillin (AMP); ciprofloxacin (CIP); erythromycin (ERY); quinupristin/dalfopristin (SYN); tetracycline (TET); tigecycline (TGC); vancomycin (VAN); salinomycin (SAL). Isolates from conventional (conv) and organic (org) origin, absolute (abs) number (n) of isolates showing resistance (Res). *E. faecalis* exhibits intrinsic resistance (Intr) against SYN.

The breakpoint for salinomycin (SAL) was previously suggested at 2 mg/L, but the sequencing results obtained in this study (presence/absence of the *narAB* operon) suggest this breakpoint should be established at 1 mg/L. This adjustment yields a considerable increase in the number of strains with a reduced susceptibility for SAL (see Table 1). For both species a substantial number of the isolates show resistance against erythromycin (ERY), tetracycline (TET) and SAL. The overall observed resistance levels for ERY and TET are in line with published monitoring data (NethMap/MARAN, 2017). Table 1 also shows that ERY and SAL resistance appear to be significantly lower in isolates from organic origin.

Multidrug resistance is defined as antimicrobial resistance against three or more classes of antibiotics (NethMap/MARAN, 2021). Figure 2 shows the percentages of isolates exhibiting resistance to 0-5 classes of antimicrobials. It is clear that SAL resistance has a large impact on this parameter. If SAL resistance is included in the calculation, the percentage of multidrug resistant isolates increases from 3.6% to 21.9%.

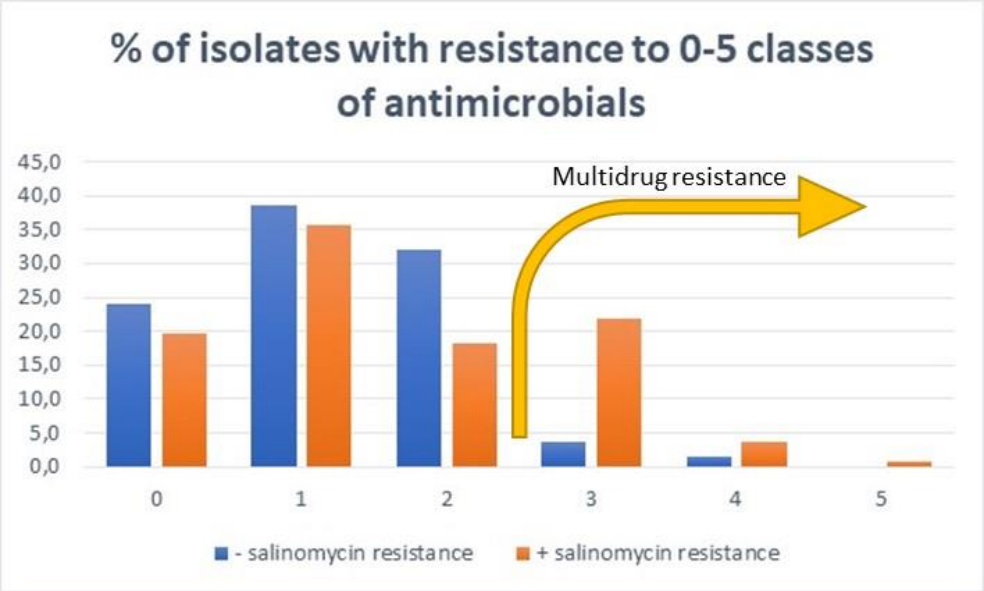

Figure 2 *Percentage of isolates resistant to 0-5 classes of antimicrobials.*

3.2 Statistical analysis of the MIC profiles

To determine whether there is a correlation between the occurrence of SAL resistance and resistance towards other classes of antimicrobials we performed a resampling strategy to obtain confidence intervals for the co-occurrence for each possible pair of antibiotic resistance. This confidence interval reflects the hypothesis that two AMRs co-occur solely on chance, based on both individual AMR occurrences frequencies in the data (sub)set. Isolates from conventional and organic origin were analysed as separate subsets. The analysis was performed using a breakpoint for SAL of >1 mg/L. Figure 3 is a graphical representation of the expected (squares) and actual co-occurrence (circles within the squares). The hight of the bars at the side represents the frequency of occurrence of resistance to an individual antibiotic. The number in a circle indicates the observed frequency of co-occurrence of resistance to the two antibiotics. In case the observed frequency of co-occurrence is higher than what could be expected just by chance, the value and circle are highlighted red.

The fact that the correlation analysis shows multiple significant co-occurrences is not very surprising, since the majority of the resistance genes are known to be located on plasmids (small transferable extrachromosomal DNA molecules), which implicates they easily co-transfer between bacteria (see Figure 3). Thus far it was generally assumed that resistance to ionophores is not plasmid-borne. However, the results of this correlation analysis show statistically significant co-occurrence of SAL resistance with several other types of resistance. In particular the co-occurrence with ERY resistance is evident for both *E. faecium* and *E. faecalis* (except for the *E. faecium* from organic production). This observation provides a strong indication that SAL resistance is occurring physically linked (viz. on the same DNA molecule) with other types of AMR, which has major implications for the (ease of) dissemination of SAL resistance but more importantly for the dissemination of the associated resistance genes.

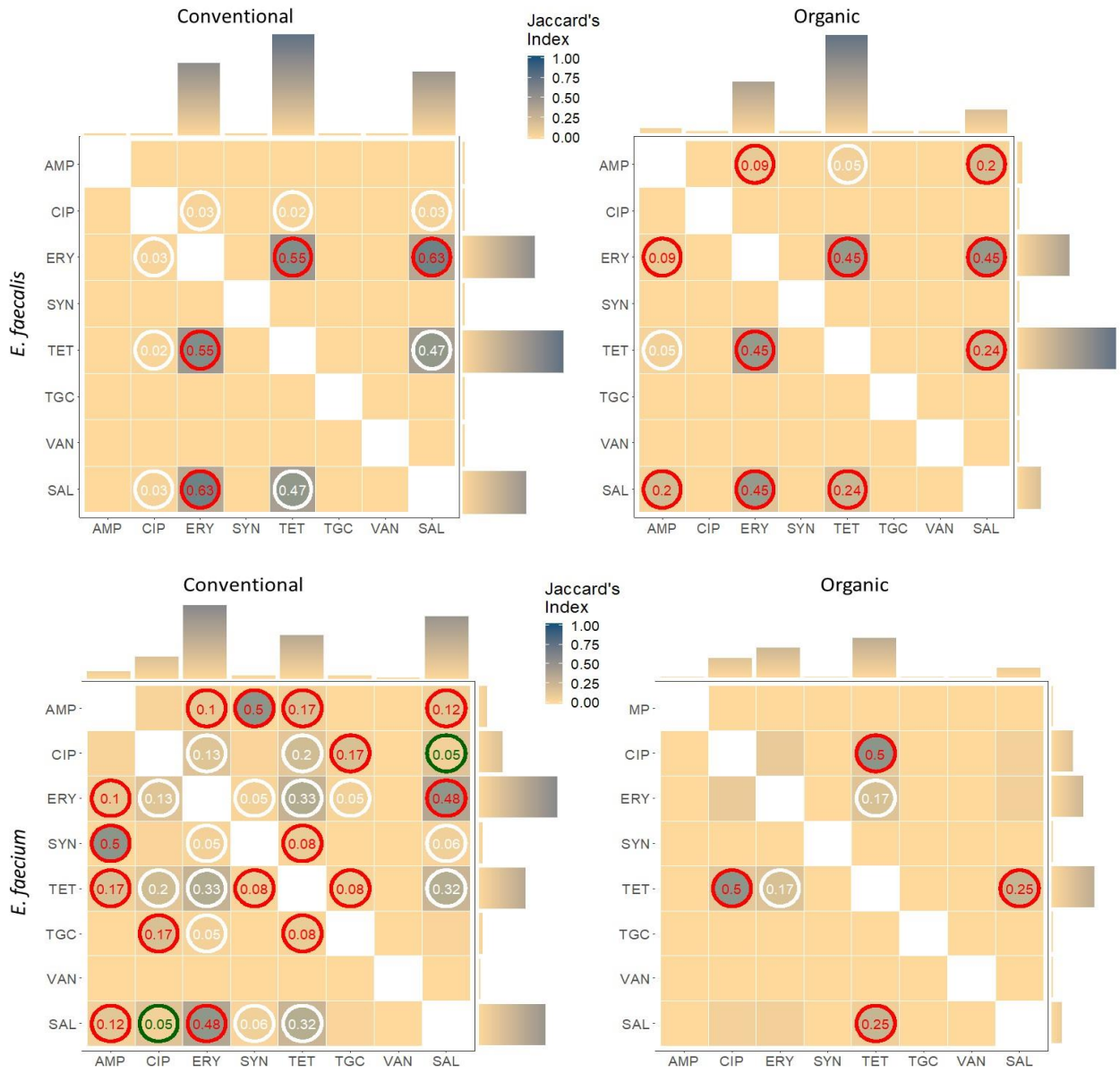

Figure 3 *Co-occurrence of antibiotic resistance in enterococcal isolates, with significance analysis. Bars top/right of plot represent the observed frequency of resistance for individual antibiotics. The squares' color represents the expected Jaccard's index (median value from resampling). The circles' fill color and printed value represent the observed Jaccard's index (no circle if observed value is 0). Red, white and green circle rims indicate >95%, within, and <5% confidence limits, respectively.*

3.3 Genome sequencing and analysis

To determine if SAL resistance is genetically linked to resistance to other antimicrobials, 15 enterococcus isolates (*E. faecium* n=4 and *E. faecalis* n=11) from the 2020 subset with MIC_{SAL} >1 mg/L were selected for genome sequencing along with five susceptible isolates (*E. faecium* n=2 and *E. faecalis* n=3) with MIC_{SAL} ≤1 mg/L.

A hybrid approach was used to reconstruct the full-length sequences of plasmids in each isolate. Both short-read sequence data for accuracy and long-read sequencing data to bridge repetitive regions were used. Short-read sequencing, whilst very accurate at nucleotide level, falls short when attempting to accurately link reads together over longer distances to reproduce entire DNA molecules with repetitive sequences. Examples of such sequences are those that code for transposases, which are common in bacterial plasmids. Long-read sequencing is less accurate at nucleotide level but can bridge across such areas.

The complement of known resistance genes determined to be present in these isolates based on Illumina short-read sequencing (Annex 2) was in agreement with the expected phenotypic resistances shown in the MIC profiles (Annex 1). Specifically, *narAB* resistance genes were determined to be present in all isolates with an elevated MIC_{SAL} and absent from all susceptible isolates (MIC_{SAL} ≤1). This is in accordance with the observations of Nilsson et al. (2012), who showed the presence of the *narAB* operon in isolates with MIC_{SAL} ≥2 mg/L in *Enterococcus faecium* isolated from Swedish broilers.

The hybrid assembly allowed us to determine the genomic background and assess the potential co-occurrence of *narA* and *narB* with other resistance genes. The majority of assembled sequences were reported as circular, which is an indicator of successful reconstruction of the full sequence of the chromosome or plasmid. Furthermore, the same resistance genes as were determined to be present using Illumina (short-read) sequencing data could be detected in the hybrid assembly, indicating that the assembly procedure had not excluded relevant sequence information. The hybrid assembly can therefore provide an overview of all known resistances that co-occur on a plasmid. Thirteen out of fifteen *narAB*-carrying sequences were unequivocally reported as circular, showing sufficiently uniform long-read coverage to suggest complete and correct assembly (see also Annex 3 and Annex 4). Two sequences showed minor artefacts. In one of these two sequences (Ef2874_2) genes are predicted to be duplicated, but sequence coverage suggests this may not be the case. In the other case (Ef2884_3) a mini plasmid with *narAB* genes is predicted besides a larger plasmid with *narAB* genes. The circularity of this plasmid is based on a single read, which is probably a sequencing artefact. It is unlikely that this predicted mini plasmid exists. The overall conclusions about gene content in these two isolates are most likely accurate, despite assembly errors that lead to duplicate predictions. A graphical overview of phenotypic resistance and genetic determinants identified in the sequenced strains is provided in Figure 4.

In all SAL-resistant isolates the *narAB* genes were found to be plasmid-borne. Most of the reconstructed plasmids encode multiple other resistance genes (Figure 4). In 10 out of 11 *E. faecalis* isolates containing *narAB,* the plasmid also carries *erm(B)* comprising ERY resistance. Of these, 9 also carry genetic determinants (either *tet(L)* and *tet(M)* or *tet(O)*) conferring TET resistance. Within the *E. faecalis* isolates pPD1-like plasmids with rep9c replicons were found to occur most often (7 out of 11 isolates). Four of these plasmids carried *ant(6)-Ia*, *aph(3')-III*, *erm(B)*, *tet(O)*, and *dfrG* AMR genes besides *narAB*. The three remaining pPD1-like plasmids carry *erm(B)*, *tet(M)*, and *tet(L)* besides *narAB*. In plasmids occurring less-frequently in *E. faecalis* different combinations of these genes and *ant(9)-Ia* were found.

In *E. faecium narAB* was most frequently found on pRE25-like plasmids (three out of four *narAB* -carrying plasmids). One of these plasmids also carried *lsa(E)*, *lnu(B)* resistance genes, and a partial *erm(B)* resistance gene.

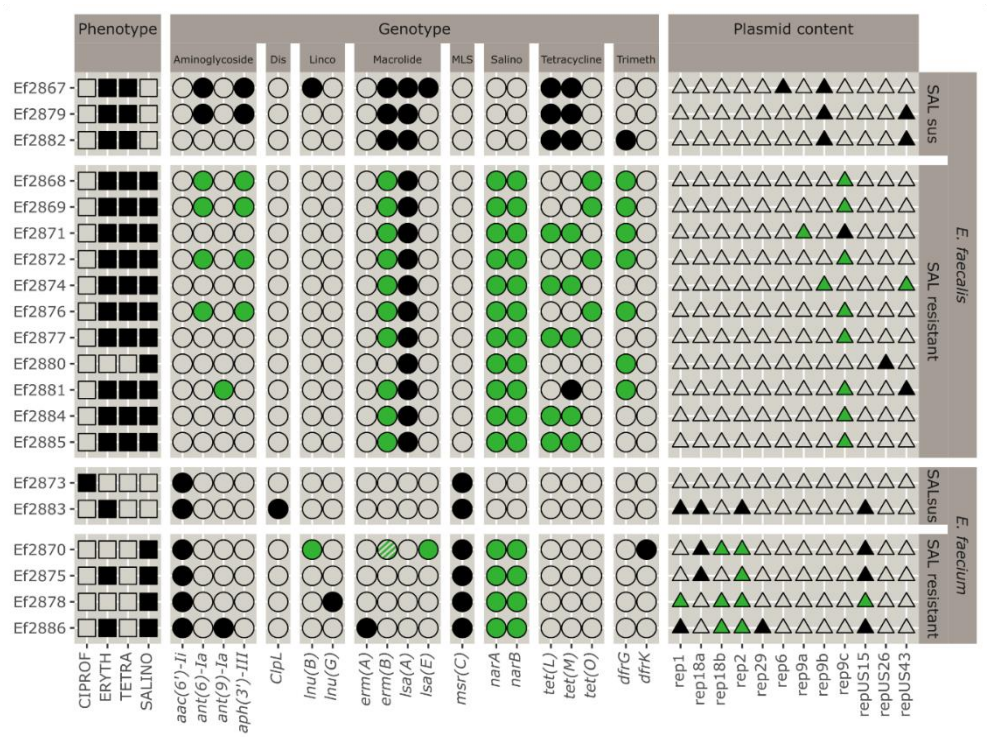

Figure 4 *Overview of antibiotic resistance phenotype (squares), genotype (circles) and plasmid content (triangles) of sequenced isolates. Black squares indicate resistance above clinical breakpoint or MICSAL > 1 mg/L. Filled circles indicate the presence of genes in the isolate, where green indicates that the resistance gene is on the same plasmid as narAB. Hatched circles indicate the presence of an incomplete and presumed non-functional copy of a resistance gene. Predicted plasmid replicons are indicated by filled triangles, where green indicates the replicon is found on the same plasmid as narAB. Abbreviations used: Ciprof – Ciprofloxacin; Dis – Disinfectant; Eryth – Erythromycin; Linco – Lincosamide, MLS – Macrolide, Lincosamide and Streptogramin B; Salino, SAL – Salinomycin; sus – susceptible; Tetra – Tetracyclin; Trimeth – Trimethoprim.*

Summarizing, the results show plasmid colocalization of *narAB* with multiple other genetic resistance determinants, in particular with genes conferring TET and ERY resistance. This implies that selective pressure caused by application of ionophores will promote the persistence of TET and ERY resistance in the enterococcal population.

Retrospectively, the observation that *narAB* and thus ionophore resistance is present in all isolates with an MIC_{SAL} >1 mg/L has significant consequences for the presumed resistance % of the enterococci in 2013, when they were last monitored for SAL resistance. Table 2 shows the MIC distributions for SAL in *E. faecalis* and *E. faecium* as reported in the MARAN report of 2014 (NethMap/MARAN, 2014). If the cut-off is lowered from >4 to >1, the resistance % in *E. faecium* raises from 38.5 to 61.9% and in *E. faecalis* from 5.6 to 61.9%.

Table 2 *MIC distributions (in %) for E. faecalis (N = 266) and E. faecium (N = 423) isolated from conventional and organic broilers in the Netherlands in 2013 (extracted from NethMap/MARAN, 2014).*

Species	N	MIC (%) distribution mg/L						R% > 4	R% > 1
		0.5	*1*	*2*	*4*	*8*	*16*		
E. faecalis	266	4.1	33.8	8.6	47.7	5.6	-	5.6	61.9
E. faecium	423	0.2	15.1	7.8	38.3	38.5	-	38.5	84.6

4 Conclusions

This pilot study shows a widespread occurrence of transferrable *narAB* and co-occurrence of *narAB* with clinically relevant types of AMR in enterococci isolated from poultry. This is an alarming observation. Opposed to previously made assumptions (Tweede Kamer der Staten-Generaal, 2020) it shows that there is a relationship between the use of (ionophore) coccidiostats and the occurrence of other types of AMR relevant for animal and human health. Our findings imply that the application of ionophores in broiler production may be a major driver for the persistence of clinically relevant AMR in enterococci within the poultry domain, and could have human health implications when resistant strains are transmitted to humans, either through food, occupational contact, or the environment.

Unfortunately, the importance assigned to enterococci in resistance monitoring in both the human and the animal domain has decreased over the last years. Harmonized EU surveillance of enterococci in the animal domain was discontinued in 2014 and is monitored only on a voluntary basis. In practice this means that AMR data are very sparse, precluding any trend monitoring. In the Netherlands no enterococcal resistance data have been reported since 2017 for the animal domain. National surveillance of enterococci in the human domain has also been downsized; molecular typing of VRE isolates was discontinued in 2018. *E. faecium* and *E. faecalis* do however account for a significant percentage of pathogens isolated from hospital patients. In 2020, 5%, 10% and 14% of the pathogens isolated from blood, wounds, and pus and urine, respectively, belonged to these two species. In blood of ICU patients they even accounted for 17% of the isolated pathogens (NethMap/MARAN, 2021).

In this study we have shown the possible impact of ionophore use on persistence of AMR in enterococci. However, considering the fact that ionophores have antimicrobial activity against Gram-positive bacteria, co-occurrence of ionophore resistance with resistance against clinically relevant classes of antimicrobials might be present in other Gram-positive pathogens, for example *Staphylococcus aureus*, as well.

The co-occurrence of narasin/salinomycin resistance and vancomycin resistance that was observed in Norway and Sweden and culminated in the discovery of these first mobile ionophore resistance genes, was not observed in our selection of enterococci. Recent information on the prevalence of vancomycin resistant enterococci from poultry is lacking, but is expected to be low. To determine possible co-occurrence of vancomycin and ionophore resistance, selective enrichment should be employed. The co-occurrence of *narAB* with erythromycin and tetracycline resistance genes, however, is equally worrying, since the use of ionophores will inevitably result in multiplication and spread of any co-occurring type of resistance. The use of ionophores is therefore counteracting efforts to reduce the prevalence of AMR in poultry by more prudent use of antibiotics.

5 Recommendations

Our study shows that, opposed to previously made assumptions, a relationship between the use of coccidiostats and the occurrence and dissemination of resistance to therapeutically used antibiotics does exist. The current study was limited to enterococci isolated from poultry meat, representing only a limited segment of the potential issue. Follow-up on this pilot study focussing on additional domains (on farm, human isolates) is therefore urgently needed.

Obviously, more research is needed into the prevalence of the co-occurrence of *narAB* with other types of AMR in enterococci throughout the entire poultry chain, as well as in human enterococcal isolates, to determine possible zoonotic transmission. In parallel, the genetic diversity of the enterococci should be investigated, to allow for an in-depth analysis of the evolutionary relatedness of these enterococci on a population level.

Given the continuous selection pressure and the fact that varying ionophore resistance profiles have been observed in enterococcal isolates, it is to be expected that *narAB* is not the only ionophore resistance gene operon occurring in poultry-associated enterococci. Research should be initiated into the possible existence of other types of ionophore resistance. This search for ionophore resistance genes should be extended to other Gram-positive bacteria (for example *S. aureus*) as these are also susceptible to ionophores and thus may also have evolved resistance mechanisms against these substances.

The Norwegian poultry industry has shown that abolishing the use of ionophores (which was limited to narasin) as a feed additive is an effective mitigation strategy. To control Eimeria infections anticoccidial vaccines were introduced which coincided with an increased focus on cleaning and disinfection. In 2014 the proportion of the *E. faecium* population showing narasin resistance was 91%, while two years after the abolishment, in 2018, the percentage had dropped to 25%, and the previously observed persistent vancomycin resistance disappeared (Simm, 2019). It is therefore likely that restricting the use of ionophores in Dutch poultry production will significantly diminish the prevalence of *narAB* (and thus SAL resistance). This is also expected to decrease the prevalence of other frequently occurring types of AMR in poultry-associated enterococci. Thus, current management practices with respect to ionophore use in poultry will need to be reconsidered. The positioning of ionophores as feed additive requires thorough review, and measures should be taken to instigate a more prudent use. The recording (by the SDa) of ionophore usage data in a manner similar to that of the other veterinary antibiotics should be pursued. Abandoning of prophylactic use of ionophores will be inevitable, therefore alternative options for the management of coccidiosis, such as vaccination, need to be explored.

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Annex 1 Results susceptibility testing

Sample	Alias ID	Year	Country of origin	Genus	Species	AMP	CHL	CIP	DAP	ERY	GEN	LZD	SYN	TEI	TET	TGC	VAN	SAL
					ECOFF *E. faecium* ->	>4	>32	>4	>8	>4	>32	>4	>4	>2	>4	>0.25	>4	(>2)
					ECOFF *E. faecalis* ->	>4	>32	>4	>4	>4	>32	>4	Intrins.	>2	>4	>0.5	>4	(>2)
1	08OT-EF0799	2008	NL	Enterococcus	faecalis	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
2	Ent12_1012	2012	NL	Enterococcus	faecium	= 2	= 8	= 8	= 4	<= 1	<= 8	= 4	= 1	<= 0.5	= 64	= 0.12	<= 1	= 0.5
3	Ent12_1358	2012	NL	Enterococcus	faecium	= 2	= 32	= 4	= 8	= 2	<= 8	= 4	= 4	<= 0.5	= 128	= 0.12	<= 1	= 8
4	EF0229	2013	NL	Enterococcus	faecalis	= 1	= 8	= 1	= 2	= 128	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	<= 0.25
5	EF0236	2013	NL	Enterococcus	faecalis	<= 0.5	= 8	= 2	= 2	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	= 2	<= 0.25
6	EF0261	2013	NL	Enterococcus	faecalis	= 4	= 8	= 2	= 2	= 2	= 16	= 2	= 8	<= 0.5	= 128	= 0.25	= 4	= 0.5
7	EF0272	2013	NL	Enterococcus	faecium	<= 0.5	= 8	= 2	= 4	<= 1	<= 8	= 4	= 4	= 1	<= 1	= 0.12	<= 1	<= 0.25
8	EF0294	2013	NL	Enterococcus	faecium	= 2	= 8	= 0.5	= 4	> 128	= 16	= 2	= 4	<= 0.5	= 128	= 0.12	<= 1	= 0.5
9	EF0343	2013	NL	Enterococcus	faecalis	> 64	= 8	= 4	= 4	> 128		= 2	= 4	<= 0.5	= 128	= 0.12	<= 1	= 2
10	EF0351	2013	NL	Enterococcus	faecalis	= 1	= 8	= 1	= 2	= 2	<= 8	= 2	= 8	<= 0.5	= 128	= 0.12	<= 1	<= 0.25
11	EF0352	2013	NL	Enterococcus	faecalis	<= 0.5	= 8	= 1	= 1	> 128	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 1
12	EF0389	2013	NL	Enterococcus	faecalis	= 4	<= 4	= 2	= 4	<= 1	= 16	= 2	= 4	<= 0.5	<= 1	= 0.06	<= 1	<= 0.25
13	Ef01959	2016	n.d.	Enterococcus	faecalis	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 1
14	Ef02013	2016	NL	Enterococcus	faecalis	= 2	= 8	<= 0.12	= 2	= 2	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 0.5
15	Ef02016	2016	NL	Enterococcus	faecalis	= 2	= 8	= 2	= 2	= 2	<= 8	= 2	= 8	<= 0.5	= 32	= 0.12	<= 1	<= 0.25
16	Ef02017	2016	NL	Enterococcus	faecalis	= 1	= 8	= 1	= 2	= 2	= 16	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	= 0.5
17	Ef02634	2018	NL	Enterococcus	faecalis	= 1	= 16	= 1	= 4	> 128	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
18	Ef02779	2018	BE	Enterococcus	faecium	= 2	= 8	= 8	= 8	<= 1	<= 8	= 4	<= 0.5	<= 0.5	= 64	= 0.06	<= 1	= 0.5
19	Ef02803	2018	NL	Enterococcus	faecium	<= 0.5	= 8	= 0.5	= 8	= 8	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.12	<= 1	= 1
20	EF0002	2013	BE	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
21	EF0005	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	> 128	<= 8	= 1	= 16	<= 0.5	= 32	= 0.12	= 2	= 2
22	EF0020	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 16	= 0.5	> 128	<= 8	= 1	= 16	<= 0.5	= 64	= 0.12	= 2	= 2
23	EF0050	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	<= 1	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
24	EF0082	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 0.5	= 1	= 2	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	<= 0.25
25	EF0088	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	= 2	= 0.5
26	EF0090	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	= 2	= 0.5
27	EF0091	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	<= 8	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	= 0.5
28	EF0124	2013	NL	*Enterococcus*	*faecalis*	= 2	= 8	= 1	= 1	> 128	<= 8	= 2	= 8	<= 0.5	= 32	= 0.12	<= 1	= 2
29	EF0149	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 1	= 2	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	<= 1	<= 0.25
30	EF0172	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 1	= 1	> 128	<= 8	= 1	= 16	<= 0.5	= 128	= 0.12	<= 1	= 4

31	EF0175	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	= 4	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	<= 0.25
32	EF0192	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 32	= 0.06	= 2	= 0.5
33	EF0197	2013	DE	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
34	EF0221	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	> 128	<= 8	= 1	= 16	<= 0.5	= 32	= 0.06	<= 1	= 2
35	EF0232	2013	DE	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	<= 8	= 1	= 32	<= 0.5	= 32	= 0.06	= 2	= 2
36	EF0240	2013	NL	*Enterococcus*	*faecium*	= 1	= 8	= 8	= 4	= 8	<= 8	= 2	<= 0.5	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
37	EF0242	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 4
38	EF0246	2013	NL	*Enterococcus*	*faecium*	= 1	= 16	= 8	= 8	= 4	<= 8	= 4	= 4	<= 0.5	= 128	= 0.12	= 2	= 4
39	EF0253	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	= 8	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 2
40	EF0298	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	= 2	<= 8	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	= 0.5
41	EF0314	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	= 16	= 2	= 16	<= 0.5	= 32	= 0.12	= 2	= 1
42	EF0329	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	= 2	= 0.5
43	EF0347	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 64	= 0.12	= 4	= 0.5
44	EF0349	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 0.5	= 2	> 128	<= 8	= 1	= 8	<= 0.5	= 32	= 0.25	= 4	= 2
45	EF0365	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 0.5	= 1	> 128		= 1	= 16	<= 0.5	= 128	= 0.12	= 2	= 2
46	EF0373	2013	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
47	EF0377	2013	NL	*Enterococcus*	*faecalis*	= 2	= 8	= 4	= 4	= 2	<= 8	= 2	= 2	<= 0.5	= 64	= 0.12	<= 1	= 4
48	EF0380	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 0.5
49	EF0388	2013	NL	*Enterococcus*	*faecalis*	<= 0.5	= 8	= 0.5	= 1	> 128	= 16	= 1	= 16	<= 0.5	= 32	= 0.06	= 2	= 2
50	Ef01908	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	= 16	= 1	= 32	<= 0.5	<= 1	= 0.06	= 4	= 2
51	Ef01912	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	<= 8	= 1	= 16	<= 0.5	<= 1	= 0.06	<= 1	= 2
52	Ef01914	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	= 16	= 1	= 16	<= 0.5	<= 1	= 0.06	<= 1	= 1
53	Ef01922	2016	n.d.	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	<= 1	= 4
54	Ef01929	2016	n.d.	*Enterococcus*	*faecalis*	= 1	<= 4	= 0.25	= 1	<= 1	= 16	= 1	= 8	<= 0.5	= 128	= 0.12	<= 1	= 1
55	Ef01932	2016	IT	*Enterococcus*	*faecium*	= 32	= 16	= 4	= 4	> 128	= 16	= 2	= 4	<= 0.5	= 128	= 0.12	<= 1	= 4
56	Ef01934	2016	NL	*Enterococcus*	*faecium*	= 2	= 8	= 2	= 4	<= 1	<= 8	= 4	<= 0.5	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
57	Ef01938	2016	NL	*Enterococcus*	*faecium*	= 1	= 8	= 4	= 4	= 2	<= 8	= 2	<= 0.5	<= 0.5	<= 1	<= 0.03	<= 1	= 0.5
58	Ef01939	2016	NL	*Enterococcus*	*faecium*	= 1	= 8	= 4	= 2	> 128	<= 8	= 2	= 1	<= 0.5	= 64	= 0.06	<= 1	= 4
59	Ef01941	2016	DE	*Enterococcus*	*faecalis*	= 1	= 8	= 2	= 2	> 128	= 16	= 1	= 16	<= 0.5	= 32	= 0.12	= 2	= 0.5
60	Ef01947	2016	NL	*Enterococcus*	*faecium*	= 1	= 8	= 2	= 4	= 8	<= 8	= 2	= 1	<= 0.5	<= 1	= 0.06	= 2	= 4
61	Ef01951	2016	NL	*Enterococcus*	*faecium*	= 4	= 8	= 8	= 2	<= 1	<= 8	= 2	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
62	Ef01952	2016	NL	*Enterococcus*	*faecium*	= 1	= 8	= 1	= 4	= 8	= 16	= 2	= 1	<= 0.5	<= 1	= 0.06	= 2	= 0.5
63	Ef01954	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 2
64	Ef01958	2016	n.d.	*Enterococcus*	*faecalis*	= 2	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	= 32	= 0.12	<= 1	= 2
65	Ef01963	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 0.5	= 2	= 4	= 16	= 2	= 8	<= 0.5	<= 1	= 0.12	<= 1	= 1
66	Ef01965	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 2	> 128	= 16	= 2	= 16	<= 0.5	= 128	= 0.12	= 2	= 4
67	Ef01971	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 2	= 4	<= 1	= 16	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	<= 0.25
68	Ef01984	2016	n.d.	*Enterococcus*	*faecalis*	= 1	= 8	= 0.5	= 2	> 128	= 16	= 2	= 8	<= 0.5	= 32	= 0.12	= 4	= 4
69	Ef01986	2016	n.d.	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 1	= 4	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
70	Ef02002	2016	NL	*Enterococcus*	*faecium*	<= 0.5	<= 4	= 8	= 4	> 128	= 16	= 1	<= 0.5	<= 0.5	= 64	= 0.06	<= 1	= 0.5

71	Ef02007	2016	NL	*Enterococcus*	*faecium*	= 2	= 16	= 4	= 8	= 128	= 16	= 2	= 4	<= 0.5	<= 1	= 0.06	= 2	= 0.5
72	Ef02009	2016	DE	*Enterococcus*	*faecium*	= 1	= 8	= 2	= 4	= 8	<= 8	= 4	= 1	<= 0.5	<= 1	= 0.12	= 2	= 0.5
73	Ef02020	2016	DE	*Enterococcus*	*faecium*	= 1	= 8	= 2	= 4	= 8	<= 8	= 4	<= 0.5	<= 0.5	<= 1	= 0.06	= 2	= 2
74	Ef02021	2016	NL	*Enterococcus*	*faecium*	= 1	= 16	= 4	= 8	> 128	<= 8	= 4	= 1	<= 0.5	= 64	= 0.12	<= 1	= 2
75	Ef02022	2016	NL	*Enterococcus*	*faecium*	= 2	= 8	= 2	= 4	= 4	<= 8	= 2	= 1	<= 0.5	<= 1	= 0.06	<= 1	= 2
76	Ef02026	2016	NL	*Enterococcus*	*faecium*	= 1	= 16	= 4	= 4	= 2	= 16	= 2	= 4	<= 0.5	= 128	= 0.12	<= 1	= 0.5
77	Ef02027	2016	NL	*Enterococcus*	*faecium*	= 2	= 8	= 2	= 4	= 4	<= 8	= 2	= 1	<= 0.5	<= 1	= 0.06	= 2	= 1
78	Ef02030	2016	NL	*Enterococcus*	*faecium*	= 1	= 16	= 4	= 4	> 128	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 2
79	Ef02031	2016	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 2	= 4	> 128	= 16	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	= 0.5
80	Ef02602	2018	NL	*Enterococcus*	*faecalis*	= 1	= 8	= 1	= 4	<= 1	= 32	= 2	= 8	<= 0.5	<= 1	= 0.25	= 2	= 0.5
81	Ef02607	2018	FR	*Enterococcus*	*faecium*	= 2	= 8	= 4	= 4	= 4	<= 8	= 4	<= 0.5	<= 0.5	= 128	= 0.12	<= 1	= 1
82	Ef02609	2018	NL	*Enterococcus*	*faecium*	= 1	= 8	= 4	= 4	= 4	<= 8	= 4	= 1	<= 0.5	<= 1	= 0.12	<= 1	= 2
83	Ef02612	2018	DE	*Enterococcus*	*faecalis*	= 1	= 8	= 2	= 2	> 128	= 16	= 2	= 16	<= 0.5	= 128	= 0.5	= 4	= 4
84	Ef02621	2018	NL	*Enterococcus*	*faecium*	<= 0.5	= 16	= 4	= 4	> 128	<= 8	= 4	= 4	<= 0.5	= 64	= 0.25	<= 1	= 4
85	Ef02630	2018	NL	Enterococcus	*faecalis*	<= 0.5	<= 4	= 0.5	= 4	= 16	= 32	= 2	= 4	<= 0.5	<= 1	= 0.12	<= 1	<= 0.25
86	Ef02651	2018	NL	Enterococcus	*faecium*	= 2	= 16	= 16	= 2	= 8	<= 8	= 4	= 4	<= 0.5	= 128	= 0.5	= 2	= 0.5
87	Ef02658	2018	NL	Enterococcus	*faecium*	> 64	= 16	= 4	= 8	> 128		= 4	= 8	<= 0.5	= 128	= 0.25	<= 1	= 4
88	Ef02669	2018	NL	Enterococcus	*faecium*	= 2	<= 4	= 0.5	= 4	= 16	<= 8	= 2	= 4	<= 0.5	<= 1	= 0.25	<= 1	= 0.5
89	Ef02678	2018	NL	Enterococcus	*faecium*	= 1	= 8	= 2	= 4	= 8	<= 8	= 4	= 1	<= 0.5	<= 1	= 0.25	= 2	= 2
90	Ef02695	2018	n.d.	Enterococcus	*faecalis*	= 1	= 16	= 2	= 2	= 32	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.25	= 2	= 1
91	Ef02721	2018	NL	Enterococcus	*faecium*	= 2	<= 4	= 0.25	= 8	= 32	<= 8	= 2	= 4	<= 0.5	<= 1	= 0.25	<= 1	= 1
92	Ef02725	2018	NL	Enterococcus	*faecalis*	= 1	= 8	= 2	= 4	> 128	= 16	= 2	= 8	<= 0.5	= 128	= 0.25	= 4	= 4
93	Ef02776	2018	NL	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	= 16	= 2	= 8	<= 0.5	<= 1	= 0.25	<= 1	= 0.5
94	Ef02777	2018	NL	Enterococcus	*faecium*	= 2	= 8	= 4	= 4	> 128	<= 8	= 2	= 1	<= 0.5	= 128	= 0.25	<= 1	= 4
95	Ef02826	2018	DE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	> 128	= 16	= 1	= 8	<= 0.5	= 32	= 0.12	= 2	= 4
96	2020-1	2020	BE	Enterococcus	*faecium*	= 2	= 8	= 4	= 4	= 2	<= 8	= 4	= 1	<= 0.5	<= 1	= 0.06	<= 1	= 1
97	2020-2	2020	BE	Enterococcus	*faecium*	= 2	= 8	= 4	= 4	= 4	= 16	= 2	= 1	<= 0.5	<= 1	= 0.06	<= 1	= 1
98	2020-3	2020	BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 16	<= 0.5	= 128	= 0.25	= 2	<= 0.25
99	2020-4	2020	BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	<= 0.5	= 16	<= 0.5	<= 1	<= 0.03	= 2	<= 0.25
100	2020-5	2020	BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 1	<= 1	<= 8	= 2	= 32	<= 0.5	= 128	= 0.12	<= 1	<= 0.25
101	**Ef2867**	2020	IT	Enterococcus	*faecalis*	= 2	= 16	= 0.5	= 2	> 128	= 16	= 2	= 32	<= 0.5	= 128	= 0.25	<= 1	= 0.5
102	**Ef2868**	2020	NL	Enterococcus	*faecalis*	<= 0.5	= 8	= 0.5	= 2	> 128	<= 8	= 2	= 16	<= 0.5	= 64	= 0.12	= 2	= 2
103	**Ef2869**	2020	NL	Enterococcus	*faecalis*	<= 0.5	= 8	= 0.5	= 1	> 128	<= 8	= 2	= 16	<= 0.5	= 32	= 0.12	= 2	= 2
104	**Ef2870**	2020	NL	Enterococcus	*faecium*	= 2	= 8	= 4	= 2	<= 1	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 4
105	**Ef2871**	2020	NL	Enterococcus	*faecalis*	= 1	= 8	= 1	= 1	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	<= 1	= 8
106	**Ef2872**	2020	NL	Enterococcus	*faecalis*	= 1	= 8	= 0.5	= 2	> 128	= 16	= 2	= 16	<= 0.5	= 64	= 0.12	= 4	= 2
107	2020-12	2020	NL	Enterococcus	*faecalis*	= 2	= 8	= 1	= 2	<= 1	= 16	= 2	= 16	<= 0.5	<= 1	= 0.12	= 2	<= 0.25
108	2020-13	2020	NL	Enterococcus	*faecium*	= 1	= 8	= 1	= 4	= 4	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
109	2020-14	2020	n.d.	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 64	= 0.12	<= 1	= 0.5
110	**Ef2873**	2020	NL	Enterococcus	*faecium*	= 1	= 8	= 8	= 4	= 2	= 16	= 4	= 4	= 1	<= 1	= 0.06	= 2	= 0.5

111	2020-16	2020	NL	Enterococcus	*faecalis*	= 2	= 8	= 1	= 2	<= 1	= 16	= 2	= 4	<= 0.5	= 64	= 0.12	<= 1	= 0.5
112	**Ef2874**	2020	DU	Enterococcus	*faecalis*	= 2	= 8	= 1	= 1	= 64	<= 8	= 1	= 16	<= 0.5	= 128	= 0.25	<= 1	= 4
113	**Ef2875**	2020	NL	Enterococcus	*faecium*	= 1	= 8	= 1	= 8	= 8	<= 8	= 2	<= 0.5	= 1	<= 1	<= 0.03	= 2	= 4
114	**Ef2876**	2020	NL	Enterococcus	*faecalis*	= 2	= 8	= 0.5	= 1	> 128	<= 8	= 1	= 8	<= 0.5	= 32	= 0.06	= 2	= 2
115	**Ef2877**	2020	NL DU BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 1	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.25	<= 1	= 4
116	2020-21	2020	NL DU	Enterococcus	*faecium*	= 2	= 8	= 2	= 8	<= 1	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.06	= 4	= 1
117	**Ef2878**	2020	NL DU	Enterococcus	*faecium*	<= 0.5	= 8	= 4	= 2	= 4	<= 8	= 4	<= 0.5	<= 0.5	<= 1	<= 0.03	<= 1	= 8
118	**Ef2879**	2020	FR	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	> 128	<= 8	= 1	= 8	<= 0.5	= 128	= 0.12	<= 1	<= 0.25
119	**Ef2880**	2020	BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	= 2	= 16	= 2	= 8	<= 0.5	<= 1	= 0.06	= 4	= 4
120	**Ef2881**	2020	BE	Enterococcus	*faecalis*	= 2	= 16	= 2	= 2	> 128	= 16	= 2	= 32	<= 0.5	= 128	= 0.12	= 2	= 2
121	2020-26	2020	NL	Enterococcus	*faecalis*	= 1	= 8	= 1	= 4	<= 1	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.06	<= 1	= 0.5
122	**Ef2882**	2020	IT	Enterococcus	*faecalis*	<= 0.5	= 8	= 1	= 2	> 128	<= 8	= 1	= 16	<= 0.5	= 128	= 0.12	= 2	= 0.5
123	2020-28	2020	IT	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 128	= 0.25	<= 1	= 0.5
124	2020-29	2020	BE	Enterococcus	*faecium*	= 2	<= 4	= 2	= 4	<= 1	= 32	= 2	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 1
125	2020-30	2020	BE	Enterococcus	*faecalis*	= 2	= 8	= 0.5	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 128	= 0.12	<= 1	= 1
126	2020-31	2020	NL	Enterococcus	*faecalis*	= 2	= 8	= 1	= 4	= 2	<= 8	= 2	= 8	<= 0.5	<= 1	= 0.06	= 2	= 0.5
127	2020-32	2020	NL	Enterococcus	*faecium*	= 2	= 8	= 4	= 4	= 2	= 16	= 4	= 1	<= 0.5	<= 1	<= 0.03	<= 1	= 0.5
128	**Ef2883**	2020	NL	Enterococcus	*faecium*	= 2	= 8	= 1	= 4	= 8	<= 8	= 4	= 4	<= 0.5	<= 1	= 0.06	<= 1	= 1
129	2020-34	2020	BE	Enterococcus	*faecalis*	= 1	= 8	= 1	= 2	<= 1	<= 8	= 2	= 8	<= 0.5	= 128	= 0.12	= 2	<= 0.25
130	2020-35	2020	IT	Enterococcus	*faecium*	= 1	= 8	= 4	= 4	= 2	= 16	= 4	<= 0.5	<= 0.5	<= 1	<= 0.03	<= 1	= 0.5
131	**Ef2884**	2020	NL	Enterococcus	*faecalis*	= 1	= 16	= 1	= 1	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	= 4	= 4
132	**Ef2885**	2020	NL DU	Enterococcus	*faecalis*	= 1	= 8	= 1	= 1	> 128	<= 8	= 2	= 16	<= 0.5	= 128	= 0.12	<= 1	= 4
133	2020-38	2020	NL DU	Enterococcus	*faecalis*	= 1	= 8	= 0.5	= 0.5	<= 1	<= 8	= 2	= 8	<= 0.5	= 128	= 0.06	= 2	= 0.5
134	2020-39	2020	NL	Enterococcus	*faecium*	= 2	= 16	= 4	= 8	= 4	<= 8	= 4	= 1	= 1	= 64	= 0.12	<= 1	= 1
135	2020-40	2020	DU	Enterococcus	*faecalis*	= 1	<= 4	= 0.25	= 1	<= 1	<= 8	<= 0.5	= 2	<= 0.5	= 64	= 0.12	= 2	<= 0.25
136	**Ef2886**	2020	NL DU	Enterococcus	*faecium*	= 1	= 16	= 4	= 4	= 64	= 16	= 2	= 4	<= 0.5	<= 1	= 0.06	= 2	= 8
137	2020-42	2020	n.d.	Enterococcus	*faecium*	= 2	= 8	= 4	= 8	= 2	<= 8	= 4	<= 0.5	<= 0.5	<= 1	= 0.06	<= 1	= 1

Organic prod origin | **Bold: sequenced strains** | > EUCAST cut-off | = experimental SAL cut-off

Annex 2 Resistance genes detected with Illumina short-read sequencing

	resistance							
Isolate	**Aminoglycoside**	**Macrolide**	**Lincosamide**	**Salinomycin**	**Tetracycline**	**Trimethoprim**	**MLS[1]**	**Disinfectant**
Ef2867	ant(6)-Ia, aph(3')-III	erm(B) lsa(A) lsa(E)	lnu(B)	-	tet(L) tet(M)[2]	-	-	-
Ef2868	ant(6)-Ia aph(3')-III	erm(B) lsa(A)	-	NarA NarB	tet(O)[2]	dfrG	-	-
Ef2869	ant(6)-Ia aph(3')-III	erm(B) lsa(A)	-	NarA NarB	tet(O)	dfrG	-	-
Ef2870	aac(6')-Ii	~~erm(B)~~[2,3] lsa(E)	lnu(B)	NarA NarB	-	dfrK	msr(C)	-
Ef2871	-	erm(B) lsa(A)	-	NarA NarB	tet(L) tet(M)[2]	dfrG	-	-
Ef2872	ant(6)-Ia aph(3')-III	erm(B) lsa(A)	-	NarA NarB	tet(O)[2]	dfrG	-	-
Ef2873	aac(6')-Ii	-	-	-	-	-	msr(C)	-
Ef2874	-	erm(B) lsa(A)	-	NarA NarB	tet(L) tet(M)[2]	-	-	-
Ef2875	aac(6')-Ii	-	-	NarA NarB	-	-	msr(C)	-
Ef2876	ant(6)-Ia aph(3')-III	erm(B) lsa(A)	-	NarA NarB	tet(O)[2]	dfrG	-	-
Ef2877	-	erm(B) lsa(A)	-	NarA NarB	tet(L) tet(M)[2]	-	-	-
Ef2878	aac(6')-Ii	-	lnu(G)	NarA NarB	-	-	msr(C)	-
Ef2879	ant(6)-Ia aph(3')-III	erm(B) lsa(A)	-	-	tet(L) tet(M)[2]	-	-	-
Ef2880	-	lsa(A)	-	NarA NarB	-	dfrG	-	-
Ef2881	ant(9)-Ia	erm(B) lsa(A)	-	NarA NarB	tet(M)	dfrG	-	-
Ef2882	-	erm(B) lsa(A)	-	-	tet(L) tet(M)[2]	dfrG	-	-
Ef2883	aac(6')-Ii	-	-	-	-	-	msr(C)	ClpL
Ef2884	-	erm(B) lsa(A)	-	NarA NarB	tet(L) tet(M)[2]	-	-	-
Ef2885	-	erm(B) lsa(A)	-	NarA NarB	tet(L) tet(M)[2]	-	-	-
Ef2886	aac(6')-Ii ant(9)-Ia	erm(A)	-	NarA NarB	-	-	msr(C)	-

[1] Macrolide, Lincosamide and Streptogramin B.

[2] This gene is reported based on assembled data; it is not detected when reads are used as input.

[3] Gene is not predicted to be full length and is presumed non-functional, but the match scores above the reporting level recommended by the EURL-Antimicrobial Resistance.

Annex 3 Reconstructed plasmids carrying *narAB*

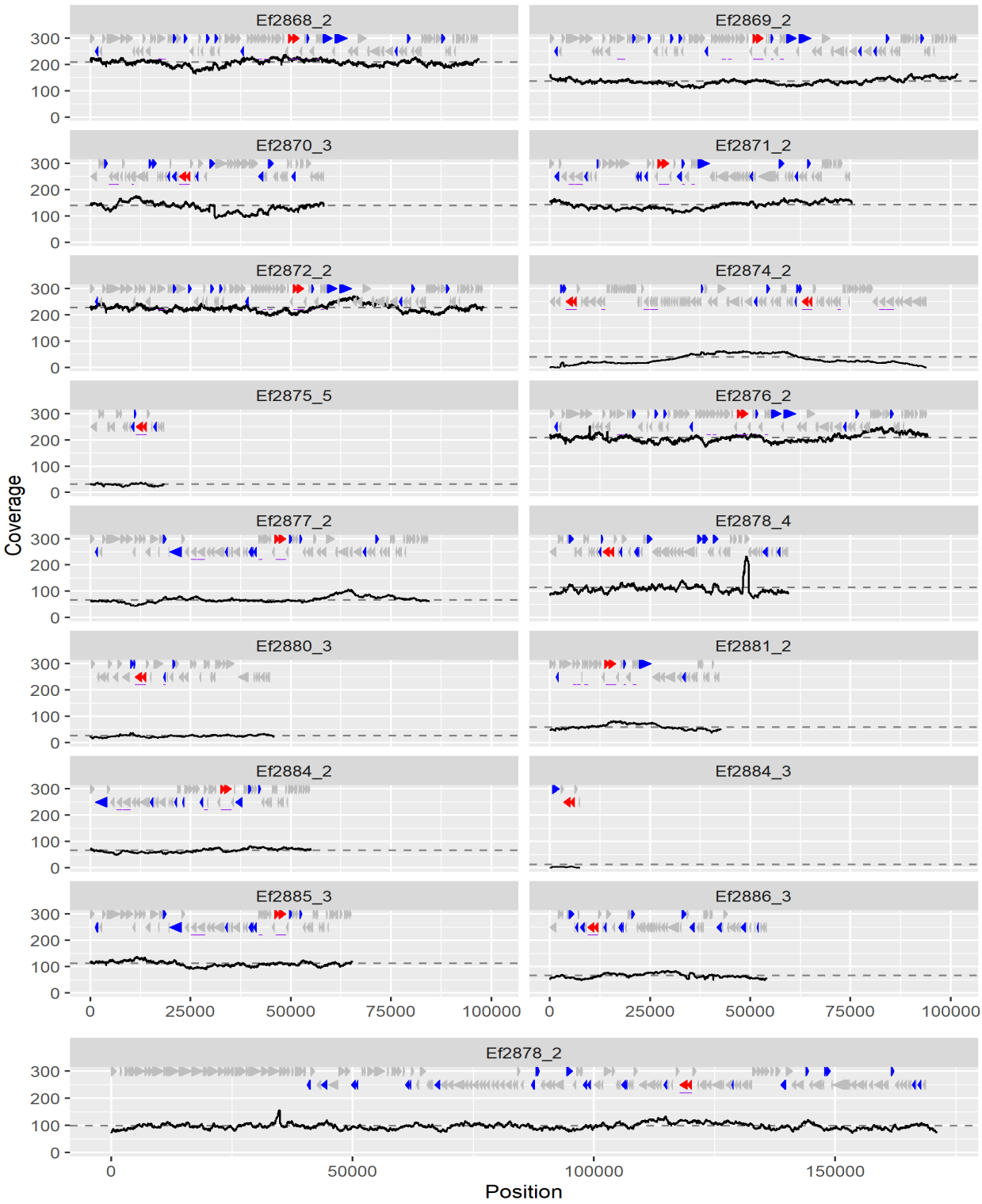
Isolate	Species (MALDI)	Sequence	Length	Replicon	Prototype plasmid	Resistance genes	Notes
Ef2868	E. faecalis	2	96896	rep9c	pPD1	ant(6)-Ia,aph(3')-III,erm(B),tet(O),dfrG,NarB,NarA	
Ef2869	E. faecalis	2	101744	rep9c	pPD1	ant(6)-Ia,aph(3')-III,erm(B),tet(O),dfrG,NarB,NarA	
Ef2870	E. faecium	3	58256	rep2 / rep18a	pRE25 / pE1p13	lsa(E),lnu(B),erm(B),NarB,NarA	
Ef2871	E. faecalis	2	75302	rep9a	pAD1	erm(B),tet(M),tet(L),dfrG,NarB,NarA	
Ef2872	E. faecalis	2	98018	rep9c	pPD1	ant(6)-Ia,aph(3')-III,erm(B),tet(O),dfrG,NarB,NarA	
Ef2874	E. faecalis	2	93957	repUS43 (x2) / rep9b (2x)	D0p1 / EF62pC	erm(B),erm(B),tet(M),tet(M),tet(L),tet(L),NarB,NarB,NarA,NarA	1
Ef2875	E. faecium	5	18443	rep2	pRE25	NarB,NarA	
Ef2876	E. faecalis	2	94206	rep9c	pPD1	ant(6)-Ia,aph(3')-III,erm(B),tet(O),dfrG,NarB,NarA	
Ef2877	E. faecalis	2	84509	rep9c	pPD1	erm(B),tet(M),tet(L),NarB,NarA	
Ef2878	E. faecium	4	59504	rep2 / rep18b	pRE25 / pE1p13	NarB,NarA	
Ef2878	E. faecium	2	171046	repUS15 / rep1	pNB2354p1 / pIP816 or pAMbeta	NarB,NarA	
Ef2880	E. faecalis	3	45783	repUS26	EFD32pB	dfrG,NarB,NarA	
Ef2881	E. faecalis	2	42635	rep9c	pTW9	ant(9)-Ia,erm(B),erm(B),erm(B),erm(B),dfrG,NarB,NarA	2
Ef2884	E. faecalis	2	55071	rep9c	pPD1	erm(B),tet(M),tet(L),NarB,NarA	
Ef2884	E. faecalis	3	7593	-	-	NarB,NarA	3
Ef2885	E. faecalis	3	65314	rep9c	pPD1	erm(B),tet(M),tet(L),NarB,NarA	
Ef2886	E. faecium	3	54096	rep2 / rep18b	pRE25 / pE1p13	NarB,NarA	

[1] Coverage profile suggests this contig is misassembled, leading to genes being duplicated.

[2] A total of four erm(B) database matches are reported for three loci.

[3] The sequence is reported to be circular, based on a single read. This sequence is likely a technical artefact, though the presence of multiple NarA/B alleles in the isolate cannot be ruled out.

Annex 4 Coverage and annotation of *narAB*-carrying plasmids



Coverage and annotation plots of narAB carrying plasmids. Black line: long-read coverage, averaged over a 50 nucleotide moving window. Dashed grey line: median long-read coverage. Triangles: gene annotations, where red indicates NarA/B and blue indicates a predicted transposase. Purple lines under the gene annotations indicate the locations of AMR genes listed in 0.

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WFSR Report 2022.005

The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 6,800 employees (6,000 fte) and 12,900 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.

To explore the potential of nature to improve the quality of life

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