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3	Supplementary for
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5	Collateral sensitivity to pleuromutilins in vancomycin-resistant
6	Enterococcus faecium
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Asia (n = 29) Europe (n = 20) North America (n=17) South America (n = 10) Oceania (n = 3)



55

## Supplementary Fig. 1 Phylogenetic tree of VRE<sub>fm</sub> genomes and types of *van* gene cluster

(a) Phylogenetic tree of prevalent *van* gene clusters in global VRE<sub>fm</sub> isolates,

including *vanA* (n = 71), *vanB* (n = 7) and *vanM* (n = 1). Color strips of the outer ring

60 show the MLST types. More details of the isolates can be found in Supplementary

- 61 Table 3.
- 62 (b) Genotypes of *van* gene clusters in 22 clinical VRE<sub>fm</sub> isolates. *E. faecium* BM4147,
- 63 E. faecium Efm-HS0661 and E. faecium 8672 are reference strains for vanA, vanM
- 64 and *vanB*, respectively.



### 66 Supplementary Fig. 2 The mode of action of pleuromutilins

- 67 (a) Chemical structures of pleuromutilins. Compound 55 in (a) was named in the
- 68 reference<sup>1</sup>.
- 69 (**b**) Modes of action of ribosome-targeting antibiotics.
- 70 (c) Binding sites of pleuromutilins in the PTC domain. No mutations were found at
- the sites in 40 *E. faeciums* isolates. More details about the binding sites were shown in
- 72 Supplementary Table 4.



#### 74 Supplementary Fig. 3 Bacteriostatic activity of lefamulin against VRE<sub>fm</sub>.

75 Time-dependent killing curves of VRE<sub>fm</sub> CAU369 in the presence of lefamulin.

VRE<sub>fm</sub> CAU369 at exponential phase were challenged with  $1 \times MIC$ ,  $10 \times MIC$  and

40×MIC lefamulin. Experiments were performed as three biologically independent

experiments, and the mean  $\pm$  S.D. (n = 3) is shown. *P* values were determined by non-

79 parametric one-way ANOVA.





82 Supplementary Fig. 4 Maintained integrity and functions of VRE<sub>fm</sub> membrane.

(a-d) Membrane permeability (a), membrane potential (b),  $\triangle$ pH (c) and membrane fluidity (d) of VRE<sub>fm</sub> CAU369 treated with lefamulin at the levels of 1×MIC, 5×MIC and 10×MIC. The arrows indicate the time points of added compounds. Nisin (100

- $\mu$ g/mL), CCCP (50  $\mu$ M) and benzyl alcohol (50 mM) were used as positive controls.
- PI (10  $\mu$ M), DiSC3(5) (1  $\mu$ M), BCECF-AM (10  $\mu$ M) and Laurdan were used as probes.
- 89 (e-f) ROS accumulation (e) and levels of intracellular and extracellular ATP (f) in
- $VRE_{fm}$  CAU369 in the presence of lefamulin. ROSup and Nisin (100  $\mu$ g/mL) were
- 91 used as positive controls.
- 92 Experiments were performed as three biologically independent experiments, and the
- mean  $\pm$  S.D. (n = 3) is shown. *P* values were determined by non-parametric one-way
- 94 ANOVA.



#### 96 Supplementary Fig. 5 Accumulated lefamulin in bacteria.

97 (**a-b**) Fold changes of lefamulin accumulation in diverse isolates of *E. faeciums* (**a**), *E.* 

98 *faecalis* and *S. aureus* (**b**). Accumulations of lefamulin in *E. faeciums* (n = 8), *E.* 

99 *faecalis* (n = 2) and *S. aureus* (n = 2) after treatments of 0.3  $\mu$ g/mL lefamulin for 1 h.

100 (**c-j**) Chromatographic and MS/MS spectra of lefamulin in four phenotypes of *E*.

101 *faeciums* isolates. No further chemical modifications were observed in all isolates.

102 Experiments were performed as three biologically independent experiments, and the

103 mean  $\pm$  S.D. (n = 3) is shown. *P* values were determined by non-parametric one-way

104 ANOVA.



#### 106 Supplementary Fig. 6 Multiple resistance genes and virulence genes in *E*.

#### 107 *faeciums* isolates

- 108 (a) Scheme of mechanisms of resistance to antibiotics in *E. faecium*.
- 109 (**b**) Multiple resistance genes and virulence genes in 40 *E. faeciums* isolates.
- 110 Phylogenetic tree of 40 E. faeciums isolates of different origins was shown (Left). Red
- 111 "S" represents that isolates are sensitive to pleuromutilins, and black "R" represents
- resistance. No *cfr* was found in these isolates.



#### 114 Supplementary Fig. 7 No methylation on A2503 in *E. faecium*.

- 115 (a) Locations of A2503 and control site in sequence of PTC.
- 116 (b) qPCR-Ct values of select  $m^6A$  of control site and test site in VRE<sub>fm</sub> CAU369.
- 117 Control site was used as non-methylated negative controls compared with test site.
- 118 Test site is not methylated. All data are presented as mean  $\pm$  S.D (n = 3).
- 119 (c) qPCR-Ct values of select  $m^6A$  of test sites in four phenotypic isolates. A2503 site
- in VRE<sub>fm</sub> CAU369 was used as non-methylated negative controls compared to other
- isolates. All data are presented as mean  $\pm$  S.D (n = 6).
- 122 *P* values were determined by non-parametric one-way ANOVA.
- 123



#### Supplementary Fig. 8 Increased binding of pleuromutilins to ribosomes in PsVr 125 VRE<sub>fm</sub>. 126

- (a) The fluorescence polarization values (FP value) between ribosomes of  $VRE_{fm}$ 127
- CAU369 and pleuromutilin-tracers (using 0.5 µM VAL-DTAF as a model). 128
- (b) FP values of 0.5  $\mu$ M pleuromutilin-tracers binding to the ribosomes (1 nM) of 129
- VRE<sub>fm</sub> CAU369 or VSE<sub>fm</sub> CAU310. 130
- Experiments were performed as three biologically independent experiments, and the 131
- mean  $\pm$  S.D. (n = 3) is shown. P values were determined by non-parametric one-way 132
- ANOVA. 133



## Supplementary Fig. 9 Collateral sensitivity in PsVR VREfm with decreased *msrC* transcription.

- 137 (**a-c**) Transcription analysis of lsaE (**a**), eatAv (**b**) and msrC (**c**).
- 138 *E. faeciums* isolates were treated with lefamulin (0.3  $\mu$ g/mL) for 1 h.
- 139 Experiments were performed as three biologically independent experiments, and the
- 140 mean  $\pm$  S.D. (n = 4) is shown. *P* values were determined by non-parametric one-way
- 141 ANOVA.
- 142



144 Supplementary Fig. 10 Transcription of *msrC* in VRE<sub>fm</sub> in the presense of

145 **lefamulin.** Transcription analysis of *msrC* in the pleuromutilin sensitive isolate

146 VRE<sub>fm</sub> CAU996 (**a**) and the resistant isolate VRE<sub>fm</sub> CAU421 (**b**). Both VRE<sub>fm</sub>

147 isolates were treated with lefamulin for 1 h. Experiments were performed as three

biologically independent experiments, and the mean  $\pm$  S.D. (n = 3) were shown. *P* 

149 values were determined by non-parametric one-way ANOVA.



152 Supplementary Fig. 11 Transcription of *msrC* in pleuromutilin resistant VRE<sub>fm</sub>

- 153 isolates increased in a dose-dependent manner.
- 154 Transcription analysis of *msrC* in pleuromutilin resistant VRE<sub>fm</sub> isolates (a-i). VRE<sub>fm</sub>
- isolates were treated with lefamulin for 1 h. Experiments were performed as three
- biologically independent experiments, and the mean  $\pm$  S.D. (n = 3) is shown. *P* values
- 157 were determined by non-parametric one-way ANOVA.
- 158



Supplementary Fig. 12 The overexpression of *msrC* induces pleuromutilins
resistance.

162 (**a-b**) Design of *msrC* expression plasmid (**a**) and scheme of transformation (**b**).

163 (c) The expression of msrC in the mutant (pAM401+msrC) in the presence of a

164 concentration gradient of lefamulin. Experiments were performed as three

biologically independent experiments, and the mean  $\pm$  S.D. (n = 3) is shown.

166 (d) Comparison of antibiotic susceptibility in wild-type *E. faecium* and mutant.

167

![](_page_14_Figure_0.jpeg)

#### 169 Supplementary Fig. 13 The *vanA* gene clusters in global plasmids.

170 Locations of the *vanA* gene clusters are shown in 14 plasmids. The plasmid pCAU369

- in  $VRE_{fm}$  CAU369 shares the same sequence of the *vanA* gene cluster with that in the
- other 13 global plasmids. The identities of the *vanA* genes are 100%.
- 173

![](_page_15_Figure_0.jpeg)

## Supplementary Fig. 14 Characterization of the conjugant with the plasmid pCAU369.

- (a) High similarity between the plasmid pCAU369 and pBM369 in the conjugant.
- pCAU369 is from VRE CAU369 (donor), pBM369 is from the conjugant BM369-1.
- (b) Growth dynamics of *E. faeciums* BM4105-RF (receptor) treated with sublethal
- 180 levels of lefamulin under oxygen rich (left) and poor (right) conditions.
- 181 (c) Growth dynamics of the conjugant *E. faeciums* BM369-1 of *E. faeciums* BM4105-
- 182 RF treated with sublethal levels of lefamulin under oxygen rich (left) and poor (right)183 conditions.
- 184 Experiments were performed as three biologically independent experiments, and the
- 185 mean  $\pm$  S.D. (n = 3) is shown. *P* values were determined by non-parametric one-way 186 ANOVA
- 186 ANOVA.

var	R promoter	-80	-63 protein bingding sites	-35	-10	
vanA	CAU369 VREF001 ISMMVRE-1 ERV196 AUS04005 CAU996	5' TAAAAAAAGAATCA' TAAAAAAAGAATCA' TAAAAAAAGAATCA' TAAAAAAAGAATCA' TAAAAAAAGAATCA' TAAAAAAAGAATCA'	ICATCTTAAGAAATTCTTAGTC ICATCTTAAGAAATTCTTAGTC ICATCTTAAGAAATTCTTAGTC ICATCTTAAGAAATTCTTAGTC ICATCTTAAGAAATTCTTAGTC ICATCTTAAGAAATTCTTAGTC	ATTTATTATGTAAAT ATTTATTATGTAAAT ATTTATTATGTAAAT ATTTATTATGTAAAT ATTTATTATGTAAAT ATTTATTATGTAAATGC	TCGGCCCTATAATCTG 3'	
vanB	<i>E. faecium</i> 8672 MLG856-2 AE12 Efm 6123	ТААААААААGAATCA ТААААААААGAATCA ТААААААААGAATCA ТАААААААAGAATCA	ТСАТСТТААВААТТСТТАВТС ТСАТСТТААВААТТСТТАВТС ТСАТСТТААВАААТТСТТАВТС ТСАТСТТААВАААТТСТТАВТС ТСАТСТТААВАААТТСТТАВТС	ATTTATTATGTAAATGC ATTTATTATGTAAATGC ATTTATTATGTAAATGC ATTTATTATGTAAATGC	TTATAAATTCGGCCCTATAATCTG TTATAAATTCGGCCCTATAATCTG TTATAAATTCGGCCCTATAATCTG TTATAAATTCGGCCCTATAATCTG TTATAAATTCGGCCCTATAATCTG	
ms	rC promoter	-176	137 bp -	35	-10	
vanA	CAU369 VREF001 ISMMVRE-1 ERV196 AUS04005	ACTGCATCGATGA/ ACTGCATCGATGA/ ACTGCATCGATGA/ ACTGCATCGATGA/ ACTGCATCGATGA/	AATTACGTTCAGTTTTTTG AATTACGTTCAGTTTTTTG AATTACGTTCAGTTTTTTG AATTACGTTCAGTTTTTTG AATTACGTTCAGTTTTTTG	CAAACAGATTTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG	CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT	
vanB	CAU996 E. faecium 8672 MLG856-2 AE12 Efm 6123	ACTGCATCGATGAA ACTGCATCGATGAA ACTGCATCGATGAA ACTGCATCGATGAA ACTGCATCGATGAA	ATTACGTTCAGTTTTTTG ATTACGTTCAGTTTTTTG ATTACGTTCAGTTTTTTG ATTACGTTCAGTTTTTTG ATTACGTTCAGTTTTTTG	CAAACAGATTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG CAAACAGATTTTTCTG	CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT CTCTTTTTTAGTATCGATAATTGTT	
	Asia 📃 Europ	be 📃 North America	South America	eania		
Supplementary Fig. 15 The <i>vanA</i> and <i>vanB</i> type <i>E. faecium</i> share the same patterns in the promoters of <i>msrC/vanR</i> . CAU369, VREF001, ISMMVRE-1,						

- 190 ERV196, and AUS04005 are *vanA* type isolates; CAU996, *E. faecium* 8672,
- 191 MLG856-2, AE12, Efm 6123 are *vanB* type isolates.

/RE <sub>fm</sub> CAU 369	VRE	E <sub>m</sub> CAU 378
ABC transporter protein		500 rikesemel protein 1.21
Phosphoglycerate mutase		Sus ribosomai protein L31
Protein of unknown function		Protein of unknown function
Protein of unknown function		
Transcriptional suppressor YefM		ABC transporter ATP-binding protein
Endoribonuclease YoeB family		Protein of unknown function
Muts2 protein		UDP pyrophosphate phosphatase
NADP oxidoreductase, coenzyme f420-dependent		Amino acid permease
Protein of unknown function		ABC transporter
Muts2 protein		TetL tetracycline efflux protein
Cdp-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferas	e	Phosphoglycerate mutase
VanR		Protein of unknown function
VanS		Protein of unknown function
Protein of unknown function		Autolysin
Sodium hydrogen exchanger		N-acetylmuramoyl-L-alanine amidase sle1 precurso
Antibiotic biosynthesis monooxygenase		Protein of unknown function
Protein of unknown function		AcpP: acvl carrier protein
Protein of unknown function		Cro/CI family transcriptional regulator
MerC: ABC transporter		TetM Tetracycline resistance protein
Mate delivered systems		Dehydrogenase related protein
Distain of unknown function		MsrC: ABC transporter
Protein of unknown function		Efp. alongation factor P
Alkylpheenhenete utilization energy protein PhpA		Dta system
Aikyiphosphonate utilization operon protein FIIIA		Pts system
		Pts system
Catalase		Gaim; aldose 1-epimerase
Domain protein		PTS system ascorbate-specific transporter subunit
Oligoendopeptidase		MarR family transcriptional regulator
ManO family		Nramp family transporter
Acetolactate synthase		ABC1 family protein
ABC transporter protein		Isochorismatase hydrolase
ABC transporter protein		YidA hypothetical protein
Catalyzes the attachment of tyrosine to tRNA (Tyr)		Protein of unknown function
Beta-glucosidase		Tagatose 1,6-diphosphate aldolase
Protein of unknown function		GspA-1; general stress protein A
N-acetylmuramoyl-I-alanine amidase		Short chain dehydrogenase
ROK family		YsnF; putative stress response protein
Protein of unknown function		Gls24 protein
PTS system		Mo <sup>2+</sup> and Co <sup>2+</sup> transporter
Protein of unknown function	4 40	
1 10 ×MIC	1 10	

## 194 Supplementary Fig. 16 Activation of *vanRS* in P<sub>s</sub>V<sub>r</sub> VRE<sub>fm</sub> with decreased *msrC*

- 195 **transcription.**
- 196 Transcriptome analysis of VRE<sub>fm</sub> CAU369 and CAU378 treated with  $1 \times$  and  $10 \times$ MIC
- 197 lefamulin for 1 h. Genes were identified as significantly different with fold changes of
- log<sub>2</sub> [FPKM] values of at fold increase or fold decrease at expression levels. The top
- 199 20 genes changed were shown from the original 2,960 genes.
- 200 Experiments were performed as three biologically independent experiments, and the
- 201 mean  $\pm$  S.D. (n = 3) is shown. *P* values were determined by non-parametric one-way
- 202 ANOVA.
- 203

![](_page_18_Figure_0.jpeg)

![](_page_18_Figure_1.jpeg)

#### Supplementary Fig. 17 Lefamulin induces activation of vanR and vanS in PsVr 205 isolates.

(a-b) Transcript ratios of the *vanR/vanS* were quantified in diverse  $P_sV_r$  (a) and  $P_rV_r$ 207

- (b) VRE<sub>fm</sub> isolates based on qRT-PCR analysis. 208
- Experiments were performed as three biologically independent experiments, and the 209
- mean  $\pm$  S.D (n = 3). is shown. *P* values were determined by non-parametric one-way 210
- ANOVA. 211

![](_page_19_Figure_0.jpeg)

#### 213 Supplementary Fig. 18 Vector construction and transfection scheme.

- 214 (a) Vector construction. Design of *vanRS* mutant plasmid.
- 215 (**b**) Transfection scheme of electroporation and conjugative transformation.
- 216 Conjugant-2 (pAM401+*vanRS*) was screened through this pathway.
- 217 (c) Screening the conjugant carring pAM401+ vanRS plasmid in E. faecium. The
- 218 conjugant shows chloramphenicol resistance.
- 219 (d) PCR amplification products of the conjugant. VRE<sub>fm</sub> CAU369 and pAM401 were
- 220 used as controls. Experiments were performed as three biologically independent
- 221 experiments
- 222

![](_page_20_Figure_0.jpeg)

224 Supplementary Fig. 19 The *vanRS* inhibits the transcription of *msrC*.

(a-b) Transcription of *vanR* (a) and *vanS* (b) in the conjugant treated with lefamulin.

(c) Comparison of *msrC* transcription in wild type *E. faecium* and conjugant in the

227 presence of lefamulin. Experiments were performed as three biologically independent

experiments, and the mean  $\pm$  S.D. (n = 3) is shown.

(d) Comparison of the MICs of multiple antibiotics in wild type *E. faecium* and

- 230 conjugant. Vancomycin and linezolid were used as controls.
- 231

![](_page_21_Figure_0.jpeg)

Supplementary Fig. 20 Lefamulin inhibits the growth of the conjugant carring *vanRS*.

- 235 Growth curves of wild type *E. faecium* and conjugants treated with a sublethal level of
- lefamulin (1/2 MIC). Conjugant-1 receives sole plasmid pAM401, whereas
- 237 Conjugant-2 receives the plasmid pAM401+*vanRS*.
- 238 Experiments were performed as three biologically independent experiments, and the
- 239 mean  $\pm$  S.D. (n = 3) were shown. *P* values were determined by non-parametric one-
- 240 way ANOVA.
- 241
- 242

![](_page_22_Figure_0.jpeg)

![](_page_22_Figure_1.jpeg)

#### 244 Supplementary Fig. 21 Pyruvate metabolism in *E. faeciums*.

245 (a) Scheme of pyruvate metabolism in *E. faeciums*, according to the reference<sup>2</sup>. Major

end-products are boxed. Genes of *E. faeciums* encoding the synthesis of enzymes are

shown as follows: *cit*, citrate lyase; *oad*, oxaloacetate decarboxylase; *ldh* L- (+) -

248 lactate dehydrogenase; *alsS*,  $\alpha$ -acetolactate synthase; *alsD*,  $\alpha$ -acetolactate

249 decarboxylase; *pfl*, pyruvate formate-lyase; *adhE*, aldehyde-alcohol dehydrogenase.

250 (b) Volcano plot showing the metabolites in  $VRE_{fm}$  CAU369 and  $VSE_{fm}$  CAU309.

251 (c) Comparison of five major metabolites in  $VRE_{fm}$  CAU369 and  $VSE_{fm}$  CAU309.

All data are presented as mean  $\pm$  S.D. (n = 12 biological replicates). *P* values were

determined by non-parametric one-way ANOVA.

![](_page_23_Figure_0.jpeg)

#### 255 Supplementary Fig. 22 Lefamulin restores the homeostasis of intestinal flora.

- 256 (**a-b**) The Chao1 index (**a**) and Shannon index (**b**) of intestinal microbiota.
- 257 (c-f) Genus counts in the mouse intestine. Bacteroides (c) Prevotella (d) Clostridium
- 258 (e) and *Escherichia/Shigella* (f) in the mice treated with lefamulin restored faster than
- that with PBS after expansion of  $VRE_{fm}$  CAU369 at the 7th day.
- 260 Experiments were performed as three biologically independent experiments, and the
- 261 mean  $\pm$  S.D. is shown. *P* values were determined by non-parametric one-way
- ANOVA.
- 263

	Antibactorial	Μ	IC <sub>50</sub>		
Class	Antibacterial	VRE	VSE	RR	Target
	agent	(n = 101)	(n = 109)		
Aminoglycoside	Gentamicin	>128	32	8	Ribosome, 30S
Amphenicol	Chloramphenicol*	32	8	4	Ribosome, 50S
	Ampicillin	>128	2	64	
β-lactam	Cefoxitin	>128	64	2	PBP
	Meropenem	>128	16	8	
Clusopontido	Vancomucin	× 100	2	61	D-Ala-D-Ala
Grycopeptide	vancomycm	>120	Z	04	dipeptide
Lincosamide	Clindamycin	>128	4	16	Ribosome, 50S
Macrolide	Erythromycin	16	8	2	Ribosome, 50S
Oxazolidone	Linezolid	4	4	1	Ribosome, 50S
	Azamulin	0.5	>16	0.0312	
	Lefamulin	0.06	>16	0.0037	
Pleuromutilin	Retapamulin	0.03	>16	0.0019	Ribosome, 50S
	Tiamulin	0.5	>16	0.0312	
	Valnemulin	0.03	>16	0.0019	
Quinolone	Ciprofloxacin	>64	2	32	DNA gyrase
Rifamycin	Rifampicin	8	4	2	RNA polymerase
Streptogramin	Virginiamycin M1*	2	4	0.5	Ribosome, 50S
Tetracycline	Tetracycline	2	4	0.5	Ribosome, 30S

# Supplementary Table 1 Antibacterial activity and collateral sensitivity in *E*. *faecium* (n = 210).

266 \*Note: The MIC<sub>50</sub> of chloramphenicol and virginiamycin M1 were calculated with 40 isolates.

	Isolates	AMP	CIP	CLI	CRO	ERY	GEN	LZD	MEM	LMU	RIF	TET	VAN
	CAU309	128	128	128	128	128	128	4	128	16	16	16	2
	CAU310	128	128	64	128	128	128	2	128	16	8	1	2
	CAU273	1	0.5	0.5	64	8	16	2	8	16	16	0.5	2
	CAU274	2	2	4	128	4	16	4	16	16	16	1	2
	CAU311	128	128	128	128	128	128	4	128	0.125	4	16	1
	CAU312	4	4	0.25	128	128	32	2	16	0.03	16	16	1
	CAU313	2	2	128	128	128	16	4	16	16	4	16	1
	CAU275	4	2	4	64	8	16	4	16	16	8	0.5	2
	CAU314	128	128	128	128	128	128	4	128	16	16	16	2
VSE <sub>fm</sub>	CAU276	8	16	32	128	16	8	4	16	16	0.25	4	2
	CAU315	128	128	128	128	128	128	2	128	0.125	4	16	2
	CAU316	4	16	128	128	128	128	4	16	16	0.25	4	1
	CAU277	4	0.3	0.25	128	8	0.25	0.25	16	0.03	0.25	1	2
	CAU278	2	2	4	128	4	16	4	16	16	4	0.5	1
	CAU279	4	4	64	128	32	32	4	32	16	16	0.25	2
	CAU280	2	1	8	8	16	16	2	16	16	32	2	1
	CAU281	0.25	1	1	4	2	32	1	0.25	16	4	0.25	1
	CAU317	2	1	64	128	16	2	4	16	16	64	4	1
	CAU282	4	16	32	128	32	8	4	32	16	16	0.25	2
	CAU359	>128	>64	>128	>128	>128	32	2	>128	0.06	16	0.5	> 128
	CAU360	>128	>64	>128	>128	>128	>128	2	>128	0.12	16	0.5	> 128
	CAU187	>128	>64	<0.25	>128	>128	16	2	>128	0.06	4	32	> 128
	CAU361	>128	>64	>128	>128	>128	8	2	>128	< 0.03	< 0.25	< 0.25	> 128
	CAU362	>128	>64	>128	>128	>128	>128	4	>128	0.03	8	1	> 128
	CAU363	>128	>64	< 0.25	>128	< 0.25	128	1	>128	< 0.03	1	< 0.25	> 128
	CAU364	>128	>64	>128	>128	>128	>128	4	>128	2	4	0.5	> 128
	CAU365	>128	64	>128	>128	>128	>128	2	>128	2	2	0.5	> 128
	CAU366	>128	64	< 0.25	>128	< 0.25	>128	2	>128	0.25	4	32	> 128
	CAU367	>128	>64	< 0.25	>128	< 0.25	>128	2	>128	0.12	4	32	> 128
VRE <sub>fm</sub>	CAU368	>128	>64	>128	16	>128	>128	2	< 0.25	< 0.03	8	32	> 128
	CAU369	>128	>64	>128	>128	>128	>128	2	>128	< 0.03	4	2	> 128
	CAU370	>128	>64	< 0.25	>128	8	16	2	>128	0.03	2	1	> 128
	CAU371	>128	>64	>128	>128	>128	16	2	>128	< 0.03	8	< 0.25	> 128
	CAU372	>128	>64	>128	>128	>128	16	2	>128	< 0.03	4	32	> 128
	CAU373	>128	>64	>128	>128	>128	>128	2	>128	< 0.03	4	32	> 128
	CAU374	>128	>64	>128	>128	64	>128	1	>128	0.12	< 0.125	< 0.25	> 128
	CAU375	>128	>64	>128	>128	>128	>128	2	>128	0.03	4	32	> 128
	CAU376	>128	>64	>128	>128	>128	>128	1	>128	< 0.03	4	< 0.25	> 128
	CAU377	>128	>64	>128	>128	>128	32	2	>128	0.12	4	16	> 128
	CAU378	>128	>64	>128	>128	>128	16	2	>128	> 16	4	32	> 128

268 Supplementary Table 2 MICs of multiple classes of antibiotics in 40 *E. faecium* (µg/mL).

- 269 Note: AMP: Ampicillin; CIP: Ciprofloxacin; CLI: Clindamycin; CRO: Ceftriaxone; ERY:
- 270 Erythromycin; GEN: Gentamicin; LZD: Linezolid; MEM: RET: Retapamulin; RIF: Rifampicin; TET:
- 271 Tetracycline; VAN: Vancomycin.

Supplement	ntary Table 3	Information ab	out VRE <sub>fm</sub>	isolates	worldwide.
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	Accession ID (Bio sample)	Strain	Source		Accession ID (Bio sample)	Strain	Source
1	14694313	CAU187		41	4397503	805447/07	
2	14693177	CAU 359		42	6330395	LIM1590	
3	14693178	CAU 360		43	6555613	LIM695	Brazil
4	14687682	CAU 361		44	6555612	LIM559	
5	14693732	CAU 362		45	5461961	ERV196	
6	14693733	CAU 363		46	5461954	ERV157	
7	14693734	CAU 364		47	5461957	ERV175	Calambia
8	14694296	CAU 365		48	5461978	ERV98	Colombia
9	14694297	CAU 366		49	5461977	ERV35	
10	14694298	CAU 367		50	5461976	ERV34	
11	14694299	CAU 368	China (This steeder)	51	5461958	ERV177	Colombia
12	14694301	CAU 369	(This study)	52	6621457	CFSAN059071	Denmark
13	14694302	CAU 370		53	6111320	GER_10_Efcm_HA-DE	Cormony
14	14694303	CAU 371		54	6111319	9_Efcm_HA-DE	Germany
15	14694304	CAU 372		55	6463381	2014-VREF-114	V
16	14694306	CAU 373		56	6472829	2014-VREF-63	Korea
17	14694307	CAU 374		57	4500826	VREr7	
18	14694308	CAU 375		58	4500822	VREr6	Malaraia
19	14694309	CAU 376		59	6885147	VREr5	Malaysia
20	14694310	CAU 377		60	4500812	VRE2	
21	14694311	CAU 378		61	5461973	MAL_ERV279	Mariaa
22	3988550	ISMMS_VRE_2		62	5461972	Y_MAL_ERV275	Mexico
23	3988560	ISMMS_VRE_5		63	10248949	EF_386	
24	3988561	ISMMS_VRE_6		64	10249139	EF_367	
25	3988626	ISMMS_VRE_8		65	10249219	EF_181	
26	7274322	VRE2014-195		66	10248944	PAK_EF_048	
27	7274321	VRE2014-7		67	10249011	PAK_EF_042	Pakistan
28	7274325	VRE2016-194		68	10248976	PAK_EF_034	
29	7274324	VRE2016-78		69	10249190	EF_033	
30	3198116	909_EFCM	America	70	10248976	EF_028	
31	3197807	607_EFCM		71	10249104	EF_006	
32	3197907	702_EFCM		72	4270932	SDW_VRE-1400294	
33	3197760	560_EFCM		73	4270930	VRE-1300937	
34	3197708	514_EFCM		74	4270929	VRE-1300911	
35	3197623	43_EFCM		75	4270928	VRE-1300900	Sweden
36	3197600	41 EFCM		76	4270927	VRE-1300899	
37	3197568	- 377 EFCM		77	4270926	VRE-1300578	
38	3197342			78	4270925	VRE-1300518	
39	8595978	AUSMDU00004055	Australia	79	8196783	UK_VREF003	U.K.
40	10273305	RBWH1					

Supplementary Table 4 Key nucleotides of 23S rRNA related to pleuromutilins resistance
in 40 E. faecium isolates.

	Copies 23S rRNA					A nucleotides					
	Isolates	of 23S rRNA	PLEs*	G2061	A2062	C2452	A2503	U2504	G2505	U2506	U2585
	CAU273	1	R	G	А	С	А	U	G	U	U
	CAU274	1	R	G	А	С	А	U	G	U	U
	CAU275	1	R	G	А	С	А	U	G	U	U
	CAU276	1	R	G	А	С	А	U	G	U	U
	CAU277	1	S	G	А	С	А	U	G	U	U
	CAU278	1	R	G	А	С	А	U	G	U	U
	CAU279	1	R	G	А	С	А	U	G	U	U
	CAU280	1	R	G	А	С	А	U	G	U	U
	CAU281	1	R	G	А	С	А	U	G	U	U
<b>VSE</b> <sub>fm</sub>	CAU282	1	R	G	А	С	А	U	G	U	U
	CAU309	1	R	G	А	С	А	U	G	U	U
	CAU310	1	R	G	А	С	А	U	G	U	U
	CAU311	1	S	G	A	C	A	U	G	U	U
	CAU312	1	S	G	A	C	А	U	G	U	U
	CAU313	1	S	G	A	C	А	U	G	U	U
	CAU314	1	R	G	А	C	А	U	G	U	U
	CAU315	1	S	G	A	C	А	U	G	U	U
	CAU316	1	R	G	A	C	A	U	G	U	U
	CAU317	1	R	G	A	C	А	U	G	U	U
	CAU187	1	S	G	А	С	А	U	G	U	U
	CAU359	1	S	G	А	С	А	U	G	U	U
	CAU360	1	S	G	А	С	А	U	G	U	U
	CAU361	1	S	G	А	С	А	U	G	U	U
	CAU362	1	S	G	А	С	А	U	G	U	U
	CAU363	1	S	G	А	С	А	U	G	U	U
	CAU364	1	S	G	А	С	А	U	G	U	U
	CAU365	1	S	G	А	С	А	U	G	U	U
	CAU366	1	S	G	А	С	А	U	G	U	U
	CAU367	1	S	G	А	С	А	U	G	U	U
VREfm	CAU368	1	S	G	А	С	А	U	G	U	U
, <u> </u>	CAU369	1	S	G	А	С	А	U	G	U	U
	CAU370	1	S	G	A	C	A	U	G	U	U
	CAU371	1	S	G	Δ	C	Δ	U	G	U	U
	CAU372	1	S	G	Λ	C C	Λ	U	G	U	U
	CAU272	1	ы с	G	л л	C	л л	U	G	U	U
	CAU375	1	3	G	A	C	A	U	G	U	U
	CAU374	1	5	G	A	C	A	U	G	U	U
	CAU375	1	S	G	А	C	А	U	G	U	U
	CAU376	1	S	G	А	С	А	U	G	U	U
	CAU377	1	S	G	А	С	А	U	G	U	U
	CAU378	1	R	G	А	С	А	U	G	U	U

\*Note: PLEs: Pleuromutilin antibiotics.

	Plasmids	Source	Length (bp)	Genbank	Reference
1	pEMA120		79797	KX853854.1	3
2	pEM19081	China	61320	NZ_KN880430.1	4
3	pVRE1		132733	CP040742.1	NCBI
4	pZB18	Japan	68058	AB611033.1	NCBI
5	p2014-VREF-63	Korea	287502	CP019989.1	NCBI
6	pIP816		34616	NC_011140.1	5
7	pVEF3		63135	AM931300	6
8	pVEF1	Norway	39626	NC_008768.1	6
9	pVEF2		39714	NC_008821.1	
10	pVEF4		44443	FN424376.1	NCBI
11	pVRE001		59226	CP018072.1	7
12	pEFA-790c	USA	35515	NZ_CP025755.1	NCBI
13	pS177		39032	HQ115078.1	8

Supplementary Table 5 Information of 13 plasmids containing vanA genes in E. faecium

# SELECT qPCR primersSequences23S-m6A1-upTAGCCAGTACCGTAGTGCGTGTCAAACTACAGTAAAGCTCCATGGGGTCTT23S-m6A1-downTCCGTCCTGTCGCGGGTAACCTGCATCTTCCAGAGGCTGAGTCGCTGCATqPCR primersSequencesqPCR-FATGCAGCGACTCAGCCTCTG

280

qPCR-R

Supplementary Tables 6 Primers for SELECT qPCR or qRT-PCR assay

TAGCCAGTACCGTAGTGCGTG

Supplementary Tables 7 Primers for RT-PCR assay

Primers	Sequences	Reference
16S - F	CCTACGGGAGGCAGCAG	9
16S - R	ATTACCGCGGCTGCTGGC	
msrC - 1F	CAGCAAACTACGGACAAGCG	This study
<i>msrC</i> - 1R	GTCGGCGAAAATGGTTCAGG	
<i>lsaE1</i> - F	AAGCCGAATGGTCTCGTTCC	This study
<i>lsaE1</i> - R	CGCTGATCTGGGTCTCCATC	
<i>eatAv</i> - 1F	TTCAGGTCCTAACGGTGCAG	This study
<i>eatAv</i> - 1R	TCCATCCCAAGCTTTCGGAG	
<i>vanS</i> - F	CCGCTGCATACAGTGAGGAT	10
<i>vanS</i> - R	CCGTATCGGAAGAACGAGCA	
<i>vanR</i> - F	GGCACAAGCGGCCTTACTAT	
<i>vanR</i> - R	TAACTCCAGTGGGCGAAAGG	

287 288	<b>Supplementary Table 8</b> MICs of multiple antibiotics in wild-type <i>E. faecium</i> and mutan $(\mu g/mL)$ .										
	Pleuromutilin antibiotics										
		LMU	RET	VAL	TIA	AZA	VAN	LIN	ERY		
	Wild-type	0.06	0.06	0.06	0.25	0.25	2	1	1	-	
	Mutant	16	16	16	32	32	2	>128	>128		

Table 9 MICs of ----14:---1. •1 1  $E f_{\alpha}$ a 1 . . : А ıt

LMU: Lefamulin; RET: Retapamulin; VAL: Valnemulin; TIA: Tiamulin; AZA: Azamulin; VAN: Vancomycin; LIN: Lincomycin; ERY: Erythromycin. 289 290

291 292 (pAM401+*msrC*)

293	Refe	References					
294							
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296		that confer high-level fosfomycin resistance to Vancomycin-resistant Enterococci. Front					
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298	2	Simons, J. A. Snoep, J. L. Feitz, S. Mattos, T. M. J. D. & Neijssel, O. M. Anaerobic 2-					
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300		culture: Involvement of the pentose phosphate pathway. J Gen Microbiol, 138,423-428					
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304		(2001).					
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307		peptidoglycan precursors in Enterococcus faecium BM4147. Bacteriol 175, 117-127					
308		(1993).					
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310		of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter. <i>Plasmid</i> 60,					
311		75-85 (2008)					
312	6	Sletvold, H. et al. Comparative DNA analysis of two vanA plasmids from Enterococcus					
313		faecium strains isolated from poultry and a poultry farmer in Noway. Antimicrob Agents					
314		Chemother <b>51</b> , 736-739 (2007).					
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321		nanoengineered antimicrobial peptide polymers. Nat. Microbiol, 16162 (2016).					
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324		Antimicrob Agents 55, 105897 (2020).					
325							