Preventive antibiotic treatment in agriculture: emergence of dysbiosis and propagation of obese state associated and mobile multidrug resistance carrying bacteria.

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Abstract.

In agriculture, antibiotics are often used to prevent livestock disease. Antibiotic treatment perturbs the bacterial composition of the gut but the extent of these changes and potential consequences for animal and human health are still debated. Six calves were housed in a controlled environment receiving the same diet. Three animals received an injection of the antibiotic florfenicol (Nuflor) and three received no treatment. Faecal samples were collected from each animal 0, 3 and 7 days after treatment and bacterial communities profiled to assess the impact of a standard antibiotic therapy on the gut microbiota over time. Phylogenetic analysis of 16S-rRNA genes established that at day 7 the microbiota of calves given Nuflor showed a 10-fold increase in facultative anaerobic *Escherichia spp*, a signature of imbalanced microbiota, dysbiosis. Analysis of the antibiotic resistome revealed a high background of antibiotic resistance genes, the abundance and diversity of which did not significantly change in response to Nuflor. However, the maintenance of *Escherichia coli* plasmid-encoded quinolone, *oqxB* and propagation of *mcr-2*, colistin resistance genes was observed and confirmed by Sanger sequencing of PCR fragments of the metagenomic DNA. The microbiota of animals treated with antibiotics had a higher population of energy harvesting bacteria, including *H₂*-producing *Prevotellaceae* and *H₂*-oxidising methanogenic *Archaea*, common to the obese microbial community. We propose that antibiotic treatment of healthy animals leads to unbalanced, disease- and obese related microbiota that promote growth of mobile-resistance carrying *E. coli*, increasing the risk of spread and transmission of resistant bacteria to humans.
Significance Statement. Antibiotics, the most important tool in fighting bacterial infections, are widely used in livestock as a prophylactic therapy and/or to enhance growth. Here we show that preventive antibiotic treatment of healthy calves resulted in unbalanced intestinal microbiota, termed dysbiosis, with two major outcomes. Firstly, the preferential growth of bacteria with high energy harvesting capacity, a key obesity determinant. Secondly, the selective growth of bacteria carrying a range of highly transmissible, clinically significant antibiotic resistance genes, crucially to antibiotics that have not been administered. We propose that the primary outcome of antibiotic treatment is dysbiosis that predisposes the host to a myriad of diseases, significantly increasing the risk of spread and transmission of multidrug-resistant bacteria to humans.
Introduction.

The intestinal microbiota is critical for homeostasis in humans and animals and represents a natural reservoir of antibiotic resistance genes (1-4). Antibiotics (AB) are commonly used in livestock farming to prevent and treat infections or to support growth, despite promoting the rapid development of multidrug resistant pathogens including those resistant to ‘antibiotics of last resort’, such as colistin (5-11). Also, it has long been thought that such treatments directly select for bacteria resistant to the prescribed antibiotic, leading to “on-target selection”. However, AB treatment appears to cause the more general imbalance of the microbiota (dysbiosis) that can have additional effects, including a disproportional increase in abundance of specific bacteria which may carry mobile resistances and/or being associated with obesity.

An overlooked aspect of the widespread use of antibiotics is how they affect the composition of the gut microbiota with relation to the host wellbeing. There is an ever-growing body of evidence to support the role of the gut flora in human and animal health and disease (12). A balanced intestinal microbiota, generally dominated by obligate anaerobes due to the hypoxic nature of the gut, is vital in maintaining homeostasis, energy metabolism and supplying essential biomolecules that are not synthesised by the host (13,14). AB treatment is known to perturb the intestinal microbiota, in part due to elevating the oxygen level in the colon (15). AB treatment in mice (16), humans (17) and pigs (18) has been associated with an expansion of facultative anaerobes, disrupting anaerobiosis and enhancing aerobic respiration. A disproportional increase in abundance of certain Proteobacteria, such as facultative anaerobes like Escherichia coli in the gut is linked to inflammation, obesity, can promote aerobic pathogen colonization and enhance permeability of the colon, compromising the ability of microbiota to maintain a balanced, protective bacterial community (12,14,19,20). Also, commensal facultative anaerobes may carry a wide range of genes responsible for genetic transfer, including resistance determinants to antibiotics that have not been administrated (18), therefore increasing the prevalence of ‘off-target’ antibiotic resistance. Changes in the composition of the gut microbiota has also been linked to weight gain in animals and humans (21-23), the effect on health however has continued to be overlooked. The bacterial gut microbiota composition of obese mammals differs from their lean counterparts and it has been shown to be critical to both fat storage and host energy balance (24,25). Interestingly, an increase in body mass may occur without changes in food consumption, strongly suggesting that the microbiota in the gut is crucial to obesity and its composition influences the amount of energy derived from the diet (26). Hence, the obesity associated gut microbiota appears to be more efficient in energy extracting than a balanced bacterial counterpart. Such evidence demonstrates that effects of the liberal use of antibiotics have been long underestimated.

One of the emerging challenges with respect to AB resistant commensal bacteria is that they may carry mobile resistance elements that had previously been thought to be associated only with chromosomal mutations, for example, resistance to colistin which has now been found to occur on mobile genetic elements (7). Whilst originally found in animals, colistin resistance has already been reported in humans, making zoonotic transmission a major public health concern. A similar pattern of resistance has emerged for fluoroquinolones (27). The use of colistin and/or fluoroquinolones in livestock to promote growth has led to the rise of the corresponding plasmid-mediated resistance determinants in E. coli (7,27-30). However, AB-driven dysbiosis may further contribute to the expansion of commensal and pathogenic bacteria carrying a wide range of ‘off-target’ plasmid-
mediated resistances which are highly mobile and capable of self-transmission between agricultural and clinical settings. Therefore, growth of mobile-resistance carrying bacteria due to dysbiosis in the human food chain following antibiotic treatments poses significant risk to animals and humans has long been overlooked.

Here, calves housed and reared under controlled conditions were exposed to standard preventive antibiotic therapy (florfenicol; trade name Nuflor) to assess the effects on resistome and the gut microbiota. This is the first report of a molecular analysis of calf gut microbiota in response to a standard preventive antibiotic therapy that predisposes animals to dysbiosis and disease, promoting the growth of bacteria that carry highly mobile clinically relevant resistances that can be transmitted to humans.

Results.

Antibiotic Treatment Leads to an Imbalanced Microbial Community in the Animal Gut. Deep sequencing of the V3-V4 hypervariable regions of the bacterial 16S rDNA was carried out. For six calves, samples were sequenced at three time points: before treatment (T_0) and at three days (T_3) and seven days (T_7) after treatment. The 18 samples produced 5,578,146 sequence reads; 2,888,547 from medicated and 2,689,599 from non-medicated calves. In each sample, up to 10 different bacterial phyla (Fig. 1A) were identified, along with methanogenic Archaea (Fig. 2). The predominant phyla were Firmicutes (64±1.7%) and Bacteroidetes (26±2.6%) (Fig. 1A, B), which have been shown to be the most abundant in human and animal guts (31,32). At lower prevalence were Saccharibacteria, Tenericutes, Spirochaetes, Actinobacteria, Proteobacteria, Verrucomicrobia and Cyanobacteria. Except Proteobacteria and Verrucomicrobia, the overall prevalence of phyla did not significantly change in response to antibiotic treatment (Fig. 1C, D).

AB treatment changed the composition of gut microbiota through a ten-fold rise in Proteobacteria at day 7 (p=0.01) compared with non-medicated samples (Fig. 3A). Such a shift was mainly due to an expansion of the genus Escherichia (T_3/T_0 v T_7, p=0.011) in response to AB (Fig. 3B). The increase in E. coli was confirmed by semi-quantitative PCR of the corresponding metagenomic DNAs targeting uidA, the gene encoding for β-glucuronidase, a marker for E. coli (Supplementary information Fig. S1). A significant increase in the prevalence of E. coli in the gut is a distinct feature of an imbalanced microbial community which in turn increases a risk of disease due to the greater production of ethanol that enhances permeability of intestinal cells and promotes colonization by obligate aerobes (20).

The population of the phylum Verrucomicrobia, which is poorly represented in obese animals and humans (33,34), significantly decreased (p=0.001) in response to AB treatment (Fig. 4A). The treatment led to selection of both methanogenic Archaea (T_0 v T_7, p=0.003) and Prevotellaceae spp (T_0 v T_3, p=0.029, T_0 v T_7, p=0.045, T_3 v T_7, p=0.022) (Fig. 4B), a distinct feature of obesity-associated microbiota due to the ability of methanogenic Archaea to enhance the production of short-chain fatty acids (34,35). We also found that the medicated animal gut microbiota is gradually enriched with Erysipelotrichaceae (phylum Firmicutes) over 7 days (p=0.05), increased abundance of which has also been associated with obesity (Fig. 4C) (35-37). This is consistent with the composition of the calves’ gut microbiota treated with Nuflor demonstrating increased in energy harvesting bacteria, resembling microbiota of obese/overweigh animals and humans (24).
Emergence of *E. coli* Carrying Mobile *mcr*-2, Maintenance of *oqxB* Genes and Resistance to Clinical Antibiotics in Response to Nuflor. Whole meta-genome sequencing of samples at day T₀ and T₇ resulted in 27,231,751 trimmed sequences for one non-medicated and 32,598,705 for two medicated animals. Diverse antibiotic resistance genes were identified in faecal samples of both non-medicated (Table S1) and medicated animals (Table S2 and S3) with, overall, no significant differences between them, with two exceptions. Firstly, at day seven, the gut microbiota of the two medicated animals contained detectable amounts of the *mcr*-2 gene DNA-seq reads, phosphoethanolamine-lipid A transferase MCR-2 (NCBI Reference: NG_051171.1) (Table S2 and S3). The emergence of *mcr*-2 alleles in response to Nuflor coincides with a 10-fold increase in the abundance of *E. coli*, *mcr*-2 was originally found on the *E. coli* plasmid pKP37-BE (NCBI Reference: LT598652.1) (38). Nested PCR followed by Sanger sequencing of the first 683 nucleotides of the gene confirmed a 100% match to the *mcr*-2 gene (Fig. S2), starting non-medicated samples at T₀ displayed no PCR products. While we were not able to confirm the presence of the pKP37-BE plasmid in the samples by PCR it is very likely that the *mcr*-2 allele occurs on this vector due to the complete match of the identified sequence to the reference.

Secondly, analysis of the prevalence of quinolone resistance genes revealed that DNA-seq reads for *oqxB*, a constituent of an RND-type multidrug efflux pump (resistance to olaquindox in *E. coli*) (29) were equally present in all tested T₀ samples. Surprisingly, the *oqxB* gene was detected by neither DNA-seq nor PCR in the non-medicated sample at day seven. However, upon the Nuflor treatment the number of *oqxB* sequences for the two medicated samples at T₀ and T₇ were being maintained at a similar level (36 ± 9.5 and 43.7 ± 0.4, respectively) (Table S4). It has been reported that the *oqxB* gene occurs on the plasmid pOLA52 (NCBI Reference: EU370913.1) (39). Sanger sequencing of the nested PCR fragments of 386 bp (48049 to 48434 nt positions within pOLA52), the sequence which is predicted to be within the drug efflux channel (40) (Fig. S3), of the metagenomic DNA of medicated samples confirms the presence of the *oqxB* gene at T₇. At the DNA level silent and missense point mutations were found that translated into four sets of mutated polypeptides (Fig. 5). The G148N amino acid substitution was the main feature of all sequenced clones which resulted in appearance of the PN motif. The G148N substitution also occurred on its own, along with L90I or T92A and alongside both L90I and D152N. Hence, polypeptides with either single, double or triple amino acid substitutions were present in the metagenomic *oqxB* gene. Analysis using a combination of molecular modelling and Monte Carlo refinement was used to examine the potential structural consequences associated with substitutions at each locus. It was revealed that the mutations can be divided into 2 clusters G148N (Fig. S4)-D152N (Fig. S5) and L90I/T91A (Fig. S6) and are located in the drug efflux channel and at the interface between OqxA and OqxB, respectively. L90I/T91A, despite the apparent physiochemical similarity between Leu and Iso the β-branched nature of Iso, can influence the secondary structure formation in β-sheets and α-helices. *In silico* mutagenesis and Rosetta refinement was used to mimic the mutation (L90I) observed in the cluster. 27 of the 30 independent Monte Carlo simulations revealed a break in the β-sheets and subsequent reorientation of a juxtaposed helical region (P56-A70). This region of helix is directly exposed to the drug efflux channel and has the potential to alter its shape. Examination of the effect of T91A which is also located in this region revealed in contrast, the *in silico* mutation did not have a marked effect on the secondary structure at this locus. However, the substitution with an Ala at this position does result in the loss of interaction through a hydrogen bond with Ser82 in a juxtaposed β-sheets and reordering of this area. This also results in the change of position in the helical region P56-A70 and subsequent
re-ordering resulting in a larger aperture in the efflux channel. G148N/D152N, the location of the second cluster of mutations sits at the interface between the membrane-bound OqxB and soluble scaffold protein OqxA of the complex. G148N is located at the interface of 2 β-sheets. The in silico substitution of Asn at this locus results in a further inter β-sheet interaction with Asp323 located on a juxtaposed β-sheet. This further hydrogen bond represents an increased stability of approximately 61kcal/mol in this region of secondary structure. For D152N, this mutation is also at the interface between OqxA and OqxB. Visual inspection of the Reference sequence reveals that this position (A152) in OqxB is juxtaposed to a charged cluster in an associated chain including an acid residue A281. The substitution of Asn may provide a better complementarity between the subunits which is reflected in the molecular modelling with the two positions becoming closer in the mutant receptor after refinement compared to the reference sequence. The additional interactions in the mutated protein sequence will not only result in local increases in stability but taken together will also increase the stability of the complex as a whole. The enhanced thermodynamic stability of the complex would be predicted to lead to an increased protein half-life and as such increase efflux capacity of the host.

The presence of the pOLA52 plasmid in the treated samples at T7 was confirmed by Sanger sequencing of nested PCRs of the metagenomic DNA targeting a randomly selected sequence of 215 bp within the plasmid. These results strongly suggest that preventive antibiotic treatment of healthy animals promotes growth of E. coli that carry clinically relevant mobile-resistances, increasing a risk of the spread of resistance within farm and human populations.

Discussion.

We investigated the impact of a preventive antibiotic (Nuflor) on the gut microbiome and antibiotic resistome of healthy calves. The results show that such therapy has a pleotropic effect on the gut microbiota composition leading to emergence of an obesogenic and dysanaerobic, disease prone bacterial community that carries clinically relevant mobile antibiotic resistance determinates. Firstly, the selection of obesity related microbes without changes in the diet was observed, suggesting that antibiotic treatments may be a key determining factor of obesity. Secondly, dysbiosis was found during the treatment with an expansion of facultative anaerobes that may trigger inflammation and affect the immune system. Finally, the observed dysbiosis was associated with a significant expansion of E. coli carrying clinically important mobile antibiotic resistance genes. This is consistent with a real risk of the spreading of E. coli carrying resistance determinants within and between environmental and clinical settings that will diminish the ability to treat bacterial infections with antibiotics. The data strongly argue that the primary outcome of AB therapy is dysbiosis, which in turn propagates obese state bacteria and facilitates the spread of a wide range of resistance genes (on- and off-target) due to enhancing the aerobic environment of the gut.

The phyla Firmicutes and Bacteroidetes comprise around 90% of the gut microbiota of the calves. These provide the animals with energy harvested from indigestible and poorly digestible polysaccharides (31, 41). Previous analyses of animal and human microbiota have shown that antibiotic treatments can cause transient changes in the composition of the gut microbiota depending upon the activity spectrum of the drug(s) (42). Antibiotics such as tetracyclines, glycopeptides and macrolides administered individually or in combination (e.g. ASP250) have often been used to promote weight gain in animals (18,22,43). Equally, weight gain in humans, both adults
and children (21,44,45), has been observed with some of antibiotics (e.g. tetracycline, macrolides, sulphonamides) however, these effects on health have been disregarded until recently. It is not clear whether the observed antibiotic-mediated weight gain is a result of improved, infection free gut microbiota, the perturbation of its composition or a combination of both. There is however mounting evidence to support the view that changes in the composition of the gut microbiota play a key role in the amount of energy produced from the diet without any changes in nutrient consumption. For example, *Verrocomicrobia* and *Akkermancia* spp are found at lower densities in obese individuals compared to those who are not, while significant increases in the abundance of both methanogenic *Archaea* and *Prevotellaceae* spp (which has been observed following antibiotic treatment in this work) is also linked to obesity in humans and animals. *Prevotellaceae* are H$_2$-producing *Bacteroidetes* (33,46) which during the fermentation process enable accumulation of H$_2$, reducing the yield of ATP and leading to a gradual decrease in fermentation efficiency. The methanogenic *Archaea* are believed to not only have a role in obesity through promoting caloric intake from otherwise indigestible polysaccharides (24,34,47), but alongside an increase in *Prevotellaceae*, can utilise the excess H$_2$ to produce acetate, a highly absorbed short chain fatty acid (48). Hence, the coexistence of *Prevotellaceae* with methanogenic *Archaea* species in the gut of obese mammals allows for greater efficiency of dietary polysaccharide fermentation and increases their conversion into short-chain fatty acids, leading to excessive energy storage. It has been suggested that *Bacteria-Archaea* syntrophy may be a novel biomarker of susceptibility to obesity (33). In addition, the animal gut microbiota treated with antibiotics is gradually enriched with *Erysipelotrichaceae*, bacteria that produce butyrate, another major energy source, an excess of which may lead to substantial energy storage (36,49). In this study the improved energy harvesting capacity of the calves’ gut microbiota was found to occur without any diet alteration. This is consistent with the idea that an increase in the body fat may occur without any increase in food intake (26) and supports the view that the bacterial composition of the gut drives the energy harvesting from the diet.

In the healthy mammalian gut, the hypoxic environment governs the composition of the bacterial community, favouring obligate anaerobes such as *Firmicutes* and *Bacteroidetes*. In turn, they utilise oxygen via β-oxidation of microbiota-produced butyrate to carbon dioxide, the main energy production pathway which is critical for the development of the host immune system. Hence, a disruption of anaerobiosis (dysanaerobiosis) due to inflammation (50-53) or treatment of infections with antibiotics (16,17,54) for example, leads to an increased oxygenation of the colon and expansion of facultative anaerobic *Proteobacteria* via aerobic respiration. During dysbiosis, energy is also obtained through anaerobic glycolysis, (55) which does not require oxygen leading to increased epithelial oxygenation (56). The resultant dysanaerobiosis, an increase of oxygen that disrupts anaerobic environment in the gut would enable selection for facultative anaerobes, including commensal and pathogenic *E. coli* by aerobic respiration. In this study, Nuflor treatment caused an abnormal escalation of *Proteobacteria* (mainly *E. coli*), resulting in dysbiosis of the gut microbial community which can have a number of consequences as it enhances the risk of disease (20,57). *Escherichia* spp continually produce ethanol which increases the permeability of the intestinal wall, characteristics linked to inflammation and obesity. This can lead to certain environmental toxins such as dimethylarsine which can be metabolized by *E. coli* to produce toxic micrometabolites, potential carcinogens of the gut (58). Increased oxygen availability which promotes an expansion of *E. coli* can also pave the way for an expansion of aerobic pathogens, hence offsetting the benefit of
using preventive AB therapies. Furthermore, *E. coli* are also a potential human pathogen that can carry antibiotic resistance determinants on mobile elements, raising a possibility of spreading resistance within- and between communities.

Taken together, an antibiotic-induced dysbiotic expansion of facultative anaerobic *E. coli* can predispose the host to disease, such as intestinal inflammation and cancer. It also can drive an *Enterobacteriaceae* enteric pathogen colonization which carries clinically relevant transmissible antibacterial resistances. Therefore, practices of a preventive antibiotic treatment of healthy animals must consider the potential threat to animal health as well as a high risk of zoonotic transfer of pathogens carrying mobile resistances to the human population.

Antibiotic therapy in livestock plays a key role in the selection of resistant bacteria (59) that can be transmitted between animals, humans and the environment (5). Recently, the *mcr*-1 allele conferring a novel plasmid-borne colistin resistance gene was discovered in food animals (7) and it has now been detected across all continents (8,60-62). The *mcr*-1 resistance gene is found on a highly transmissible plasmid, namely IncX4 which is a narrow host range mobile element of *Enterobacteriaceae* with a lack of fitness burden on bacterial host (7). This allows the cell to maintain the plasmid even in the absence of antibiotic pressure, suggesting a stable colonization of *mcr*-1 carrying bacteria in the intestine (63), an expansion of which may be triggered in response to antibiotic pressure or other factors, such as bowel inflammation that enhances the level of oxygen in the intestine. Recent evidence confirms transfer of *mcr*-1 from animals to humans (8). Subsequently *mcr*-2, another plasmid-mediated colistin resistance gene has been identified in porcine and bovine colistin resistant *E. coli* (38). Like *mcr*-1, *mcr*-2 is a phosphoethanolamine transferase which also occurs on an IncX4 plasmid (pKP37-BE) that makes this resistance highly transmissible. While we have not been able to confirm the presence of pKP37-BE in the samples where PCR clones containing the *mcr*-2 sequences were identified (probably due to still low abundance of the DNA), it is very likely that *mcr*-2 found in this study is also occurring on pKP37-BE or another IncX4 plasmid.

Due to the constant movement of livestock across continents it is feasible that *mcr*-2 may have already spread between different countries and emergence is only a matter of time. Hence an urgent introduction of *mcr*-2 screening within livestock is essential (38) as this study is the first example of emergence of *mcr*-2 in livestock in response to antibiotic treatment. It is imperative to understand the scale of its dissemination within the agricultural environment to prevent its spread from animals to humans and the food chain.

Efflux pumps are associated with resistance to multiple antimicrobial agents due to their ability to efficiently extrude a broad range of chemicals from the bacterial cell, thereby lowering the intracellular level of antibiotics. This mechanism of resistance is seen as the first line of defence which plays a key role in the selection of novel resistance genes. This gives the cell a time to develop a targeted (multi)drug resistance to higher concentrations of the antibiotic and/or drives the accumulation of additional gain-of-resistance mechanisms. Moreover, efflux pump genes can occur on mobile elements that can be transmitted to pathogenic bacteria (64-68). The cloned and sequenced region of 312 nts, from 271 to 583 nts, of the metagenomic *oqxB* gene is a part of the predicted efflux pump domain. Based on the crystal structure of the *E. coli* AcrB protein, the *E. coli* chromosomal efflux system AcrAB-ToIC (69) we have developed a model of the OqxA/B drug efflux transporter. The V89-N194 sequenced region of the *oqxB* spans two regions of the protein which are critically involved with drug recognition/removal and intra/inter subunit stability. The positions
Leu90 and Thr91 are located in the RGD region of the protein which is responsible for substrate recognition and ultimately antibiotic removal from the host. Mutations in this region may alter the poly-substrate specificity of the transporter but based on the increase diameter of the efflux channel a higher rate of antibiotic removal may be predicted. In addition to this, further mutations at positions Gly148 and Asp152 in OqxB result in enhanced thermodynamic and kinetic stability of the complex as a whole through additional intra and inter molecular interactions. Collectively these mutations would give rise to an efflux pump which is predicted to have both a longer half-life and also an enhanced ability to remove drugs from the host. The oqxAB operon carries a resistance-nodulation-cell division type efflux pump that confers resistance to the growth promoter olaquindox as well as to ampicillin and chloramphenicol (39). In this study the identified oqxAB gene of the oqxAB-encoded pump resistance determinant occurs on an IncX1 plasmid, pOLA52 which is also highly mobile due to the presence of an IS26 element and a T3 transposon (70). Alarmingly, oqxAB-positive commensal E. coli isolates have been found among farmworkers who have undergone no previous antibiotic treatment or been admitted to the hospital, suggesting horizontal transfer of the oqxAB resistance (27,71). Direct transmission of oqxAB-positive E. coli between humans and livestock has also been reported (72). Thus, maintenance of E. coli carrying oqxAB in animals in response to Nuflor and possibly to other antibiotics may enhance its spread to humans. This, in turn may increase the risk of horizontal transfer of resistance to human pathogens.

**Implications.** Nuflor treatment of calves resulted in changes in the gut microbiota, so that it resembles the one of obese humans/animals without any diet alteration. Obesity is a major public and clinical health concern affecting around 400 million people globally. Increased fat storage is the hallmark of obesity due to excessive energy intake over expenditure. While diet contributes to the diversity of the gut microbiota (73) and affects body mass, however it is shown that this increase may occur without changes in food consumption. For example, the gut microbiota of normal mice transplanted into germ-free rodents leads to an increase in the body fat of recipients without any increase in food consumption, strongly suggesting that the microbiota in the gut can mediate obesity (26). Hence, the improved energy harvesting capacity of the healthy gut microbiota in response to the antibiotic found in this work argues that antibiotic therapy may contribute to enhanced energy intake while maintaining the same food intake. Therefore its potential impact on obesity is to be taken into consideration by health professionals during the treatment of clinical infections.

As a result of dysbiosis, a significant enrichment in the prevalence of facultative anaerobes such as E. coli in response to a preventive antibiotic treatment increases the risk of aerobic pathogen colonization in the healthy host gut and predisposition to a number of diseases (e.g. cancer via activation of anti-inflammatory responses of the immune system). Additionally, dysbiosis changes permeability of the colon and decreases the defence mechanisms of the host against bacteria-driven toxins. Therefore, a positive outcome of antibiotic preventive therapies, namely protecting the host against potential infection may inadvertently lead to a number of severe side effects. Of clinical concern, the observed expansion of E. coli coincides with the emergence and preservation of E. coli mobile-mediated resistance determinants, a colistin resistance allele mcr-2 and a member of the multidrug efflux pump oqxAB operon, the oqxAB gene (29,38) respectively. These genes confer resistance to antibiotics of first choice (e.g. quinolones) and last resort (e.g. colistin) to treat multidrug resistant hospital bacterial infections. The emergence of the novel mcr-2 gene on Belgian
commercial farms where the AB therapy is strictly regulated (38) showed primary evidence of E. coli carrying mobile elements with stable resistances, such as the mcr allele (26) to preserve genes which might confer the host bacteria novel adaptive properties, e.g. ability for stable colonization in strictly anaerobic environment and/or survive in response to AB pressure. The presence of mcr-2 in the gut of animals on a small rural farm where non-colistin prophylactic antibiotic therapy is commonly used is consistent with the previous findings and its emergence coincides with expansion of E. coli carrying this resistance suggesting that mcr alleles are important for adaptive responses. E. coli carrying efflux pump resistance determinants, such as oqxAB can be less susceptible to a broad range of antibiotics due to acquiring new mutations, hence enhancing their surviving properties. Furthermore, the recently discovered plasmid-born mcr-1 already is found on extended-spectrum β-lactamase multidrug resistance plasmids (e.g. 74) while plasmid-mediated multidrug efflux cassettes are also linked to multidrug resistant infections in the hospital setting (75). While it is shown that originally these resistance elements have been selected due to a direct effect of polymixin and olaquindox to promote animal growth their enrichment in the animal gut in response to Nuflor, a non-cognate class of antibiotic was alarming. The unexpected ‘off-target’ emergence of resistance to ABs that have not been administered shows that stable resistance determinants, such as mcr may be involved in as yet unknown mechanisms that confer a more broad, antibiotic-independent adaptive response. Nevertheless, this represents a collateral effect of antibiotic therapies that may lead to an increase in resistance to non-administered drugs (18) driving the development and spread of a wide range of clinical resistance regardless of the specificity of antibiotics; mobilized colistin resistance determinants have already been identified in clinical settings (38). Identification of the plasmid mediated mcr-2 and oqxAB resistances mark a paradigm shift in our understanding of the spread and development of resistance as until recently they were sequestered to the chromosome (hence, were less likely to be associated with multidrug resistant plasmid-mediated infections which in turn were relatively easy to treat with drugs that target the chromosomal genes) and capable of vertical transmission only. Hence, routine screening for mobile resistance genes in livestock must be considered as a prerequisite for any farm using antibiotics as a preventative therapy on food animals in order to contain the spread of plasmid-borne resistance determinants. Additionally, both resistance determinants have originally been identified on highly transmissible plasmids, IncX4 and IncX1 which impose no fitness burden to bacterial hosts allowing the cells to maintain the plasmid which can be easily spread to other pathogens. These plasmids carry IS (insertion sequence elements) and transposons making them highly transmissible between animals and humans. Based on our results, we would argue that the short-term benefit of a given preventive antibiotic therapy must be carefully assessed and weighed against the collateral effects on promoting antibiotic resistance and that novel screening policies for clinically important bacteria occurring in agricultural environments are urgently required.

Materials and Methods

Samples. Six seven-month old calves were divided into two groups of three. One group (‘medicated’) received an injection of Nuflor® (containing florfenicol, a fluorinated synthetic analogue of thiamphenicol, 40 mg/kg body weight) while the other group (‘non-medicated’) received no antibiotics. The calves were housed in decontaminated and highly controlled setting at the Moreton Morrell College Farm, Warwickshire College. The Warwickshire College Board approved the use of
college animals for this study. Animals were treated under the supervision of the Farm veterinarian and in accordance with the Moreton Morrell College Farm guidelines. All animals were fed the same diet (*ad libitum* hay and silage and 1kg of concentrates/animal/day) throughout the study and were not exposed to any antibiotic treatment prior to the study. A week before the study, the medicated and non-medicated calves were segregated into two separate rooms to prevent cross-contamination. Detail of sample handling is described in *SI Material and Methods*.

**Genomic DNA isolation and sequencing.** Total genomic DNA (gDNA) was extracted using the QIAamp® DNA Stool kit (QIAGEN®). 0.2 g of cell pellet was re-suspended in 180 μL of 1xPBS and processed according to the manufacturer’s instructions. The concentration of purified gDNA was measured by spectrophotometry at A_{260}. 1 μg of gDNA was sequenced using an Illumina 2500. Details of Illumina primer sequences are provided in *SI Material and Methods*.

**Metagenomic profiling of bacterial communities.** For each sample, 2x300 bp sequencing read pairs from 16S amplicons were aligned to each other using flash v1.2.11 (76). Resulting sequences were processed to identify and remove amplification primer sequences from their 5’ and 3’ ends, retaining only those with an identified amplification primer sequence at each end, using cutadapt v1.9.1 (77). Processed sequences containing undefined nucleotides (N’s) and those of unexpected size (< 350 bp or > 450 bp) were removed. To balance sample sizes for each time-course, read sets were randomly down-sampled to match the size of the smallest of the 3 libraries prior to clustering. In each size-matched read set, sequences were de-replicated by clustering identical sequences and clusters of size 1 (likely to be enriched for erroneous sequences) were removed. The remaining filtered, de-replicated reads were clustered at 97 % sequence identity. De-replication and clustering were performed using vsearch v1.1.1 (78). Clusters were further filtered to remove putative chimeric sequences using the “uchime denovo” option in vsearch. The remaining, putatively non-chimeric, clusters were given a taxonomic assignment using SINA (79) using default parameters, the ‘search and classify’ option and the SILVA database as the primary source of taxon labels. In each case, results from SINA were processed by trimming taxonomic labels to the required rank and summing the read numbers for clusters with identical labels.

**Profiling the bacterial community ‘resistome’ using whole genome metagenomic shotgun sequencing.** To assay the ‘resistome’ in each sample, sets of resistance-associated genes were obtained from Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) and used to make a set of databases suitable for use with BLAT (80). Reads were processed by removing sequencing library adapter sequences using cutadapt v1.9.1 (77), retaining only reads longer than 50 bp and containing no ‘N’s after trimming. Trimmed reads were converted to FASTA format and ‘de-replicated’ (identical reads collapsed into one read), using vsearch v1.1.1 (79). This was done to (i) make later analysis more computationally efficient and (ii) enable artefacts such as PCR duplicates to be accounted for in later analyses. De-replicated reads from each sample were searched against each gene set using BLAT (80). All reads and target genes were translated in all 6 reading frames and aligned as proteins in order to capture reads from species more distantly related from the ‘reference’ sequences. Reads matching ‘reference’ sequences in each ‘database’ were quantified as the number of unique sequence matches (i.e. de-replicated reads) and the total number of matches (i.e. including multiple identical reads) and expressed as counts and as proportions of the total library size.
Semi-quantitative PCR amplification of 16S rRNA and *uidA* genes. A significant increase in the DNA-seq reads for *Escherichia* spp. at T7 was validated by semi-quantitative PCR for the *uidA* gene and using the 16S rRNA gene as a normalisation control. PCR conditions are presented in *SI Material and Methods*.

**PCR, cloning and Sanger sequencing of the *mcr-2* and *oqxB* genes.** To confirm the DNA-seq data with regards to the emergence of the *mcr-2* gene and presence or absence of *oqxB* visualisation of nested PCR bands followed by cloning and Sanger sequencing was carried out. Details of cloning approaches are described in *SI Material and Methods*.

**Acknowledgements.** DD was supported by NERC grant to IM (NE/N019288/1) and MTFL was supported by the Health Life Sciences Faculty research Centre for Sport, Exercise and Life Sciences, Coventry University. We would like to thank S. Tompsett and S. Andrews for technical assistance.


Figure legends

Fig. 1. Phylogenetic analysis of the composition of faecal animal gut microbiota. (A) Phylum-level composition of non-medicated, control (s77c, s61c and s62c) and medicated, injected with Nuflor (s63m, s66m and s76m) faecal microbiota samples before treatment (T0) and after the antibiotic treatment, day 3 (T3) and day 7 (T7). The saccharolytic bacteria, Firmicutes and Bacteroidetes are the most abundant phyla, followed by Saccharibacteria, Tenericutes, Spirochaetes with minor contributors Actinobacteria, Proteobacteria, Verrucomicrobia and Cyanobacteria. (B) Firmicutes and Bacteroidetes phyla composition in injected and control samples. (C) Bacterial composition of less abundant phylum in injected and (D) control samples over time course, T0, T3 and T7. The Y axis is the percentage of rDNA-seq reads over the total number of reads. rDNA-seq reads from three medicated and non-medicated faecal samples were quantified and the data represented graphically (± SD). Statistical significance (*) of differences between time courses (T0 before AB treatment, T3 and T7 – days after treatment) was measured by the t-test, one-way ANOVA (SPSS v.24), with p-values < 0.05 considered significant for all comparisons.

Fig. 2. Abundance of methanogenic Archaea (Methanobacteria) spp is significantly increased by T7 in response to the Nuflor treatment (*, T0/T7, p<0.05 (0.003)). (A) Time course over seven days for injected (medicated) and control (non-medicated) samples were plotted. (B) Euryarchaeota (Archaea) composition, percentage of sequences normalised to the total number of reads shown.

Fig. 3. Nuflor treatment leads to dysbiosis, an expansion of Proteobacteria. (A) Phylum-level composition in the gut (*, T0/T3 v T7, p<0.05 (0.01)). (B) Genus-level composition of Escherichia (*, T0/T3 v T7, p<0.05 (0.011)). Details are as in Fig.1.

Fig. 4. Nuflor treatment leads to selection of obese related microbiota. (A) Nuflor treatment leads to a significant decrease in the relative abundance of Verrucomicrobia at T7. Details are as in Fig. 1. *, T0 v T7, p=0.001. (B) Nuflor treatment leads to a significant increase in relative abundance of the family Prevotellaceae (phylum Bacteroidetes). Details are as in Fig. 1. *, T0 v T3, p=0.029, T0 v T7, p=0.045, T1 v T7, p=0.022. C. The animal gut microbiota treated with AB is gradually enriched with the family Erysipelotrichaceae (phylum Firmicutes) (p=0.05). Details are as in Fig. 1.

Fig. 5. The protein sequence of the metagenomic OqxB gene found on the E. coli plasmid pOLA52 (EU370913.1:48049-48434 nucleotide positions). The cloned DNA Sanger sequences were translated into amino acid sequences by using the NCBI ExPaSy Translation Tool. The 128 amino acid translated sequence of the N-terminus (from the 67th to 194th amino acid) is presented. The four different types of amino acid mutations found in the metagenomic sequences are shown in red with superscript letter showing the amino acid substitutions, L90I, T92A, G148N and D152N. The G148N mutation (underlined) was found in all sequenced clones.
Fig. 1A
Fig. 1B
Fig. 1C
Fig. 1D
Fig. 2A
Fig. 2B
Fig. 3A

The graph shows the percentage of Proteobacteria over time for two conditions: Injected and Control. The x-axis represents days (T0, T3, T7), and the y-axis represents the percentage. The Injected condition shows a significant increase in Proteobacteria at T7 compared to T0 and T3. The Control condition remains relatively constant. The asterisks indicate statistical significance.
Fig. 3B
Fig. 4A

Verrucomicrobia, Injected
Verrucomicrobia, Control

Days
To T3 T7

*
Fig. 4B

The graph shows the percentage of Prevotellaceae in samples taken at different time points (T0, T3, T7). The data is represented for both injected and control groups. Significant differences are indicated by asterisks (*) on the graph.
Fig. 4C
67-LEEAINGVENMMYMKSAGSGVLTVTVFRPGTDQAQVQVQRVAQAEARLPEDVR

LGITTQKQSPPTLTLLFVHLFSG

\[\text{DPN-194}\]

Fig. 5
**Supplementary Information (SI).**

**Material and Methods.**

**Sample preparation.** Freshly passed faeces was collected from medicated and non-medicated animals immediately before the treatment (T₀) and at 3 (T₃) and 7 (T₇) days after the treatment. Around 40g of faecal samples were collected and split into four 50 mL Falcon® tubes equally, around 10 g per tube and re-suspended in 40 ml of cold, sterile phosphate buffered saline (1xPBS, 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na₂HPO₄ and 0.24 g/l KH₂PO₄ pH7.4), followed by low speed centrifugation at 300×g (1,400 rpm) for 10 min at 4°C to remove debris, including undigested food. The bacterial cells were pulled down by centrifugation of the resultant supernatant at 3,000×g (4,400 rpm) for 30 min at 4°C. The pellets of bacterial cells were washed with 45 ml of cold 1xPBS and cleared further by centrifugation at 3,000×g as described above. The faecal debris/pellets from the first spin were washed again with 40 ml of cold 1xPBS and processed as above in order to pull down any co-precipitated cells. Pellets of bacterial cells were combined, re-suspended in 10 ml of cold 1xPBS and filtered through Miracloth (Calbiochem®) to clear the cell fraction further. Cells were collected from the flow-through by centrifugation at 3,000×g 30 min at 4°C and stored at -80°C.

**Genomic DNA sequencing.** The V3 and V4 regions of the bacterial 16S rRNA gene were amplified with standard Illumina primers, forward primer: 5'-CCTACGGGNGGCWGCAG-3' and reverse primer: 5'-GACTACHVGGGTATCTAATCC-3' to produce 550 bp products for sequencing (Vertis, Germany). Sequencing of the metagenome for antibiotic resistome analysis, gDNA of T₀ and T₇ for one non-medicated and two medicated animals were used for Illumina NextSeq 500 sequencing (Vertis, Germany).

**Semi-quantitative PCR amplification of 16S rRNA and uidA genes.** The *uidA* gene, encoding the β-glucuronidase (a marker gene for *E. coli*) was targeted with forward primer, 5’-CGAACTGAACTGCGAGAC-3’ and reverse primer, 5’-AATGCGAGGTACGGTAGG-3’ as described previously (1) while the 16S RNA gene was amplified with forward primer, 5’-AGAGTTTGTACCTGGTCTCAG-3’ and reverse primer, 5’-ATTACCGCGGCTGCTGG-3’. For each assay, 240ng of T₀ and T₇ gDNA samples were diluted in a 2-fold series, amplified (3 min at 95°C followed by 15 cycles of 1 min at 95°C, 45 sec at 56°C and 1 min at 72°C) with 10 μM primers, 10 μL of 2xPCR Mastermix (Roche) and analysed on 1% TAE agarose gels. Independent triplicate samples were analysed in parallel.

**PCR, cloning and Sanger sequencing of the mcr-2 and oqxB genes.** 240 ng of gDNA was used for the first PCR with 10 μM primers, 10 μL of 2xPCR Mastermix (Roche), 3 min at 95°C and 40 cycles of 1 min at 95°C, 45 sec at 50°C for mcr-2 or 60°C for oqxB and 1 min at 72°C, and a final elongation step for 10 min at 72°C. Nested PCR with 2 μL of the first PCR reactions as the template was performed as described above with the corresponding annealing temperature for each primer set. mcr-2 was targeted with forward primer 5’-CATCACATCACCTTGGT-3’ and reverse primer 5’-TTGAACATGCGACGCGTCA-3’ (5-761 nts of the gene) followed by a nested PCR for the 44-726 nts region with forward primer 5’-GTGCTCATGTTGTGTTG-3’ and reverse primer 5’-GAGACAGACACACACTAG-3’ at 55°C for annealing. A part of the oqxB gene was amplified with forward primer 5’-CTGGAGAGAGCGATCAAC-3’ and reverse primer 5’-ATTGGATCCAGCCAGAC-3’ (199-582 nts of the gene) followed by amplification with the nested forward primer 5’-GTACCACCGTCACCTC-3’ and the same reverse primer (312-582 nts of the gene) and annealing
temperature at 60°C. To confirm the presence of the pOLA52 plasmid in the gDNA of the medicated samples at day 7 nested PCRs were carried out as described for the mcr-2/oqxB gene. The first set of PCRs was conducted with forward primer 5’-TCAAGAGAGCGCCGCTT-3’ and reverse primer 5’-TGAACGACAGACGCTTGA-3’ at the annealing temperature of 59°C. The nested PCRs targeted the region between 11,464 and 11,700 nts with the same forward primer and reverse primer 5’-CGTCAGGACTGCTTAC-3’ with the annealing temperature of 55°C. The PCR products were visualised on 1% agarose gel, extracted from the gel using QIAquick® Gel Extraction Kit (Qiagen), cloned into pGEM®-T Easy Vector using pGEM®-T Easy Vector Systems (Promega) according to the manufacturer’s protocol and transformed into JM109 competent cells (Promega). Ten randomly selected clones for each set were used for Sanger sequencing (GATC, UK).

**Molecular Modelling.** Molecular models of OqxA and OqxB were generated using the crystal structure of AcrAB-ToLC Multidrug Efflux Pump (PDB 5V5S.pdb) using Modeller (2). 100 models were generated and initially ranked using OPUS_PSP (3). The top scoring model went through 30 cycles of refinement using Rosetta (4). Changes in secondary structure and relative motions of secondary structure elements were analysed using an in-house python script.


**SI Figure legends.**

**Fig. S1.** Assessment of an increase in the *E. coli* level in response to Nuflor treatment. A series of dilutions of the same sets of gDNAs (240 ng, 120 ng or 60 ng) at T₀ and T₇ for the 16S RNA- and *uidA* genes were used for the semi-quantitative PCR amplification. 1 and 3, a 573 nts PCR fragment of *uidA* at T₇ and T₀, correspondently; 2 and 4, a 500 nts PCR fragment of the 16S RNA gene at T₇ and T₀, respectively. From this analysis it was apparent that the T₀ *uidA* sample produced a signal <2% of the Nuflor treated sample at day 7.

**Table S1.** Change in the number/proportion of matches over the time course for ‘non-medicated’.

¹ n=number of unique sequences matching at least one of the reference sequences; p=proportion (x10⁶) of the total unique reads in the library (12,513,980 for T₀ and 14,717,771 for T₇); ² n=total number of sequences matching at least one of the reference sequences; p=proportion (x10⁶) of the total reads in the library (13,892,099 for T₀ and 16,016,605 for T₇).

**Table S2.** Change in the number/proportion of matches over the time course for ‘medicated 1’.
\(^1\) \(n=\) number of unique sequences matching at least one of the reference sequences; \(p=\) proportion \((\times 10^6)\) of the total unique reads in the library \((7,789,067\) for \(T_0\) and \(9,520,799\) for \(T_7\)); \(^2\) \(n=\) total number of sequences matching at least one of the reference sequences; \(p=\) proportion \((\times 10^6)\) of the total reads in the library \((7,948,869\) for \(T_0\) and \(9,962,515\) for \(T_7\)).

**Table S3.** Change in the number/proportion of matches over the time course for ‘medicated 2’.

\(^1\) \(n=\) number of unique sequences matching at least one of the reference sequences; \(p=\) proportion \((\times 10^6)\) of the total unique reads in the library \((7,231,715\) for \(T_0\) and \(8,057,124\) for \(T_7\)); \(^2\) \(n=\) total number of sequences matching at least one of the reference sequences; \(p=\) proportion \((\times 10^6)\) of the total reads in the library \((7,523,723\) for \(T_0\) and \(8,390,249\) for \(T_7\)).

**Table S4.** Changes in the DNA-seq \(OqxB\) reads in response to Nuflor over the time course.

Numbers of \(OqxB\) DNA sequence reads per tested animal gut (from the sequencing of the metagenome for the antibiotic resistome) normalised to the corresponding total number of reads of the sample presented.

**Fig. S2.** Sanger sequencing of the nested PCR fragments for first 683 nucleotides of the \(mcr-2\) gene confirmed the DNA-seq data for the emergence of the \(mcr-2\) allele. Alignment of a representative sequence of the cloned DNA which were BLASTed against bacterial nucleotide database presented.

**Fig. S3. (A)** A molecular model of the \(OqxA/B\) efflux pump as shown parallel to the membrane. Highlighted and coloured by protein chain are the multiple subunits of the efflux pump, blue represents TolC and yellow is \(OqxA\). The individual components \(OqxA\), \(OqxB\) and TolC were shown. **(B)** The drug efflux transporter as viewed from the extracellular side.

**Fig. S4.** The G148N mutation in \(OqxB\). **(A)** In silico mutation at position 148 (the location of the mutated region is within the circle) to introduce an Asp in \(OqxB\) resulted in an increased stability as measured by FoldX. **(B)** Analysis of the simulated mutant substitution revealed that a further hydrogen bond (Asn148-Asp323) was generated to both juxtaposed regions secondary structure elements and also to backbone atoms in the neighbouring sequence.

**Fig. S5.** The D152N mutation in \(OqxB\). **(A)** Figure legends as in Fig. S3 and S4. **(B)** The in silico analysis of the mutation revealed a new hydrogen bond interaction between Asp281 in \(OqxA\) and Asp152 in \(OqxB\) which is absent in the WT structure. This is consistent with the increased stability as defined by FoldX.

**Fig. S6.** The effect of mutations L90I and T92A on the efflux channel. **(A)** The three subunits of \(OqxB\) coloured according to chain (as in Fig. S3) and viewed from above. At the interface of the 3 subunits the position of the drug efflux pore is shown as a circle in the diagram. **(B)** The rearrangement of secondary structure elements as a result of the mutations to positions L90I and T92A (Blue WT, Orange mutated). The backbone for remainder of the protein (shown in white) and was largely unaffected by the mutations and overlaid well with the WT structure. TM shown for orientation of \(OqxB\).
Fig. S1
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<td>Phenicol</td>
<td>30 (4.1484)</td>
<td>46 (5.7092)</td>
<td>30 (3.9874)</td>
<td>61 (7.2703)</td>
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<td>Quinolone</td>
<td>26 (3.5953)</td>
<td>49 (6.0816)</td>
<td>26 (3.4557)</td>
<td>50 (5.9593)</td>
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<tr>
<td>Rifampicin</td>
<td>0 (0.0000)</td>
<td>0 (0.0000)</td>
<td>0 (0.0000)</td>
<td>0 (0.0000)</td>
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<tr>
<td>Sulphonamide</td>
<td>0 (0.0000)</td>
<td>2 (0.2482)</td>
<td>0 (0.0000)</td>
<td>2 (0.2384)</td>
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<tr>
<td>Tetracycline</td>
<td>722 (99.8380)</td>
<td>730 (90.6030)</td>
<td>749 (99.5518)</td>
<td>768 (91.5348)</td>
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<tr>
<td>Trimethoprim</td>
<td>7 (0.9680)</td>
<td>3 (0.3723)</td>
<td>7 (0.9304)</td>
<td>4 (0.4767)</td>
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Table S3
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<th>$T_0$</th>
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<th>$T_7$</th>
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<td>s63, Injected</td>
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<td>24</td>
<td>13</td>
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<tr>
<td>s66, Injected</td>
<td>49</td>
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<tr>
<td>s76, Injected</td>
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Table S4
Fig. S2

LAUGN output for 700_5-M13-FP/RP.seq vs. Mcr2:

Escherichia coli KPS7 pKPS7-BE Mcr-2 gene for phosphoethanolamine-lipid A transferase Mcr-2, complete CDS (100% match) NCBI Reference Sequence: NC_051171.1

Fig. S2
Fig. S3A

TolC, Subunit A

OqxA, Subunit A

TolC

OqxAB

OqxB
Fig. 3B

Drug Efflux Channel

TolC, Subunit A

OqxA, Subunit A
Fig. S4B

G148N

323 Asp
Fig. S5A
Fig. S5B

D152N OqxB

Asp\textsuperscript{281} OqxA
Drug efflux pore

Fig. S6A