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RESEARCH TOPICS

# LOW-DOSE ANTIBIOTICS: CURRENT STATUS AND OUTLOOK FOR THE FUTURE

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Joshua D. Nosanchuk, Jun Lin,
Robert P. Hunter and Rustam I. Aminov

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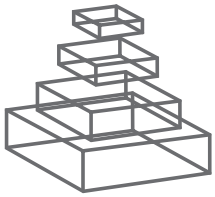

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# LOW-DOSE ANTIBIOTICS: CURRENT STATUS AND OUTLOOK FOR THE FUTURE

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Antimicrobial therapy is a key factor in our success against pathogens poised to ravage at risk or infected individuals. However, we are currently at a watershed point as we face a growing crisis of antibiotic resistance among diverse pathogens. One area of intense interest is the impact of the application of antibiotics for uses other than the treatment of patients and the association with such utilization with emerging drug resistance. This Research Topic "Low-dose antibiotics: current status and outlook for the future" in Frontiers in Microbiology: Antimicrobials, Resistance and Chemotherapy details various aspects of the wide ranging effects of antimicrobial therapy from areas such as the regulation of host responses to modulation of bacterial virulence factors to acquisition of antibiotic resistance genes.

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# Low-dose antibiotics: current status and outlook for the future

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Antimicrobial therapy is a key factor in our success against pathogens poised to ravage at risk or infected individuals. However, we are currently at a watershed point as we face a growing crisis of antibiotic resistance among diverse pathogens. One area of intense interest is the impact of the application of antibiotics for uses other than the treatment of patients and the association with such utilization with emerging drug resistance. This Research Topic "Low-dose antibiotics: current status and outlook for the future" in *Frontiers in Microbiology: Antimicrobials, Resistance, and Chemotherapy* details various aspects of the wide ranging effects of antimicrobial therapy from areas such as the regulation of host responses to modulation of bacterial virulence factors to acquisition of antibiotic resistance genes.

A remarkable and often overlooked fundamental of antibiotics is that they have biological activities beyond microbial killing. The host modulatory aspects of macrolides, tetracyclines, and beta-lactams are reviewed by Aminov (2013a) underscoring how, for example, macrolides such as azithromycin are routinely used for immunomodulation in patients with chronic pulmonary disease rather than for an antimicrobial effect. Azithromycin is also used as a tool by Imperi et al. to detail how non-conventional thinking about regulating virulence factors or modifying host inflammatory cascades are useful to combating major pathogens such as *Pseudomonas aeruginosa* (Imperi et al., 2014). Along this line, Morita and colleagues carefully detail the pleotropic responses of *P. aeruginosa* to sub-therapeutic levels of several antibacterials and propose avenues to pursue to combat this pathogen, such as developing efflux pump inhibitors (Morita et al., 2014). In their article, Charlebois et al. show *Clostridium perfringens* biofilm can be regulated by certain antibiotics at low concentrations (Charlebois et al., 2014). For example, low dose bacitracin significantly enhances biofilm formation whereas low dose penicillin reduces biofilm. This work underscores how there are untoward effects that are not predictable when antimicrobials are administered at low concentrations. Providing a view on specific host effector pathways with antimicrobials, Mihu et al. detail how antifungal medications effectively stimulate host responses via engagement with toll-like receptors (Mihu et al., 2014). In light of the expanding difficulties with drug resistance and a lack of therapeutics to combat them, Clark presents a cogent call for pursing $Ca^{2+}$ modulating strategies where by host $Ca^{2+}$ homeostasis is modulated to block pathogens from effectively utilizing this essential element (Clark, 2013).

An important focus in this Research Topic is the use of antibiotics as growth enhancers in animals. Sorensen and colleagues provide key insights into the effects of scientific evidence on the policy decisions on the use of low-dose antimicrobials in livestock for growth promotion and disease prevention particularly delineating how data have led to the European Union's ban of low-dose antimicrobials whereas their use in the United States of America remains in flux (Sorensen et al., 2014). The bottom line is that there is an urgent need to develop policy based on well derived data, with this data being easily and widely available to independent parties. The articles by Cheng et al. (2014), Chattopadhyay (2014), and Hao et al. (2014) all further underscore critically important facets of the continued utilization of antibiotics in animal husbandry. Looft and colleagues detail their research on how the use of the in-feed antibiotic carbadox cases dramatic short- and long-term effects on the composition of porcine gut microbiota (Looft et al., 2014). Diarra and Malouin specifically describe the impact of antibiotics in Canadian poultry production and describe the use of alternatives, such as bioactive molecules from cranberries, that should not drive antibiotic resistance (Diarra and Malouin, 2014). Similarly, Rendondo et al. provide thoughtful insights into the use of tannins in lieu of antibiotics for improving health in poultry (Redondo et al., 2014). Lin details that the effective of antibiotics as growth promoters is linked to decreased activities of bile salt hydrolase, which thus makes targeting this enzyme directly a promising method for removing antibiotics for use as growth enhancers (Lin, 2014).

You and Silbergeld critically discuss the effects of antimicrobials as drivers of resistome expansion (You and Silbergeld, 2014), a major secondary effect due to environmental pollution. The effects of antibiotics permeating our environment are highlighted by Conro and colleagues who present their findings that the presence of antibiotics in aquatic environments can induce co-aggregation of bacterial species as an effective mechanism to combat the effects of the antimicrobials (Corno et al., 2014), which can lead to extensive resistance through the transfer of resistance genes among these aggregated bacteria. It is a small leap for these microbes to then impact humans and other organisms. Aminov provides the example of the rampant use of tetracyclines for non-medical purposes as driving the penetration of *tet*(X) into pathogenic microbial communities (Aminov, 2013b). Chowdhury and colleagues eloquently discuss the import of surveillance strategies for critically elucidating the emergence of drug resistant pathogens in the context of low-dose antibiotic use in animal husbandry (Roy Chowdhury et al., 2014).

In summary, the articles within this Research Topic serve as a "call to arms" for scientists, policy makers and the public to be increasingly vigilant about the use of antimicrobials, particularly in low-dose or where they can become widespread in the environment, in order to maintain our capacity to effectively care for individuals with infectious diseases. The articles also provide new concepts for approaches for the development of antimicrobials as well as for novel growth enhancers for the use in animal husbandry.

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# Biotic acts of antibiotics

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Biological functions of antibiotics are not limited to killing. The most likely function of antibiotics in natural microbial ecosystems is signaling. Does this signaling function of antibiotics also extend to the eukaryotic – in particular mammalian – cells? In this review, the host modulating properties of three classes of antibiotics (macrolides, tetracyclines, and β-lactams) will be briefly discussed. Antibiotics can be effective in treatment of a broad spectrum of diseases and pathological conditions other than those of infectious etiology and, in this capacity, may find widespread applications beyond the intended antimicrobial use. This use, however, should not compromise the primary function antibiotics are used for. The biological background for this inter-kingdom signaling is also discussed.

**Keywords: tetracyclines, macrolides, β-lactam, inflammation, respiratory, cardiovascular, neuroprotection, cancer**

## INTRODUCTION

We are all familiar with the use of antibiotics for treatment of infectious diseases. But antibiotics do not only kill bacteria, their original role possibly involved signaling functions (Davies et al., 2006; Linares et al., 2006; Yim et al., 2006, 2007; Martínez, 2008; Aminov, 2009; Romero et al., 2011). These functions, usually performed at lower concentrations, are different from those leading to cell death, and they are realized through different sets of molecular targets in the cell. While many aspects of this communication in the microbial world remain elusive, there is a large body of information regarding the signaling effects of low-dose antibiotics on humans and animals beyond the intended antimicrobial activities. Thus the intention of this article is to undertake an interdisciplinary coverage of and familiarize biologists with this aspect of non-antimicrobial antibiotic use in clinical research and practice. The results covered in this review have been collected in various animal models, tissue cultures, and pre-clinical and clinical trials, with little or no involvement of microbiology, and, therefore, might have escaped the attention of microbiologists. I believe this interdisciplinary coverage is highly important to close the gap in non-antimicrobial use of antimicrobials for a number of reasons. First of all, it is the specifics of this type of therapy, with the use of low-dose antibiotics for very extended periods of time measured in weeks, months, and even years. Second, while in clinical microbiology a great deal of attention is paid to the appearance of antibiotic resistance as a side effect of antibiotic therapy, this aspect has had a relatively low priority and has been largely overlooked in the low-dose long-term antibiotic treatment trials. Another aspect that may need more careful consideration in this type of therapy is the role of commensal microbiota, which is also an important player in human metabolism and physiology. Antibiotics act not only on the targets in the human body but also on the microbiota, which is the integral part of human metabolism and physiology. And as we know, the role of commensal microbiota in human health and disease is immense, affecting almost every aspect of it. Thus the antibiotic effects have to be evaluated from both sides of their activities, including the direct interaction of antibiotics with the host cells as well as indirect, through the modulation of microbiota and, correspondingly, microbial metabolites, macromolecules, and other biologically active components of microbiota that affect the host. And finally, it is intriguing to recognize how many molecular targets for antibiotics are in the human body. Is it by chance that they have such pleiotropic properties that are affecting almost every organ or system in the human body? Only three classes of antibiotics are covered in this review because of space restraints. These are the macrolides, tetracyclines, and β-lactams. For the same reason, only few most important examples for each antibiotic class and for each group of diseases are given. These are followed by a discussion of various implications of the effects and consequences of the non-antimicrobial antibiotic use.

## MACROLIDES

There are many examples of antibiotic signaling effects on the host beyond the intended antimicrobial activity. The use of macrolides for treatment of non-infectious diseases has the earliest history among other antibiotics. A considerable amount of information regarding the therapeutic potential of macrolides for non-antimicrobial use has been collected beginning from the late 1980s. Since then, a great number of animal experiments have been performed, many representatives of this class of antibiotics have gone through clinical trials, and a number of drugs in this group have been approved and are currently commonly used in clinical practice for non-antimicrobial purposes.

The use of macrolides has been especially successful in the management of various chronic respiratory diseases not only in the role of antimicrobial agents but also due to their anti-inflammatory and pro-kinetic properties. The positive effect of long-term low-dose administration of erythromycin to patients with diffuse panbronchiolitis was demonstrated by Japanese researchers more than two decades ago, thus suggesting other than antimicrobial nature of erythromycin action (Kudoh et al., 1987; Nagai et al., 1991). From this point, the use of macrolides for non-antimicrobial

purposes has become one of the mainstream choices for treatment of chronic respiratory diseases.

In cystic fibrosis (CF), the main bacterium associated with the pulmonary disease is *Pseudomonas aeruginosa,* which produces biofilms resistant to antibiotic treatment within the airways (Singh et al., 2000). Although *P. aeruginosa* is naturally resistant to macrolides, these antibiotics, even at subinhibitory concentrations, can suppress quorum sensing necessary for biofilm formation (Tateda et al., 2007). This mechanism possibly contributes to the heightened sensitivity of non-susceptible *P. aeruginosa* toward a variety of anti-pseudomonal agents in biofilms when exposed to macrolides at subinhibitory concentrations (Lutz et al., 2012). In addition, low-dose macrolides display immunomodulatory properties influencing cytokine production and altering polymorphonuclear cell functions (Schultz, 2004). This prevents excessive uncontrolled inflammation and associated tissue damage. Another benefit of the macrolide use in the management of CF is the reduced chronic airway hypersecretion (Tamaoki et al., 1995).

Treatment of other respiratory diseases such as asthma may benefit from the dual action of macrolides because asthma is a result of interaction of genetic and environmental factors. The presence of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in asthmatics best identifies the macrolide responsive phenotype because of the antimicrobial and anti-inflammatory properties of macrolides covering the infection and genetic predisposition continuum (Good et al., 2012).

Chronic obstructive pulmonary disease (COPD) remains one of the important causes of morbidity, mortality, and health-care costs worldwide (Mannino and Buist, 2007). Although smoking is the most important risk factor for the disease, it also has a substantial genetic component (Wain et al., 2012). Pathogenesis in COPD is largely driven by dysregulated responses of the innate and adaptive immune systems to the environmental cues leading to an exaggerated inflammatory response, which results in permanent inflammation, tissue damage, and lung function decline (Holloway and Donnelly, 2013). A well-designed, randomized, 1-year trial of erythromycin, at a dose of 250 mg twice daily, has found a significant reduction in COPD exacerbations compared to the placebo group (Seemungal et al., 2008). Long-term administration of azithromycin by outpatients with severe COPD has appeared to be safe and effective, with reduced exacerbations, hospitalizations, and improved quality of life (Blasi et al., 2010). Another trial with a daily azithromycin for 1 year for prevention of exacerbations of COPD has demonstrated decreased frequency of exacerbations and improved quality of life but has caused hearing decrements in a small percentage of subjects (Albert et al., 2011). A recent review of controlled clinical studies focusing on the prevention of COPD exacerbations with long-term azithromycin, erythromycin, or clarithromycin treatment suggests that it is effective, safe, and cost-efficient (Simoens et al., 2013). Other chronic respiratory diseases may also be treated by macrolides, but better designed trials are necessary to confirm their efficacy (Suresh Babu et al., 2013).

Novel effects of macrolides on cardiovascular diseases have been discovered recently. In animal models, clarithromycin has suppressed the development of myocarditis, cardiac rejection, and myocardial ischemia (Nakajima et al., 2010; Suzuki et al., 2012). The positive effect of clarithromycin in cardiovascular diseases may be due to the alteration of inflammatory factors and matrix metalloproteinases (MMPs). MMPs as a part of the extracellular matrix participate in a number of normal physiological processes, which contribute to tissue structure, function, and remodeling, including the myocardium (Spinale et al., 2013). Both the expression and activity of MMPs are regulated by the tissue inhibitors of matrix metalloproteinases (TIMPs), and the MMPs/TIMPs balance is crucial for the normal maintenance of myocardial interstitial homeostasis. Misbalance and the resulting involvement of MMPs in disease, however, have been shown for a number of pathologies spanning from cancer to cardiovascular diseases and to neurodegeneration (Sbardella et al., 2012). The protective effect of clarithromycin in the case of autoimmune myocarditis appears to be implemented through the inhibition of the MMP-9 activity (Hishikari et al., 2010). In the long run, however, a short-term clarithromycin administration in patients with coronary heart disease for clearance of suspected infections results in increased risk of mortality (Gluud et al., 2008).

Immunosuppressive activities of macrolides have been known for almost four decades now. The first macrolide with this activity, rapamycin (also called sirolimus), was discovered by Brazilian researchers during a screening program for antifungal compounds produced by soil bacteria (Vézina et al., 1975). But its use as an antifungal antibiotic has been abandoned due to potent immunosuppressive and antiproliferative activities. Its antiproliferative action is realized through the formation of an active complex with its cytosolic receptor protein, FKBP12, and targeting of a putative lipid kinase termed target of rapamycin (TOR; Brown et al., 1994; Sabers et al., 1995; Wiederrecht et al., 1995). The loss of TOR function leads to the inhibition of G1- to S-phase progression in various sensitive cells. The immunosuppressive activity of rapamycin is also realized via the same protein kinase inhibition pathway affecting cell-cycle proliferation of lymphoid cells (Abraham, 1998).

The TOR complexes regulate cell growth and metabolism in response to environmental and intracellular cues and are comprised of two distinct multiprotein complexes: TOR complex 1 (TORC1), which is sensitive to rapamycin, and TORC2, which is not (Wullschleger et al., 2006). Dysregulation of these complexes is associated with various pathologies, including cancer, cardiovascular diseases, autoimmunity, metabolic disorders, and neurodegenerative diseases. Thus rapamycin and its derivatives can be used for treatment of a variety of diseases (Cruzado, 2008). It is also potentially useful for treatment of substance abuse conditions, alcohol abuse in particular, since inhibition of TORC1 by rapamycin disrupts alcohol-associated memory reconsolidation, leading to a long-lasting suppression of relapse (Barak et al., 2013). Currently it is approved for prevention of transplant rejection, and its latest derivative, everolimus, is widely used to prevent the rejection of heart, lung, kidney, or liver allografts (Gurk-Turner et al., 2012). Since TOR complexes are evolutionary conserved and involved in very fundamental biological processes in the cell, pharmacological inhibition of TOR signaling by rapamycin increases the lifespan of yeasts and higher eukaryotes (Powers et al., 2006). The use of rapamycin in humans as an anti-aging agent is uncertain because of side effects; this use will require the

development of safer derivatives, termed rapalogs (Lamming et al., 2013).

Another macrolide compound with a potent immunosuppressive activity was discovered in 1984 during the screening program of Fujisawa Pharmaceutical Company aimed at compounds that reduce the risk of transplant rejection (Kino et al., 1987). This 23-membered macrolide lactone, isolated from *Streptomyces tsukubaensis* and called FK-506 (later also called tacrolimus or fujimycin), was initially approved in 1994 for the prophylaxis of liver transplant rejection, and since then the range of its use has expanded dramatically. In fact, it is considered as a cornerstone of modern immunosuppressive therapy and is used to treat allograft rejections that are resistant to other immunosuppressants (Rath, 2013). The effect of this class of macrolide immunosuppressants is also based on targeting the evolutionary conserved signal transduction pathways but via a mechanism other than that of rapamycin. In particular, FK506/FKBP complex inhibits the cytosolic phosphatase calcineurin, a key enzyme regulating the translocation of cytosolic components of various nuclear factors into the nucleus. Thus the blocked translocation of the cytosolic component of the nuclear factor of activated T cells (NF-AT) leads to its inability to activate a number of genes necessary for the proliferation of T cell such as IL-2 as well as for B cell help such as IL-4 (Ho et al., 1996).

Besides the extensive use in prophylaxis of transplant rejection, tacrolimus has demonstrated its efficacy and safety for a number of other inflammatory conditions. It has been successfully used in patients with inflammatory bowel disease, in particular for the treatment of severe cases (Baumgart et al., 2006) and for the induction of remission in refractory disease (Baumgart et al., 2008). In dermatology, the success of this drug is due to its topical effectiveness while cyclosporine, a drug with a similar mechanism of action, is topically non-responsive (Mrowietz, 1992). In general, many macrolide immunosuppressants, due to their chemical structure, are highly efficient in the topical form and are used extensively to treat many dermatological disorders (Mrowietz, 1999). They lack the skin-thinning side effect of corticosteroids and, therefore, can be used for extended periods of time and in areas with thin skin.

Macrolides are among the safest antibiotics in clinical use, with very few severe side effects (Periti et al., 1993). The most frequent manifestation is gastrointestinal disturbance occurring in 15–20% of patients on erythromycins and in 5% or fewer patients treated with novel macrolides. Transient deafness and allergic reactions are highly unusual and usually associated with the older macrolides. Clarithromycin and erythromycin may potentiate calcium-channel blockers by inhibiting cytochrome P450 isoenzyme 3A4 (Wright et al., 2011). Therefore, the concomitant use of calcium-channel blockers and these antibiotics may result in significant hypotension and shock (Wright et al., 2011; Henneman and Thornby, 2012). Although the risk is small, it can greater among the elderly and patients with multiple comorbidities.

In a subset of patients the use of tacrolimus for the management of hematopoietic allogeneic stem cell or solid organ transplantation is associated with a rare complication, posterior reversible encephalopathy syndrome (PRES; Wong et al., 2003; Bartynski et al., 2008; Hodnett et al., 2009; Wu et al., 2010; Hammerstrom et al., 2013). The main manifestations of PRES include altered mental status, seizures, visual abnormalities, and high blood pressure. While it is not clear how to manage the central nervous system (CNS)-related side effects, PRES associated with high blood pressure should include adequate blood pressure control (Hammerstrom et al., 2013).

Substantial progress has been made in identification of mammalian cell targets of macrolides. As discussed above, there is an extensive variety of targets for macrolides in the human body, and the list continues to grow. For instance, the immunomodulatory activities of macrolides can be mediated via the inhibition of production of many proinflammatory cytokines, the formation of leukotriene B4 (a neutrophils attractant), the formation of adhesion molecules necessary for neutrophil migration, and the release of superoxide anion by neutrophils (Tamaoki et al., 2004). The ketolide antibiotic telithromycin exerts powerful immunomodulatory and anti-inflammatory effects through NF-κB inhibition and enhancement of inflammatory cell apoptosis (Leiva et al., 2008a,b).

The macrolide antibiotic-binding human p8 protein has been cloned and identified using the phage display library approach (Morimura et al., 2008). This is a nuclear DNA-binding protein, which is strongly activated in response to several stresses, and, on the basis of functional similarity to HMG-I/Y-like proteins, it has been suggested that p8 may be involved in transcription regulation (Encinar et al., 2001; Hoffmeister et al., 2002). It plays an important role in such a basic biological process as ontogeny and hence is involved in a variety of developmental processes, such as pancreatic development in rats (Mallo et al., 1997), temporal expression of the beta subunit of luteinizing hormone (LHB) during gonadotroph development in mice (Million Passe et al., 2008), and mediation of gene expression in the diapause-destined crustacean *Artemia franciscana* (Qiu and MacRae, 2007).

In pathologies, the p8 protein is crucial for tumor development (Vasseur et al., 2002), and it is also involved in stress responses imposed by inflammation, tissue damage, and remodeling. Thus the list of pathologies includes diseases with an inflammatory component, acute pancreatitis for instance (Mallo et al., 1997). In cardiac pathology, p8 is broadly involved in cellular events leading to cardiomyocyte hypertrophy and cardiac fibroblast MMPs production, both of which take place in heart failure (Goruppi et al., 2007). Since clarithromycin, erythromycin, and azithromycin inhibit the binding of recombinant p8 protein to double-stranded DNA (Morimura et al., 2008), the anti-inflammatory effect of macrolides discussed above may be explained, at least in part, by the down-regulation of transcription of genes involved in the proinflammatory network. Interestingly, the same inhibitory effect has been observed with a structurally unrelated antifungal antibiotic dechlorogriseofulvin (Morimura et al., 2008), suggesting a potential overlap in recognition of structurally different antibiotic ligands by a single human molecular target.

The important difference between the antibiotic therapy of "classical" infectious diseases and chronic conditions such as cystic CF is the duration of antibiotic treatment. With the exception of *Mycobacterium tuberculosis* and few other difficult-to-eradicate infections, the antibiotic treatment period for infectious diseases is relatively short, while the maintenance therapy for chronic conditions is a long-term and perhaps life-long endeavor. One

of the consequences of the maintenance therapy might be the selection for, and maintenance of, antibiotic resistance genes. In recent clinical trials evaluating the efficiency of the long-term azithromycin and erythromycin maintenance treatments in patients with non-CF bronchiectasis, the level of macrolide resistance significantly increased, despite the subinhibitory concentrations used (Altenburg et al., 2013; Serisier et al., 2013). Due to the importance of other than antibacterial activities of macrolides as well as to reduce the possibility of antibiotic selection for resistance, efforts have been made to design macrolide molecules with better anti-inflammatory activities (Kobayashi et al., 2013). Another approach to lessen the resistance burden is the design of macrolides that have their antimicrobial activities completely abolished but have other activities retained. It appears that the antimicrobial and anti-inflammatory activities of macrolides are independent and can be separated, thus opening the possibility of designing macrolide-based anti-inflammatory drugs lacking antimicrobial activities (Bosnar et al., 2012).

Thus the recent macrolide development efforts have bifurcated into two directions that are focused on designing and modification of macrolides better suited either for the antimicrobial or non-antimicrobial use. Historically, antibiotics (macrolides included), as their name implies, have been selected primarily for their antimicrobial activities, while other activities such as anti-inflammatory went unnoticed for a long time. In a recent non-antimicrobial antibiotic development, a novel macrolide, solithromycin, has displayed the capability to inhibit NF-κB and demonstrated better anti-inflammatory activities *in vitro* compared to more conventional macrolides used in the clinic such as erythromycin, clarithromycin, azithromycin, and telithromycin (Kobayashi et al., 2013). A better anti-inflammatory profile of this macrolide makes it a good candidate for the management of chronic respiratory diseases. Another macrolide with a significantly diminished antibiotic activity, 2′-desoxy-9-(S)-erythromycylamine, prevents neutrophil elastase-induced mucus stasis and dehydration and, therefore, may be used for the management of CF and COPD theoretically without affecting antibiotic resistance profile (Tarran et al., 2013). Synthetic mimetics of actin-binding macrolides may provide a range of designer compounds to treat actin-associated diseases (Perrins et al., 2008). On the other hand, there are continuous efforts to modify the existing macrolides to contain pathogens that are becoming resistant to older macrolides. Recent developments, for example, have been based on the use of azalide scaffold (Ištuk et al., 2011; Sugimoto et al., 2012), which was originally implemented in 9-dihydro-9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin A (azithromycin), the antibiotic with outstanding pharmacokinetic properties (Amsden, 2001; Mutak, 2007).

## TETRACYCLINES

Tetracycline family of antibiotics is one of the best-studied examples of non-antimicrobial effects of antibiotics on the host. Tetracyclines possess multiple and potent biological activities, and minocycline, the best exemplary compound of this class, displays anti-inflammatory, neuroprotective, anti-proteolytic, and anti-apoptotic properties as well as inhibits angiogenesis and metastatic growth (Garrido-Mesa et al., 2013). In addition, it displays antioxidant activity, inhibits several enzyme activities, and regulates immune cell activation and proliferation.

Similar to the macrolides discussed above, tetracyclines are able to inhibit MMPs; this discovery was actually made in 1983, i.e., before the discovery of the corresponding activity among macrolides (Golub et al., 1983). As mentioned before, MMPs are involved in a number of pathologies, including metastatic growth, cardiovascular diseases, neurodegeneration, and a variety of inflammatory conditions (Sbardella et al., 2012). Thus the inhibition of MMPs may be a valuable option in treatment of a broad spectrum of diseases. Interestingly, the inhibitory effect on MMPs is realized through several targets/mechanisms ranging from indirect effects of regulatory network to down-regulation of expression and to direct interference with enzymatic activity. These activities can include binding divalent cations such as $Ca^{2+}$ and $Zn^{2+}$, inhibition of neutrophil migration and degranulation, and suppression of synthesis of oxygen radicals (Gabler and Creamer, 1991). Administration of minocycline to diabetic rats, for example, normalized the activity of four MMPs, while in *in vitro* assays minocycline inhibited only collagenase and gelatinase activities, with no inhibition of elastase and β-glucuronidase (Chang et al., 1996). The inhibitory effect of tetracycline on stromelysin is mediated via transcriptional inhibition involving sequences upstream of the activating protein complex 1 binding site (Jonat et al., 1996). But doxycycline, for example, down-regulates MMP-8 induction at both the mRNA and protein levels (Hanemaaijer et al., 1997). It also disrupts the conformation of the hemopexin-like domain of MMP-13 and the catalytic domain of MMP-8 (Smith et al., 1999).

Presently the only MMPs-targeting tetracycline that has been approved by the US Food and Drug Administration (FDA) and other national regulatory agencies in Canada and Europe is the low-dose formulation of doxycycline for the adjunctive treatment of chronic periodontal disease (Gu et al., 2012). Other conditions for potential application of low-dose tetracyclines to inhibit the pathological effects of MMPs are: (i) cardiovascular diseases such as coronary artery disease (Payne et al., 2011), hypertension (Castro et al., 2011), atherosclerosis (Gu et al., 2011), and abdominal aortic aneurysm (Abdul-Hussien et al., 2009); (ii) pulmonary diseases such as acute respiratory distress syndrome (ARDS) for which there is no approved medication (Roy et al., 2011), and COPD (Dalvi et al., 2011); (iii)metastatic cancers (Lokeshwar, 2011; Richards et al., 2011); and (iv) systemic bone loss conditions (Payne and Golub, 2011).

In many studies tetracyclines have demonstrated excellent anti-inflammatory activities achieved through the inhibition of chemotaxis, granuloma formation, nitric oxide production, and protease activities (Weinberg, 2005; Webster and Del Rosso, 2007). Positive effects of minocycline have been observed in animal models of rheumatoid arthritis (RA; Sewell et al., 1996), and this effect has been confirmed in several clinical trials as well (Greenwald, 2011). Tetracycline treatment of RA, however, is not widespread because of the almost universal use of methotrexate. Based on the results of successful clinical trials, subinhibitory concentrations of doxycycline and minocycline were approved by the FDA for the treatment of skin conditions and infections that have a

substantial inflammatory component (Del Rosso, 2007). Another FDA approval, after successful clinical trials, has been obtained for the long-term management of chronic periodontitis by subantimicrobial doses of doxycycline (SDD) (Caton and Ryan, 2011). This host modulatory therapy is directed against excessive MMPs activities that are implicated in degradation of connective tissue collagen surrounding and supporting the teeth. This effect may have broader implications for other bone loss conditions (Payne and Golub, 2011).

Following the first reports on the neuroprotective effects of minocycline in animal models of cerebral ischemic injury (Yrjanheikki et al., 1998, 1999), doxycycline was proposed as a candidate for clinical trials of acute neurologic injury (Elewa et al., 2006). Among other tetracyclines, the neuroprotective potential of the second-generation antibiotic, minocycline, is remarkable. Its effect has been confirmed in experimental models of ischaemia, traumatic brain injury and neuropathic pain, and of several neurodegenerative conditions including Parkinson's disease (Wu et al., 2002), Huntington's disease (Chen et al., 2000; Wang et al., 2003), Alzheimer's disease (Choi et al., 2007), amyotrophic lateral sclerosis (ALS; Tikka et al., 2002; Zhu et al., 2002), multiple sclerosis (MS; Metz et al., 2004; Zabad et al., 2007), and spinal cord injury (Marchand et al., 2009).

Presently, the results of several clinical trials aimed at the estimation of neuroprotective effects of minocycline are available, although with rather discouraging outcomes compared to animal studies. A phase III randomized trial of minocycline in ALS patients actually demonstrated a harmful effect of minocycline (Gordon et al., 2007). A futility study of minocycline in Huntington's disease precluded proceeding with a phase III clinical trial (Schwarz et al., 2010). A prospective study with a cohort of multiple-system-atrophy Parkinson-type patients failed to show a clinical effect of minocycline on severity of symptoms (Dodel et al., 2010). Although the results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury did not establish efficacy, several outcome measures had a tendency toward improvement (Casha et al., 2012).

Treatment of a psychiatric illness relies on a combination of psychological and biological approaches. The latter have been for a long time focused on pharmacological interventions targeting mainly the neurotransmitter systems, but there is a growing body of evidence that these conditions are system-wide and include oxidative stress, inflammation, changes in glutamatergic pathways and neurotrophins as well (Dean et al., 2012). Minocycline is known as a modulator of glutamate-induced excitotoxicity and, in addition, it possesses antioxidative, anti-inflammatory, and neuroprotective properties. Pleiotropic properties of minocycline targeting multiple proteins and cellular processes implicated in the pathoetiology of mood disorders make it a suitable candidate for treatment of depression (Soczynska et al., 2012). It may be a valuable adjunctive therapeutic agent to antipsychotic medication in patients with schizophrenia as well (Miyaoka, 2008). In a clinical ad-on trial, minocycline treatment of early-phase schizophrenia patients improved negative symptoms and cognitive functions (Levkovitz et al., 2010). A recent preliminary open-label study has suggested that minocycline, in combination with antidepressants, is effective and well-tolerated in the treatment of unipolar psychotic depression (Miyaoka et al., 2012). In a mouse model of Fragile X syndrome (FXS), an inherited disorder with intellectual disability and behavior at the extreme of the autistic spectrum, minocycline showed potential to treat mental retardation and associated behavior (Bilousova et al., 2009; Rotschafer et al., 2012). Recent FXS clinical trials have indicated that minocycline may be effective in treating human patients as well (Siller and Broadie, 2012). In a number of cognitive impairment models minocycline treatment demonstrated promising results (Jin et al., 2013; Kong et al., 2013; Li et al., 2013). In a recent clinical trial evaluating the effect of minocycline on HIV-associated cognitive impairment, however, no significant improvement in cognitive functions has been found (Nakasujja et al., 2013).

Microglia are glial cells, the only resident immune cells in the CNS that respond to infections and brain injury and are actively involved in brain development and function as well as in neurodegenerative disease (Miyamoto et al., 2013). In the normal brain, these cells contribute to neuronal proliferation and differentiation, pruning of dying neurons, synaptic remodeling, and clearance of debris and aberrant proteins (Harry, 2013). Analogous to the activities of immune cells, activated microglia release cytokines, chemokines, nitric oxide, and reactive oxygen species (Harry, 2013). Dysfunctions in the homeostatic role of microglia, however, can affect neuronal functions such as cognition, personality, and information processing (Miyamoto et al., 2013).

Minocycline is known as the only drug capable of inhibiting the activation and proliferation of microglia (Tikka et al., 2001). In pathologies such as global brain ischemia, suppression of microglial activation by tetracyclines has a neuroprotective effect with a much better survival rate of CA1 pyramidal neurons (Yrjanheikki et al., 1998). The neuroprotective effects of tetracyclines in brain hypoxia are mainly due to the selective down-regulation of proinflammatory cytokines and compounds in the microglia (Lai and Todd, 2006). Overly rapid correction of chronic hyponatremia can lead to a severe demyelination disease, and the inhibition of microglial activation by minocycline prevents neurologic impairment and improves the survival rate (Suzuki et al., 2010). Minocycline also protects against microglial activation, neuronal death, and cognitive impairment caused by severe hypoglycemia in diabetic patients (Won et al., 2012). In the normal brain, the corresponding activity of minocycline modulates human social behavior leading to a more situation-oriented decision-making, possibly by suppressing the effects of personality traits (Kato et al., 2012). Minocycline also significantly reduces the risky trusting behavior in human economic exchange (Watabe et al., 2013).

This, not exhaustive but still impressive, list of conditions and diseases that can be treated with tetracyclines suggests the pleiotropic effects and the presence of multiple targets and receptors for which tetracyclines are ligands. In addition to the discussed above, the known anti-inflammatory activities of minocycline are achieved through the inhibition of expression of nitric oxide synthases (Amin et al., 1996), suppression of B and T cell function (Sewell et al., 1996), reduction of cyclooxygenase-2 expression and prostaglandin E(2) production (Yrjanheikki et al., 1999), and up-regulation of IL-10 (Ledeboer et al., 2005). Anti-apoptotic effects are believed to be due to inhibition of activity

of caspase-1 and caspase-3 (Chen et al., 2000), inhibition of the phosphorylation of p38 mitogen-activated protein kinase (MAPK) (Du et al., 2001; Joks and Durkin, 2011), and inhibition of mitochondrial permeability-transition-mediated cytochrome *c* release (Zhu et al., 2002). By virtue of its molecular structure, minocycline is also an effective antioxidant with a radical scavenging potency similar to vitamin E thus providing excellent protection against oxidative stress (Kraus et al., 2005).

Tetracyclines are generally well-tolerated but under particular environmental conditions and in a subset of certain age and disease cohorts may provoke adverse reactions. One of the most known and well-studied side effects of tetracycline administration are cutaneous adverse events due to the increased photosensitivity of the skin, typically to the UVA spectrum of light (Drucker and Rosen, 2011; Glatz and Hofbauer, 2012). Clinically these effects can be divided into two groups, phototoxic and photoallergic reactions. The former is due to the formation of reactive oxygen species, with the impairment of many cellular macromolecules, thus leading to inflammation and apoptosis, while the latter is a type IV hypersensitivity reaction resembling eczema. The preventive measures include avoiding direct sunlight and the use of sunscreens. The culprit drug can be withdrawn if the reactions persist (Glatz and Hofbauer, 2012).

Tetracyclines are generally not recommended for pediatric patients because these compounds chelate calcium ions, which are incorporated into teeth, resulting in discoloration of both the primary and permanent dentitions (Sánchez et al., 2004). There are many case reports on the association of tooth, bone, nail, and scleral pigmentation following minocycline administration in adults as well but no systematic studies have been done in this area.

Many inflammatory diseases such as Alzheimer's, Parkinson's, Huntington's, familial Mediterranean fever, and others tend to display a substantial protein deposition bias, where a normally soluble protein is deposited in an insoluble amyloid form (Carrell and Lomas, 1997). The deposits interfere with cellular functions, eventually leading to the cell death (Thomas et al., 1995). Tetracyclines are known to inhibit the deposition process (Sirangelo and Irace, 2010) but at the cost, by keeping the amyloid protein in a pre-fibrillar, highly cytotoxic state (Malmo et al., 2006). So care should be exercised to avoid the toxic effects of oligomeric species during tetracycline therapy.

In a development analogous to the search for non-antimicrobial macrolides there have been a series of works aimed at designing tetracycline derivatives with diminished antimicrobial activities while retaining or enhancing other activities important for their non-antimicrobial use. It needs to be noted here that the works with non-antimicrobial tetracycline derivatives have been actually initiated earlier than those with macrolides, i.e., shortly after the discovery of the host modulating properties of low-dose tetracyclines 30 years ago (Golub et al., 1983). However, a closer look into literature shows that the host modulating effects of antibiotic derivatives were detected even earlier, in 1950 (Stokstad and Jukes, 1950). In one of their experiments, the discoverers of growth-promoting antibiotics found that the cultural supernatant of *S. aureofaciens*, in which the antibiotic activity of aureomycin is destroyed by alkaline hydrolysis, still enhanced the growth and improved survival of chicks when added to feed (Stokstad and Jukes, 1950). Thus, the loss of antimicrobial activity of aureomycin has not compromised its other biological activities such as growth promotion. It is unfortunate that this interesting observation has been left without attention it deserves for so many years.

Chemically modified tetracyclines with no antimicrobial activity may have numerous applications without the associated risk of selecting for antibiotic resistance. For example, chemical conversion of tetracycline hydrochloride to the analog with no antimicrobial activity, de-dimethylaminotetracycline, has not compromised the collagenase inhibitory activity of the original molecule (Golub et al., 1987), while not affecting the tetracycline resistance profiles of gut and oral microbiota (Golub et al., 1991). COL-3, a chemically modified tetracycline with a MMP inhibitor activity, has showed promising results in the treatment of AIDS-related Kaposi's sarcoma (Dezube et al., 2006). Moreover, the use of modified tetracyclines has showed promising results in many fields, including ophthalmologic diseases (Federici, 2011), dentistry (Grenier et al., 2002; Gu et al., 2012), cardiovascular pathologies (Salo et al., 2006; Gu et al., 2011), various types of cancer (Lokeshwar, 1999, 2011; Syed et al., 2004; Zhao et al., 2013a), and other conditions with excessive MMPs activities (Golub, 2011).

Another direction in the development of tetracyclines is understandably focused on the design of drugs with better antimicrobial activities and pharmacokinetic properties. Despite being highly efficient upon introduction in the clinical practice in the 1950s, the widespread resistance to the first- and second-generation tetracyclines made them essentially useless for treatment of many serious infectious diseases. One of the successful drug discovery programs resulted in a third-generation tetracycline called tigecycline (the minocycline derivative 9-tert-butyl-glycylamido-minocycline). The antibiotic is highly efficient against a broad range of pathogenic bacteria, including those resistant to the first- and second-generation tetracyclines (Bertrand and Dowzicky, 2012). Although it is on the list of reserve drugs, its use is steadily increasing (Huttner et al., 2012). Regrettably, similar to the fate of other antibiotics, the efficiency of tigecycline may start to deteriorate due to the penetration of tigecycline resistance into pathogenic microbiota (Aminov, 2013).

## β-LACTAMS

One of the most remarkable breakthroughs in the search for the therapy of neurodegenerative diseases has identified β-lactams as a very promising group of drugs. In a large screening effort involving 1,040 FDA-approved drugs and nutritionals, it has been discovered that the only drugs capable of regulating the expression and modulating the activity of the glutamate transporter subtype 1 (GLT-1) are β-lactams (Rothstein et al., 2005). Glutamate is a principal excitatory neurotransmitter in the CNS and contributes to learning and memory (Shigeri et al., 2004). The concentration of glutamate is mainly handled by GLT-1 (excitatory amino-acid transporter 2, EAAT2, responsible for 90% of glutamate uptake; Danbolt, 2001). Impairment of EAAT2 function leads to excess of glutamate and associated glutamate excitotoxicity destroying neurons and leading to neurodegenerative diseases such as ALS, epilepsy, and others (Maragakis and Rothstein, 2001). No practical pharmaceuticals modulating EAAT2 expression and activity

were known until the discovery of such activity among β-lactams (Rothstein et al., 2005). A multi-phase randomized trial of ceftriaxone for treatment of ALS has been recently finalized (Berry et al., 2013).

Similar to other antibiotics, β-lactams can target many components of the eukaryotic cellular machinery, and the effects of β-lactams are not limited solely to the modulation of expression and activity of EAAT2. In a recent investigation of ceftriaxone as a potential therapy using a murine model of spinal muscular atrophy, the effects, in addition to the increase of EAAT2, also included the increase of the nuclear factor (erythroid-derived 2)-like 2, Nrf2, and the spinal muscular atrophy protein SMN (Nizzardo et al., 2011). The treatment resulted in significant amelioration of the neuromuscular phenotype and increased survival consistent with the protection of neuromuscular units through the activation of antioxidant response pathway governed by Nrf2. Another work has pointed to this target of ceftriaxone as well: together with the induction of the cystine/glutamate transporter SLC7A11 (formerly xCT), the neuroprotective effect of ceftriaxone *in vitro* is combined with the induction of Nrf2 consistent with the activation of the antioxidant defense system of the cell (Lewerenz et al., 2009).

In various models of brain injury ceftriaxone demonstrates strong neuroprotective effects, mainly via the up-regulation of GLT-1. In an experimental model of focal cerebral ischemia, the administration of ceftriaxone induces ischemic tolerance resulting in a better functional recovery of animals (Chu et al., 2007). A dramatic survival improvement can be seen in a rat model of stroke, if the animals are treated by a single injection of ceftriaxone 90 min after the middle cerebral artery occlusion (Thöne-Reineke et al., 2008). Pre-treatment with ceftriaxone also confers a significant neuroprotection in a cerebral ischemia/reperfusion injury (Verma et al., 2010). In a neonatal rat model of hypoxic–ischemic encephalopathy, pre-treatment with the antibiotic significantly reduces the brain injury scores and apoptotic cells in the hippocampus, restores myelination in the external capsule, and improves the posttraumatic learning and memory deficits (Lai et al., 2011). The neuroprotective effects of ceftriaxone are realized not only through the regulation of expression and activity of GLT-1, SLC7A11, and Nrf2. In a rat model of traumatic brain injury the antibiotic also significantly reduces the level of proinflammatory cytokines (Wei et al., 2012). Thus the improvement of cognitive functions and mitigation of brain edema after a brain injury, which is treated by posttraumatic administration of ceftriaxone, is a combined effect of reduced excitotoxicity and suppressed inflammation.

The potent immunomodulatory properties of β-lactam antibiotics, including inflammation control, are not limited to the sole example given above (Wei et al., 2012). In a mouse model of MS, ceftriaxone treatment indirectly hampered T cell proliferation and secretion of proinflammatory cytokines thus attenuating the disease course and its severity in this model of autoimmune CNS inflammation (Melzer et al., 2008). Interestingly, ceftriaxone has had no impact on the EAAT2 protein expression levels in several brain areas as well as on the glutamate uptake rate suggesting that in this model the positive effects of the antibiotic are not mediated through the modulation of glutamate concentration. Moreover, it seems that even the individual antibiotics within the β-lactam group may display differential immunomodulatory properties (Mor and Cohen, 2013). This mechanism operates via covalent binding of various β-lactams to cellular albumin and subsequent modulation of T cell function and gene expression.

β-lactams may be considered as valuable candidates for treatment of alcohol and other drug dependencies due to the capability of normalizing glutamate transmission, which is affected in addiction (Kalivas et al., 2009). To start with the simplest model: in planarians, ceftriaxone attenuates both the development of physical dependence and abstinence-induced withdrawal from cocaine, amphetamine, methamphetamine, and benzodiazepine (Rawls et al., 2008). In rats, the administration of ceftriaxone may suppress cue- and cocaine-induced relapses to cocaine-seeking behavior via up-regulation of GLT-1 and SLC7A11 (Sari et al., 2009; Knackstedt et al., 2010). Ceftriaxone also precludes cocaine sensitization and provides a long-term attenuation of cue- and cocaine-primed reinstatement of cocaine-seeking behavior, even after the cessation of antibiotic administration (Sondheimer and Knackstedt, 2011). In general, the antibiotic normalizes many aspects of glutamate homeostasis disrupted by the use of cocaine and, therefore, has the potential to lessen relapse episodes in human cocaine addicts (Trantham-Davidson et al., 2012). Currently there is no approved medication for the treatment of cocaine addiction, and the promising results obtained in the animal model experiments described above suggest that ceftriaxone (and possibly other β-lactams) can be considered as good candidates for clinical trials.

The biggest substance abuse problem is associated with excessive ethanol consumption, and β-lactams hold the potential to contribute to this problem as well. The biochemistry of ethanol addiction is more complicated compared to other drug dependencies, but one of its components discussed above, i.e., changes in glutamate transmission, is affected analogous to other drug addictions (Rao and Sari, 2012). Possibly the same mechanism of normalization, through the activation of GLT-1 by ceftriaxone, contributes to reduced ethanol consumption as well as to attenuation of relapse-like ethanol-drinking behavior in male alcohol-preferring rats (Sari et al., 2011; Qrunfleh et al., 2013). Reduction in the acquisition and maintenance of ethanol-drinking habit by ceftriaxone has also been verified for adolescent and adult female alcohol-preferring rats (Sari et al., 2013).

Enhanced glutamatergic transmission is a primary mediator of opiate dependence, and the counteractive effect of ceftriaxone prevents the development of morphine physical dependence in rats (Rawls et al., 2010a). Through the same remedial mechanism, the antibiotic reduces morphine analgesic tolerance (Rawls et al., 2010b) and suppresses opioid-induced hyperalgesia (Chen et al., 2012). The efficacy of ceftriaxone against drug dependence-related behavior extends to amphetamine (Rasmussen et al., 2011) and nicotine (Alajaji et al., 2013), as well as to the prevention of cannabinoid tolerance (Gunduz et al., 2011).

In general even the higher doses of ceftriaxone are tolerated well but care should be taken in neonates, especially those with hyperbilirubinemia and those receiving intravenous calcium solutions (Monte et al., 2008). There is no support for such restrictions in patients >28 days old (Steadman et al., 2010). The adverse

reactions to ceftriaxone are caused by rapid intravenous injection, unlabeled use, and past history of allergic reactions to cephalosporins or penicillins (Shalviri et al., 2012). These risk factors can be easily managed under the normal clinical settings.

The host modulating properties of β-lactams have been discovered recently, and there is yet no data regarding the development of β-lactams with abolished antimicrobial activity. Unlike the macrolides and tetracyclines, the non-antimicrobial effects of β-lactams have been studied mostly with a single representative, ceftriaxone, and it is not clear if other representatives of this class of drugs possess similar properties. Another problematic area in ceftriaxone application for non-antimicrobial purposes is the concentrations used. The concentrations may be even higher than those for infectious disease treatment thus exerting potent selective pressure on commensal and pathogenic microbiota. This third-generation cephalosporin still remains a valuable therapeutic option for treatment of pneumonia, bacterial meningitis, Lyme disease, typhoid fever, and gonorrhea. Although the drug is losing its position as the last remaining option for first-line empiric treatment of, for example, *Neisseria gonorrhoeae* (Unemo and Nicholas, 2012), there are still some pathogens that have not acquired the corresponding resistance as yet. In this situation, exerting additional selective pressure by the non-antimicrobial use of ceftriaxone may complicate the management of a number of infectious diseases.

## DISCUSSION

Based on many promising results of the non-antimicrobial use of antimicrobials, the use of antibiotics for this purpose is expected to rise dramatically. Although being considered in the most recent clinical trials, a possible side effect of this therapy, such as the emergence and dissemination of antibiotic resistance among commensals and pathogens, has not received any considerable attention, and, in fact, the majority of clinical trials cited here have not monitored the occurrence of antibiotic resistance. There are some indications, however, that even low, non-selective concentrations of antibiotics used for a long time may affect the antibiotic resistance profile of human microbiota. For example, in a recent clinical trial of long-term, low-dose erythromycin on pulmonary exacerbations among patients with non-CF bronchiectasis, the proportion of macrolide-resistant oropharyngeal streptococci at the end of therapy has been substantially higher in the treatment group compared to the placebo group, 27.7 vs. 0.04%, respectively (Serisier et al., 2013). The use of azithromycin for the same purpose has resulted in the macrolide resistance rate of 88% compared to 26% in the placebo group (Altenburg et al., 2013). In this regard, the use of modified antibiotics with abolished antimicrobial activities may be helpful to circumvent such undesirable side effects of this type of therapy (Golub et al., 1991). Efforts to design newer antibiotic derivatives, which have no antimicrobial properties must be continued, especially for β-lactams that are used at the concentration range far exceeding those employed in infection control. For example, the recommended dose of ceftriaxone for treatment of gonorrhea has been increased from 125 to 250 mg due to the increasing resistance of *N. gonorrhoeae*, where for the majority of other infections the range rarely exceeds 1–2 g per day. But the most efficient management of, for example, ALS may require dosages up to 4 g/day (Berry et al., 2013). In combination with a generally long-term treatment required for this type of therapy (i.e., 20 weeks used in Berry et al., 2013), the total quantity of ceftriaxone consumed during a single course (560 g) might easily exceed the corresponding values used in a typical infection treatment by approximately 30–60 times. This may contribute to a significantly broader spread of resistance against third-generation cephalosporins among human commensals and, possibly, pathogens.

This clearly powerful selective pressure of ceftriaxone to be used for non-antimicrobial purposes brings forward another consideration for a more careful assessment of the biotic effects of antibiotics: that is, whether the effects of antibiotics are exerted solely via the human receptor-antibiotic ligand mechanisms. While the *in vitro* models largely have no issues with the influence of an indirect factor such as commensal microbiota, the corresponding experiments with animal models or human volunteers may need more careful interpretation because of a possible interfering effect of this often neglected variable. Our views on the role of commensal microbiota in human health and disease have undergone cardinal changes during the last decade from viewing it as a fairly passive bystander with a limited contribution to the host nutrition to an active organ of our body involved in many aspects of our metabolism, physiology, immunity, and disease. The field of host–microbiota research is overloaded with many works revealing the role of commensal microbiota in various pathologies ranging from inflammatory disorders to metabolic syndrome and to autism. It is, however, not possible to extend the frames of this review to include this fascinating area as well.

It is interesting to note, though, that there is an overlap between the range of diseases that can be treated by the biotic action of antibiotics and the diseases that have a substantial commensal microbial component. In Parkinson's disease subjects, for example, the integrity of the intestinal lining is compromised thus allowing translocation of proinflammatory bacteria and bacterial products leading to the formation of the pathological hallmark of the disease, i.e., Lewy bodies with alpha-synuclein protein (Forsyth et al., 2011). As discussed above, the neuroprotective effects of minocycline may include reduced mitochondrial calcium uptake, stabilized mitochondrial membranes, reduced release of apoptotic factors, up-regulation of the anti-apoptotic protein Bcl-2, direct scavenging for reactive oxygen species, and inhibition of MAPKs (Orsucci et al., 2009). On the other side, minocycline may affect and modulate the microbiota, both commensal and translocated, thus reducing the load of proinflammatory bacteria and bacterial products. The effect on microbiota may be particularly profound for ceftriaxone, the biotic use concentration of which is much higher than typical antimicrobial concentrations used for infectious diseases. At concentrations used for typical antibacterial therapy, the ceftriaxone-induced dysbacteriosis may significantly change the fecal metabolome and affect the populations of T lymphocytes, their subpopulations in Peyer's patches, and expression of various cytokines (Gao et al., 2012; Zhao et al., 2013b). Thus ceftriaxone considerably modifies the gut microbiome with the subsequent alteration of the host metabolome and immunity. It is currently unknown to what extent these secondary alterations contribute to the observed therapeutic effects of the non-antimicrobial

ceftriaxone use in various diseases and pathologies. Because of the dual role of antibiotics, further research on biotic effects of antibiotics should incorporate both of these aspects in study designs.

Similarly, in the use of antibiotics as antimicrobial agents, their biotic effects are rarely considered as significant. Almost all infectious diseases, however, have a substantial inflammatory component, and, if left unchecked, the host's proinflammatory responses may cause more harm than a pathogen would. In this regard, the anti-inflammatory activities of tetracyclines and macrolides discussed above may help to alleviate the overly aggressive inflammatory responses and prevent the resulting excessive tissue damage. In general, the number of diseases with a significant proinflammatory component, as exemplified above in this review, is on the rise, and one of the possible explanations for this phenomenon is offered by the hygiene hypothesis (Strachan, 1989). There used to be substantial exposure of humans to environmental bacteria before the advent of industrial food production resulting in essentially sterile food protected by preservatives, conservatives and freezing and generally higher hygienic standards of living. This exposure seems to have been an essential component of the immune system education and the lack of it results in an unbalanced immune system development, with a prominent proinflammatory bias. Thus antibiotics like tetracyclines and macrolides not only help to clear infections but also prevent generally excessive immune responses to infections characteristic for modern humans. Other antibiotics, however, may display the opposite biotic effects. For example, rifampin, a major drug used in tuberculosis treatment, increases inducible nitric oxide synthase expression and NF-κB activation and decreases PPARgamma expression (Yuhas et al., 2009), thus displaying strong proinflammatory properties.

The ligand and signaling activities of antibiotics that are beyond their intended antimicrobial use may explain the phenomena outside the human disease domain. Many are probably familiar with the growth-promoting effect of antibiotics on food animals. The growth-promoting antibiotics were completely phased out in the EU countries in 2006 because of their alleged contribution to the spread of antibiotic resistance, but it is still a legal practice in many countries. Despite the fact that the growth-promoting phenomenon was discovered more than 60 years ago, its mechanisms are still poorly understood. One of the possible explanations proposed has been the suppression of subclinical infections. But the concentrations of antibiotics used for growth promotion are well below the minimal inhibitory concentration (MICs) for the majority of pathogens. The irony of the situation is that it has been clear from the beginning that the growth-promoting effects of antibiotics are not due to antimicrobial activities. Since the very early days of the field it has been shown that even if the antibiotic activity of aureomycin in the culture supernatant of *S. aureofaciens* is destroyed by alkaline hydrolysis, it still retains the complete growth-promoting potential (Stokstad and Jukes, 1950). Thus Robert Stokstad and Thomas Jukes were the first to witness the biotic activity of the modified antibiotic but without realizing this. In the context of results discussed in this review, it is probable that the growth-promoting effect of antibiotics is largely due to the biotic signaling. This may affect the host and microbial components and can be realized through the modulation and pleiotropic regulation of the physiology and metabolic state of an entire microbiota as well as the regulatory mechanisms operating in the host animal. The regulatory effect of antibiotics on the microbial component has been discussed elsewhere (Davies et al., 2006; Linares et al., 2006; Yim et al., 2006, 2007; Martínez, 2008; Aminov, 2009; Romero et al., 2011). Other regulatory effects can be instigated through signaling to the host cells as has been demonstrated in this review. This could be modulation of immunity by suppressing subclinical inflammatory processes, which drain the host resources. It is probably not a coincidence that the vast majority of growth-promoting antibiotics belong to macrolides and tetracyclines that exert potent anti-inflammatory activities.

And finally, moving from the applications to the fundamentals, it remains to be discussed whether this multiplicity of antibiotic targets in the human/animal body we have just seen is merely accidental. Our perception of antibiotics as instruments of warfare in microbial communities is mainly influenced by the extrapolation of antibiotic use in clinical and veterinary microbiology, where high concentrations of antibiotics are used to eradicate bacterial infections in humans and animals. Several lines of evidence gathered in recent years suggest that antibiotic concentrations occurring in natural ecosystems are too low to kill the neighbors and, instead, they may play signaling and regulatory roles in microbial communities (Davies et al., 2006; Linares et al., 2006; Yim et al., 2006, 2007; Martínez, 2008; Aminov, 2009; Romero et al., 2011). The concept of antibiotics serving as signaling molecules in microbial ecosystems has gained considerable attention also in the context that it may explain the effect of low-dose antibiotics on the expression of genes regulating virulence, colonization, motility, stress response, biofilm formation, gene transfer, secondary metabolite production, and many other functions. If the sole role of antibiotics were limited to killing, then no effect of antibiotics would be seen below the MIC levels, and the effect would be neutral. However, it is not the case.

Are eukaryotes involved in this microbial signaling network? Before the advent of the multicellular organization about 0.5 billion years ago, the single-celled eukaryotes were probably well-incorporated into the then-existing microbial ecosystems, with the corresponding ancient signaling systems, and were also producing signaling substances themselves. They probably retained this capability even after the shift to multicellular organization, as may be exemplified by some of the ancient eukaryotic organisms having survived until the present days such as sponges which produce plethora of biologically active compounds, including antibiotics (Burkholder and Ruetzler, 1969; Laport et al., 2009). Further developments in multicellular organization in the eukaryotic world have led to a certain degree of isolation from the environment and the invention of intra-organismal signaling by hormones, but interaction and cross-talk with microbiota has remained an integral part of the lifestyle of eukaryotes. As discussed above, the microbiota is involved in many aspects of our metabolism, physiology, immunity, and disease through signaling to the host, but this is indeed a cross-talk with the microbiota also listening to the host signals (Burton et al., 2002; Sperandio et al., 2003; Clarke et al., 2006; Karavolos et al., 2008, 2013).

The importance of this host–microbe communication has been exemplified above by the hygiene hypothesis that explains a dramatic increase in the number of allergies and asthma in more developed countries due to limited exposure to environmental microbiota in the modern world. Another good example of the malfunctioning host–microbiota communication is the periodontal disease that affects 10–15% of adult population globally (Petersen and Ogawa, 2012). Although many suspected pathogens have been implicated in this disease, the underlying cause is more fundamental and mainly owes to the disrupted host–microbe interaction (Armitage, 2013; Bartold and Van Dyke, 2013). This disruption is the result of drastic changes in the lifestyle and diet imposed by the conversion to farming ca. 10,000 years ago and especially by the industrial revolution beginning from the 18th century. These changes shifted the oral microbial community to a disease-associated configuration (Adler et al., 2013), thus provoking the host to overly aggressive immune responses that result in tissue damage and disease progression. As discussed above, the situation can be somewhat corrected by the use of low-dose tetracyclines and tetracycline derivatives that suppress these responses, but this is a temporal solution which does not tackle the fundamental issue of the disease. The way of solving this is through reinstatement of oral microbiota from a less stable and diverse state, which would allow restituting the proper host–microbiota communication that has been selected and fine-tuned during prior long-term co-evolution of the host and microbiota.

Antibiotic signaling network is possibly one of the most ancient forms of inter-domain communication. It is not a coincidence that there are so many distinct molecular targets and receptors in eukaryotic cells for which antibiotics serve as ligands. Although therapeutic potential of this molecular cross-talk is well-understood and proven in certain pathologies and diseases, the effect on healthy individuals needs further elaboration. This is particularly important because the current exposure of humans to antibiotics, even in generally healthy populations, could be substantial but the consequences of this are poorly understood. For example, the phenomenon of the accelerated physiological development and tendency to be overweight in modern humans: is it the same effect of antibiotics similar to the growth promotion in food animals? The existing regulations require that the level of antibiotics for instance in meat products entering the human food chain should be below a certain tolerance level. But how the tolerance levels are defined? For example, detection of antibiotics and sulfonamides in bob veal calf carcasses are performed with the calf antibiotic and sulfonamide test (CAST) where *Bacillus megaterium* ATCC 9885 is used as the indicator organism (Dey et al., 2005). In order for a tissue sample to be considered safe, a zone of inhibition around a swab should be less than 18 mm. Under the identical incubation conditions antibiotic disks with 8.0 μg of sulfamethazine produce slightly smaller zones of inhibition, 17 mm in diameter (Dey et al., 2005). Moreover, the samples may contain the metabolized antibiotic residues that lost their antimicrobial activity but may still retain other biotic signaling properties as discussed in this review.

The use of antibiotics has been predominantly studied from the antimicrobial activity perspective and focused mostly on infection control and prevention of antibiotic resistance among pathogens. The advent and rapid expansion of non-antimicrobial application of antibiotics may enhance the already existing selective pressure of antibiotics and contribute to the present antibiotic resistance problem. Better understanding of biotic functions of antibiotics may help to design newer antibiotic derivatives with abolished antimicrobial activities and improved biotic functions.

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# Antivirulence activity of azithromycin in *Pseudomonas aeruginosa*

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Antibiotics represent our bulwark to combat bacterial infections, but the spread of antibiotic resistance compromises their clinical efficacy. Alternatives to conventional antibiotics are urgently needed in order to complement the existing antibacterial arsenal. The macrolide antibiotic azithromycin (AZM) provides a paradigmatic example of an "unconventional" antibacterial drug. Besides its growth-inhibiting activity, AZM displays potent anti-inflammatory properties, as well as antivirulence activity on some intrinsically resistant bacteria, such as *Pseudomonas aeruginosa*. In this bacterium, the antivirulence activity of AZM mainly relies on its ability to interact with the ribosome, resulting in direct and/or indirect repression of specific subsets of genes involved in virulence, quorum sensing, biofilm formation, and intrinsic antibiotic resistance. Both clinical experience and clinical trials have shown the efficacy of AZM in the treatment of chronic pulmonary infections caused by *P. aeruginosa*. The aim of this review is to combine results from laboratory studies with evidence from clinical trials in order to unify the information on the *in vivo* mode of action of AZM in *P. aeruginosa* infection.

**Keywords: antibiotic, cystic fibrosis, inflammation, macrolide, regulation, virulence**

## INTRODUCTION

Antibiotics are used as first line drugs for the treatment of bacterial infections, but the widespread resistance to these agents combined with the shortage of novel antimicrobial compounds developed by the pharmaceutical industry results in an urgent need for new strategies to combat bacterial infections (Fernebro, 2011). Virulence factors are essential for bacterial pathogens to cause infection. Hence, suppression of virulence factor production, i.e., antivirulence therapy, has become an attractive anti-infective approach. In-depth understanding of the mechanisms by which pathogens cause disease has been essential for the recognition of suitable targets for antivirulence drugs (Cegelski et al., 2008; Rasko and Sperandio, 2010). Target-based rational design and screening of chemical libraries allowed the identification of a variety of virulence inhibitors (Clatworthy et al., 2007; Law et al., 2013). However, none of the antivirulence compounds developed so far have entered into clinical practice.

The unpredicted antivirulence activity observed *a posteriori* among macrolide antibiotics prompted revisiting of laboratory and clinical data to assess the potential of these compounds as antivirulence drugs. In this review, the clinical impact of azithromycin (AZM) on patients suffering from *Pseudomonas aeruginosa* infection are discussed in the light of the biological activities exerted by AZM on both the pathogen and the host.

## THE MULTIFARIOUS BIOLOGICAL ACTIVITIES OF MACROLIDES

Macrolides are polyketide compounds characterized by the presence of a 14- (e.g., erythromycin), 15- (e.g., AZM, **Figure 1**), or 16- (e.g., josamycin) membered macrocyclic lactone to which one or more amino and/or neutral sugars are attached.

Macrolides have many important biological characteristics including antibacterial, antifungal and immunomodulatory properties. Erythromycin is the progenitor of this class of antibiotics and has served as the scaffold for the generation of newer semisynthetic macrolides (Washington and Wilson, 1985; Pal, 2006). AZM was launched in 1991 and rapidly became one of the most frequently used antimicrobials for outpatients (Hicks et al., 2013). A number of favorable pharmacological properties contributed to the success of AZM as an antibiotic, including acid resistance, a short time to achieve peak concentrations with an up to 800-fold accumulation in phagocytes at the infection site, and long half-life allowing a large single oral dose to maintain bacteriostatic activity in the infected tissue for 4 days (Girard et al., 1987; Foulds et al., 1990; Blumer, 2005).

Macrolide antibiotics inhibit bacterial growth by binding the 23S rRNA in the 50S subunit of the bacterial ribosome, thereby preventing the transfer of tRNA from the A to the P site of the ribosome. Binding to the A site prevents addition of an incoming amino acid-charged tRNA to the nascent polypeptide chain, ultimately aborting polypeptide growth (Retsema and Fu, 2001; Poehlsgaard and Douthwaite, 2005).

Some macrolides (e.g., rapamycin) lack antibacterial activity but possess potent immunosuppressive or immunomodulatory properties, and are therefore used in the therapy of autoimmune disorders and proliferative diseases. They act through different mechanisms at the level of the immune system, ultimately interfering with lymphocyte activation and cytokine production (McAlister et al., 2002; Ferrer et al., 2011; Salmond and Zamoyska, 2011).

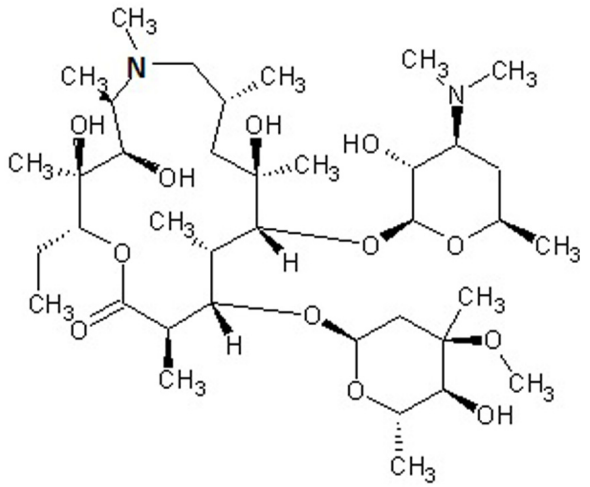

**FIGURE 1 | Chemical structure of azithromycin, a drug belonging to the azalide subclass of macrolides.** The 15-membered lactone ring is derived from erythromycin, upon incorporation of a methyl-substituted nitrogen atom (bold).

Starting from the late 1960s, evidence has been obtained showing that also macrolide antibiotics have anti-inflammatory and pro-kinetic effects which play a prominent role in some infections (Itkin and Menzel, 1970). These effects have extensively been reviewed in the recent literature (Amsden, 2005; Giamarellos-Bourboulis, 2008; Kanoh and Rubin, 2010; Steel et al., 2012; Aminov, 2013).

Macrolide antibiotics are typically bacteriostatic at therapeutic concentrations (Retsema et al., 1987). Different from cell-disrupting agents (e.g., β-lactams), they are unlikely to cause bacterial lysis and release of cell-associated pro-inflammatory molecules, thereby avoiding the induction of a detrimental inflammatory response (Spreer et al., 2003; Anderson et al., 2007). Sub-inhibitory concentrations of macrolides cause substantial inhibition of the synthesis of virulence factors in both Gram-positive and Gram-negative bacteria (Steel et al., 2012).

*Pseudomonas aeruginosa* is a paradigmatic example of a microorganism with intrinsic resistance to multiple classes of antibiotics, including macrolides. Nonetheless, a number of clinical studies have demonstrated that patients suffering from both intermittent and chronic *P. aeruginosa* infection, e.g., cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and diffuse panbronchiolitis (DPB), benefit from AZM treatment (reviewed by Steel et al., 2012 and Aminov, 2013). Hereafter, the many effects of AZM on *P. aeruginosa* virulence and their impact on infection are discussed.

## EFFECT OF AZITHROMYCIN ON *P. aeruginosa* CELLS

The pathogenic potential of *P. aeruginosa* relies on the production of cell-surface components with pro-inflammatory and/or adhesion activity, and a huge arsenal of virulence factors (Driscoll et al., 2007). Moreover, its ability to adopt the biofilm lifestyle is critical in chronic infections. In *P. aeruginosa*, the extracellular polysaccharides (EPSs) Psl, Pel and alginate play an important role in maintaining the biofilm structure and in resistance to antibiotics and to the host immune system (Wei and Ma, 2013).

AZM is not approved for the treatment of infections caused by *P. aeruginosa* and there are no published breakpoints for this species. The AZM minimum inhibitory concentrations (MICs) for *P. aeruginosa* range from 8 to 512 μg/ml, depending on the strain and the testing procedure (e.g., Kita et al., 1991; Tateda et al., 1996; Nicolau et al., 1999; Morita et al., 2001). Early studies showed that sub-inhibitory AZM concentrations (sub-MIC AZM) suppressed motility and the production of several virulence factors, including proteases, pyocyanin, exotoxin A, phospholipase C (PLC), and EPSs in *P. aeruginosa* (Kita et al., 1991; Molinari et al., 1992; Molinari et al., 1993; Ichimiya et al., 1996; Nagino and Kobayashi, 1997; Favre-Bonté et al., 2003; Gillis and Iglewski, 2004). Since in *P. aeruginosa* the expression of many virulence factors is activated at the transcriptional level by the 3-oxo-C12-homoserine lactone (3OC12-HSL) and butyryl-homoserine lactone (C4-HSL) quorum sensing (QS) signal molecules, some studies focused on the effect of sub-MIC AZM on these two QS systems.

AZM (2 μg/ml) reduces the production of both 3OC12-HSL and C4-HSL. Transcriptional repression of the corresponding synthase/receptor genes *lasI*/*lasR* and *rhlI*/*rhlR* contributes to this effect (Tateda et al., 2001). Accordingly, transcriptomic and proteomic analyses confirmed that AZM down-regulates the expression of many QS-dependent genes, as those encoding the pilus, flagellum, and oxidative stress response proteins (Nalca et al., 2006; Skindersoe et al., 2008; Kai et al., 2009). In *P. aeruginosa*, the AZM-affected transcriptome largely overlaps with the Gac/Rsm regulon, which also includes both *las* and *rhl* QS genes (Pérez-Martínez and Haas, 2011). In the Gac/Rsm regulatory pathway, the trans-membrane histidine kinase GacS phosphorylates the response regulator GacA in response to an unknown signal. Phosphorylated GacA activates the transcription of the two small regulatory RNAs (srRNAs) RsmY and RsmZ. At high concentrations, these srRNAs sequester the mRNA-binding protein RsmA, which acts as a translational repressor. RsmA directly or indirectly affects the expression of many virulence genes, including those implicated in QS regulation (Coggan and Wolfgang, 2012; Frangipani et al., 2014). Sub-MIC AZM reduced the expression of several genes in the Gac/Rsm regulon, and inhibited the transcription of the still uncharacterized ORFs PA0588–PA0584, which are required for full expression of *rsmZ* and *rsmY* (Kai et al., 2009; Pérez-Martínez and Haas, 2011). Therefore, AZM-dependent repression of 3OC12-HSL and C4-HSL synthesis could, at least in part, be explained by a cascade mechanism in which AZM represses expression of the PA0588–PA0584 genes which are required for full transcription of *rsmZ* and *rsmY*, resulting in down-regulation of QS gene expression. However, the effect of AZM on *rsmZ* and *rsmY* transcription was not completely abrogated in a PA0588–PA0584 deletion mutant, suggesting that AZM affects the Gac/Rsm system and QS also via alternative pathways (Pérez-Martínez and Haas, 2011; **Figure 2**). Allied to this, AZM also repressed transcription of genes for the synthesis of 3OC12-HSL and C4-HSL precursors (Kai et al., 2009).

Sub-MIC AZM has many pleiotropic effects on *P. aeruginosa* that cannot be explained only by its interference with the Gac/Rsm and QS systems. Examples are (i) the inhibitory effect on alginate

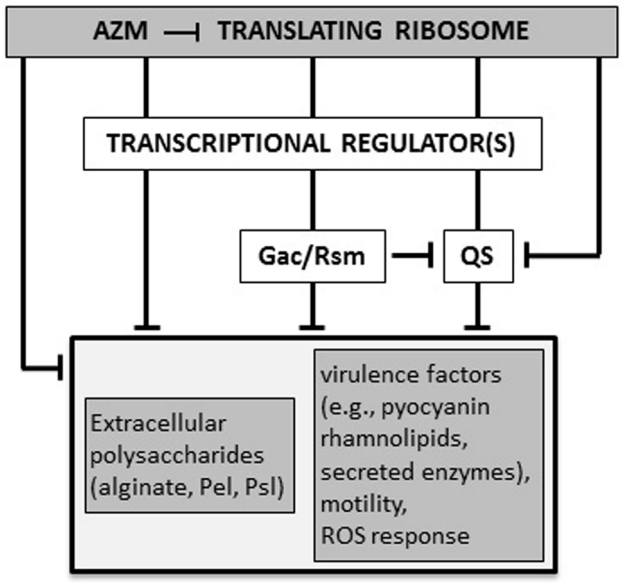


**FIGURE 2 | Proposed molecular mechanisms for AZM-mediated inhibition of *P. aeruginosa* virulence.** AZM, even at sub-inhibitory concentrations, interacts with the 50S ribosomal subunit and selectively affects the expression of a specific subset of genes, such as those for the Gac/Rsm and quorum sensing (QS) systems, and other unidentified transcriptional factors involved in regulation of virulence genes. Some genes (e.g., the QS regulator gene *rhlR*) may be affected by AZM via independent and overlapping regulatory pathways, both at the transcriptional and post-transcriptional level. The final outcome of this cascade is the suppression of a number of virulence-related phenotypes (see text for details). T-shaped lines represent negative controls.

production (Ichimiya et al., 1996; Favre-Bonté et al., 2003; Lutz et al., 2012), (ii) increased susceptibility to serum bactericidal activity, probably due to alterations of cell-surface structures such as lipopolysaccharides and outer membrane proteins (Tateda et al., 1993, 1994), (iii) increased susceptibility to some antimicrobials, due to down-regulation of the MexAB-OprM efflux pump (Sugimura et al., 2008), and (iv) killing of stationary-phase and biofilm-forming cells (Tateda et al., 1996; Imamura et al., 2005).

Some effects of sub-MIC AZM are dependent on a direct interaction with the ribosome. Indeed, heterologous expression of a *Clostridium perfrigens* 23S rRNA methylase gene in *P. aeruginosa* increased AZM resistance, counteracted inhibition of virulence factors production, and alleviated killing of stationary-phase cells (Kohler et al., 2007).

Macrolides block elongation of the nascent peptide chain and cause premature dissociation of the tRNA-charged growing polypeptide. Increased release of these abortive peptidyl-tRNAs, a phenomenon known as "drop-off", impairs the normal turnover of tRNAs, affecting the overall protein translation rate (Retsema and Fu, 2001; Gödeke et al., 2013). Interestingly, overexpression in *P. aeruginosa* of the Pth peptidyl-tRNA hydrolase, an enzyme which releases uncharged tRNA from the peptidyl-tRNA, partially restored tRNAs turn-over and reversed some phenotypes caused by sub-MIC AZM, such as the stationary-phase killing and inhibition of pyocyanin and rhamnolipid production (Gödeke et al., 2013). Therefore, some phenotypes induced by sub-MIC AZM are determined by the increased peptidyl-tRNAs drop-off and defective turn-over of tRNAs. Since production of pyocyanin and rhamnolipids is dependent on the Rhl QS system, Gödeke et al. (2013) by analysing the *rhlI and rhlR* coding sequences found that the second codon of *rhlR* (AGG, encoding Arg) is very rarely used in *P. aeruginosa*, suggesting that translation of this gene could be particularly susceptible to defects in tRNAs turn-over. Accordingly, replacement of this codon with the preferentially used codon CGC reverted AZM-mediated inhibition of rhamnolipids and pyocyanin production (Gödeke et al., 2013). This result suggests that sub-MIC AZM may selectively affect the expression of distinct subset of genes, depending on their codon usage. Thus, the pleiotropic effects of sub-MIC AZM on *P. aeruginosa* are mainly due to AZM interaction with the ribosome and interference with protein synthesis. Differential codon usage in *P. aeruginosa* might explain the selective activity of AZM in translation of specific proteins. Besides RhlR, translation of still unidentified global regulators could also be affected by sub-MIC AZM concentrations, explaining AZM effects on *P. aeruginosa* transcriptome and physiology (Nalca et al., 2006; Skindersoe et al., 2008; Kai et al., 2009).

## EFFECT OF AZITHROMYCIN ON *P. aeruginosa* INFECTION

In Japan, macrolides have been used since the 1980s to treat DPB, a rare inflammatory lung disease that mainly affects elderly Asian people, in which chronic *P. aeruginosa* lung infection is associated with a poor outcome (Schultz, 2004). In the late 1990s, the similarities between DBP and CF drove Jaffé et al. (1998) to use AZM as a last resort agent for treatment of a teenager with CF on the waiting list for heart–lung transplantation; AZM treatment almost doubled the patient's pulmonary function, leading to his removal from the list. This promising finding was confirmed by an open-label study on seven CF children infected by *P. aeruginosa* not responding to conventional therapy (Jaffé et al., 1998). Thereafter, several clinical trials have been conducted to validate AZM efficacy in CF. A recent meta-analysis of ten studies, including almost 1,000 patients, showed that AZM therapy is associated with a small but consistent improvement in respiratory function at 6 months, and has a good safety profile (Southern et al., 2012).

Hereafter, results from laboratory studies will be combined with evidence from clinical trials in order to summarize the information on the mode of action of AZM in *P. aeruginosa* infection. As described above, sub-MIC AZM exerts multiple effects on *P. aeruginosa*, including virulence inhibition, killing of stationary-phase and/or biofilm-forming cells, and synergism with other antimicrobials and with serum complement (Tateda et al., 1996; Imamura et al., 2005; Hoffmann et al., 2007; Lutz et al., 2012). In two chronic lung infection models of CF mice challenged with mucoid (alginate-producing) *P. aeruginosa* isolates, AZM suppressed QS-regulated virulence factors, drastically reduced the bacterial load in the lung, and improved lung pathology (Hoffmann et al., 2007; Tsai et al., 2009). However, in only one study AZM reduced *P. aeruginosa* associated mortality (Tsai et al., 2009). It was also found that AZM attenuated the inflammatory response and promoted macrophage phagocytic activity (Tsai et al., 2009), as previously reported for AZM-treated COPD

patients (Hodge et al., 2008). The strongly reduced bacterial load in the lungs of AZM-treated mice could be explained by concomitant factors, including killing of biofilm-forming cells, improved phagocytic activity of macrophages, and/or increased susceptibility of QS-attenuated *P. aeruginosa* to the inflammatory/immune response (Hoffmann et al., 2007; Tsai et al., 2009). However, a reduced bacterial load was not observed for other antivirulence drugs capable of protecting mice from lethal *P. aeruginosa* lung infections (Miyairi et al., 2006; Imperi et al., 2013), suggesting that AZM suppresses the infection by targeting both *P. aeruginosa* and the immune system. Accordingly, relevant anti-inflammatory effects of AZM were also observed in CF mice that were not infected with *P. aeruginosa*, where AZM treatment resulted in attenuated cellular infiltration and reduced cytokine release (Legssyer et al., 2006). Therefore, the anti-inflammatory properties of AZM in the lung are also independent of its anti-*Pseudomonas* activity. It should be noted that previous work using non-CF murine models of lethal sepsis or pneumonia caused by non-mucoid and mucoid *P. aeruginosa* isolates, respectively, failed to show protective effects of AZM alone, although AZM acted synergistically with ceftazidime in both infection models (Nicolau et al., 1997, 1999). This suggests that experimental conditions have a considerable impact on the outcome of AZM treatment in animal infection models and/or that special features of CF lungs could contribute to improved AZM activity on *P. aeruginosa*.

The therapeutic efficacy of AZM in CF has been proven in many clinical trials. Beneficial effects were observed in CF patients chronically-infected with *P. aeruginosa* and, to a lesser extent, in uninfected CF patients (reviewed in Southern et al., 2012). The latter observation is consistent with the finding that AZM significantly reduced various serum inflammatory markers in CF patients not infected with *P. aeruginosa* (Ratjen et al., 2012), confirming again that AZM has anti-inflammatory effects independent of its antivirulence activity. However, some pulmonary function parameters, including forced expiratory volume in 1s (FEV1), were slightly less improved in patients without chronic *P. aeruginosa* infection compared to chronically-infected patients (Southern et al., 2012). Whether different outcomes are related primarily to the anti-*Pseudomonas* activity of AZM or to the early stage of lung disease in young patients uninfected with *P. aeruginosa* (Clement et al., 2006; Saiman et al., 2010) cannot be established at present.

Regarding the antivirulence activity of AZM in humans, a retrospective study observed a correlation between the inhibitory effect of AZM on PLC production by *P. aeruginosa* strains isolated from CF patients and the observed FEV1 improvement after AZM therapy (Nguyen et al., 2007), suggesting that *in vivo* PLC production is a main target of AZM. This finding fits well with the relevant role of *P. aeruginosa* PLC in the impairment of lung function in a mouse infection model (Wargo et al., 2011). A more recent study in intubated patients colonized with *P. aeruginosa* attempted to directly correlate the clinical effect of AZM on the patient with its antivirulence activity (van Delden et al., 2012). Although no relevant differences were observed between AZM-treated and untreated patients with regard to the occurrence of ventilator-associated pneumonia (VAP), a lower incidence of VAP was reported in a small sub-group ($n = 5$) of AZM-treated patients infected by *P. aeruginosa* strains producing high levels of rhamnolipids compared with the corresponding untreated group. Although preliminary, this observation suggests that AZM could be more effective in individuals infected by these highly virulent strains (van Delden et al., 2012).

Only few studies measured AZM levels during administration to CF patients. Data so far available suggest a wide range of AZM concentrations in sputa (0.6−79.3 μg/ml), depending on the individual patient and the dosing regimen (Baumann et al., 2004; Wilms et al., 2006). As discussed above, the growth inhibitory activity of AZM is strongly influenced by culture conditions. Since AZM MICs for *P. aeruginosa* are low (2–16 μg/ml) when determined in eukaryotic cell media or in mouse bronchoalveolar lavage fluid (Buyck et al., 2012), it may be possible that AZM also exerts some inhibition of *P. aeruginosa* growth in CF lungs. However, the insignificant differences in the frequency and concentration of *P. aeruginosa* in sputa from AZM-treated and untreated patients (Equi et al., 2002; Saiman et al., 2003; Clement et al., 2006) argue against this possibility.

## CONCLUSION

From a microbiological perspective, the therapeutic efficacy of an antimicrobial compound results mainly from its ability to impair bacterial growth, and this assumption has driven antibiotic research until now. AZM provides a clear example of how the therapeutic efficacy of an antimicrobial cannot exclusively be attributed to growth impairment. Anti-inflammatory and antivirulence properties likely predominate in the treatment of infections involving AZM-resistant pathogens, as in the case of *P. aeruginosa* pulmonary infections. Although there is strong evidence of antivirulence activity *in vitro*, it is not possible to assess the contribution of this activity to the efficacy of AZM *in vivo* because of concomitant anti-inflammatory activity and bactericidal effects under certain conditions. According to the current model, both antibacterial and antivirulence activities are based on the interaction of AZM with the ribosome, so that tightly interwoven effects on bacterial viability and production of virulence factors are hardly distinguishable *in vivo*.

Beneficial effects have so far been documented in CF patients treated with AZM for up to 6 months, while reduced efficacy was associated with longer treatment duration (Southern et al., 2012; Fleet et al., 2013). Loss of efficacy could be explained by the emergence of *P. aeruginosa* subpopulations that become insensitive to the antivirulence activity. This hypothesis could be verified by testing the virulence properties and the response to AZM in serial *P. aeruginosa* isolates collected during long-term AZM therapy. Under this condition, the emergence of macrolide resistance among both commensal bacteria and co-infecting pathogens is a matter of concern and deserves further study (Aminov, 2013).

In conclusion, AZM offers a unique model to reconsider the central dogma of antibiotic activity, but further research is needed to gain more insight into the effects of AZM on both the pathogen and the host.

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# Responses of *Pseudomonas aeruginosa* to antimicrobials

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Infections caused by *Pseudomonas aeruginosa* often are hard to treat; inappropriate chemotherapy readily selects multidrug-resistant *P. aeruginosa*. This organism can be exposed to a wide range of concentrations of antimicrobials during treatment; learning more about the responses of *P. aeruginosa* to antimicrobials is therefore important. We review here responses of the bacterium *P. aeruginosa* upon exposure to antimicrobials at levels below the inhibitory concentration. Carbapenems (e.g., imipenem) have been shown to induce the formation of thicker and more robust biofilms, while fluoroquinolones (e.g., ciprofloxacin) and aminoglycosides (e.g., tobramycin) have been shown to induce biofilm formation. Ciprofloxacin also has been demonstrated to enhance the frequency of mutation to carbapenem resistance. Conversely, although macrolides (e.g., azithromycin) typically are not effective against *P. aeruginosa* because of the pseudomonal outer-membrane impermeability and efflux, macrolides do lead to a reduction in virulence factor production. Similarly, tetracycline is not very effective against this organism, but is known to induce the type-III secretion system and consequently enhance cytotoxicity of *P. aeruginosa in vivo*. Of special note are the effects of antibacterials and disinfectants on pseudomonal efflux systems. Sub-inhibitory concentrations of protein synthesis inhibitors (aminoglycosides, tetracycline, chloramphenicol, etc.) induce the MexXY multidrug efflux system. This response is known to be mediated by interference with the translation of the leader peptide PA5471.1, with consequent effects on expression of the PA5471 gene product. Additionally, induction of the MexCD-OprJ multidrug efflux system is observed upon exposure to sub-inhibitory concentrations of disinfectants such as chlorhexidine and benzalkonium. This response is known to be dependent upon the AlgU stress response factor. Altogether, these biological responses of *P. aeruginosa* provide useful clues for the improvement and optimization of chemotherapy in order to appropriately treat pseudomonal infections while minimizing the emergence of resistance.

**Keywords: *pseudomonas aeruginosa*, anti-bacterial agents, stress responses, multidrug efflux systems, biofilms**

## INTRODUCTION

*Pseudomonas aeruginosa*, a motile, non-fermenting Gram-negative bacterium, is an opportunistic pathogen implicated in respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media, and bacteremia in patients with compromised host defenses [e.g., cancer, burn, HIV, and cystic fibrosis (CF)]. These infections often result in significant morbidity and mortality. In the 21st century, when the life expectancy of highly susceptible immunocompromised groups has been extended in most countries, *P. aeruginosa* plays an increasingly prominent role in hospital infections.

This organism is a ubiquitous and metabolically versatile microbe that flourishes in many environments. The bacterium grows under both aerobic and anaerobic conditions, and possesses numerous virulence factors that contribute to its pathogenesis (Schurek et al., 2012). Moreover *P. aeruginosa* possesses an intrinsic resistance to many antimicrobials because of the bacterium's outer-membrane barrier, the presence of multidrug efflux transporters, and endogenous antimicrobial inactivation (Poole, 2011). Although anti-pseudomonas agents (e.g., carbapenems) have been discovered and developed, *P. aeruginosa* readily acquires resistance to individual agents via chromosomal mutations and lateral gene transfer (Poole, 2011).

*Pseudomonas aeruginosa* possesses multifactorial mechanisms of responses and resistance to antimicrobials. While antimicrobials were originally developed and used to kill bacteria, recent work reveals that the biological functions of antibiotics are not limited to bactericidal (killing) or bacteriostatic (growth inhibition) effects (Linares et al., 2006; Aminov, 2013). The most likely function of antibiotics in natural ecosystems is in intercellular "signaling," with specific consequences on the collective behavior of the bacterial population (Linares et al., 2006; Aminov, 2013). Improved genetic tools and cutting-edge technologies (e.g., DNA microarrays) have revolutionized our understanding of microbial physiology (Wecke and Mascher, 2011). Here, we summarize and discuss how *P. aeruginosa* responds to various antimicrobials and survives against its competitors.

## RESPONSES TO β-LACTAMS

β-lactams bind to cell wall transpeptidases [penicillin binding proteins (PBPs)], blocking an important step in peptidoglycan biosynthesis (Poole, 2004). Penicillins (e.g., ticarcillin, piperacillin),

cephalosporins (e.g., ceftazidime, cefepime), monobactams (e.g., aztreonam), and carbapenems (e.g., imipenem, meropenem, and doripenem) are commonly used to treat pseudomonal infections. *P. aeruginosa* is intrinsically resistant to most β-lactams due to the interplay of the inducible β-lactamase AmpC and the resistance nodulation cell division (RND) multidrug efflux systems (e.g., MexAB-OprM; Masuda et al., 1999). Benzyl-penicillins (e.g., amoxicillin) and narrow-spectrum cephalosporins are labile to hydrolysis and are strong inducers of *ampC*, leading to antibiotic degradation, whereas ureidopenicillins (e.g., piperacillin) and extended-spectrum cephalosporins are labile but are weak inducers of *ampC* (Livermore, 1995). The *mexAB-oprM* operon is constitutively expressed in wild-type cells under usual laboratory conditions, where the operon contributes to *P. aeruginosa*'s intrinsic resistance to most β-lactams (except for imipenem) and many other antimicrobial agents, including quinolones, tetracycline, chloramphenicol, and macrolides (Morita et al., 2001). Blocking of *dacB*-encoded non-essential PBP4 determines a highly efficient and complex β-lactam resistance response, triggering the overproduction of AmpC and the specific activation of the CreAB (BlrAB) two-component regulator (Moya et al., 2009).

Carbapenems are an important class of anti-pseudomonal β-lactams. Carbapenems are strong inducers that are marginally labile (imipenem) or effectively stable (meropenem; Livermore, 1995). Imipenem also has been shown to strongly induce *ampC* gene expression in biofilms (Bagge et al., 2004). In addition, *P. aeruginosa* biofilms exposed to imipenem exhibit elevated expression of genes coding for alginate biosynthesis, causing thicker and more robust biofilms (Bagge et al., 2004).

Ceftazidime, a PBP3 inhibitor, does not induce *ampC* gene expression, but is rather a substrate of AmpC. Ceftazidime impacts the transcription of a large number of genes in *P. aeruginosa,* including those encoding the SOS response repressor LexA-like proteins, causing induced mutagenesis and decreasing ciprofloxacin toxicity (Blazquez et al., 2006). In addition, this antimicrobial shows quorum sensing (QS) inhibitory activity, decreasing the production of a range of QS-regulated virulence factors, in contrast to piperacillin, another PBP3 inhibitor (Skindersoe et al., 2008) These results imply that the QS inhibitory activity of ceftazidime likely is not PBP3-dependent (Skindersoe et al., 2008).

## RESPONSES TO FLUOROQUINOLONES

Fluoroquinolones, particularly ciprofloxacin, are commonly used for the treatment of *P. aeruginosa* infections (Poole, 2011). This class of agents interacts with complexes composed of DNA and either of the two target enzymes, DNA gyrase and/or topoisomerase IV. Primary intrinsic resistance of the wild-type *P. aeruginosa* to fluoroquinolones is due to MexAB-OprM as well as to MexXY-OprM (Morita et al., 2001). The four RND-type multidrug efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) are well recognized as significant determinants of fluoroquinolone resistance in lab and clinical isolates (Poole, 2011), although there are several additional chromosomally encoded efflux pumps that are able to recognize fluoroquinolones (e.g., MexHI-OpmD, MexVW-OprM, the NorM ortholog; Morita et al., 1998; Li et al., 2003; Sekiya et al., 2003; He et al., 2004).

The formation of static biofilms increases when *P. aeruginosa* cells are incubated in the presence of sub-inhibitory concentrations of ciprofloxacin, tobramycin, or tetracycline, while no such sub-inhibitory effect is detected with members of other antibiotic classes, such as carbenicillin, chloramphenicol, or polymyxin (Hoffman et al., 2005; Linares et al., 2006). However, in the presence of a sub-inhibitory concentration of ciprofloxacin (but not of tetracycline or tobramycin), *P. aeruginosa* shows a reduction in swimming and swarming, both of which are important systems of bacterial motility and probably related to the pathogenic process in CF patients (Linares et al., 2006).

Transcriptional responses of *P. aeruginosa* to sub-inhibitory and inhibitory concentrations of ciprofloxacin demonstrate the induction or repression of 100s of genes (Brazas and Hancock, 2005; Cirz et al., 2006; Brazas et al., 2007). Surprisingly, genes for bacteriophage-like pyocins are up-regulated and mediate fluoroquinolone susceptibility (Brazas and Hancock, 2005). At least one-third of up-regulated genes occur in regulons that are likely controlled by LexA-like SOS response repressor proteins in response to inhibitory concentrations of ciprofloxacin, while down-regulated genes appear to involve virtually every facet of cellular metabolism (Cirz et al., 2006; Brazas et al., 2007). The Lon protease modulates SOS response and consequently ciprofloxacin susceptibility (Breidenstein et al., 2012).

The overall pattern of expression of the DNA replication enzymes suggests a shift from canonical DNA replication enzymes to inducible polymerases in response to inhibitory ciprofloxacin concentrations (Cirz et al., 2006). These inhibitory concentrations of ciprofloxacin create selection pressure in favor of mutants with increased *ampC* expression (Wolter et al., 2007), while sub-inhibitory levels of ciprofloxacin or ofloxacin enhance the frequency of mutation to carbapenem (especially meropenem) resistance (Tanimoto et al., 2008).

## RESPONSES TO AMINOGLYCOSIDES

Aminoglycosides bind to the 30S ribosomal subunit and interfere with protein synthesis, causing mistranslation and ultimately cell death without lysis (Davis, 1987). APH(3′)-IIb, a chromosomal *aphA*-encoded aminoglycoside phosphoryltransferase, is likely responsible for the general non-susceptibility of *P. aeruginosa* to kanamycin (Hachler et al., 1996), and APH(3′)-II predominates in clinical isolates resistant to kanamycin (Poole, 2005). Anti-pseudomonas aminoglycosides (e.g., amikacin, gentamicin, and tobramycin) therefore can be used in the treatment of *P. aeruginosa* infections (Poole, 2005). Aminoglycoside uptake and subsequent action within bacterial cells is a complex process that involves Lipopolysaccharides (LPS) binding and outer-membrane permeation, cytoplasmic membrane traversal driven by membrane potential, and ribosome disruption, leading to the production of membrane-damaging mistranslated polypeptides (Davis, 1987; Krahn et al., 2012). Primary intrinsic and adaptive resistance to aminoglycosides is due to the MexXY multidrug efflux system in laboratory and clinical isolates (Morita et al., 2012b). The antagonism of aminoglycosides by divalent cations $Mg^{2+}$ and $Ca^{2+}$ is well documented in *P. aeruginosa*, and occurs via a process

that requires the MexXY multidrug efflux system (Mao et al., 2001; Morita et al., 2012a). In wild-type *P. aeruginosa* cells, the MexXY efflux system is inducible by sub-inhibitory concentrations of aminoglycoside- and ribosome-targeting antimicrobials (e.g., chloramphenicol and tetracycline), a process shown to be involved in expression of the PA5471 gene product [recently renamed ArmZ, for anti-repressor MexZ (Morita et al., 2006; Hay et al., 2013)] The PA5471 system also is inducible through interference via translation of the gene's leader peptide, PA5471.1 (Morita et al., 2009). Very recently a clinical strain overproducing MexXY was reported also to harbor a 7-bp deletion in the coding sequence of the leader peptide involved in ribosome-dependent, translational attenuation of *PA5471* expression (Guénard et al., 2013).

Transcriptomic analyses confirm that aminoglycosides impact the expression of a myriad of genes (Kindrachuk et al., 2011). While prolonged exposure to sub-inhibitory concentrations of tobramycin causes increased levels of expression, predominantly of the *mexXY* efflux pump genes, the greatest increases in gene expression levels in response to lethal concentrations of tobramycin involve a number of *P. aeruginosa* heat shock genes (e.g., *htpG*, *ibpA*, *groES*, and *asrA*; Kindrachuk et al., 2011). Under these conditions, the likely intracellular ATP-dependent AsrA protease is noteworthy because of its modest positive impact on aminoglycoside resistance (Kindrachuk et al., 2011). The Lon protease also is inducible by aminoglycosides (Marr et al., 2007).

Sub-inhibitory concentrations of aminoglycosides, especially tobramycin, induce biofilm formation in *P. aeruginosa* (Hoffman et al., 2005). Notably, the aminoglycoside tobramycin also induces both swimming and swarming of *P. aeruginosa* (Linares et al., 2006). Also induced is the *aminoglycoside response regulator* (*arr*) gene, which is predicted to encode an inner membrane phosphodiesterase. The Arr substrate is cyclic di-guanosine monophosphate (c-di-GMP), a bacterial second messenger that regulates cell surface adhesiveness; c-di-GMP is essential for this induction and contributes to biofilm-specific aminoglycoside resistance (Hoffman et al., 2005).

## RESPONSES TO POLYMYXINS

Owing to the increased prevalence of multidrug-resistant *P. aeruginosa,* polymyxin B and colistin (also called polymyxin E), belonging to a family of antimicrobial cyclic oligopeptides, have returned to favor as a last-resort treatment option, although these agents have strong side effects (e.g., nephrotoxicity) with high incidence (Poole, 2011). Very recently, the polymyxin mutant prevention concentrations (MPCs) for *P. aeruginosa* were shown to be very high (≥64 μg/ml), even for susceptible isolates (i.e., with minimum inhibitory concentration (MIC) ranges of 1–2 μg/ml; Choi and Ko, 2013). In the MPC studies, mutation to polymyxin resistance apparently can result from a single mutation (Choi and Ko, 2013).

The mechanism of action of polymyxins involves an initial stage of interaction with the lipid A of the LPS, leading to self-promoted uptake of polymyxins across the membrane, followed by cell death (Zhang et al., 2000; Fernandez et al., 2013). The most common mechanism of resistance to polymyxin has been shown to arise from modification of LPS lipid A with 4-amino-L-arabinose, a process that has been seen both in *in vitro*-selected mutants and in CF isolates; other unknown mechanisms remain under investigation (Miller et al., 2011; Poole, 2011; Moskowitz et al., 2012). This modification is carried out by the products of the *arnBCADTEF-ugd* operon, otherwise known as *pmrHFIJKLM-ugd* (McPhee et al., 2003; Yan et al., 2007). The two-component ParRS regulator leads to the induction of the LPS modification operon in response to sub-inhibitory concentrations of polymyixin B and colistin (Fernandez et al., 2010).

Approximately 0.5% of genes showed significantly altered expression upon exposure to sub-inhibitory concentration of colistin (Cummins et al., 2009), a frequency that is no less dramatic than that seen with the other anti-pseudomonas agents (e.g., ceftazidime, ciprofloxacin, and tobramycin) described above. The most striking alterations were up-regulation of the *Pseudomonas* quinolone signal (PQS) biosynthetic genes such as the *pqsABCDE* operon, the phenazine biosynthetic operon, and the *arn* operon (Cummins et al., 2009).

## RESPONSES TO THE MAJOR HUMAN CATIONIC HOST DEFENSE PEPTIDE, LL-37

The major human cationic host defense peptide LL37 [a.k.a. hCAP-18, FALL-39, or cathelicidin antimicrobial peptide (CAMP)], a 37-amino-acid, 18-kDa peptide, is encoded by the cathelicidin gene (CAMP) and was originally identified in humans (Kosciuczuk et al., 2012). Sub-inhibitory concentration of LL-37 (1/4–1/128 of the MIC of 64 μg/ml) were shown to prevent biofilm formation by decreasing the attachment of *P. aeruginosa* cells, stimulating twitching motility, and influencing two major QS systems (Las and Rhl), leading to the down-regulation of genes essential for biofilm development (Overhage et al., 2008). Similar results were obtained using the bovine neutrophil peptide indolicidin, but no inhibitory effects on biofilm formation were detected using sub-inhibitory concentrations of the mouse peptide CRAMP (67% identical with LL-37), polymyxin B, or the bovine bactenecin homolog Bac2A (Overhage et al., 2008).

## RESPONSES TO MACROLIDES

Macrolides such as erythromycin and azithromycin are widely used antibiotics that block translation by binding to the 50S ribosomal subunit. *P. aeruginosa* cells are intrinsically resistant to macrolides when tested in standard broth culture; for instance, the MIC of erythromycin for *P. aeruginosa* PAO1 is about 512 μg/mL in Mueller-Hinton broth (e.g., Morita et al., 2001; Buyck et al., 2012). Nonetheless, low-dose macrolides such as azithromycin are effective treatments in patients with chronic lung infections (Jaffe et al., 1998; Kudoh et al., 1998). Even at concentrations (e.g., 2 μg/ml of azithromycin) far below the MIC, macrolides inhibit the QS circuitry of *P. aeruginsoa* strain PAO1, leading to a reduction in virulence factor production (Tateda et al., 2001). Low-dose azithromycin shows bactericidal activity for *P. aeruginosa* biofilms, but selects for *nfxB* mutants, which overproduce the MexCD-OprJ efflux pump (Mulet et al., 2009). Notably, the AmpC β-lactamase produced by *nfxB* mutants is protective in biofilm growth, although over expression of the MexCD-OprJ pump is known to impair *P. aeruginosa*'s intrinsic resistance, which is dependent on the MexXY/MexAB-OprM efflux pump and the AmpC (Mulet et al., 2011).

Genome-wide approaches have revealed the QS antagonistic activities of azithromycin (e.g., inhibition of QS, reduction of virulence factor production, and strong induction of type-III secretion systems; Nalca et al., 2006; Skindersoe et al., 2008). This modulation causes decreased expression of the genes encoding the MexAB-OprM efflux pump in *P. aeruginosa* (Sugimura et al., 2008). Azithromycin inhibits expression of the small RNAs *rsmY* and *rsmZ*, a process that depends on the GacA/Rsm signal transduction pathway; this pathway is known to positively control *P. aeruginosa* QS (Perez-Martinez and Haas, 2011). Both effects of azithromycin on QS (quorum factor-dependent virulence factor production and cell death) require azithromycin to interact with ribosomes (Kohler et al., 2007). The stationary-phase killing of azithromycin is further enhanced by the production of rhamnolipids, which likely facilitate macrolide uptake (Kohler et al., 2007). The mode of action of azithromycin *in vivo* also is demonstrated through mutations in 23S rRNA that confer azithromycin resistance in bacterial isolates of *P. aeruginosa* in chronically infected CF patients (Marvig et al., 2012). The clinical efficacy of macrolides in treating pseudomonal infections can be partially explained by the increased susceptibility of *P. aeruginosa* to these compounds in eukaryotic cell culture media and biological fluids, due to decreased *oprM* expression and increased outer-membrane permeability (Buyck et al., 2012).

## RESPONSES TO TETRACYCLINES AND CHLORAMPHENICOL

Tetracyclines are bacteriostatic antibiotics based on a hydroanthracene nucleus, which contains four fused rings. The class also includes glycylcyclines (e.g., tigecycline), a group of semisynthetic tetracycline derivatives containing a glycylamido substitution at position 9 (Yao and Moellering, 2011). Tetracyclines enter bacteria by an energy-dependent process and bind reversibly to the 30S ribosomal subunit, preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor A-site in the RNA-ribosome complex (Yao and Moellering, 2011). *P. aeruginosa* is intrinsically resistant to tetracyclines and glycylcyclines due to the MexAB/MexXY efflux systems (Morita et al., 2001; Dean et al., 2003). Sub-inhibitory concentrations of tetracycline and tigecycline induce the MexXY RND efflux system via a mechanism dependent on the ribosomal inhibitor-inducible PA5471 gene product (Dean et al., 2003; Morita et al., 2006).

Tetracycline increases biofilm formation as well as ciprofloxacin and tobramycin, as described above (Linares et al., 2006). Surprisingly, incubation with tetracycline at concentrations (∼1 μg/ml) that do not decrease the growth rate of *P. aeruginosa* increases the expression of type-III secretion system (T3SS) genes *exsA* and *exoS*, and the presence of tetracycline increases the cytotoxicity of *P. aeruginosa* by nearly 4-fold (Linares et al., 2006). The T3SS is a mechanism by which bacterial pathogens can deliver effectors directly into the cytoplasm of the eukaryotic host cell (Hauser, 2009). Expression of genes forming the T3SS regulon is triggered by ExaA (Hauser, 2009), a transcriptional activator that autoregulates its own expression by a feedback mechanism (Hauser, 2009). ExoS corresponds to a T3SS-secreted toxin (Hauser, 2009).

Chloramphenicol is a bacteriostatic agent that inhibits protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit and preventing the transpeptidation process of peptide chain elongation (Yao and Moellering, 2011). *P. aeruginosa* is usually intrinsically resistant to chloramphenicol, in part due to the MexAB-OprM efflux system (Morita et al., 2001). In addition, sub-inhibitory concentrations of chloramphenicol induce the MexXY efflux system via a mechanism dependent on the ribosomal inhibitor-inducible PA5471 gene product (Morita et al., 2006). This effect is reminiscent of the induction of the MexEF-OprN efflux system (via the MexT activator) in response to chloramphenicol and nitrosative stress (Fetar et al., 2011). Chloramphenicol is a nitro-aromatic antimicrobial and resembles a nitrosative stress product (Fetar et al., 2011).

## RESPONSE TO BIOCIDES

*Pseudomonas aeruginosa* also has been reported to contaminate disinfectants (e.g., chlorhexidine, benzalkonium, and triclosan) in hospital or other such environments, thereby compromising the disinfectant's ability to reduce or eliminate bacterial contamination. Chlorhexidine and benzalkonium are a cationic biguanide and a nitrogen-based quaternary ammonium compound, respectively. These biocides function by affecting the cell membrane, resulting in lysis and the loss of cytoplasmic material (Poole, 2002). The RND-type MexCD-OprJ multidrug efflux pump is induced by sub-inhibitory concentrations of disinfectants such as benzalkonium chloride or chlorhexidine (Morita et al., 2003), a process that is dependent upon the AlgU stress response factor (Fraud et al., 2008). Global transcriptome response to chlorhexidine includes up-regulation of the *mexCD-oprJ* and *oprH-phoPQ* operons and down-regulation of genes encoding proteins involved in membrane transport, oxidative phosphorylation, electron transport, and DNA repair (Nde et al., 2009). A *P. aeruginosa* variant highly adapted to benzalkonium showed increased resistance to fluoroquinolones, owing to mutations in the quinolone resistance-determining region of *gyrA* and to mutations in genes (*mexR* and *nfxB*) that encode repressors of *mexAB-oprM* and *mexCD-oprJ,* respectively (Mc Cay et al., 2010). Development of chlorhexidine-tolerant sub-populations in *P. aeruginosa* biofilms also is dependent on the *mexCD-oprJ* genes (Chiang et al., 2012).

Triclosan specifically inhibits fatty acid synthesis through inhibition of bacterial enoyl–acyl carrier protein reductase, although *P. aeruginosa* is intrinsically resistant to triclosan due to the structure of the pseudomonal FabV protein (a triclosan-resistant enoyl–acyl carrier protein reductase) and active efflux. This innate resistance stems from at least five RND efflux pumps, including MexAB-OprM (Mima et al., 2007; Zhu et al., 2010). In *P. aeruginosa* mutant cells lacking the *mexAB-oprM* genes, sub-inhibitory concentrations of triclosan lead to alterations in the expression of almost half the genome, with 28% of genes being significantly up-regulated and 16% being significantly down-regulated (Chuanchuen and Schweizer, 2012). QS-regulating genes are among the most strongly down-regulated, and surprisingly, iron homeostasis is completely blocked in triclosan-exposed cells, thus mimicking conditions with excess iron (Chuanchuen and Schweizer, 2012).

## SUMMING UP AND FURTHER PROSPECTS

The primary biological responses of *P. aeruginosa* to sub-inhibitory concentrations of antimicrobials are summarized

**Table 1 | Biological responses of *P. aeruginosa* exposed to various antimicrobials at levels below the inhibitory concentrations.**

| Antimicrobials | Biological responses | References |
|---|---|---|
| β-lactams | Induction of the AmpC β-lactamase(some β-lactams are inducers, but others are not) | Livermore (1995) |
| Carbapenems | Formation of thicker and more robust biofilms(induction of alginate biosynthesis) | Bagge et al. (2004) |
| Ceftazidime | Induction of mutagenesis and decreasing ciprofloxacin toxicity | Blazquez et al. (2006) |
| | Inhibition of quorum sensing | Skindersoe et al. (2008) |
| Fluoroquinolones | Induction of biofilm formation | Linares et al. (2006) |
| | Reduction in swimming and swarming | Linares et al. (2006) |
| | Induction of SOS response | Brazas and Hancock (2005) |
| | Up-regulation of the bacteriophage-like pyocins | Brazas and Hancock (2005) |
| | Shift from canonical DNA replication enzymes to inducible polymerases | Cirz et al. (2006) |
| | Enhancement of mutation frequency to β-lactam resistance. | Wolter et al. (2007), Tanimoto et al. (2008) |
| Protein synthesis inhibitors | Induction of the MexXY efflux system | Morita et al. (2012b) |
| Aminoglycosides | Induction of heat shock genes | Kindrachuk et al. (2011) |
| | Induction of biofilm formation | Hoffman et al. (2005) |
| | Induction of swimming and swarming | Linares et al. (2006) |
| | Induction of the Lon protease | Marr et al. (2007) |
| Macrolides | Quorum sensing antagonistic activity(reduction in virulence factor) | Tateda et al. (2001) |
| | Induction of the T3SS | Nalca et al. (2006) |
| | Down-regulation of the MexAB-OprM pump | Sugimura et al. (2008) |
| Tetracycline | Induction of biofilm formation | Linares et al. (2006) |
| | Induction of the T3SS and cytotoxicity | Linares et al. (2006) |
| Chloramphenicol | Induction of the MexEF-OprN efflux pump | Fetar et al. (2011) |
| Polymyxins | Modification of LPS lipid A with 4-amino-L-arabinose | Fernandez et al. (2010) |
| | Up-regulation of the PQS biosynthetic genes | Cummins et al. (2009) |
| Chlorhexidine | Induction of the MexCD-OprJ efflux pump | Morita et al. (2003) |
| | Induction of the oprH-phoPQ operon | Nde et al. (2009) |

in **Table 1**. Interestingly, clinically useful anti-pseudomonas agents (carbapenems, fluoroquinolones, and aminoglycosides) are known to efficiently kill *P. aeruginosa* planktonic cells when used properly, but lead to more severe biofilm development upon exposure at sub-inhibitory concentrations. This pattern means that optimization of anti-pseudomonas chemotherapy is critical; in the absence of such optimization, chemotherapy fails to treat the infection and readily selects multidrug-resistant *P. aeruginosa*. By contrast, macrolides (which are not effective against *P. aeruginosa* planktonic cells because of the planktonic cells' intrinsic resistance) provide activity that is antagonistic to QS, thereby reducing pseudomonal virulence. These findings regarding responses to antimicrobial agents suggest a route toward conquering *P. aeruginosa* infections. For example, macrolides have been shown to augment the *in vitro* activity of anti-pseudomonas agents against biofilms (Lutz et al., 2012).

We are aware of potential molecular targets for novel anti-pseudomonas agents, including essential genes (Morita et al., 2010). Novel class anti-pseudomonas agents should be expected to minimize a severe situation in which few agents are effective against these organisms. However, it is a much more difficult task to develop novel class antimicrobials for *P. aeruginosa* because of the presence of low membrane permeability and the RND multidrug efflux pumps. In addition this organism has the ability to adapt to various stresses, including sub-inhibitory antimicrobial exposure, by recruiting antimicrobial resistance mechanisms, notably that of RND efflux systems such as the MexXY system. While many laboratories are currently screening, so far no efflux pump inhibitors have been made available for clinical settings. Screening for novel antibacterial agents, including efflux pump inhibitors, is currently in progress in many laboratories, including our own.

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# Biofilm formation of *Clostridium perfringens* and its exposure to low-dose antimicrobials

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*Clostridium perfringens* is an opportunistic pathogen that can cause food poisoning in humans and various enterotoxemia in animal species. Very little is known on the biofilm of *C. perfringens* and its exposure to subminimal inhibitory concentrations of antimicrobials. This study was undertaken to address these issues. Most of the *C. perfringens* human and animal isolates tested in this study were able to form biofilm (230/277). Porcine clinical isolates formed significantly more biofilm than the porcine commensal isolates. A subgroup of clinical and commensal *C. perfringens* isolates was randomly selected for further characterization. Biofilm was found to protect *C. perfringens* bacterial cells from exposure to high concentrations of tested antimicrobials. Exposure to low doses of some of these antimicrobials tended to lead to a diminution of the biofilm formed. However, a few isolates showed an increase in biofilm formation when exposed to low doses of tylosin, bacitracin, virginiamycin, and monensin. Six isolates were randomly selected for biofilm analysis using scanning laser confocal microscopy. Of those, four produced more biofilm in presence of low doses of bacitracin whereas biofilms formed without bacitracin were thinner and less elevated. An increase in the area occupied by bacteria in the biofilm following exposure to low doses of bacitracin was also observed in the majority of isolates. Morphology examination revealed flat biofilms with the exception of one isolate that demonstrated a mushroom-like biofilm. Matrix composition analysis showed the presence of proteins, beta-1,4 linked polysaccharides and extracellular DNA, but no poly-beta-1,6-*N*-acetyl-D-glucosamine. This study brings new information on the biofilm produced by *C. perfringens* and its exposure to low doses of antimicrobials.

**Keywords: *Clostridium perfringens*, biofilm formation, antibiotic prophylaxis, anticoccidials, biofilms, anaerobes, low dose antibiotics**

## INTRODUCTION

The predominant organizational state of bacteria in nature is biofilms. They have been defined as a structured community of bacterial cells enclosed in a self-produced extracellular matrix composed primarily of exopolysaccharides (Costerton et al., 1999). The process of biofilm formation involves the following stages: attachment, maturation and dispersion (Davey and O'Toole, 2000; Hall-Stoodley and Stoodley, 2009). Features of cells in biofilms include: aggregation in suspension or on solid surfaces, increased antibiotic resistance, protection from phagocytosis and immune cells, and resistance to physical and environmental stresses (Davey and O'Toole, 2000; Davies, 2003; Hall-Stoodley and Stoodley, 2009). In *Pseudomonas aeruginosa*, extracellular DNA present in the matrix was shown to chelate cations and to induce the expression of antibiotic resistance (Mulcahy et al., 2008). Also, promotion of biofilm formation was observed through the ability of extracellular DNA to chelate $Mg^{2+}$ (Mulcahy and Lewenza, 2011). Recently, current knowledge of bacterial biofilms in animal pathogens was reviewed (Jacques et al., 2010) and surprisingly, very little is known about the biofilm formed by *Clostridium perfringens*.

*Clostridium perfringens* is a Gram-positive anaerobic bacterium that causes numerous human and animal diseases, primarily as a result of its ability to produce many different toxins (Markey et al., 2013). A classification based on the production of four major toxins (alpha, beta, epsilon, and iota) divides the *C. perfringens* into five toxigenic biotypes (A to E; Petit et al., 1999). *C. perfringens* has been ranked by the Center for Disease Control and Prevention as one of the most common bacterial causes of food-borne illness in the United States, causing nearly a million cases each year, and is also classified as a class-B bioterrorism agent (Scallan et al., 2011). Recently, an increasing number of reports have implicated the organism in antibiotic–associated diarrhea and sporadic diarrhea cases in humans, as well as diarrhea cases in animals (Smedley et al., 2004; Uzal et al., 2012; Banaszkiewicz et al., 2013). In chickens and swine, *C. perfringens* causes enteritis, a disease of economic importance to the worldwide animal-food producing industry in terms of both animal loss and vaccination costs (Markey et al., 2013). Isolates of animal origin constitute a risk for transmission to humans through the food chain. Recent studies have shown early signs of acquired antibiotic resistance in *C. perfringens* indicating that antibiotic resistance is now emerging (Lyras et al., 2009; Soge et al.,

2009; Slavić et al., 2011; Charlebois et al., 2012). *C. perfringens* has been the subject of considerable investigations in the past several years; however, an area of substantial uncertainty relates to biofilm formation with, or without exposure to low-doses of anticoccidials and antibiotics. Anticoccidials are ionophores such as monensin, narasin, and salinomycin that are used to prevent/treat infections caused by coccidia, an obligate intracellular parasite, and in some cases for their effect against *C. perfringens*. These compounds are also used as growth promoters in some countries (Callaway et al., 2003; Lefebvre et al., 2006; Diarra et al., 2007).

Only a few studies have reported on *C. perfringens* biofilm formation (Varga et al., 2008; Donelli et al., 2012). Type IV pilus (TFP)-dependent gliding motility and the catabolite control protein (CcpA), a key regulator of the response to carbohydrate limitation, were both shown to be necessary for efficient biofilm formation (Varga et al., 2008). In the same study, the authors also observed that biofilm cells demonstrated 5- to 15-fold-increased survival rate over planktonic cells after exposure to penicillin G and an increased survival to oxygen stresses. It is suspected that biofilms of *C. perfringens* may play an important role in resistance to environmental stresses (Varga et al., 2008). In a study by Ledder et al. (2008) on coaggregation among numerically and ecologically important intestinal bacteria, and between intestinal bacteria and oral isolates, *C. perfringens* scored the highest overall for coaggregation among the gut species tested (Ledder et al., 2008). To our knowledge, no other data is available on biofilms of *C. perfringens*.

Studies demonstrating that low-doses of antibiotics induce bacterial biofilm formation have recently been reviewed by Kaplan (Kaplan, 2011). In contrast, low dose of a fluoroquinolone caused a significant decrease in adhesion and biofilm formation by *Stenotrophomonas maltophilia* (Pompilio et al., 2010). Also, lower cell densities within biofilms have been reported with sub-MIC of dicloxicillin, a β-lactam antibiotic, for *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (Cerca et al., 2005). The effect of sub-MIC of bacitracin, virginiamycin, lincomycin, tylosin, and ionophores on biofilm formation has not yet been described in *C. perfringens*. These antibiotics and ionophores are largely used in swine and poultry production for therapy, prophylaxis purposes, or as feed additives (low-dose usage) in some countries.

The aims of this study were to evaluate the biofilm formation of field isolates of *C. perfringens* of various sources, to determine biofilm tolerance to oxygen and antibiotics, and to investigate the effect of low doses of antibiotics and ionophores on biofilm formation.

## MATERIALS AND METHODS

### BACTERIAL ISOLATES

Commensal isolates of *C. perfringens* from poultry and swine were recovered from the normal intestinal microbiota of animals taken at seven (five poultry and two swine) processing plants located in the province of Québec, Canada. Isolates were identified and typed by PCR as previously described (Charlebois et al., 2012). Clinical isolates of animal origin were provided by the Clinical Laboratory of Molecular Diagnostic of Université de Montréal (St-Hyacinthe, QC, Canada) and human isolates were provided by the Infectious Disease Research Center of Université Laval (Québec, Canada). Thawed isolates were grown on Columbia agar with 5% sheep blood (Oxoid, Nepean, ON, Canada) and then incubated in anaerobic condition at 37°C.

### BIOFILM GROWTH AND QUANTIFICATION

Different temperatures, incubation times and growth media with and without glucose, as well as three different isolates (ATCC 13124, c1261A, FMV-CP12) were used to standardize the biofilm formation assay. More specifically, overnight blood agar cultures of *C. perfringens* were resuspended at a density of 0.5 MacFarland in Trypticase-peptone-glucose (TPG), fluid thioglycolate (FTG), tryptic soy broth (TSB; BD, Mississauga, ON, Canada) supplemented or not with 10 mM of filter-sterilized glucose (Sigma, Oakville, ON, Canada), or Brain Heart Infusion (BHI) supplemented or not with 10 mM of filter-sterilized glucose. 100 μL of cultures were added in 96-well polystyrene tissue culture plates (Costar® #3595, Corning Incorporated, Corning, NY, USA) which were then incubated anaerobically at 30, 35, or 44°C for 1, 3, or 6 days in a sealed container. All isolates recovered in this study were tested for biofilm formation. These experiments were done in triplicate and repeated three times. Optimized biofilm growth conditions were as follows: bacterial cells were inoculated in TSB medium supplemented with 10 mM of filter-sterilized glucose and plates were incubated for 6 days at 44°C in an anaerobic environment. To quantify the biofilm formation, the crystal violet assay was used as described elsewhere (Varga et al., 2008). *C. perfringens* ATCC 13124 was used as positive control because this strain was previously shown to produce biofilm (Varga et al., 2008).

Isolates were categorized as described previously (Stepanovic et al., 2007). Briefly, isolates were divided into the following categories: no biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer, based upon the previously calculated optical density (OD) values measured at 570 nm: $OD \leq ODc$ = no biofilm producer; $ODc < OD \leq 2\times ODc$ = weak biofilm producer; $2\times ODc < OD \leq 4\times ODc$ = moderate biofilm producer; $4\times ODc < OD$ = strong biofilm producer. ODc is defined as three standard deviations (SD) above the mean OD of the negative control. Isolates were also compared in regards to a few parameters: clinical or commensal and animal sources. A subgroup of *C. perfringens* isolates ($n = 18$) was randomly selected for further characterization (**Table 1**). These were from the collection of isolates recovered in this study from seven processing plants. To those was added *C. perfringens* ATCC 13124 as a positive control.

### ANTIMICROBIAL SUSCEPTIBILITY TESTING

The subset of isolates was tested for MICs as previously described in the CLSI M11-A8 document (CLSI, 2012). Briefly, colonies of *C. perfringens,* from a 24 h culture grown at 37°C on blood agar plates (Oxoid) under anaerobic conditions, were resuspended into 5 mL of saline to achieve a 0.5 McFarland turbidity. This adjusted inoculum was then diluted 1:75 in supplemented Brucella broth. 50 μL of this suspension was then transferred to individual wells on microplates containing antibiotics. The microplates were sealed and incubated in an anaerobic chamber for 48 h at 37°C. Antimicrobials tested were penicillin, lincomycin, virginiamycin,

**Table 1 | *Clostridium perfringens* selected isolates and their MIC (μg/mL) to the antibiotics and anticoccidials tested in this study.**

| | | | | MIC (μg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Strains** | **Origin** | **Biofilm formation** | **Bacitracin** | **Virginiamycin** | **Penicillin** | **Tylosin** | **Lincomycin** | **Salinomycin** | **Narasin** | **Monensin** | **Source** |
| Poultry | c1261_A | Commensal | Weak | 512 | 0.12 | 0.03 | 0.5 | 16 | 0.12 | 0.12 | 0.25 | This study |
| | c2188_B | Commensal | Moderate | 512 | 0.12 | 0.03 | 0.5 | 32 | 0.12 | 0.06 | 2 | This study |
| | c3336_B | Commensal | No | 4 | 1 | 0.06 | 128 | 512 | 0.12 | 0.03 | 1 | This study |
| | c3342_A | Commensal | Moderate | 2 | 1 | 0.12 | 128 | 8 | 0.25 | 0.12 | 0.25 | This study |
| | c3342_B | Commensal | High | 256 | 1 | 0.0075 | 128 | 64 | 0.25 | 0.12 | 1 | This study |
| | c3437_A | Commensal | Moderate | 6 | 2 | 0.015 | 128 | 256 | 0.25 | 0.06 | 2 | This study |
| | c3807_A | Commensal | No | 256 | 1 | 0.03 | 64 | 8 | 0.25 | 0.06 | 2 | This study |
| | STF2003-1256 | Clinical | Weak | 4 | 1 | 0.06 | 0.5 | 256 | 0.25 | 0.06 | 0.5 | Dre Martine Boulianne |
| | 2006-4758 | Clinical | Weak | 256 | 2 | 0.03 | 0.5 | 16 | 0.06 | 0.03 | 0.5 | Dre Martine Boulianne |
| | SHY07-383 | Clinical | High | 3 | 0.5 | 0.0019 | 0.5 | 8 | 0.015 | 0.03 | 0.015 | Dre Martine Boulianne |
| | CP4 | Clinical | Moderate | 1.5 | 0.5 | 0.06 | 1 | 8 | 1 | 0.25 | 2 | Dr. John F. Prescott |
| | JGS4143 | Clinical | Moderate | 8 | 2 | 0.015 | 1 | 32 | 0.5 | 0.06 | 1 | Dr. Glenn Songer |
| Human | CCRI-16276 | Clinical | Moderate | 16 | 0.5 | 0.03 | 2 | 8 | 0.5 | 0.25 | 2 | Dr. Maurice Boissinot |
| | ATCC 13124 | Clinical | Weak | 2 | 0.5 | 0.03 | 1 | 0.5 | 0.06 | 0.12 | 0.015 | ATCC |
| Swine | FMV-CP4 | Clinical | Moderate | 0.75 | 1 | 0.06 | 2 | 8 | 0.5 | 0.12 | 2 | This study |
| | FMV-CP23 | Commensal | No | 1 | 0.5 | 0.12 | 1 | 16 | 0.25 | 0.06 | 1 | This study |
| | FMV-CP71 | Commensal | No | 0.75 | 0.12 | 0.12 | 4 | 128 | 0.25 | 0.12 | 0.25 | This study |
| | 1285414 | Clinical | Moderate | 16 | 2 | 0.12 | 0.5 | 512 | 0.5 | 0.12 | 0.25 | This study |
| | 1304504 | Clinical | Moderate | 2 | 1 | 0.03 | 2 | 4 | 0.12 | 0.03 | 0.015 | This study |

tylosin, salinomycin, narasin, and monensin (all from Sigma). For bacitracin susceptibility testing, the Etest technique was used as described earlier (Charlebois et al., 2012). Isolates with MIC higher than 256 μg/mL by Etest were tested with the microdilutions broth technique to determine the exact MIC. *C. perfringens* ATCC 13124 was used as a control. Breakpoint for penicillin (2 μg/mL) is available from CLSI M11-A8. There is a previously published breakpoint for bacitracin (16 μg/mL) that is widely used in the literature (Chalmers et al., 2008; Charlebois et al., 2012). No other breakpoints are available for the antimicrobials tested in this study.

#### BIOFILM TOLERANCE TO OXYGEN, ANTIBIOTICS AND ANTICOCCIDIALS

A subgroup of *C. perfringens* isolates ($n = 18$) was randomly selected from the collection of isolates recovered in this study from seven processing plants. These isolates were tested for their tolerance to oxygen and antibiotics when grown as biofilms. Biofilms were cultured in triplicates as described above and the initial ATP levels were measured with the BacTiter Glo kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. After 6 days of incubation, the supernatants were removed and replaced with TSB-glucose in the biofilm cultures. The supernatants of the biofilm containing the planktonic cells were centrifuged and resuspended in fresh TSB-glucose medium. Planktonic cells were then transferred to fresh tissue culture plates. For both the biofilm cultures and planktonic cells, the antibiotic and oxygen tolerance assays were performed. Briefly, plates were incubated anaerobically at 44°C with 1.5 mg/mL of bacitracin, 20 μg/mL of penicillin, 512 μg/mL of lincomycin, 4 μg/mL of virginiamycin, 256 μg/mL of tylosin, 1 μg/mL of narasin, 2 μg/mL of salinomycin, or 4 μg/mL of monensin (all antimicrobials from Sigma) for 6 or 24 h. These concentrations of antimicrobials correspond to two times the highest MIC found among the isolates of the random group. The effects of different combinations of anticoccidials and antibiotics (monensin, narasin, or salinomycin with bacitracin, tylosin, or virginiamycin) on pre-formed biofilms were also analysed. For the oxygen tolerance assay, plates were incubated aerobically at 44°C for 6 or 24 h. After the oxygen and antimicrobial treatments, the ATP levels were measured with the BacTiter Glo kit. Percentages of survival were calculated by dividing the final ATP level by the initial ATP level. Treatment with penicillin was used as a positive control. The effect of low doses of antibiotics and anticoccidials on biofilm formation was assessed on the randomly selected *C. perfringens* isolates ($n = 18$). Briefly, biofilms were cultured as described above with the exception that antibiotics were added at a concentration of 0.1× the MICs of each isolate before incubation. All plates were incubated anaerobically at 44°C for 6 days in a sealed container. All isolates were done in triplicates. To quantify the biofilm formation, the crystal violet assay was used as described above. Control wells were incubated with medium only.

#### EFFECTS OF ENZYMATIC TREATMENTS ON BIOFILM FORMATION

Biofilms of the selected *C. perfringens* isolates ($n = 18$) were grown for 6 days in TSB supplemented with 10 mM of glucose as described above. Wells were washed 2x with distilled water and then filled with 100 μL of PBS containing 20 μg/mL of dispersin B (Kane Biotech Inc., Winnipeg, MB, Canada) as described by Izano et al. (2007), or 100 μg/mL of DNase I or proteinase K (Kaplan et al., 2004; Grasteau et al., 2011). Plates were incubated at 37°C for 5 min for the dispersin B or 1 h for the DNase I and the proteinase K. For the cellulase treatment, wells were filled with 120 U/mL of cellulase and then incubated at 45°C for 72 h (Jain and Bhosle, 2008). After incubation, wells were washed once with distilled water and stained with crystal violet. *Staphylococcus aureus* ATCC 25923 was used as a positive control for all treatments.

#### SCANNING LASER CONFOCAL MICROSCOPY

Biofilms of the selected *C. perfringens* isolates ($n = 18$) were grown in 96-well plates as described above with or without low doses of bacitracin. Plates were incubated for 6 days at 44°C under anaerobic conditions. After incubation, wells were washed two times with PBS to remove unattached cells. 100 μL of PBS containing the fluorescent FM 1-43 stain (Invitrogen, Burlington, ON, Canada), was added to each well to allow visualization of individual bacteria by laser confocal microscopy. In addition, 50 μg/mL of calcofluor white dye (Sigma), which is specific for beta-1,3 and beta-1,4 linkages in polysaccharides, was added to each well. This stain was selected because it was previously shown to bind to *C. perfringens* biofilm matrix (Varga et al., 2008). After incubation at room temperature for 30 and 15 min, respectively, wells were washed with 200 μL of sterile distilled water. Before readings, 100 μL of sterile distilled water was added to each well. An Olympus FV1000 IX81 laser confocal microscope was used to collect three-dimensional images of the biofilms. An argon laser set at 472 nm was used to excite the FM 1-43 dye (emission green) and a UV laser at 364 nm to excite the calcofluor white dye (emission blue). The images were processed using Fluoview software (Olympus). For the matrix composition, the biofilms were cultured as described above. The fluorophores assay was done as described previously (Wu et al., 2013a). Briefly, after the incubation, the wells were filled with 100 μL of Wheat Germ Agglutinin (WGA) – Oregon Green 488, Sypro Ruby Red or BOBO-3 (all from Invitrogen). WGA was diluted 1/100 in PBS whereas BOBO-3 was diluted 1/1500 in water. Sypro Ruby Red was used as described by the manufacturer. Plates were incubated for 30 min at room temperature in the dark then washed once with water and filled with 100 μL PBS. The plates were observed by confocal microscopy. The excitation/emission wavelengths for the fluorophores were as follow: 496/524 nm (WGA), 450/610 nm (Sypro Ruby Red), and 570/602 nm (BOBO-3). The images were processed using Fluoview software (Olympus). *Staphylococcus aureus* ATCC 25923 was used as a positive control for all fluorophores.

#### STATISTICAL ANALYSIS

The statistical significance ($p$ value) of differences in biofilm between the animal origin or commensal and clinical isolates were calculated with an unequal variance linear model. A Student $t$-test was used for the biofilm tolerance to oxygen and antibiotics assays, the low-dose and the enzymatic treatments. A $p < 0.05$ was considered to be significant. Statistics were done with the SAS software v.9.1. (Cary, NC, USA).

## RESULTS

### BACTERIAL ISOLATES

A total of 277 *C. perfringens* isolates of poultry (clinical, $n = 14$; commensal, $n = 136$), swine (clinical, $n = 34$; commensal, $n = 50$), human (clinical, $n = 9$), and other animal origins [clinical isolates from cows ($n = 12$), sheep ($n = 10$), goats ($n = 3$), horses ($n = 3$), deer ($n = 1$), duck ($n = 1$), alpaca ($n = 1$), cat ($n = 1$), dog ($n = 1$), and hare ($n = 1$)] were isolated for this study. A total of 273 isolates were of type A and 4 of type D. Type D isolates were found in clinical samples from bovine ($n = 1$) and ovine ($n = 3$).

### BIOFILM GROWTH AND QUANTIFICATION

Most of the *C. perfringens* isolates tested were able to form biofilm ($n = 230/277$) in the conditions used in this study. The OD values at 570 nm ($OD_{570}$) ranged from 0.009 to 0.489 (**Figure 1**) indicating that biofilm formation can vary among tested isolates. Of those, strong ($n = 7$), moderate ($n = 42$), and weak ($n = 181$) biofilm producers were observed. Biofilm formation was compared among clinical and commensal isolates of *C. perfringens* of animal origin to test whether there is a difference between these two groups of isolates. The mean OD value of biofilm formed by clinical isolates originating from swine was significantly higher ($p < 0.05$) than the mean OD value of their commensal counterparts (**Figure 1**). However, no difference in biofilm formation was observed between clinical and commensal isolates of poultry origin ($p > 0.05$). The low number of clinical poultry isolates was likely not sufficient to allow a statistical significance to be seen. Also, biofilm formation was compared among isolates of *C. perfringens* of different animal origins. No significant difference in biofilm formation was observed between isolates recovered from swine, poultry, human, and other animal origins ($p > 0.05$). Again, the low number of human and other animal species isolates was likely not sufficient to allow a statistical significance to be seen. A subgroup of *C. perfringens* isolates ($n = 18$) were randomly selected for further characterization (**Table 1**). These were from the collection of isolates recovered in this study from seven processing plants. To those, we included the *C. perfringens* ATCC 13124. The following categories were covered in this subgroup: commensal, clinical, swine, poultry, human, and different levels of biofilm productions. However, *C. perfringens* isolates of other animal species from this study are not represented in this subgroup.

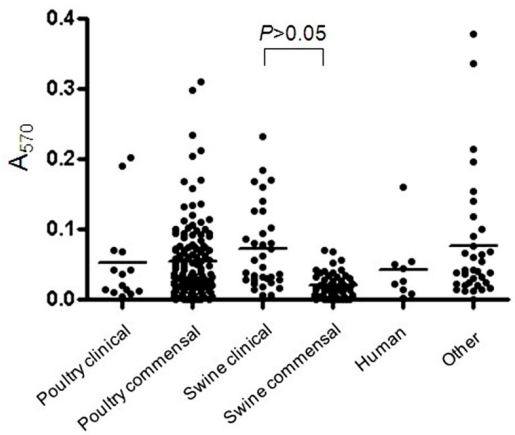


**FIGURE 1 | Biofilm formation by *C. perfringens* isolates.** Biofilm formation of *C. perfringens* strains in 96-well plates, measured after 6 days as described in Section "Materials and Methods." The *p* values were calculated using a linear model for unequal variances. Each dot represents the mean of the replicates for each strain. The bars represent the mean values of each group.

### ANTIMICROBIAL SUSCEPTIBILITY TESTING

Isolates tested ($n = 19$) demonstrated low MICs to penicillin (0.002–0.12 μg/mL), virginiamycin (0.12–2 μg/mL), narasin (0.03–0.25 μg/mL), salinomycin (0.015–1 μg/mL), and monensin (0.015–2 μg/mL; **Table 1**). For bacitracin, 13 isolates were susceptible (0.75–16 μg/mL) and 5 were resistant (256–512 μg/mL). For lincomycin, MICs between 0.5 and 512 μg/mL were observed whereas for tylosin, MICs between 0.5 and 128 μg/mL were obtained.

### BIOFILM TOLERANCE TO OXYGEN, ANTIBIOTICS AND ANTICOCCIDIALS

In the atmospheric oxygen tolerance assays, the mean viability rates of planktonic cells after exposure to oxygen for 6 and 24 h were 63 and 7.4%, respectively (**Figure 2A**). However, the viability rates were higher (80.6% of viable cells after 6 h and 61% after 24 h) when *C. perfringens* cells were organized in biofilm. Data between planktonic cells and biofilm, obtained after an incubation of 24 h, were significantly different ($p < 0.05$) (**Figure 2A**). For the antibiotic and anticoccidials tolerance assays, planktonic cells had between 7.0 and 69.1% of viability after 6 h exposure and between 1.2 and 20.7% of viability after 24 h exposure to antibiotics or anticoccidials. On the other hand, cells in biofilm had between 32.7 and 65.0% of viability, and between 14.3 and 47.1% of viability for the same periods of time, corresponding to a 0.6- to 9-fold-increased survival rate after 6 h and to a 0.8- to 36-fold-increased survival rate after 24 h over planktonic cells. Viability rates for cells in biofilm were significantly higher ($p < 0.05$) than those observed for planktonic cells (**Figures 2B–I**). Antibiotics and anticoccidials were also used in combination to determine if there was a synergistic activity toward cells of *C. perfringens* ATCC 13124 within a biofilm (**Table 2**). A higher activity was observed for six combinations but these results were not significant ($p > 0.05$).

### BACTERIAL EXPOSURE TO LOW DOSES OF ANTIBIOTICS AND ANTICOCCIDIALS

After exposure of isolates ($n = 19$) to low doses of antibiotics and anticoccidials, no clear trend was observed for bacitracin, tylosin, virginiamycin, and monensin (**Figures 3A,B,D,H**) but a few isolates showed an increase in biofilm formation when exposed to those compounds. However, exposure to low doses of penicillin, lincomycin, salinomycin, and narasin tended to lead to a diminution of the biofilm formed (**Figures 3C,E,F,G**). This phenomenon was particularly observed within the poultry clinical group of isolates. No other trends could be observed. Statistically significant results are indicated in **Figure 3** ($p < 0.05$).

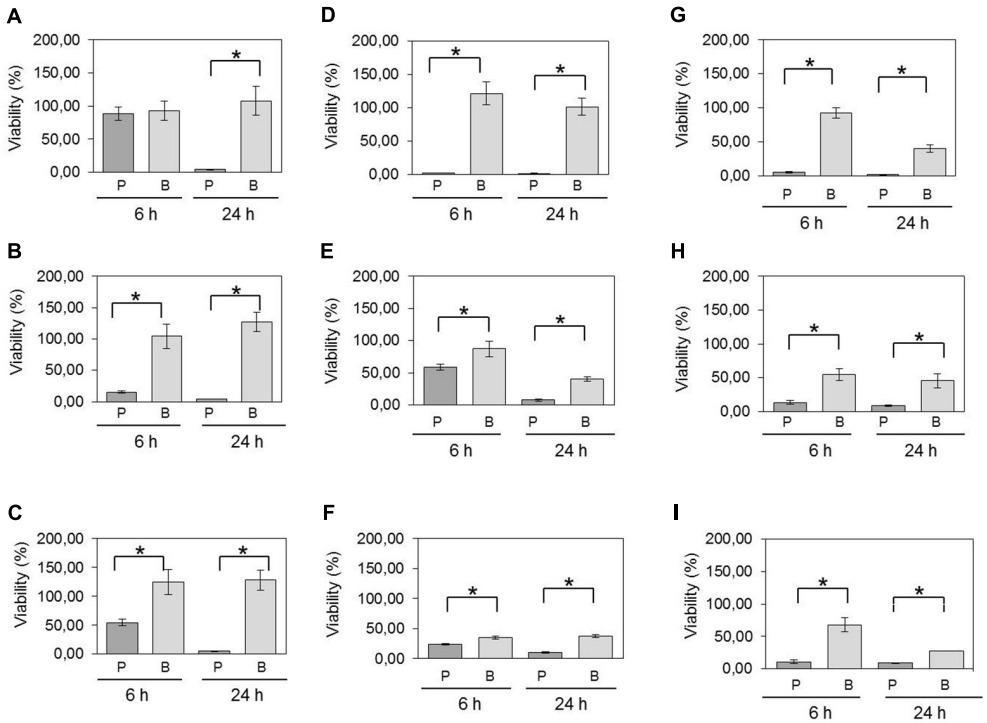


**FIGURE 2 | Viability of planktonic cells compared to cells in biofilm following exposure to oxygen and antimicrobials.** *C. perfringens* 6-days-old biofilms and planktonic cultures were exposed to **(A)** atmospheric oxygen, **(B)** 1.5 mg/mL of bacitracin, **(C)** 20 μg/mL of penicillin, **(D)** 512 μg/mL of lincomycin, **(E)** 4 μg/mL of virginiamycin, **(F)** 256 μg/mL of tylosin, **(G)** 4 μg/mL of monensin, **(H)** 2 μg/mL of salinomycin, or **(I)** 1 μg/mL of narasin for the times indicated. Differences in survival of planktonic cells [P] versus biofilm [B] at each time were compared using Student's *t*-test. $*p < 0.05$. The error bars represent standard deviations. Results presented are for strain *C. perfringens* ATCC 13124. For all isolates, statistically significant increases in viabilities were observed for bacteria in biofilms compared to planktonic cells.

## SCANNING LASER CONFOCAL MICROSCOPY

Using laser confocal microscopy, biofilms of tested isolates ($n = 19$) were stained with the fluorescent dyes SYPRO Ruby Red, WGA and BOBO-3 in order to visualize extracellular proteins, poly-beta-1,6-*N*-acetyl-D-glucosamine (PNAG) exopolysaccharide and DNA, respectively. Extracellular proteins and DNA were visible in biofilms of all tested isolates (**Figure 4**). However, PNAG was absent from the biofilm formed by *C. perfringens* isolates. Binding of calcofluor white to the biofilm matrix was observed. Calcofluor white binds to polysaccharides with beta-1,3 and beta-1,4 linkages. Six isolates (*C. perfringens* c1261_A, c3807_A, ATCC 13124, SHY07-383, FMV-CP23 and c3437_A) were randomly selected to further analyse their biofilms in the presence of low dose of bacitracin (0.3 × MIC). The biofilms of these isolates grown in the presence of bacitracin were approximately between 35 and 80 μm in height, whereas biofilms formed without bacitracin were thinner with elevations between 30 and 60 μm (**Table 3**). It was also found that five of them showed an increase in the area occupied by bacteria in the biofilm following exposure to low doses of bacitracin (**Table 3**). Laser confocal microscopy images showed that the biofilm formed by the *C. perfringens* isolates ($n = 6$) were mainly flat (**Figure 5A**) with the exception of one isolate demonstrating a mushroom-like biofilm (**Figure 5B**).

**Table 2 | Viability (%) of *C. perfringens* strain ATCC 13124 in biofilm after 24 h incubation with antibiotics and anticoccidials alone or in combinations.**

| | Alone | Monensin | Salinomycin | Narasin |
|---|---|---|---|---|
| Alone | 100 | 65.5 | 79.5 | 61.8 |
| Bacitracin | 74.2 | 74.4 | 67.4 | 59.0 |
| Virginiamycin | 70.5 | 62.9 | 58.4 | 72.1 |
| Tylosin | 69.1 | 77.8 | 55.9 | 59.8 |

*Values underlined: values of the combination lower than the antimicrobials used alone.*

## ENZYMATIC TREATMENTS ON BIOFILM FORMATION

Dispersion of the biofilm matrix of *C. perfringens* isolates ($n = 19$) was not observed with dispersin B enzymatic treatment confirming that PNAG is absent from the matrix formed by the tested *C. perfringens* isolates. However, proteinase K, cellulase, and DNase I enzymatic treatments significantly dispersed the preformed biofilm ($p < 0.05$) indicating the presence of proteins, beta-1,4 linked polysaccharides and extracellular DNA in the matrix (**Figure 6**).

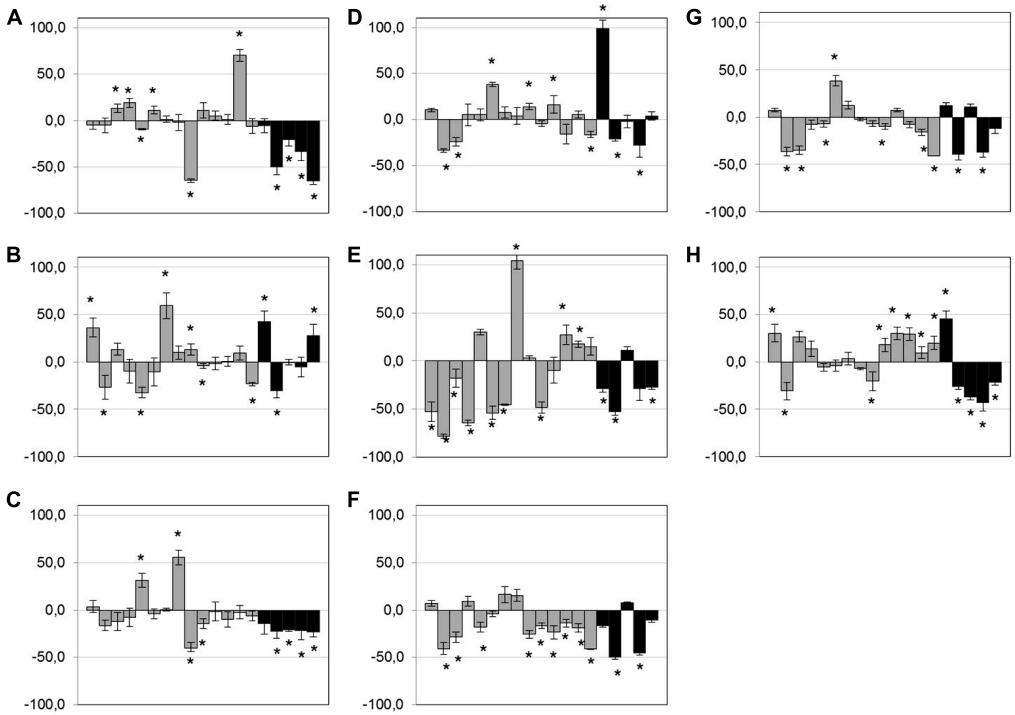


**FIGURE 3 | Effect of low-dose antimicrobials on biofilm formation.** Biofilm formation of *C. perfringens* strains in 96-well plates in presence of 0.1× MIC of bacitracin **(A)**, tylosin **(B)**, penicillin **(C)**, virginiamycin **(D)**, lincomycin **(E)**, salinomycin **(F)**, narasin **(G)**, or monensin **(H)**, measured as described in Section "Materials and Methods." Strains are in the following order: c1261_A, c2188_B, c3336_B, c3342_A, c3342_B, c3437_A, c3807_A, ATCC 13124, CCRI-16276, FMV-CP4, FMV-CP23, FMV-CP71, 1285414, 1304504, SHY07-383, STF2003-1256, 2006-4758, CP4, and JGS 4143. Results are expressed as percentages of the control not exposed to antibiotics. Conditions were compared to the control biofilm using Student's *t*-test.*$p < 0.05$. The error bars represent standard deviation. Black bands represent poultry clinical isolates.


**FIGURE 4 | Matrix composition of *C. perfringens* biofilm.** Representative results of the matrix composition of *C. perfringens* biofilm observed with fluorescent probes. *Staphylococcus aureus* ATCC 25923 was used as a positive control for all fluorophores. FM1-43: bacterial cells; BOBO-3: extracellular DNA, WGA: PNAG; Ruby Red: proteins; Calcofluor white: beta-1,3 and beta-1,4 linked polysaccharides.

## DISCUSSION

Most of the tested isolates in this study which originated from various animal species were able to form biofilm at various degrees in the conditions used. Optimized biofilm growth conditions were different than those previously described (Varga et al., 2008; Donelli et al., 2012). However, results of this study are in accordance with the ones obtained by Varga et al. (2008) where all sequenced strains of *C. perfringens* (biotypes A of ATCC 13124, 13, and SM101), as well as representatives of type C, D, and E strains which cause infections in animals were shown to form biofilms with OD values between 0.07 and 0.5 (Varga et al., 2008). Donelli et al. (2012) were able to obtain a higher biofilm formation for *C. perfringens* strain CpeBs31 in their study, with a mean OD of 3.2, which classified this isolate as strongly adherent under the following conditions: BHI supplemented with 1% glucose and 48 h incubation at 37°C (Donelli et al., 2012). This strain was not tested in our study.

In the present study, clinical isolates recovered from swine formed significantly more biofilm than their commensal counterparts. In *Salmonella typhymurium*, it was found that clinical, outbreak-associated and retail product isolates produced thicker

**Table 3 | Effect of subinhibitory concentrations of bacitracin on biofilm formation as analyzed by scanning laser confocal microscopy.**

| | Isolates | Total height (μm) | Matrix (μm) | Cells (μm) |
|---|---|---|---|---|
| Increased biofilm | c1261_A | 35 | 18 | 17 |
| | c1261_A + bacitracin | 60 | 40 | 20 |
| | c3807_A | 45 | 35 | 10 |
| | c3807_A + bacitracin | 75 | 40 | 35 |
| | c3437_A | 30 | 22 | 8 |
| | c3437_A + bacitracin | 80 | 50 | 30 |
| | SHY07-383 | 50 | 25 | 25 |
| | SHY07-383 + bacitracin | 55 | 20 | 35 |
| Decreased biofilm | ATCC 13124 | 45 | 33 | 12 |
| | ATCC 13124 + bacitracin | 40 | 23 | 17 |
| | FMV-CP23 | 60 | 35 | 25 |
| | FMV-CP23 + bacitracin | 35 | 20 | 15 |

biofilms compared to isolates recovered from food (Castelijn et al., 2012). Reisner et al. (2006) did not observe an increased biofilm formation in human clinical strains of intestinal *Escherichia coli*. Skyberg et al. (2007) also found that isolates of *Escherichia coli* recovered from healthy birds produced significantly stronger biofilms than isolates from cases of avian colibacillosis. In *Listeria monocytogenes*, it was found that isolates from food formed more biofilm than clinical isolates recovered from cases of listeriosis (Barbosa et al., 2013). For *C. perfringens*, biofilm formation could play a role in the development of the disease because biofilm can help bacteria adhere to surfaces, and this facilitates colonization and infection. Moreover, the ability to grow as a biofilm favors survival of bacteria in the environment (Semenyuk et al., 2014). In this study, it was observed that clinical isolates of swine *C. perfringens* formed more biofilms suggesting these isolates might survive longer in the environment.

*C. perfringens* is known to be an aerotolerant bacterium capable of surviving in soil or water (Rood and Cole, 1991). Results obtained with the atmospheric oxygen tolerance assays showed that the biofilm could protect *C. perfringens* cells from oxygen stress. Varga et al. (2008) obtained similar results in their study. The involvement of biofilm in oxygen tolerance has already been described in *Fusobacterium nucleatum*, another anaerobic bacterium (Gursoy et al., 2010). The present study also showed that the biofilm could protect *C. perfringens* from high concentrations of antibiotics and anticoccidials. Increased survival of *C. perfringens* cells in biofilm following penicillin G exposure have been described elsewhere (Varga et al., 2008). Interestingly, virginiamycin, tylosin, and the three anticoccidials (namely monensin, narasin, and salinomycin) had good activity against cells in biofilm, decreasing the viability below 50%. Virginiamycin has already been found to be active against biofilms formed by some strains of *Lactobacillus* spp. (Rich et al., 2011) but to our knowledge, the effects of tylosin and anticoccidials on biofilm have never been described. Results could not be subdivided between bactericidal and bacteriostatic antimicrobials because bacteriostatic antimicrobials used at high concentrations likely become bactericidal (Pankey and Sabath, 2004). The reverse is also reported, bactericidal agents used at low doses likely become bacteriostatic (Pankey and Sabath, 2004). In the biofilm tolerance assay, antimicrobials were used at high concentrations indicating that tylosin and lincomycin are both likely acting as bactericidal in this experiment. In the bacterial exposure to low doses of antimicrobials assay, all antimicrobials likely became bacteriostatic due to the low doses used in this experiment. Moreover, in the present study, different combinations of antibiotics and anticoccidials were assessed for their activity against cells in biofilm. In general, the viability in the biofilm tended to be lower when exposed to a combination

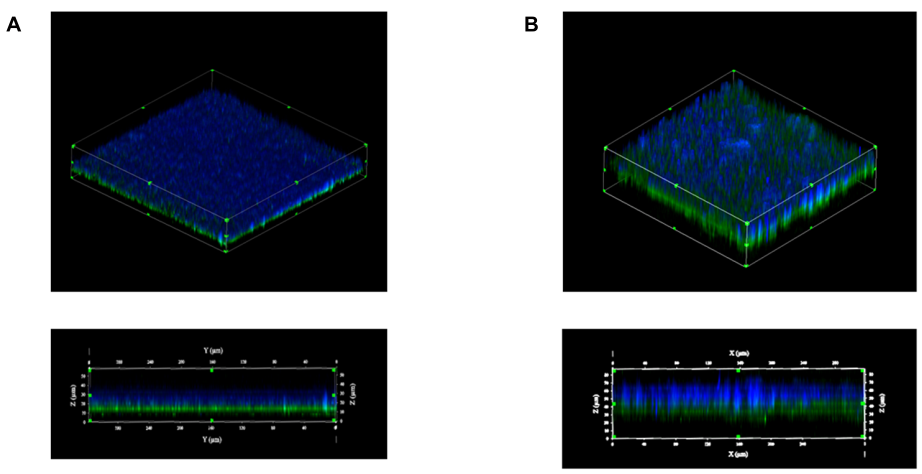


**FIGURE 5 | Biofilm morphologies observed by scanning laser confocal microscopy.** Biofilms formed by the *C. perfringens* c3437_A isolate **(A)** or by the *C. perfringens* SHY07-383 isolate **(B)** after 6 days of incubation. Blue: exopolysaccharides (Calcofluor white); Green: bacteria (FM 1–43).

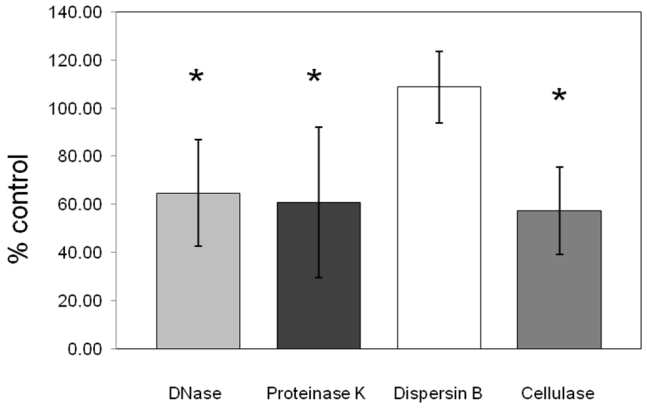


**FIGURE 6 | Effect of enzymatic treatments on pre-formed *C. perfringens* biofilm.** DNase, proteinase K, cellulase, and dispersin B effects on pre-formed biofilm of *C. perfringens*. Data were measured as described in Section "Materials and Methods." Results are the average of all strains tested and are expressed as a percentage of the control. The error bars represent standard deviation. $*p < 0.05$.

compared to either component used alone. Different combinations of antibiotics have already been described to be active against biofilm of *Staphylococcus epidermidis*, methicillin-resistant and -susceptible *Staphylococcus aureus* (MRSA and MSSA), *P. aeruginosa*, and *Enterococcus faecalis* (Saginur et al., 2006; Tre-Hardy et al., 2008; Holmberg et al., 2012; Wu et al., 2013b). This tolerance to antimicrobial agents observed in biofilm makes the treatment of these infections generally ineffective. For *C. perfringens*, it has been hypothesized that biofilm formation by this organism in the small intestine could contribute to antibiotic-associated diarrhea, a form of non-food-borne enteritis associated with antibiotic use, by facilitating in bacterial persistence through antibiotic treatment (Varga et al., 2008).

Because it has been described that low doses of antibiotics can either reduce or increase the biofilm production in other bacteria (Cerca et al., 2005; Hoffman et al., 2005; Majtan et al., 2008; Kaplan, 2011), the effect of low concentrations of antibiotics and anticoccidials on *C. perfringens* biofilm formation was studied by microplates assays. No clear trend was observed when exposed to low doses of antibiotics with the exception of penicillin, lincomycin, salinomycin, and narasin. In these cases, less biofilm was detected in the majority of isolates. Also, the effect of the antibiotics and anticoccidials on the biofilm varied depending on the strain tested indicating that this phenomenon is strain dependent. These variations among strains have also been observed for *Staphylococcus epidermidis* exposed to 0.5× the MIC of cefazolin, vancomycin, and dicloxacillin, and for *Streptococcus pyogenes* exposed to 0.015× to 0.5× the MIC of fluoroquinolones (Henriques et al., 2005; Balaji et al., 2013). To further analyse the effect of low doses of bacitracin on *C. perfringens* biofilms, laser confocal microscopy was used. An increase in the area occupied by bacteria was observed in biofilms exposed to low doses of bacitracin. This increased area occupied by bacteria has also been observed in *Staphylococcus epidermidis* biofilms exposed to 0.25× the MIC of erythromycin, in *P. aeruginosa* biofilms exposed to 0.25X the MIC of imipenem and in *Streptococcus intermedius* biofilms exposed to sub-MICs of ampicillin, ciprofloxacin, and tetracycline (Bagge et al., 2004; Ahmed et al., 2009; Wang et al., 2010).

To analyse the structure of *C. perfringens* biofilms, laser confocal microscopy was used and results obtained were consistent with the ones found in the study of Varga et al. (2008). In this study, most of the biofilms analysed in confocal microscopy were flat with one exception that showed a mushroom-like structure. To our knowledge, this is the first description of a mushroom-like biofilm in *C. perfringens*. For the matrix, it was found that the biofilm of *C. perfringens* contained polysaccharides, proteins, and extracellular DNA. Proteins and carbohydrates have already been described as components of *C. perfringens* biofilms (Varga et al., 2008). In addition to polysaccharides and proteins, extracellular DNA was also found to be a part of the matrix. The presence of extracellular DNA in the matrix of the biofilm has been described in other Gram positive bacteria (Barnes et al., 2012; Domenech et al., 2012; Kaplan et al., 2012) but to our knowledge, not in Clostridia. The mechanisms by which the DNA is released in biofilms are poorly understood but autolysis of cells has been hypothesized to mediate DNA release (Bayles, 2007; Ma et al., 2009). In *Enterococcus faecalis*, the release of extracellular DNA by autolysis is regulated by the action of the two proteases GelE and SprE (Thomas et al., 2008) whereas in *Staphylococcus aureus*, a finely tuned holin/antiholin system is thought to mediate cell lysis and programmed cell death (Bayles, 2007; Rice and Bayles, 2008). A previous study has revealed no extracellular DNA in biofilms of both *Bordetella bronchiseptica* strain 276 and *Escherichia coli* strain ECL 17602 using confocal laser scanning microscopy (Wu et al., 2013a), indicating that extracellular DNA is not a component of all biofilms. The presence of PNAG in the biofilm was studied because it is one of the most common and extensively studied matrix EPS (Jacques et al., 2010). In this study, PNAG was absent from *C. perfringens* biofilm matrix. This has been observed in other bacteria (Wu et al., 2013a). Binding of calcofluor white to the biofilm indicated that polysaccharides with beta-1,3 and beta-1,4 linkages, such as cellulose, are part of the matrix. To confirm this hypothesis, biofilms were treated with cellulase (Jain and Bhosle, 2008). It was observed that this enzyme could disperse the biofilm of *C. perfringens* confirming, for the first time, the presence of beta-1,4 linked polysaccharides in the matrix.

In conclusion, this study reports for the first time the presence of extracellular DNA and beta-1,4 linked polysaccharides in the matrix of *C. perfringens* biofilms. This study also demonstrated that virginiamycin, tylosin, and anticoccidials were active against *C. perfringens* cells in biofilm. Exposure to low doses of penicillin, lincomycin, salinomycin, and narasin tended to lead to a diminution of the biofilm formation. Further studies are needed to characterize the exopolysaccharides found in the matrix and to identify the genes involved in the biofilm formation in *C. perfringens*.

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# The impact of antifungals on toll-like receptors

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Fungi are increasingly recognized as major pathogens in immunocompromised individuals. With the increase in the number of fungal infections each year and the development of resistance to current therapy, new approaches to treatment including stimulation of the immune response in addition to concurrent pharmacotherapy is ongoing. The most common invasive fungal infections are caused by *Candida* spp., *Aspergillus* spp., and *Cryptococcus* spp. Amphotericin B (AmB) has remained the cornerstone of therapy against many fulminant fungal infections but its use is limited by its multitude of side effects. Echinocandins are a newer class of antifungal drugs with activity against *Candida* spp. and *Aspergillus* spp. and constitutes an alternative to AmB due to superior patient tolerability and fewer side effects. Due to their oral delivery, azoles continue to be heavily used for simple and complex diseases, such as fluconazole for candidal vaginitis and voriconazole for aspergillosis. The objective of this paper is to present current knowledge regarding the multiple interactions between the broad spectrum antifungals and the innate immune response, primarily focusing on the toll-like receptors.

**Keywords: antifungals, amphotericin B, echinocandins, caspofungin, voriconazole, toll-like receptors**

## INTRODUCTION

Fungal species are ubiquitous in the environment an estimated 1.5 million are known to exist (Hube, 2009). Only a few species are actually true pathogens in humans. Opportunistic fungi can cause life threatening infections ranging from superficial to deep seated infections in immunocompromised patients. In developing countries fungal infections affect both immunocompromised and immunocompetent individuals in areas that are endemic to mycoses (Brown et al., 2012). Fungal infections have increased over the last few decades and this can be correlated to increased invasive medical management, immunosuppressed patients either from acquired infections or from treatment induced deficiencies (Pfaller and Diekema, 2007). Interestingly, fungi are the fourth main cause of hospital acquired infections in populations "at-risk" despite the availability of antifungal treatment. This point illustrates the need for further study to identify more efficient ways to combat these interesting pathogens. It is often challenging to treat fungal infections because current methods to identify particular species are not always reliable or accurate resulting in delayed or inappropriate treatment (Perlin, 2011; Pfaller, 2012).

Amphotericin B (AmB) is a polyene antifungal agent first isolated from *Streptomyces nodosus* in 1955, from Venezuelan soil samples near the Orinoco River region (Dutcher, 1968). AmB is selectively toxic toward fungal cells, displaying high affinity for ergosterol, subsequently destabilizing fungal membranes (refer to **Table 1** – "Commonly used systemic antifungal drugs" for mechanism of action of common antifungals). It is primarily used in systemic fungal infections caused by *Histoplasma, Coccidioides, Candida, Blastomyces, Rhodotorula, Cryptococcus, Sporothrix, Mucor and Aspergillus spp.* and the drug has remained the cornerstone of the therapy against fulminant fungal infections (Ellis, 2002). AmB also has activity against some protozoans, and prions (Adjou et al., 1997; Kafetzis et al., 2005). AmB is amphoteric as well as amphipathic, has a low therapeutic index, and is associated with significant dose-related nephrotoxicity (Fanos and Cataldi, 2000), as well as acute, infusion-related febrile reactions (Khoo et al., 1994). Lipid-based formulations of AmB have allowed patients to receive higher doses while sparing toxicity (Hiemenz and Walsh, 1996). AmB also stimulates the production of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8), chemokines (MCP-1, MIP-1β, IL-8), prostaglandins, and nitric oxide (Cleary et al., 1992; Arning et al., 1995; Razonable et al., 2005). In addition to its direct antifungal activity, AmB activates toll-like receptors (TLRs), which contribute to the cytokine responses.

Echinocandins are a newer class of antifungal drugs that display a unique mechanism of action, inhibiting the synthesis of 1,3-β-D-glucan in the cell wall, through the inhibition of the enzyme 1,3-β glucan synthase (Morris and Villmann, 2006; Fera et al., 2009). Besides having a structural role, β-D-glucan also demonstrates potent immunostimulatory properties mediated by the innate immune receptor Dectin-1, as well as TLRs and C-type lectin receptors, which are expressed on host cells (Brown, 2006; Wheeler et al., 2008). Following binding, echinocandins induce the activation of phagocytic and proinflammatory responses (Dennehy and Brown, 2007). The echinocandins' antimicrobial spectrum includes most of the *Candida* spp. strains, *Aspergillus spp.*, and has some activity against *Pneumocystis jiroveci* (Denning, 2002). The advantages of echinocandins include long half-life allowing daily dosing, no dose adjustment in renal impairment or hemodialysis, minimal adverse effects, and limited drug interactions (Denning, 2002).

Human TLRs are closely related to the toll receptors in *Drosophila melanogaster*, and they are important for defense

**Table 1 | Commonly used systemic antifungal drugs [as reviewed by Lewis (2011)].**

| Mechanism | Class | Drugs |
|---|---|---|
| **Cell membrane** | Azoles (14-α demethylaseinhibitors) | **Triazoles** |
| Ergosterol inhibitors/binders | | Fluconazole, itraconazole, voriconazole, posaconazole, |
| | Polyenes (ergosterol binding) | Amphotericin B |
| | Allylamines (squalene monooxygenase) | Terbinafine |
| **Cell wall** | Echinocandins (β-1,3 D-glucan synthesis inhibitors) | Anidulafungin, caspofungin, micafungin |
| B-1,3 D-glucan synthesis | | |
| **Intracellular** | Pyrimidine analogs/thymidylate synthase inhibitor | Flucytosine |
| | Mitotic inhibitor | Griseofulvin |

against microbial infection (Medzhitov et al., 1997). TLRs are a class of proteins that play a critical role in the immune systems response to invading pathogens. They are found in tissues involved in immune function, as well as in tissues exposed to the external environment (the respiratory and the gastrointestinal tract). Ten TLRs have been identified in humans (TLR1–TLR10). They recognize structural repeating sequences known as pathogen-associated microbial patterns which are expressed by microbial pathogens, or danger-associated molecular patterns that are endogenous molecules released from necrotic cells and stimulate the release of inflammatory cytokines (Newton and Dixit, 2012). Some of the most important TLRs are TLR1 which recognize pathogen-associated molecular pattern with a specificity for Gram-positive bacteria, TLR2s that recognize many bacterial, fungal, viral, and certain endogenous substances, TLR4 which detects lipopolysaccharide from Gram-negative bacteria and TLR9, which is expressed by numerous cells of the immune system such as dendritic cells, B lymphocytes, monocytes, and natural killer cells and recognizes unmethylated CpG sequences in DNA molecules.

## AMPHOTERICIN B AND TLRs

AmB stimulates multiple TLRs, namely TLR1, TLR2, TLR4. Sau et al. (2003) demonstrated that TLR2 and CD14 receptors play an important role in the release of the inflammatory cytokines TNF-α and IL-8. Moreover, TLR2 has a key role in the release of IL-1β. Peritoneal macrophages, isolated from murine cells lacking TLR2, failed to release TNF-α, IL-1β, and IL-8 in response to AmB stimulation, in comparison with peritoneal macrophages isolated from TLR2 positive mice, which displayed increased inflammatory cytokine production. Furthermore AmB induced TNF-α production was suppressed in peritoneal macrophages that expressed mutant, non-functional TLR4. However, this effect was observed only at higher AmB concentrations. The authors of the study also demonstrated that TLR response to AmB was CD14 dependent. Therefore, CD14 positive cells produced TNF-α when stimulated by AmB, whereas those which were CD14 negative did not. Interestingly, lipid formulations of AmB did not elicit significant cytokine production and release from murine peritoneal macrophages, possibly due to the low concentration of unbound, non-lipid associated AmB (Sau et al., 2003).

The essential role of TLR1 in AmB induced cell activation was proven by Razonable et al. (2005) using THP1 monocytic cell line. The preincubation of THP1 cells with murine anti-human TLR1 monoclonal antibody (anti-TLR1 MAb) reduced the production of IL-6, IL-8, and TNF-α in response to AmB. Anti-TLR1 MAb also inhibited IL-8 secretion in response to the TLR2–TLR1 ligand Pam-3-*Cys*. Additionally, IL-8 inhibition with anti-TLR1 MAb was superior than with anti-TLR2 MAb and the addition of anti-TLR1 MAb augmented the degree of IL-8 inhibition by anti-TLR2 MAb (Razonable et al., 2005).

To further characterize the influence of AmB on TLRs, Bellocchio et al. (2005) showed that the expression of TLR2 and TLR4 was activated upon exposure of neutrophils (human and mice) to *Aspergillus* conidia. However, TLR4 was only stimulated upon exposure to the fungal hyphae. AmB increased the expression of TLR2, while liposomal AmB increased the expression of TLR4 in neutrophils. Using purified murine neutrophils, the authors were able to demonstrate that both TLR4 activation and liposomal AmB deter production of pro-inflammatory cytokines and stimulate anti-inflammatory cytokines. Additionally, in the absence of TLR4, liposomal AmB acts like deoxycholate AmB, stimulating the release of inflammatory cytokines (Bellocchio et al., 2005).

In another study published by Matsuo et al. (2006) using monocyte-like cell lines, the authors demonstrated that AmB phosphorylates p65 of nuclear factor-kappaB. Further evidence in the study suggested that this leads to stimulation of proinflammatory cytokine production, mediated by receptors including TLR2 and NF-kappaB (Matsuo et al., 2006).

## ECHINOCANDINS AND TLRs

The influence of echinocandins on the innate immune receptors is achieved through the influence of these antifungals on β-D-glucan. A report published by Moretti et al. (2012) demonstrated that caspofungin influenced TLR2/Dectin-1 interactions, as wells as Dectin-1 engagement with TLR4 and TLR9. Using an invasive aspergillosis model in which two different strains of Dectin-1/TLR2 deficient murines were treated with caspofungin, the authors found that at lower concentration of the drug (0.1 mg/kg) the restricting activity on fungal growth was preserved, as well as the inflammatory cell recruitment. However, this was dependent on the genetic background of the host (C57BL/6 responded to treatment with caspofungin but BALB/c did not). At higher doses (5 mg/kg) both types of mutant mice had significant restriction of fungal growth and reduction of inflammatory cell recruitment. Both the protective

(at 0.1 mg/kg) and the exacerbating (at 5 mg/kg) effects of caspofungin were lost in TLR2 deficient mice, indicating that TLR2 is required for the antifungal activity of echinocandins against aspergillosis. Furthermore, using TLR4 and TLR9 deficient mice with invasive aspergillosis, the authors showed that TLR4 contributes to the protective effect and TLR9 contributes to the exacerbating effect of caspofungin (Moretti et al., 2012).

Similarly, using a murine model of infective aspergillosis, Moretti et al. (2013) studied the immunomodulatory activity of echinocandins. Micafungin controlled cytokine response to *A. fumigatus* by decreasing the expression of TNF-α and increasing IL-10 release. The anti-inflammatory activity of micafungin required IL-10 and occurred through signaling via the TLR2/dectin-1 and TLR3/TRIF pathways (Moretti et al., 2013).

In another article published by Salvenmoser et al. (2010), caspofungin treatment resulted in the highest upregulation of TLR2 by *A. fumigatus* whereas exposure of *C. albicans* to caspofungin let to the significant upregulation of TLR4 and TLR9.

## AZOLES AND TLRs

There seems to be a similar interaction of azoles on TLR and subsequent immunomodulation, however, the data is limited. In previously mentioned article published by Salvenmoser et al. (2010), voriconazole treatment upregulated TLR2, TLR4, and TLR9 by *A. fumigatus*. In another study by Simitsopoulou et al. (2008) an additive antifungal effect was demonstrated when voriconazole was combined with monocytes and *A. fumigatus* hyphae. Both *A. fumigatus* hyphae and voriconazole induced increased expression of TLR2 as well as TNF-α in monocytic cells compared to untreated cells. The effects were seen when both were used independently but more significantly when used in combination. In contrast, TLR4 expression was not increased by either voriconazole or fungal hyphae. In addition, significantly more NF-kappaB was translocated to monocyte cell nuclei treated with voriconazole than untreated cells. The study suggests that TLR2 signaling, TNF-α, and NF-kappaB activation in the presence of voriconazole proposes an immunomodulation effect leading to a more efficient response to *A. fumigatus* (Simitsopoulou et al., 2008).

## DISCUSSION

Fungi are increasingly recognized as major pathogens in immunocompromised individuals. Risk factors for invasive fungal infections include prolonged neutropenia, hematological malignancy, transplantation (particularly bone marrow transplant), cytotoxic drugs, and steroid therapy (Enoch et al., 2006).

The most common invasive fungal infections are candidiasis, followed by aspergillosis and *cryptococcosis* (Shoham and Marr, 2012). Disseminated candidiasis is associated with a mortality in excess of 25% (Kibbler et al., 2003), and represents the fourth most common cause of nosocomial blood stream infection in United States (Wenzel and Edmond, 2001). Invasive aspergillosis is also associated with significant morbidity and mortality, lung, and heart–lung transplant recipients being at greatest risk of infection, affecting 14–18% of patients (Hagerty et al., 2003). AmB remains the most effective drug against fulminant fungal infections but its use is limited by the multitude of side effects including nephrotoxicity, infusion related toxicity, electrolyte abnormalities, and others. Echinocandins are a newer class of antifungal drugs with activity against *Candida* spp. and *Aspergillus* spp. and constitutes an alternative to AmB due to a superior toleration profile and less side effects. The azoles used for systemic fungal infections are triazoles and include fluconazole, itraconazole, voriconazole, and posaconazole. They inhibit the cytochrome P450 dependent enzyme lanosterol 14-alpha-demethylase, thus inhibiting the synthesis of ergosterol, which represents a vital component of the cellular membrane of fungi (Zonios and Bennett, 2008). As outlined in this review, AmB, echinocandins, and some of the studied azoles have long been used and their mechanisms of action against fungi are well established. However, these drugs also act on components of the innate immune system aiding in the body's natural defense against the infecting pathogens. As outlined in this review, TLRs seem to be significant components in this setting. The innate immune response has physical barriers that provide protection from the environment which include the skin and mucus membranes of the respiratory, gastrointestinal, and genito-urinary tracts. Once fungi have invaded these barriers they encounter a multitude of innate defenses that include phagocytes, natural killer cells, T cells, B cells, and endothelial cells (Blanco and Garcia, 2008). Generally the interaction between antifungal therapy and the immune system is synergistic. Chemotactic factors are produced at the site of fungal infections, leading to activation of the complement pathway. The synthesis of these chemotactic factors is stimulated by pathogen associated molecular patterns, (PAMPs) that are recognized by pattern recognition receptors (PRRs). PRRs include TLRs. PRRs and TLRs activate PAMPs which signal the synthesis and release of pro-inflammatory cytokines that activate the adaptive immunity. A fungal pathogen activates multiple PRRs, which alerts the immune system to respond with a broad range of possibilities (Janeway and Medzhitov, 2002; Roeder et al., 2004; Romani, 2004). Antimicrobial peptides are other components of the innate immune system that have an antimicrobial effect against fungi. There exact mechanism is not known but they are likely to activate and mediate the innate and adaptive immune response in infection and inflammation. They also inactivate fungi by directly affecting their membrane (Ganz, 2003; Aerts et al., 2008, Steinstraesser et al., 2008).

Polymorphisms in TLR genes have been associated with a susceptibility to fungal infections. The type of infection depends on which TLR has mutated. Recognition of *Candida* normally occurs through PRRs such as TLRs. Plantinga et al. (2012) analyzed that TLR single nucleotide polymorphisms (SNPs) [R80T, S248N, 1602S] on TLR1 were associated with candidemia in white populations. This was not present in African American populations but this was attributed to the lower power in the smaller study population. These polymorphisms also impaired cytokine release by monocytes (Plantinga et al., 2012).

Invasive aspergillosis is a particular concern in patients that have had hematopoietic-cell transplants, with its incidence rate increasing. Despite the availability of new medications (azoles and echinocandins) their outcome remains poor (Marr et al., 2002). Aspergillosis activates the immune system through TLR4.

Bochud et al. (2008) analyzed that donor TLR4 haplotypes (S3, S4) increased the risk of invasive aspergillosis among recipients of allogenic hematopoietic-cell transplants.

The ability of antifungals to simultaneously elicit an efficient immune response should be researched further for the potential development of new drugs to induce effective activation of the innate immune system via TLRs and subsequent pathways that may synergistically help clear fungal infections.

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# Biotic activity of $Ca^{2+}$-modulating non-traditional antimicrobial and -viral agents

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## INTRODUCTION

Combined serendipitous and rational drug-design and -retasking approaches continue to identify many natural and synthetic substances with multipurpose therapeutic properties (Clark, 2013a). Among these substances are $Ca^{2+}$ modulators capable of attenuating the transmission and severity of viral, bacterial, fungal, and protozoal infections (Clark and Eisenstein, 2013; Clark et al., 2013). The majority of purported $Ca^{2+}$-modulating antiinfective compounds belong to the functional drug class termed $Ca^{2+}$-channel blockers, including traditional synthetic 1,4-dihydropyridines, phenylalkylamines, and benzodiazepines long approved and marketed for various human and animal cardiovascular and neurological indications (Clark and Eisenstein, 2013; Clark et al., 2013). Additional $Ca^{2+}$-modulating (putative) antiinfective substances, such as artemisinin, caloxin, dantrolene, cyclosporin A, and FK506, can be further categorized within a broader set of natural and synthetic compounds that affect operation of $Ca^{2+}$ channels, transporters, exchangers, and/or protein sensors of both hosts and infectious agents (Clark and Eisenstein, 2013; Clark et al., 2013). Notably, depending on chemical structure, site, and mechanism of chemical action, and delivered chemical concentrations, these and other non-traditional antimicrobial and -viral compounds, many of which are expressed by pathogens themselves, may instead exert helpful trophic effects on hosts, their symbiotic microbiota, and harbored mutualistic copathogens. The reasons for such biphasic drug-response profiles partly derive from how pathogens evolved to parasitize host $Ca^{2+}$-dependent functions and resources, yielding insights into devising better antiinfective treatment regimens and new valued probiotic medicines.

## PATHOGEN USURPATION OF HOST $Ca^{2+}$ SYSTEMS

Viruses, bacteria, fungi, and protozoa evolved the strong obligate parasitic strategy of hijacking host systems to augment their comparatively primitive genomic, epigenomic, and somatic capabilities, thereby facilitating infectious disease adaptation and propagation. Though infectious agents coopt many different host systems, few are more significant than host intracellular $Ca^{2+}$ signaling pathways. Free intracellular $Ca^{2+}$ serves as an intermediate between sensory input and response output for all known cellular life. Its ubiquitous presence within cells of diverse phylogeny and function makes $Ca^{2+}$ an essential messenger for controlling host-cell stress responses, fate and death, synaptic plasticity, homeostasis, motility, bioenergetics, growth, morphogenesis, immunodefenses, protein modification and transport, cytoskeletal polymerization, endosome formation, and various other host processes (Clark and Eisenstein, 2013; Clark et al., 2013). Therefore, the ability of microbes to preferentially control host intracellular $Ca^{2+}$ pathways enables them to optimize the timing and effectiveness of infection stages against barriers to invasion, pathogenesis, proliferation, and release (Moreno and Docampo, 2003; TranVan et al., 2004; Kozubowski et al., 2009; Zhou et al., 2009; Clark and Eisenstein, 2013; Clark et al., 2013).

Pathogens, mainly via toxic proteins and lipopolysaccharides, manipulate host intracellular $Ca^{2+}$ systems by modulating (1) ligand- [e.g., N-methyl-D-aspartate receptors (NMDAr)] and voltage-gated (e.g., L-, N-, P/Q-, R-, and T-type receptors and Bsc1, Cch1, and NaChBac receptors) channels that permit $Ca^{2+}$ entry from extracellular spaces, (2) upstream first or second messengers (e.g., inositol 1,4,5-trisphosphate ($IP_3$), AMP-activated protein kinase, and mitogen-activated protein kinase pathways), (3) ion- (e.g., $Ca^{2+}/H^+$ and $Na^+/Ca^{2+}$ exchangers) and ATP-dependent (e.g., sarcoplasmic-endoplasmic-reticulum (SERCA) and plasma-membrane (PMCA) ATPases) $Ca^{2+}$ pumps that sequester or extrude free cytosolic $Ca^{2+}$, (4) ligand-gated channels (e.g., $IP_3$ and ryanodine receptors) and peptidergic porins (e.g., amoebaporins, aquaporins, and PorB) responsible for store-operated $Ca^{2+}$ mobilization and leakage, and (5) downstream host $Ca^{2+}$ binding proteins and sensors (e.g., calmodulin, calrectulin, calcineurin, calnexin, and annexin) (Clark and Eisenstein, 2013; Clark et al., 2013). The wide range of host intracellular $Ca^{2+}$ systems influenced by pathogen factors gives microbes remarkable control over the behavior and well-being of humans and animals, including, but not limited to, mental function and psychological state, voluntary and involuntary motor performance, and gastrointestinal absorption and metabolism. Yet, for microbes, the advantages of pathogen-mediated regulation of host intracellular $Ca^{2+}$ systems

extend beyond the impact on host health. In the case of viruses, increased host free cytosolic $Ca^{2+}$ levels may promote viral adsorption, structural stability, capsid uncoating, enzymatic activity, replication, assembly, transport, and fusion (cf. Zhou et al., 2009; Clark and Eisenstein, 2013). Whereas, in cases of bacteria, fungi, and protozoa, alterations of host intracellular $Ca^{2+}$ homeostasis is critical for pathogen sensory transduction, cell energetics, infection sequences, stress adaptation, gene expression, toxin biosynthesis and secretion, molecular biomimicry, conjugation and true sexual reproduction, cell motility and tropisms, growth, biofilm formation and cell aggregation, antigenic variation, and morphogenesis and lifecycle transitions (cf. Cyert, 2003; Moreno and Docampo, 2003; TranVan et al., 2004; Kozubowski et al., 2009; Clark et al., 2013).

## PATHOGEN SELECTIVE MANIPULATION OF HOST $Ca^{2+}$ SYSTEMS

To coordinate pathogen needs with operation of host cells, infectious agents must precisely change their host environment to maximize survival, proliferation, and spread with a repertoire of social-like (e.g., cell-cell communication, biofilm formation, cooperative, and competitive coinfection, etc.) and non-social (e.g., phenotypic variation, biomimicry, etc.) phenomena sometimes interpreted as pathogen intelligence (cf. Crespi, 2001; Casadesus and D'Ari, 2002; Ben-Jacob et al., 2004; Hellingwerf, 2005; Marijuán et al., 2010; Clark, 2013b). In regard to host intracellular $Ca^{2+}$ homeostasis, pathogens rely on certain toxins that may either increase or decrease intracellular $Ca^{2+}$ levels depending on stages of infection and host status. Such fine-tuned aptitude for altering host $Ca^{2+}$ systems confers both advantages and disadvantages on hosts in relation to proper cell function and fate. Although most pathogens have evolved suites of toxins to manipulate host processes, including $Ca^{2+}$-mediated ones, the selective fitness of surprisingly numerous single toxin molecules achieves multiplexed pathogen attacks on their host niche. This kind of pathogen intelligence conserves viral, bacterial, fungal, and protozoal resources for highly efficient and integrated host invasion and exploitation.

For example, overexpression of the multifunctional Hepatitus B Virus (HBV) protein HBx activates caspase-dependent cleavage of host $Ca^{2+}$ PMCA, elevating free intracellular $Ca^{2+}$ concentrations (Chami et al., 2003) as well as $IP_3$ production and mitochondrial $Ca^{2+}$ uptake during virus replication (Gearhart and Bouchard, 2010a,b; Yang and Bouchard, 2012). Unless competitively antagonized by $IP_3$-receptor-inhibitors dantrolene and FK506 or other drug types, temporary stimulation of the endoplasmic reticulum/mitochondrial interface by $IP_3$ boosts ATP synthesis and transport for energy-dependent cell processes required during early viral infection stages. However, when mitochondrial $Ca^{2+}$ uptake subsequently exceeds buffering capacity, HBx advances mitochondrial swelling and fragmentation (Chami et al., 2003), making host cells more vulnerable to free radical generation, metabolic stress, and apoptosis prior to viral release. While sequalae are treatable with non-traditional compounds, including dual-active Beta Cell Lymphoma (Bcl)-related proteins (Clark and Eisenstein, 2013), HBV obviously evolved to carefully manage host-cell operation through well-timed, titrated levels of a single toxin, with lower concentrations of HBx causing long-term/short-term positive outcomes for virus/host and higher concentrations of HBx largely causing positive/negative outcomes for virus/host. This sort of versatility for single viral toxins to exploit host $Ca^{2+}$ systems is observed for other viruses, including Human Immunodeficiency Virus type 1 (HIV-1). HIV-1, via the transcription factor Tat, for instance, potentiates $Ca^{2+}$ influx through dihydropyridine-sensitive voltage-gated L-type $Ca^{2+}$ (Lannuzel et al., 1995) and NMDAr channels (Prendergast et al., 2002; Self et al., 2004), leading to host-cell cytotoxicity. By means of the same $Ca^{2+}$ channels, Tat also evokes production of the tumor necrosis factor (TNF)-alpha cytokine, an important compound for HIV-1 replication and pathogennesis (Contreras et al., 2005). Each harmful effect on host cells may be mitigated by voltage-gated L-type $Ca^{2+}$ (e.g., nifedipine) and NMDAr channel antagonists (e.g., memantine). In contrast, Tat, similar to verapamil, inhibits cytotoxic release of serine esterases by blocking the phenylalkylamine-binding site of voltage-gated $Ca^{2+}$ channels (Zocchi et al., 1998). As with protein HBx of HBV, Tat therefore affords HIV-1 with the ability to either facilitate or guard against host-cell death depending on infection stage and location (e.g., molecule-binding site, cell type, and organ). Moreover, besides direct influence over host condition, both HBx and Tat may act synergistically on HBV and HIV-1 infections (Li et al., 2012) as well as provide opportunistic copathogens, such as mycobacteria (Pathak et al., 2010; Toossi et al., 2012), herpesviruses (Huang et al., 2001; Guo et al., 2004; Caselli et al., 2005), and commensal host fungi (Cassone and Cauda, 2012) and coliform bacteria (cf. Diniello et al., 1998; Mani et al., 2007), an (probiotic) enriched or (anti-infective) hostile host habitat affecting communicable disease progression.

Only two among many instances of viral proteins were discussed above to illustrate the powerful biphasic regulation of pathogen toxins in modifying host and infectious agent physiology (cf. Clark and Eisenstein, 2013). A large number of pathogen-associated $Ca^{2+}$-modulating factors exist for bacteria, fungi, and protozoa as well (cf. Clark et al., 2013). These endo- and exotoxins, of which just a few exemplars will be described here for protists, often allow microbes to evade host defenses by usurping membrane repair systems, down-regulating redox immunological responses, mimicking proinflammatory chemokine and cytokine mobilization, and initiating irreversible host programmed cell death. In addition to purely selfish pathogen infective, survival, and reproductive strategies, such compounds may render trophic support and protective immunity for hosts and their microbiota. Prime examples, similar to those also reported for obligate parasitic *Chlamydia*, *Rickettsia*, and *Toxoplasma* species (cf. Romano et al., 2013), come from intracellular protozoan trypanosomes, etiogenic agents of Chagas' disease, sleeping sickness, and other human and animal illnesses. Several substances, a serine endopeptidase, also called a proteolytically generated trypomastigote factor, Tc-Tox, an acidic pore-forming protein, and acidic sphingomyelinase, synthesized and secreted by *Trypanosoma cruzi* induce host plasma-membrane

damage, extracellular $Ca^{2+}$ entry, $IP_3$ formation, transient store-operated cytosolic $Ca^{2+}$ liberation, and/or cytoskeletal reorganization to assist in parasite internalization and trafficking (Tardieux et al., 1994; Burleigh and Andrews, 1995; Rodríguez et al., 1995; Burleigh et al., 1997; Fernandes et al., 2011). These compounds are only produced during the infective stage of trypanosome lifecycles, when $Ca^{2+}$-dependent, energy-expensive lysosome and endosome recruitment works to restore integrity of pathogen-injured host plasma membranes. To a limited extent, toxin activation of store-operated $Ca^{2+}$ release can be decreased by $IP_3$-receptor blockers. But by directly commandeering host membrane-repair systems and subverting intracellular innate immune-surveillance and potent inflammatory signaling pathways, trypomastigotes ensure successful host invasion and maintenance of host structural and biotic reliability for persistent cryptic and latent trypanosome and copathogen disease states, such as those involving multiple trypanosome strains, symbiotic enterobacteria and other Gram-negative bacteria, and entomopathic double-stranded DNA viruses (Peacock et al., 2007; Alam et al., 2012; Lowry et al., 2013). In turn, these processes, directed by identical toxin concentrations used for trypanosome benefit, can present formidable obstacles to other infectious agents, including convergent trypanosome strains (Ulrich and Schmid-Hempel, 2012) and possible *Encephalitozoon* (cf. Leitch et al., 2001) and *Toxoplasma* parasites (cf. Meirelles and De Souza, 1983), which compete for limited shared host resources and/or must overcome toxin-modified host immunoresponses.

## PROSPECTIVE $Ca^{2+}$-MODULATING PROBIOTIC AND OTHER TREATMENT STRATEGIES

Repurposed medications which target pathogen capacities to alter host $Ca^{2+}$ homeostasis and vital cell functions, such as traditional $Ca^{2+}$-channel blockers, SERCA-inhibitor artemisinins, PMCA-inhibitor caloxins, and the $IP_3$-receptor-inhibitors dantrolene, FK506, and Bcl antiapoptotic compounds (Clark and Eisenstein, 2013; Clark et al., 2013), show efficacious antiinfective effects against both treatable and previous drug-resistant pathogens. Given examples of HBV, HIV-1, and trypanosome infections readily demonstrate how these drugs exert their chemotherapeutic properties through disruption of pathogen attack, reinforcement of compromised host immunity, and trophic support for host operation. Perhaps more significantly, toxins encoded by pathogens also show non-traditional antiinfective and probiotic traits, oftentimes in a concentration-dependent manner. Such highly adaptive cooperative and competitive traits evolved so pathogens can invade, inhabit, and abandon host niches. Many of these multipurpose pathogen toxins modulate $Ca^{2+}$ systems of host cells and host microbiota, including aforementioned viral and protozoan toxins, HBx, Tat, and Tc-Tox, and different pathogen virulence factors, such as mycobacterial (macolide) mycolactone and lipoarabinomannan (Rojas et al., 2000; Snyder and Small, 2003; Vergne et al., 2003; Boulkroun et al., 2010), staphylococcal leukotoxins (Jover et al., 2013), coliform heat-stable enterotoxin B (Dreyfus et al., 1993), and saccharomycete and ascomycete gliotoxins (Niide et al., 2006), to name a few. In some cases, predictable antiinfective properties of pathogen toxins result from mechanisms known for antibiotic drugs, including the streptomycin-analogous (Diniello et al., 1998) polyamine-starving characteristics of Tat (Mani et al., 2007), or from entirely novel mechanisms. Regardless, pathogen toxins with combined antiinfective and biotic qualities provide exciting substrate to begin developing new medicines of broad therapeutic potential and lifespan.

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# Interplay between policy and science regarding low-dose antimicrobial use in livestock

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Since the introduction of penicillin in the 1940s, antimicrobial resistance (AMR) has become an ever-increasing threat to human and animal health (Carlet et al., 2012; ITFAR, 2012). The low-dose antimicrobial use in food-producing animals for production purposes, i.e., disease prevention and growth promotion, has been documented to drive AMR, though the risk to human health from such uses has been a source of a heated debate among stakeholders including industry members, citizen advocacy groups, academics, and public health workers (Davis and Rutkow, 2012). In the United States (US), it remains common practice to use medically-important antibiotics[1] for production purposes. Since the 2000s, the European Union (EU) has banned many low-dose antimicrobial uses. The crucial element to the debate has been whether scientific evidence supports similar action in the US. Industrial food animal production advocates, henceforth "industry," claims that scientific evidence is currently lacking, while other stakeholders argue that a convincing and substantial body of evidence exists to support policy change.

The US and the EU have taken vastly different approaches to AMR surveillance and research, and these choices have led to a wide variance in the current policy climate regarding antimicrobial use and AMR. We argue that the EU has prioritized scientific research and surveillance efforts that target uses of antimicrobials in animals. The US has not invested deeply in either research funding or surveillance programs that include such uses. If policy shifts will occur in the US, changes in AMR surveillance and allocation of scientific research funding are required.

## CURRENT US PRACTICES

The US National Antimicrobial Resistance Monitoring System (NARMS) is a joint effort of the US Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Centers for Disease Control and Prevention (CDC) for AMR surveillance. The three arms of NARMS—VetNet (USDA), PulseNet (CDC), and retail (FDA)—collect samples from animal carcasses at the time of slaughter, human food-borne infections, and retail meat samples, respectively. Techniques in each arm vary in regards to both isolates collected and laboratory definitions for resistance, making data comparison across branches challenging (US GAO, 2011). Collection of data on antimicrobial use and sales in food animals in the US is limited, though the FDA has made recent efforts to obtain more data. The Government Accountability Office, in a report published in 2011, commented on the limited scope of sampling techniques in the US:

> This non-random sampling method means the NARMS data obtained … are not representative of food animals across the country and cannot be used for trend analysis because bacteria tested by NARMS are now collected at greater rates from slaughter plants that are not in compliance with food safety standards. According to FDA officials, due to this sampling method, the resulting data are skewed for NARMS purposes (US GAO, 2011).

The lack of harmonization within NARMS limits the interpretation of these data, with resulting impacts on evidence available to support science-driven policy.

Current NARMS funding is approximately $7.8 million, bringing the per-capita investment in this surveillance tool to $0.025 (US GAO, 2011). Lack of funding is routinely cited as the primary cause of limited government-led research and lack of advancement in surveillance techniques, though the lack of coordination within the current approach suggests that changes could be made to use existing budget funds more effectively (Pew Trust, 2008; US GAO, 2011; IDSA, 2012).

## CURRENT EUROPEAN PRACTICES

The European approach to AMR surveillance, research, and policy development began with individual country efforts, with Denmark and Sweden as leaders. In recent years, the EU has begun to compile data from numerous countries through the functions of the European Centers for Disease Control, European Antimicrobial Susceptibility Surveillance in Animals Center, and the European Animal Health Study. In general, European efforts have been more systematic than US efforts, including sampling of healthy and diseased animals and people, monitoring antimicrobial usage patterns through novel techniques such as geomapping, and using veterinarians to obtain samples from a wide variety of regions and animals,

[1]Based on definitions for critically-important antimicrobials used by the Food and Agriculture Organization of the United Nations, World Health Organization, and World Organization for Animal Health (OIE), and definitions of medically-important antibiotics in the Preservation of Antibiotics for Medical Treatment Act, first introduced to the U.S. Congress in 1999.

including pets (DANMAP, 2011; Davis and Rutkow, 2012; IDSA, 2012; ITFAR, 2012; de Jong et al., 2013).

Selective bans on antimicrobials for growth promotion were instituted in the 1990s, and a complete ban of drugs for non-therapeutic uses was established in Denmark and the EU in 2000 and 2006, respectively (DANMAP, 2011; US GAO, 2011). Research is prioritized and is typically led by industry rather than government; this approach may reflect differences in cultural or social norms between the EU and US. Indeed, industry participation in such programs has been largely voluntary, and the additional cost is thus often internalized to industry rather than externalized to the public through government expenditures.

## COMPARISON OF APPROACHES

According to the 2011 GAO audit of NARMS, policy in the EU, "… has been built around the precautionary principle, which states that where there are threats of serious or irreversible damage, lack of scientific certainty should not postpone cost-effective measures to reduce risks to humans" (US GAO, 2011). In contrast to the US approach of risk assessment, the EU method is buoyed by consumer concern and voluntary industry measures. At a recent meeting, the Infectious Disease Society of America (IDSA) released a statement comparing current US and EU efforts:

> Even given the value [National Health Safety Network], [Emerging Infections Program], Prevention Epi-centers, NARMS, and [Multidrug Resistant Repository and Surveillance Network] provide, IDSA remains deeply concerned about the lack of detailed, publicly available data on both resistance trends and human antimicrobial use in humans, food animals and other areas of agriculture and food production in the United States. The U.S. is far behind other countries in collecting and benefiting from data on antibiotic consumption and resistance (IDSA, 2012).

In the same vein, the 2011 GAO report noted that CDC did not routinely publish data on AMR patterns in pathogens associated with outbreaks of foodborne illness (US GAO, 2011). Such statements indicate the low political priority assigned to AMR surveillance and program evaluation in the US. Evaluation is a vital part of any system, and as the 2011 GAO report indicated, NARMS, FDA, USDA, and CDC have conducted few evaluations. Without regular evaluation, programs and policies that are neither cost-effective nor impactful can waste money and prevent new and more efficacious systems from being put in place.

DANMAP was established in 1995 with 2.4 million kroner ($0.3 million) and in 2000 further supported with 2.6 million kroner resulting in a total of 5 million kroner ($0.7 million) of initial investment across that time period[2]. This program was one of the pioneers in European surveillance efforts, predating EU-wide efforts (Davis and Rutkow, 2012). Currently, DANMAP is not funded by earmarked money but is a part of the general tasks of Statens Serum Institut and Danish Technological University (DTU-Food) financed by Danish Ministry of Health and the Danish Ministry of Food, Agriculture, and Fisheries. Although the budget for DANMAP changes relative to general fund availability and is subject to annual decreases, the budget from the Ministry of Food, Agriculture and Fisheries alone for all food research (not just application to AMR) was 246 million kroner ($44.8 million), or a per-capita investment of $8 for 2014[3]. Funding across multiple federal agencies places US food research investment at an estimated $1863 million, or a per-capita investment of less than $6 for 2013[4]. Given similar per-capita budgets, the food safety and DANMAP programs have more effectively influenced AMR policy in Denmark than parallel programs have in the US. This example suggests that the choice of where and how to apply funding may be more important than total expenditure in the generation of scientific evidence to support policy decisions.

The role of industry in the US is complex, and the 2008 report of the Pew Commission on Industrial Farm Animal Production noted, "We found significant influence by the industry at every turn: in academic research, agriculture policy development, government regulation, and enforcement" (Pew Trust, 2008). Industry itself is not held accountable for ensuring public health, nor is it charged with contributing peer-reviewed research to support or refute its current or preferred practices, yet it remains able to exercise tremendous influence in government and academia, especially among land grant universities where industry funding has largely replaced public research funding (Pew Trust, 2008).

Hence, policy success, defined as both action by agencies and reductions in AMR rates, in the EU has been driven in part by strategic allocation of funding support, adherence to the precautionary principle, involvement of industry, and periodic mandatory participation of EU members in region-wide data collection efforts (DANMAP, 2011; IDSA, 2012; Silley et al., 2012). In contrast the US has not seen the same returns relative to funding efforts, has not applied the precautionary principle in formulating policy, has not required participation of producers or benefited from voluntary support by industry, and has conducted limited and flawed surveillance for antimicrobial use in livestock and AMR.

## RECOMMENDATIONS

In an era of resource constraints for government agencies, we do not expect AMR surveillance efforts in the US to experience significant budget increases. The EU approach, including the specific example of Denmark, offers lessons for change in the US. We propose that efforts be made to provide minimal increases in funding to obtain more representative and comparable data, and to analyze data currently being collected in a more effective way. For example, NARMS data in the US are available online, but manipulation of the data

[2]Based on a year 2000 exchange rate of 7.3855kr/US dollar. With a year 2000 Danish population of 4.8 million, the per-capita investment was roughly $0.14. We thank Robert Skov for his assistance in obtaining this DANMAP funding information.

[3]Based on a December 1, 2013 exchange rate of 5.489810kr/US dollar. With a year 2013 Danish population of 5.6 million, the per-capita investment was $8.

[4]Based on the sum of food safety budget estimates of $42 million (NIFA), $108 million (ARS), $1 million (ERS), $1425 (FDA), and $287 (NIH), for a total of $1863 million (Ohlhorst et al., 2013). With a year 2013 US population of 320.6 million, the per-capita investment was $5.8.

is not feasible (Silley et al., 2012). Allowing independent researchers and their students to use the NARMS data for analysis may lead to more rapid reports that can be used for comparison with data from other countries, while supplying a no-cost source of analysis and enhancing educational opportunities. In particular, provision of data at the sample level, allowing for analysis of cross-resistance patterns, would inform both scientific and policy efforts to understand and combat the rise of AMR pathogens.

Additionally, a shift in industry's role toward a more harmonized partnership with government and other stakeholders would assist national efforts to address the problem of AMR. Voluntary FDA guidance to phase-in veterinary oversight and phase-out growth promotion uses of antimicrobials in livestock is a first step toward this goal (FDA, 2013). Following the EU example for industry involvement in research efforts would ultimately offer a solution to meet the needs for representative sampling within a country as large and diverse as the US. While industry may be faced with higher costs, all stakeholders would benefit from improvements in data collection—and hence data interpretation—in driving evidence-based policy change.

Ultimately, changes need to occur within the US to encourage industry accountability, research efforts, and government investment in adequate surveillance systems. Some improvements within the US surveillance system, such as reporting of data already collected, would require minimal funding adjustments. AMR is a pressing problem that threatens the health and well-being of humans and animals, with impacts through the food system. Fundamental shifts in the governmental and industry approaches, including structural changes to enhance data collection and dissemination, are urgently needed to generate science-based policies and then understand the impact of the policies on AMR.

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# Obesity in the United States – dysbiosis from exposure to low-dose antibiotics?

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The rapid increase in obesity prevalence in the United States in the last 20 years is unprecedented and not well explained. Here, we explore a hypothesis that the obesity epidemic may be driven by population-wide chronic exposures to low-residue antibiotics that have increasingly entered the American food chain over the same time period. We propose this hypothesis based on two recent bodies of published reports – (1) those that provide evidence for the spread of antibiotics into the American food chain, and (2) those that examine the relationship between the gut microbiota and body physiology. The livestock use of antimicrobial agents has sharply increased in the US over the same 20-year period of the obesity epidemic, especially with the expansion of intensified livestock production, such as the concentrated animal feeding operations. Observational and experimental studies support the idea that changes in the intestinal microbiota exert a profound effect on body physiology. We propose that chronic exposures to low-residue antimicrobial drugs in food could disrupt the equilibrium state of intestinal microbiota and cause dysbiosis that can contribute to changes in body physiology. The obesity epidemic in the United States may be partly driven by the mass exposure of Americans to food containing low-residue antimicrobial agents. While this hypothesis cannot discount the impact of diet and other factors associated with obesity, we believe studies are warranted to consider this possible driver of the epidemic.

**Keywords: obesity, antibiotic residues, intestinal microbiota, food chain, CAFOs, animal husbandry, polysaccharide diet**

## INTRODUCTION

Obesity in non-elderly adults is defined as a body mass index (BMI = weight in kilograms divided by height in meters squared) of 30 or higher (1). Obesity has emerged as an epidemic, especially in the United States, accompanied by a variety of chronic medical problems, including diabetes, hypertension, and dyslipidemia. In the United States, the increase in overweight and obesity prevalence has particularly accelerated in the last 20 years. The overweight prevalence doubled from a mean of 15.1% in 1976–80 to 31.2% in 2001–2004 (2). In 2000, none of the states had an obesity prevalence exceeding 30%; by 2010, 12 states reported an obesity prevalence of >30% (3). Although the prevalence appears to be leveling off, it had increased to 35.7% among adults by 2009–10 (4). A Centers for Disease Control and Prevention (CDC) survey of 3141 counties in the US in 2007 revealed distinct geographic clustering of obesity prevalence, with the highest prevalence (>30.9%) occurring in the Southeastern states and in the Appalachian counties of Tennessee and Kentucky (5).

Many putative causes of obesity and its epidemic in the US have been suggested (6). Most reports attribute the obesity epidemic to factors such as excess food energy intake, changes in diet and eating behavior, and increasing sedentary life style. Undoubtedly, these factors contribute, but can they all account for the rapid increase in this problem that occurred over the last two decades? Recently, a series of experimental and observational studies have described the role and impact of intestinal microbial population structure (microbiota) on body metabolism and energy balance, and how its disruption (dysbiosis) could adversely affect body physiology and health (7–9). If such a mechanism – disruption of the intestinal microbiota – occurred at the population level, it could potentially explain the obesity epidemic.

As with infectious disease epidemics, the obesity epidemic affecting large segments of a population within a short time frame suggests common population-wide exposures. What such common exposures could alter the gut microbiota at the population level? Here, we review a body of literature to support a hypothesis that the American human intestinal microbiota may have been disrupted by chronic, widespread exposures to antimicrobial residues that have increasingly entered our food chain and the environment over the last 20 years. These exposures may be contributing to the obesity epidemic.

Our hypothesis is based on review of relevant literature, performed as follows. We first searched PubMed by cross-referencing the word "obesity" with the following terms: epidemic, prevalence, diet, calorie intake, nutrients, physical activity, lifestyle, host factors, genetics, antibiotics/antimicrobial agents, antibiotic residues in food, antibiotics in plants, antibiotic growth promoters, environmental release of antibiotics, animal husbandry, animal feed, gut microbiome/microbiota, and metagenome. We then found additional references by reviewing the cited references from the primary articles. We excluded abstract reports or conference proceedings. Articles not available electronically were sought at the

University of California library collections. The search was limited to publications in English up to August 2013.

## ANTIBIOTICS IN THE ENVIRONMENT AND FOOD

What is the evidence that American food contains antimicrobial agents or that food can become contaminated with them? One major potential source of antibiotics that enter the food chain in the US is the food animal reservoir. The intensification in livestock production and animal feeding operations (AFOs) greatly expanded in the 1990s, which also greatly expanded the therapeutic and prophylactic use of antibiotics (10). In the US, at least 17 classes of antimicrobial agents are approved for use in animal husbandry (11). The estimates of antibiotic use in food animals prior to 2008 are not readily obtainable, but various sources report the use in the US to range from 20.5 million pounds for all purposes in 1999 (12) to 24.6 million pounds a year for non-therapeutic purposes alone (13). An Institute of Medicine report in 1989 estimated that more than 50% of all antimicrobial agents produced in the US are used for livestock (14). The Food and Drug Administration (FDA) reported that in 2011, the sales and distribution of antimicrobial agents approved for use in food-producing animals were 13,542,030 kg (15), which is about four times the total sold and distributed for use to treat human infections (15). Chlortetracycline alone had the highest annual estimated use at 533,973 kg just for swine production in the US (16).

About 75% of antibiotics given to feedlot animals are not absorbed by the body and hence excreted in waste (manure and urine) (17, 18). In 2002, 185 million swine sold in the US generated about 280 million tons of fresh manure; in 2006, chicken produced even more (460 million tons), while, in 2007, beef cattle produced 3.6 million tons of manure (19–21). Environmental pollution from animal waste results from direct discharges, open feedlots, pastures, storage lagoons, stockpiles, and land application fields. Manure is converted into fertilizers and spread over crops. From these sources, contamination can occur on surface water, groundwater, and soil. Based on the amount of antibiotics estimated to be used for growth promotion in animal husbandry, 7.5–18 million pounds of these antimicrobial drugs could be released into the environment annually (12, 13).

In the last 20 years, the above practices as well as the shift from integrated farming operations to concentrated animal feeding operations (CAFOs) have led waste-related pollution to be concentrated in certain geographic regions of the US (18). The largest increase in broiler chicken CAFOs occurred in the Southeastern states in the last 20 years (22) – practically overlapping with the counties with the highest obesity prevalence in the US (5).

Antibiotics released into the environment have varied biodegradability, depending on their chemical property, soil composition, climactic conditions, and other environmental factors (10). Some antibiotics released into soil by manure may be detected up to 5 months (23). Yang and Carlson analyzed tetracyclines and sulfonamides along the Cache la Poudre River in Colorado, and found that while no drugs were found in the pristine, mountain stretch of the river, sulfonamides were found along the remaining stretch and tetracycline concentrations progressively increased downstream from urban areas (24). The tetracycline concentrations correlated with agricultural activity (24). In another study of 139 streams sampled in the US between 1999 and 2000, trimethoprim, erythromycin, lincomycin, and sulfamethoxazole were detected in more than 15% of the samples (25).

Antibiotics can also accumulate in plant tissue. Dolliver et al. showed experimentally that sulfamethazine in manure-amended soil accumulated in corn, lettuce, and potato at concentrations of 0.1–1.2 mg/kg dry weight (26). Others have shown accumulation of low concentrations of antibiotics in carrot roots, green onion, and cabbage (23, 27).

In addition to livestock sources, antimicrobial agents are released into the environment from aquaculture (e.g., shrimp and fish farms) (28–30), spraying fruit orchards and vegetables (31, 32), and from discarded expired drugs, hospital effluents, and other human activities (33). Thus, there is ample evidence that antibiotics can enter our food chain from a variety of sources, and that humans are chronically exposed to these drugs (**Table 1**). These exposures have greatly increased in the US in the last 20 years, overlapping closely both in time and place with the increasing prevalence of obesity.

Of course, we acknowledge that exposure does not necessarily indicate cause and effect, and the evidence to date showing the association is largely ecologic. Furthermore, the impact of changes in dietary intake that also took place in the US in the last 20 years cannot be completely discounted. The average per capita per day energy levels available in the US food supply was 3,400 kcal in 1909–1919, 3,600 kcal in 1990–1999, and 3,900 kcal in 2004 (59). National Center for Health Statistics through series of surveys (National Health Examination Surveys from 1959 to 1970 and the National Health and Nutrition Examination Surveys from 1971 to 2004) shows that the prevalence of overweight and obese Americans increased slowly through the middle of 1970s and then sharply began to rise thereafter (2). Thus, the period of accelerated increase in weight gain overlaps with both increased dietary caloric intake and antibiotic exposures in food. Here, we argue that the effect of diet and exposure to low-residue antibiotics in food on weight gain may be related and that they cannot be easily disassociated. We provide below the biological plausibility evidence for how antibiotics may influence body physiology and how diet contributes to this effect in ways that had not been previously considered.

## ANTIBIOTICS AS GROWTH PROMOTERS

In 1946, Moore et al. reported that the administration of low-dose streptomycin and sulfasuxidine in chicken feed caused increased weight gain in chicks (60). Stokstad et al. reported in 1949 that chlortetracycline-containing mash had a growth-promoting effect on poultry (61). In 1950, Luecke et al. reported that streptomycin in combination with vitamin B12 included in a basal diet caused 40% weight gain in pigs (62). These and other similar observations led the food animal industry to gradually adopt the practice of administering subtherapeutic doses of antibiotics as growth promoters and to enhance feed efficiency (63, 64). Debate concerning the economic benefits versus health hazards of this practice began in the 1960s and continues to this day (64–72). Much of the debate has focused on whether or not this practice contributes significantly to the selection of pathogens that cause human drug-resistant infectious diseases. Here, we propose a new

**Table 1 | Food, water, and environmental sources found to contain residues of antimicrobial agents.**

| Source | Antimicrobial agents found | Concentrations | Country | Reference |
|---|---|---|---|---|
| **FOOD** | | | | |
| Shrimp | Fluoroquinolones | 0.1–1 ng/g | USA | (28) |
| Salmon, trout, shrimp tissues | Fluoroquinolones | 0.28–16 ng/g | Canada | (29) |
| Swine, chicken, shrimp tissues | Fluoroquinolones | 1–100 ng/g | China | (34) |
| Bob veal, heavy calves, heifers, market hogs, non-formula-fed veal, roaster pig, sows | Sulfonamides | 0.1–1 ppm | USA | (35) |
| Bull meat | Moxidectin (milbemycin) | 89.13 ppb | USA | (35) |
| Goat meat | Oxytetracycline | 4.66 ppm | USA | (35) |
| Market hog, roaster pig meat | Carbadox | 47–110 ppb | USA | (35) |
| Catfish, basa | Fluoroquinolones | 1.9–6.5 ppb | China | (36) |
| Honey | Erythromycin | 50–1776 ng/g | Turkey | (37) |
| Corn, green onion, cabbage | Chlortetracycline | 2–17 ng/g | USA | (27) |
| Pig farm waste water | Sulfonamides | 20 μg/mL | Vietnam | (38) |
| Sewage samples | Cefalexin, cefotaxime | >1 μg/mL | Hong Kong, Shenzhen | (39) |
| Swine farm lagoon | Chlortetracycline | 68–1000 μg/L | USA | (40) |
| Wastewater treatment plant effluent | Minocycline, epitetracycline, tetracycline, doxycycline | 95.8–915.3 μg/L | Portugal | (41) |
| Wastewater treatment plant final effluents | Erythromycin, ciprofloxacin, sulfamethoxazole, tetracycline | 0.08, 0.118, 0.243, 0.151 μg/L | Canada | (42) |
| Wastewater | Chlortetracycline, ciprofloxacin, erythromycin, sulfamethoxazole, tetracycline, trimethoprim | 0.69, 0.03–0.14, 0.9–1.7, 0.05–1.9, 0.05–0.85, 0.05–0.71 μg/L | USA | (25, 43) |
| Cache la Poudre River | Macrolides | 0.06–0.17 μg/L | USA | (44) |
| Wastewater | Sulfamethoxazole | 232–9000 ng/L | Austria, Switzerland, USA, Spain, Germany | (45) |
| Elbe and Saal rivers | Erythromycin, sulfamethoxazole, trimethoprim | 30–70, 30–70, <30–40 ng/L | Germany | (46) |
| Po river | Macrolides | 0.7–68.3 ng/L | Italy | (47) |
| Wastewater treatment plant effluents | Quinolones | 40–580 ng/L | France, Italy, Sweden, Greece, Switzerland | (48, 49) |
| Wastewater treatment plant effluent | Sulfamethoxazole, trimethoprim, ofloxacin, erythromycin | 310–400, 180–320, 110 ng/L, 2.5 μg/L | USA, Germany | (50, 51) |
| Rio Grande river | Sulfamethoxazole | 300 ng/L | USA | (51) |
| Surface water | Erythromycin, sulfamethoxazole | 150, 30 ng/L | Germany | (50) |
| Cattle manure | Chlortetracycline | 7.73 mg/L | USA | (52) |
| Cattle, turkey manure | Monensin | 1–4.4, 1.2–1.5 mg/L | USA | (53) |
| Swine manure | Chlortetracycline | 27 mg/L | USA | (54) |
| Swine slurry | Tetracycline | 5–24 mg/L | Germany | (55) |

*(Continued)*

**Table 1 | Continued**

| Source | Antimicrobial agents found | Concentrations | Country | Reference |
|---|---|---|---|---|
| **OTHERS** | | | | |
| Hospital effluent sludge | Oxofloxacin, ciprofloxacin | 0.7–2.0 mg/kg | Sweden | (56) |
| Hospital effluent | Ciprofloxacin, ampicillin | 0.7–124.5, 20–80 μg/L | Germany | (57) |
| Hospital effluent | Minocycline, epitetracycline, tetracycline, doxycycline | 8.1–531.7 μg/L | Portugal | (41) |
| Hospital effluent | Ciprofloxacin, metronidazole, sulfamethoxazole, trimethoprim, doxycycline | 3.6–101, 0.1–90.2, 0.4–12.8, 0.6–7.6, 0.6–7.6 μg/L | Sweden | (58) |
| Hospital effluent | Sulfamethoxazole, trimethoprim, ofloxacin, ciprofloxacin, lincomycin, penicillin G | 400–2100, 2900–5000 ng/L, 25.5–35.5 μg/L, 850–2000, 300–2000, 850–5200 ng/L | USA | (51) |
| Dairy plant effluent | Lincomycin | 700–6600 ng/L | USA | (51) |

hypothesis that this practice may also contribute to the human obesity epidemic.

Several hypotheses to explain the mechanism of weight gain in antibiotic-fed animals have been proposed: (1) suppression of subclinical infections and overt diseases which promotes general health of the animal and hence better nutrition, (2) stimulation of growth of bacteria in the gut that synthesize essential nutrients, (3) suppression of microbes that compete with the host for nutrients, and (4) improvement in intestinal absorption of nutrients (73, 74).

The possibility that humans are exposed to subtherapeutic doses of antibiotics led Ternak to first propose in 2004 that human exposure to low-dose antibiotics may be contributing to weight gain in humans (75). Raoult in 2008 proposed that the gut microbiota modifiers, such as antibiotics and probiotics used in animal husbandry as growth promoters, may contribute to human body weight gain (76). Thus, if humans are indeed chronically exposed to low-dose antibiotics from the environment, there is a good reason to believe that they too can gain weight.

## CHANGE IN INTESTINAL MICROBIAL POPULATION STRUCTURE AND ITS EFFECT ON BODY PHYSIOLOGY

A recent series of reports has shown that the structure of the total human intestinal microbial population (microbiota) has a profound influence on body physiology (8, 77–89). The total number of bacterial cells in the human intestine (~100 trillion) is estimated to exceed the total number of somatic and germ cells of the human body by 10-fold (90). Thus, the gut microbiota requires an external supply of energy and nutrients for its own long-term residence in the intestine. It also has to share these nutrients with the human body. In fact, this homeostatic competition, if not disrupted, benefits the host in many different ways. The gut microbiota may even be considered as another vital human organ.

Anaerobic bacterial species that ferment dietary polysaccharides produce short-chain fatty acids (SCFAs) that are taken up by the host as an energy source (91). It is estimated that ~4–10% of the food energy intake of the human body, or 80–200 kcal/day, is derived from these SCFA produced by colonic bacterial fermentation (92, 93). Other bacterial populations produce vitamins (e.g., B and K) and amino acids essential for the host (94). The microbiota also protects the host against pathogens by competitive exclusion (95). Finally, it is needed for the proper development of the intestinal immune system (96, 97). Over a course of tens of thousands of years, the intestinal bacterial population evolved with the *Homo sapiens* body to share and compete for energy and nutrient supply and, in the process, has come to establish a stable equilibrium state. This intestinal microbial equilibrium state contributes to homeostatic maintenance of body weight. A disruption of this steady state (dysbiosis) could affect nutrient and energy utilization by the human body and therefore its physiology.

The human gut microbiota is predominantly composed of four bacterial phyla – Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, with the first two accounting for about 90% of the gut phylotypes (98). Cultivation-independent methods (e.g., 16S rDNA sequence analysis) have revealed that the total number of bacterial species in the gut could be in the range 15,000–36,000 (99). The largest number of bacteria is found in the colon with up to $10^{11}$ microorganisms/gram of feces (100). A study of 22 samples of intestinal microbiomes from four countries identified three predominant clusters (enterotypes) that differ in species and functional composition, independent of nation or continent (101).

Studies of mammalian host gut microbiota have shown that its alteration can affect body weight (7, 77–79, 81–83, 85). The impact of dysbiosis on obesity and metabolic disorders has been recently reviewed in detail by Harris et al. (9). Backhed et al. first reported that conventionally reared (CONV-R) mice fed polysaccharide-rich diet weighed about 40% more than its germ-free (GF) counterpart fed the same diet (77). When the gut microflora from the CONV-R mice was transferred to the GF mice, the latter underwent a 60% weight gain in 2 weeks (77). The increase in weight of the lean mice was attributed to the gut microflora that allowed energy to be salvaged from otherwise indigestible polysaccharide diet. Ley et al. have shown that obese mice, due to a homozygous

mutation in the leptin gene (*ob/ob*), have a 50% reduction in the Bacteroidetes population with a proportional increase in Firmicutes (102). Turnbaugh et al. reported that a transfer of the microbiota from obese mice to GF mice resulted in greater increase in total body fat in the latter (7).

Differences in gut microbiota have also been documented in obese versus lean human subjects (8, 80, 86, 87). One study characterized the fecal microbial communities of obese and lean pairs of adult female monozygotic and dizygotic twins and their mothers and found that obesity was associated with phylum-level changes in the microbiota and reduced bacterial diversity (8). A study by Schwiertz et al. showed that rather than Firmicutes, increased Bacteroidetes to Firmicutes population ratio was associated with obesity (88). Ley et al. followed obese subjects on weight-reducing diet for 1 year and found Bacteroidetes proportion to increase relative to Firmicutes (80). Other authors have observed that weight-reducing diets in obese subjects can alter the gut microbiota species composition, but no major changes in the ratio of Bacteroidetes to Firmicutes between lean and obese subjects were found (84). The study that examined the three enterotypes of human gut microbiota did not find any correlation between Bacteroidetes/Firmicute ratio and BMI (101). Jumpertz et al. prospectively measured ingested and stool calories of lean volunteers, and found that a 20% increase in Firmicutes with a corresponding decrease in Bacteroidetes correlated with an increased energy harvest of ~150 kcal (103). The above studies that compared intestinal microbiomes at the phylum-level show mixed and conflicting results. However, when the intestinal microbiota is examined at the functional group level, correlations with host characteristics begin to be revealed. Several studies have found that obese mice and human subjects have a larger amount of SCFA in their stool than do lean individuals (7, 82, 88). Arumugam et al. reported that while the three human gut enterotypes did not show any correlation with BMI, there was strong correlation between F-type ATPase abundance and increasing BMI (101). Cani et al. showed that lipopolysaccharide purified from *E. coli* injected subcutaneously into mice induced obesity as well as insulin resistance (82).

Another report showed that endotoxin-producing Enterobacter given to GF mice on high-fat diet became obese, while control GF mice fed the same diet did not gain weight (104). Mice given Enterobacter and normal mouse chow did not gain weight. The Enterobacter was obtained from a morbidly obese volunteer subject, whose intestinal microbiota was largely (35%) comprised of this bacterial genus (104). With shift to a diet comprised of whole grain and traditional Chinese medicinal food, the volunteer's weight decreased >57 kg after 23 weeks, and the Enterobacter population decreased to 1.8% of the intestinal microbiota (104). Indeed, Zhang et al. recently reported that in mice, diet changes explained 57% of the population variation in the intestinal microbiota, whereas genetic mutation accounted for no more than 12% (105).

More recently, Ridaura et al. transplanted fecal microbiota from human monozygotic twins discordant for obesity into GF mice and showed that mice given the microbiota from the obese member of the twin increased in adiposity (89). Then, mice transplanted with fecal microbiota from the co-twins were cohoused. Mice given obese co-twins' microbiota were prevented from gaining adiposity when cohoused with mice given lean co-twins' microbiota (89). This transformation was associated with invasion of Bacteroidales from lean into obese microbiota. Mass spectrometry analysis of sera revealed significant increases in branched chain amino acids in mice that received fecal microbiota from obese co-twins, while the microbiota transplanted from lean co-twins exhibited a significantly higher expression of genes involved in plant-derived polysaccharides and a higher concentration of SCFAs (butyrate and propionate)(89). These observations demonstrate that the interactions between diet and gut microbiota can influence body physiology. Such interactions can certainly be profoundly affected by antibiotics.

The next relevant question would be, how does exposure to antibiotics contribute to alteration in intestinal microbial population?

## EVIDENCE OF ANTIBIOTIC-INDUCED CHANGES IN THE HUMAN GUT MICROBIOTA AND BODY PHYSIOLOGY

The intestinal microbiota serves as an interface between the human body and its environment. Its perturbation is bound to affect body physiology. We argue that low-dose antibiotics in food are a major contributor to this perturbation. The analysis of the intestinal microbiota or body physiology in humans exposed to antibiotics has been largely confined to those studies of subjects receiving therapeutic drug doses. Blaser and Falkow have suggested that the accelerated disappearance of the normal human microbial community, such as *Helicobacter pylori* in the stomach, due in part to the human use of antibiotics to treat peptic ulcer disease, may be contributing to post-modern conditions, including obesity (106). In a 10-month prospective experimental study of three human subjects given two courses of ciprofloxacin, Dethlefsin and Relman showed that the gut microbiota was rapidly altered with the antimicrobial drug exposure (107). The composition of the microbiota stabilized after 10 months, but in an altered state (107). A study in France of patients with infective endocarditis showed that a 6-week treatment course with vancomycin and gentamicin, but not other antibiotics, was associated with a significant increase in BMI among males >65 years of age (108). Trasande et al. examined the long-term effect of antibiotic exposures in the first 2 years of life of children born in Avon, United Kingdom between 1991 and 1992 (109). They found that exposure to antibiotics during the first 6 months of life, but not during 6–14 or 15–23 months was consistently associated with a later increase in BMI (109).

Thus, even short-term exposures to antibiotics appear to have an effect on BMI in humans. If indeed, a large proportion of Americans are exposed to low-residue antibiotics in the food they eat, chronic exposures to such sources could certainly disrupt the normal steady state gut microbiota, which should be reflected by changes in body physiology that occur at the community and population level. It is interesting to note that adult obesity prevalence increased only slightly from the 1950s through the mid-1970s (involving cohort of people born in the 1930–50s, who would have had no or low-dose exposure to antibiotics in food) (2). The sharp rise began after the 1970s, with the greatest increase occurring after 2000, when obesity among children and teenagers also had the greatest increase (4) (involving cohort of people born during the period of sharpest increase in CAFOs and other forms

of intensified livestock production and AFOs). These population-based data do not show a causal effect, but the fact that low-dose antibiotics do cause weight gain in food animals provides a strong rationale to examine the effect of this type of exposure at the human population level.

### FUTURE RESEARCH

This new hypothesis raises several research questions. To date, the demonstration of the link between intestinal microbiota with changes in body physiology has been experimental, involving mice or selected human subjects. The impact of antibiotic exposures on the gut microbiota ecology and body physiology needs to be examined at the population level. The comparison of gut microbiota of populations residing near CAFOs versus those living away from such places may reveal microbiota population structures associated with differences in body physiology. Antibiotic concentrations in the water and food these populations ingest could be measured for some class of drugs. Serial analysis of the gut microbiome of children at risk for weight gain followed prospectively may reveal changes associated with weight gain. Clearly, obesity has multifactorial causes. Prospective, population-based studies can be conducted to determine the relative proportion of the obesity prevalence that may be contributed by antibiotic exposure in food, if any. Obesity prevalence is also increasing in other countries, especially in the emerging economy nations. The practice and trend of use of antibiotics as growth promoters in animal husbandry can be assessed to examine the relevance of this hypothesis to the obesity epidemic in these other regions of the world. Finally, a more systematic and comprehensive analysis of antimicrobial residues in food and the environment is needed.

## CONCLUSION

There is accumulating evidence that changes in gut microbiota can affect body physiology. While the exact mechanism remains unsolved, animal experimental and human observation studies provide tantalizing evidence that these changes in the intestine can be triggered by exposures to antimicrobial agents, especially in the context of certain dietary nutrients introduced into the gut, and that these changes can contribute to weight gain. What has been lacking is the epidemiologic evidence in support of this idea. Today, the core gut microbiota of many Americans may be substantially different from that of most Americans living before the 1950s. We propose this idea to be considered to explain part of the obesity epidemic in the US. Clearly more observational as well as experimental studies are necessary to support the hypothesis.

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# Antibiotic alternatives: the substitution of antibiotics in animal husbandry?

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It is a common practice for decades to use of sub-therapeutic dose of antibiotics in food-animal feeds to prevent animals from diseases and to improve production performance in modern animal husbandry. In the meantime, concerns over the increasing emergence of antibiotic-resistant bacteria due to the unreasonable use of antibiotics and an appearance of less novelty antibiotics have prompted efforts to develop so-called alternatives to antibiotics. Whether or not the alternatives could really replace antibiotics remains a controversial issue. This review summarizes recent development and perspectives of alternatives to antibiotics. The mechanism of actions, applications, and prospectives of the alternatives such as immunity modulating agents, bacteriophages and their lysins, antimicrobial peptides, pro-, pre-, and synbiotics, plant extracts, inhibitors targeting pathogenicity (bacterial quorum sensing, biofilm, and virulence), and feeding enzymes are thoroughly discussed. Lastly, the feasibility of alternatives to antibiotics is deeply analyzed. It is hard to conclude that the alternatives might substitute antibiotics in veterinary medicine in the foreseeable future. At the present time, prudent use of antibiotics and the establishment of scientific monitoring systems are the best and fastest way to limit the adverse effects of the abuse of antibiotics and to ensure the safety of animal-derived food and environment.

**Keywords: antibiotics, antibiotic alternatives, application, limitation**

## INTRODUCTION

Since the discovery and application of penicillin in 1940s, antibiotics have played unparalleled roles in the prevention, control, and treatment of infectious diseases for humans and animals. It is also proved that the use of antibiotics in animal feeds is an important way to enhance feed efficiency, to promote animal growth, and to improve the quality of the animal products. Recent studies showed that the growth-promoting effect of antibiotics was correlated with the decreased activity of bile salt hydrolase, an intestinal bacteria-produced enzyme that exerts negative impact on host fat digestion and utilization (Lin, 2014). Therefore, antibiotics are effective tools for ensuring the development of intensive and large-scale farming industry. However, the unreasonable use of antibiotics has given rise to a fear of the development of resistant bacteria (Aarestrup et al., 1998) that may lead to the transfer of resistant bacteria and its resistant factors from animals to humans (Stanton, 2013). Non-therapeutic antimicrobial uses are also linked to the propagation of multidrug resistance (MDR), including resistance against drugs that were never used on the farm (Marshall and Levy, 2011). Due to this concern, Sweden firstly prohibited the use of some of the antibiotics in animal feeds in 1986 (Castanon, 2007), and European Union (EU) member nations banned all antibiotic growth promoters in 2006 according to European Parliament and Council Regulation EC No. 1831/2003.

However, the ban of in-feed use of antibiotics has brought unintended impacts on animal production industries in EU, such as the increase of infections in animals and the decrease of animal production. Meanwhile, the total usage amount of antibiotics in animals increased because the use of therapeutic antibiotics and disinfectants was significantly increased due to the fact that the high incidence of diseases occurred resulted from the ban. Unlike its golden age when a lots of antibiotics were discovered and commercialized, the discovery and development of new antibiotics dramatically decreased for decades (Stanton, 2013). The antimicrobial shortages increased by 283% in 2006∼2010 (Borchardt and Rolston, 2013). The lack of novel core moiety of antibiotics potentially compensate for the resistance to existing antibiotics and owes to the high cost and risk associated with the development and application of such products (Cooper and Shlaes, 2011).

To overcome the increased rate of mortality and morbidity due to the ban of in-feed antibiotics, a number of alternatives/replacements have been proposed (Seal et al., 2013). They are antibacterial vaccines, immunomodulatory agents, bacteriophages and their lysins, antimicrobial peptides (AMPs), pro-, pre-, and synbiotics, plant extracts, inhibitors for bacterial quorum sensing (QS), biofilm and virulence, and feed enzymes, etc. (Millet and Maertens, 2011). Are these antibiotic alternatives really as effective as antibiotics to control the diseases in animals?

The development and application of the alternatives to antibiotic was reviewed, and the possibility of the alternatives to antibiotics was discussed in this paper.

## IMMUNITY MODULATING AGENTS

The development of an infection is the interaction between the pathogen and the immune system of the host. The immune system protects the body against the disease by recognizing and neutralizing the pathogen. The innate immune response includes both humoral and cellular defense such as the complement system and the processes played by granulocytes and macrophages. Immunity modulating agents (immunomodulators) are used for immunotherapy, which is defined as treatment of disease by inducing, enhancing, or suppressing an immune response. Vaccine is one of the most important immunomodulators, and some pharmaceutical agents could also be used as immunomodulators.

### ANTIBACTERIAL VACCINES

Traditional vaccines are generally classified into live-attenuated and inactivated/killed vaccines. Bacterin is a suspension of killed or weakened bacteria used as a vaccine. Live-attenuated bacteria, replicating transiently in the host, are capable of expressing a full repertoire of antigens. Take *Salmonella* vaccine for an example. Many live *Salmonella* vaccine strains have been tested with varying degrees of efficacies (Desin et al., 2013). However, the major drawbacks of the live strains is that they persist in the animal body for a longer time (Tan et al., 1997) and have a high risk of reverting to full virulence (Barrow, 2007; Gast, 2007). Although various *Salmonella* live-attenuated vaccines have been reported, not all of them have been tested under field conditions. In addition, they do not induce sufficient cross protection against other non-host-adapted serotypes (Desin et al., 2013).

Killed vaccines are safer than the live vaccines. It is made by killing *in vitro*-grown bacterial cultures and packing with oil-based adjuvants to enhance immune responses (Potter et al., 2008). They are quite inexpensive in production and stable in storage. The inactivated autogenous poultry vaccines currently include *Pasteurella multocida*, *Salmonella*, *Mycoplasma*, *Ornithobacterium*, *Haemophilus*, *Staphylococcus*, *Escherichia coli*, and *Bordatella Bacterins*. However, killed vaccines have numerous disadvantages such as the lack of relevant protective antigens (PAs) due to *in vitro* growth conditions and killing processes (Barrow, 2007; Gast, 2007), antigenic competition between non-protective and protective components, a lack of safety due to potentially harmful components such as lipopolysaccharide, and a lack of broad-spectrum protection. In addition, killed vaccines require the use of adjuvants which limits the delivery options for the vaccines. Moreover, most of the killed vaccines are injectable products and are not routinely used in intensive broiler operations.

With the increasing use of bacterins, there are concerns that this may lead to the increasing virulence of bacteria. As an alternative, subunit vaccine is composed of either a single antigen or multiple defined antigens (predominantly proteins). This kind of vaccines lack the regulatory and biological complications associated with the living organisms. On the other hand, subunit vaccines are usually poorly immunogenic, requiring formulation with appropriate adjuvant(s) (Mutwiri et al., 2011). Although *Salmonella* subunit vaccines are under development, it is hard to conclude that one class of vaccines is more efficacious than another (Desin et al., 2013). Besides, the use of oral subunit vaccines in large animals remains problematic due to the degradation of the antigens and poor absorption in guts (Potter et al., 2008).

DNA vaccines offer another promising improvement to conventional vaccines (Haygreen et al., 2005). DNA vaccine is made up of a small, circular piece of bacterial DNA (called a plasmid) that has been genetically engineered to include the DNA sequence(s) encoding the antigen(s) from a pathogen. When the vaccine DNA is injected into the cells of the body, the host cells "reads" the DNA and converts it into pathogenic proteins which would trigger a range of immune responses. Nevertheless, DNA vaccine is limited in its protective capacity to the encoded proteins on the vector and may pose a risk of integrating the genetic elements of the vector into the host genome. Most of the literatures dealing with DNA vaccines have described the use of viral antigens delivered in mouse models. However, when they are used in large animals, results are often disappointing (Potter et al., 2008). Therefore, DNA vaccines are unlikely to reach the market until the plasmid dose can be controlled and the problems of effective delivery are solved.

Unlike the anti-viral vaccine market which is quite mature, the available antibacterial vaccines are still rare in market. For example, no vaccines are commercially available for either *C. jejuni* or *E. coli* O157 although immunization against both of these strains has been respectively demonstrated in chicken and cattle (Potter et al., 2008). In the case of *E. coli* O157, immunization with bacterins has not shown any protection effect. Although vaccines against *E. coli* and *Brachyspira hyodysenteriae* for treating swine dysentery have been reported (Francis and Willgohs, 1991; Song et al., 2009), safety and efficacy data are still lacking, hindering the commercialization process of these biological products (Ruan et al., 2011). *S. aureus* vaccines are developed for bovine mastitis, but a systematic review evaluating 24 *in vivo* studies suggests that the methodological differences and a lack of more rigid scientific criteria (such as double blind protocols) in some cases hinder the assessment of the efficacy of these vaccines (Pereira et al., 2011). Moreover, the protection rates of animals by antibacterial vaccine in the market are low (Buckley et al., 2010; Crouch et al., 2010).

Last but not least, the development of a vaccine that is both practical and inexpensive so that it can be affordable for use in poor countries is still a key problem (Zhang and Sack, 2012). As for poultry vaccines, the most important challenge for mass immunization is the cost of vaccine as well as the ability in most cases. While vaccines may lessen our reliance on the use of antibiotics, they are complementary rather than a replacement.

### OTHER IMMUNOMODULATORS

Immunomodulators, mainly immunostimulants, are able to non-specifically enhance the innate immune function and to improve the host's resistance to diseases. The use of immunotherapy in infectious diseases may resulting in modulating the immune response to a microbe (e.g., by using cytokines and cytokine

inhibitors), modifying a specific antigen-based response (e.g., using interferons) and minimizing end-organ damage using non-specific anti-inflammatory agents (e.g., steroids; Masihi, 2000). β-Glucans, bacterial products, and plant constituents could directly initiate activation of innate defense mechanisms acting on receptors and triggering intracellular gene(s) that may result in the production of antimicrobial molecules.

There is a variety of immunostimulants, no less than a dozen categories with hundreds of varieties (**Table 1**). Since 1990s, nucleotides, thymosin, and oregano oil have mainly been used as immunostimulants. Later, probiotics, herbs and their extracts have also become subjects to immunostimulant studies (Thacker, 2010). Studies in animals exhibit significant health benefits by using β-1,3/1,6-glucan (from yeast cell walls) as a feed ingredient to protect animals against microorganisms (Williams et al., 1996). It is suggested that the use of immunostimulants as feed additives can improve the innate defense of animals, providing resistance against pathogens during periods of high stress, such as grading, reproduction, transfer, and vaccination (Bricknell and Dalmo, 2005).

Many factors affect the efficacy of immunostimulants. Immunostimulants exhibit different effects in different animal species. They do not reveal a linear relationship between dose and effect, usually more efficient during or prior to infection. Besides the beneficial effects, immunostimulants have so broad effects among which they inhibit the protective aspects of the host immune system (Thacker, 2010). When immunogenic stimulation persists or autoregulatory immune mechanisms cease, adaptive immunologic events can result in immune-mediated processes detrimental to systemic or organ-specific homeostasis (Moore, 2004). It has been proposed in larval fish aquaculture that the delivery of immunostimulants as a feed additive could be of considerable benefit in boosting the animals' innate defense with little detriment to the developing of fishes. Conversely, immunomodulating a neotenous animal before its immune system is fully formed may adversely affect the development of a normal immune response (Bricknell and Dalmo, 2005). Importantly, most immunomodulators just enhance the immune system of animals, rather than directly kill the bacteria.

Presently, there are no uniform standards for evaluating the efficacy and safety of immunostimulants. It was reported that a reputed immunostimulant composed of *Propionibacterium acnes* extract, *Ochrobactrum intermedium* lipopolysaccharides and Proclin® did not affect the immune system of goats (Morales-delaNuez et al., 2009). The widespread notion of immunostimulatory plant natural products and their potential therapeutic use is rather obscure, suggesting that the product is some sort of "tonic" for the immune system without actually specifying the mechanisms (Gertsch et al., 2011). It is argued that the paradigm of oral plant immunostimulants lacks clinical evidence, originating from primary *in vitro* studies. No conclusive data on orally administered immunostimulants can be found in the scientific literatures up to now. Overall, immunotherapy to modulate the immune response just can be used as an adjunct to the antimicrobial therapy (Kak et al., 2012).

**Table 1 | Classification of immunostimulants.**

| Category | Variety |
|---|---|
| Mineral substances | Selenium, zinc, etc. |
| Vitamins | Vitamin A, vitamin E, vitamin C, etc. |
| Amino acids | Arginine, leucine, ubenimex, etc. |
| Chinese herbal medicines | *Astragalus*, *Echinacea*, etc. |
| Plant polysaccharides | *Astragalus* polysaccharide, lentinan, algal polysaccharides, ganoderan, *Polyporus* polysaccharide, chitosan, etc. |
| Oligosaccharides | Mannan-oligosaccharides, fructooligosaccharide, etc. |
| Microbial preparations | BCG vaccine, corynebacterium seedlings, *Lactobacillus*, cholera toxin B subunit, *Mycobacterium phlei*, muroetasin, prodigiosin, etc. |
| Immunologic adjuvants | Aluminum adjuvant, propolis, liposome, Freund's adjuvant, etc. |
| Hormones and hormone-like substances | Growth hormone, thymosin, metallothionein, thymopentin, etc. |
| Nucleic acid preparations | Polynucleotide, immune ribonucleic acid, etc. |
| Anthelmintics | Levomisole, metronidazole, etc. |
| Chemical synthetics | Levomisole, cimetidine, sodium houttuyfonate, imiquimod, pidotimod, ubenimex, tilorone, polyinosinic acid, etc. |
| Bacterial extracts | β-Glucan, peptidoglycan, lipopolysaccharide, etc. |
| Biological (cytokines) | Interferon, transfer factor, interleukin, immune globulin, etc. |
| Others | Bee pollen, bursa extracts, gamma globulin, heat shock protein, poly IC, glycyrrhizin, etc. |

## BACTERIOPHAGES AND THEIR LYSINS

### BACTERIOPHAGES

Bacteriophages are viruses that are parasitic on bacteria, and they have been considered as one of the types of agents to treat bacterial infections for a long time (Wittebole et al., 2014). They were first discovered by Frederick Twort in UK in 1915 and by Félix d'Herell in France in 1917. The first study on the clinical use of phage was published in Belgium in 1921 by Bruynoghe and Maisin who injected staphylococcus-specific phage near the base of the cutaneous boils to treat cutaneous furuncles and carbuncles. The commercial phages was introduced by two companies in the United States and France in 1940s. Recent animal studies show that phage therapy is worth of recognition (O'Flaherty et al., 2009). It is reported that phages has certain preventive effects on pathogens as *E. coli* O157:H7, *Salmonella* and *Campylobacter* (Huff et al., 2005; Johnson et al., 2008). In 2006, a phage cocktail designated LMP-102$^{TM}$ containing six types of pure bacteriophages was approved by US-FDA as a food additives for prevention of meat contamination with *Listeria*. In 2007, United States Department of Agriculture (USDA) approved another phage product to be used for disinfection of *E. coli* in hidden parts of cattle. Nonetheless, most of the bacteriophage products to date are still in the research stage.

Bacteriophages can replicate in host cells and are able to produce new lytic phages to keep pace with the mutation of pathogens. However, the replacement of antibiotics by phages in treatment of bacterial diseases encounters controversy because of the following characteristics (Pirnay et al., 2011):

(1) Since phages have strict host strain specificity, the precise etiological microorganism causing infection needs to be determined with accuracy before the use of phage therapy (Allen et al., 2013). Also, the narrow host range impedes the ability of a single kind of phages to be used as replacement of antibiotics, which are typically broader in their antibacterial spectrum;
(2) Since they are viruses, phages can be seen by the immune system of the host as a potential invader and may therefore rapidly be eliminated from the systemic circulation by reticulo-endothelial system clearance before they are accumulated in the target sites, or, they may be inactivated by the adaptive immune defense mechanisms (Dabrowska et al., 2005), which may lead to the treatment failure (Merril et al., 1996);
(3) Another concern of phage therapy is the potential ability of bacteriophages to transfer their DNA from a bacterial cell to another. This could be responsible for the transfer of pathogenicity determinants and virulence factors, leading to the development of a new microbe or even more resistant bacteria (Brabban et al., 2005; Maiques et al., 2007). Therefore, the use of phages that are unable to package extra host DNA or of phages that use the host DNA to synthesize its own DNA would be preferred (Gorski et al., 2009). In addition, under certain conditions, lytic phages would be transformed into lysogenic phages, making it possible to transfer their own virulence factors to the host bacteria (Brussow, 2007);
(4) Pharmacokinetic characteristics of phages are barely known. Optimal dose, route of administration, frequency, and duration of treatment still need to be defined before widespread clinical trials are contemplated;
(5) Phage therapy is time-sensitive. Use phages early in a disease setting could obtain a better therapeutic effect. For example, when the phages were given immediately after the infection of *E. coli* O18ac:K1:H7 ColV+, the efficacy was 100%; whereas when phage treatment was carried out 16 h after infection, the therapy failed (Smith and Huggins, 1982);
(6) Phages can cause the release of toxins, e.g., endotoxin (LPS), in large quantities from bacteria, especially Gram-negative bacteria. This may account for several side effects on the host such as the development of an inflammatory cascade leading to a multiple organ failure;
(7) Bacteria can obtain resistance to phages by mutation. The mutation rates for antibiotics and phages are $10^{-7}$ and $10^{-6}$, respectively (Carlton, 1999). There are at least four mechanisms that may be involved in bacterial resistance to a specific phage. Loss or lack of receptor (Liu et al., 2002), structural modification (Riede and Eschbach, 1986) and/or masking of the receptor (Drulis-Kawa et al., 2012) may prevent phage adsorption to the bacteria and prevent further an ability to generate new phages. The other mechanisms include the prevention of phage DNA integration by superinfection exclusion system (Sie), the degradation of phage DNA by restriction–modification defense system or by clustered regularly interspaced short palindromic repeats (CRISPR), and the blockage of phage replication, transcription, translation, or virion assembly by abortive infection system (Drulis-Kawa et al., 2012);
(8) Some phages can only survive in the intestines when bacteria counts reach certain numbers. Phages can only reduce but not completely eliminate *S. typhimurium* in the animal intestines (Berchieri et al., 1991; Callaway et al., 2011);
(9) Preparation of phages should be at a low temperature (Burrowes et al., 2011), and this kind of biological products are not stable.

Currently, the main challenge for the promotion of phage preparations is the lack of data obtained from large-scale clinical trials, thus hindering the universal application of them. Regulatory loopholes remain another major hurdle. In addition to the inherent safety concern, neither the US-FDA nor the European Medicines Agency has an approval process in place that can easily accommodate the ever-changing combinations of phages that accompanies the need to continuously develop the product in order to stay one step ahead of evolving MDR bacteria (Miedzybrodzki et al., 2012).

### ENDOLYSINS

Endolysins, including glucosidase, amidase, endopeptidase, and transglycosylase, are generated at the late phage lytic cycle,

degrading bacterial peptidoglycan to facilitate the release of new phages from the infected bacteria. Endolysins were first discovered in the 1950s (Ralston et al., 1955), and revealed antibacterial activity against *Staphylococcus*, *Bacillus anthracis*, *L. monocytogenes*, and *Clostridium butyricum* in the 1990s (Low et al., 2005). Endolysins can treat sepsis and a few Gram-positive bacteria infections, such as *Enterococcus faecalis*, *C. perfringens*, and Group B *Streptococcus* (Fenton et al., 2010). Endolysin PAL is able to kill Group A *Streptococcus* which cause tonsillitis and other infections. Amidase PAL and endopeptidase Cpl-1 from phage Cpl-1 is capable of synergistically reducing the incidence of local and systemic pneumococcal disease (Loeffler et al., 2003; Fischetti, 2005). Endolysins LysK from phage K could kill nine *Staphylococcus*, including methicillin-resistant *S. aureus* (MRSA; O'Flaherty et al., 2005). Endolysins PlyV12 shows a good lytic activity against *Enterococci*, vancomycin-resistant *E. faecalis* and *E. faecium* (Yoong et al., 2004). Endolysins isolated from phage phi3626 can treat *Clostridium* infections (Courchesne et al., 2009).

Endolysins can quickly kill susceptible strains with wider antibacterial spectrum than phages and the activities are easier to be detected. Long-term evolution of endolysins makes them target only for some of the key elements on bacterial cell walls (Loeffler et al., 2003) thus cause bacteria lysed fast. Therefore, there is not enough time for bacteria to develop a resistance (Fischetti, 2005). However, the conventional methods for endolysin production are complicated, making the cost higher than that of the phage production. The use of recombinant DNA technology can reduce the costs (Loeffler et al., 2001), but it cannot guarantee the correct structure and full activity of the enzymes. Meanwhile, endolysins are easily degraded and lose activities during use and storage; hence they should be used with a full dose. In addition, the efficacy of endolysins is poor against Gram-negative bacteria; thus the use of single type of endolysin limits the scope of application. Future studies will focus on the chimeric endolysin (O'Flaherty et al., 2009), which could widen the antibacterial spectrum of this kind of enzymes. Presently, there are no published clinical studies concerning the endolysins.

### VIRION-ASSOCIATED PEPTIDOGLYCAN HYDROLASES

Bacteriophage virion-associated peptidoglycan hydrolases (VAPGHs) are a kind of phage lyases that hydrolyze bacterial peptidoglycan to assist the entry of phages into the bacterial cells (Rodriguez-Rubio et al., 2013). Many VAPGHs have been discovered and proved exhibiting antibacterial activities. Protein HydH5 from phage phiIPLA88 shows higher activity against *S. aureus* in the early logarithmic growth phase (Rodriguez et al., 2011). Protein 17 from phage P68 and protein gp61 from phage phiMR11 exhibit lytic activity against *S. aureus* including MRSA (Takac and Blasi, 2005; Rashel et al., 2008). Protein P5 from phage Φ6 possess the antibacterial activity against *Pseudomonas*, *E. coli*, *S. typhimurium*, *Proteus vulgaris*, and other Gram-negative bacteria whose outer membrane structures are unstable (Caldentey and Bamford, 1992). Protein Gp181 from phage ΦKZ demonstrates a lytic activity against the above mentioned Gram-negative bacteria as well as *Ralstonia solanacearum* and *Yersinia*. Protein gp36 of phage ΦKMV is effective against *P. aeruginosa* and *E. coli*, and exhibits a high thermal stability as the enzyme retained 26% of activity after incubation at 100°C for 2 h (Lavigne et al., 2004).

Currently, the researches on purified VAPGHs are limited. Since bacterial resistance caused by endolysin has not been reported to date, this may also be the case for VAPGHs (Rodriguez-Rubio et al., 2013). VAPGHs from Gram-negative bacterial phages have broad antimicrobial spectrum while the antibacterial spectrum of VAPGHs from Gram-positive bacterial phages only confine to specific host bacteria. VAPGHs are also effective against some antibiotic-resistant pathogens, increasing their application prospects (Paul et al., 2001; Rodriguez-Rubio et al., 2012). Many VAPGHs exhibit thermal stability, retaining activity at high temperatures generally associated with food technology. Taking into account that extracellular action can reduce the intracellular action which may trigger widespread bacterial resistance mechanisms, such as efflux pumps, VAPGHs provides an alternative source for bacterial lyases except for endolysins (Rodriguez-Rubio et al., 2013).

## ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are classified into two categories, non-ribosomally synthesized AMPs and ribosomally synthesized AMPs, according to the peptide synthesis mechanism. The non-ribosomal AMPs, mainly produced by bacteria, are synthesized by peptide synthetases and structural modifications. They include gramicidin, polymyxin, bacitracin, and sugar-peptide. Polymyxin is from *B. polymyxa*, playing bactericidal effect by destroying the bacterial cell membranes. It is effective against many Gram-negative bacteria, such as *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Haemophilus*, and *Salmonella*. Bacitracin is a cyclic peptide produced by *B. subtilis* and *B. licheniformis*, and it functions by inhibiting the synthesis of cell wall peptidoglycans and glycoprotein core oligosaccharides in Gram-positive bacteria. Bacitracin shows bactericidal effects against Gram-positive bacteria, Gram-negative cocci and spirochetes. The USA and China have approved the use of bacitracin zinc and bacitracin methylene salicylic acid as feed additives in 1960 and 1990, respectively. These two bacitracins have a wide compatibility with other medicines.

Ribosomally synthesized AMPs can be further classified according to the sources of the peptides, such as mammals, amphibians, insects, plants, bacteria, viruses, etc. These AMPs are not only anti-bacterial or anti-mycotic, but also anti-protozoal, anti-viral, or anti-neoplastic, with broad application prospects. The positive charges of AMPs could form electrostatic adsorption with negatively charged phospholipid molecules on the bacterial cell membranes, resulting in structural damage of the membranes.

There are many reports on the protective effect of AMPs on humans (Guani-Guerra et al., 2010) and animals (Leonard et al., 2012). Here, bacteriocins which are produced by bacteria are taken as an example. Bacteriocins are defined into four classes as lantibiotics, the small heat-stable peptides (SHSPs), the large heat-labile proteins (LHLPs), and undefined mixture proteins with lipids and carbohydrates (Bierbaum and Sahl, 2009). Bacteriocins can also be subdivided on the basis of their modifications into class I (modified) and class II (unmodified or circular; Cotter et al., 2013). There

have been lots of identified bacteriocins such as nisin, lactacin, lactocin, helveticin, fermenticin, sakacin, lacticin, plantacin, subticin, etc. *In vitro* tests show that bacteriocins have strong killing and suppressive effects on a variety of pathogens, including resistant pathogens (Field et al., 2011). In 1988, nisin received the US-FDA approval as food additive for the first time. Pediocin PA-1 from *Pediococcus* is on the market now. However, pure bacteriocins have so far only few and limited authorized uses in foods.

Bacteriocins have been found to have many distinct mechanisms of action. In addition to punch holes in the cell membrane like other AMPs do (Cotter et al., 2005), nisin, several lantibiotics and some class II bacteriocins, target lipid II (Bierbaum and Sahl, 2009), a key intermediate in the peptidoglycan biosynthesis machinery. Besides, bacteriocins can kill bacterial cells by interfering with DNA, RNA, and protein metabolism. MccB17 functions by inhibiting DNA gyrase-mediated DNA supercoiling, thereby interfering with DNA replication (Parks et al., 2007). MccC7-C51 inhibits aspartyl-tRNA synthetase, thus blocking mRNA synthesis (Metlitskaya et al., 2006). Nocathiacins and several other thiopeptides target the bacterial ribosome, binding the 23S rRNA of the 50S ribosomal subunit (Bagley et al., 2005).

Although AMPs are with good bactericidal effects and easily digested by bodies without adverse effect to the taste of feed or polluting the environment, a number of constraints have accompanied with the deepening research concerning AMPs.

(1) The high production cost limits the use of AMPs as effective antibiotic alternatives to livestock. Nowadays, bacteriocins are produced traditionally by culturing the wild strains, but the yield is low and the purification process is complex;
(2) Scientists begin to use genetic engineering techniques to synthesize bacteriocins owing to their peptide nature because they are directly encoded by genes (Cotter et al., 2013). AMPs can be modified by protein engineering to improve their efficacy. Site-saturation mutagenesis approach is used to create a bank of nisin A derivatives to screen the ones exhibiting enhanced bioactivity (Molloy et al., 2013). However, it cannot guarantee that the spatial structures of AMPs obtained by genetic engineering are consistent with the those of natural AMPs, which often causes differences in activities (Field et al., 2011);
(3) Many natural AMPs, such as melittin (a peptide which is the principal active component of bee venom), buthotoxin (a scorpion venom polypeptide) and plant AMPs, are potentially toxic to eukaryotic cells due to their hemolytic effects. Recently, a potent *E. coli* displaying multimeric AMPs on the cell surface was constructed (Shin et al., 2013). The multimeric AMPs can be converted into active AMP monomers by the pepsin in the stomach of livestock;
(4) Antibacterial spectrum of most bacteriocins is narrow, only effective to the related bacterial species (Lee and Kim, 2011);
(5) Bacteria can still develop resistance to AMPs by reducing the corresponding receptors (Piper et al., 2009), changing the composition of cell wall (Kramer et al., 2006), or changing the primary target of bacteriocin (del Castillo et al., 2001). Some bacteria can also utilize immune genes (Draper et al., 2009) or bacteriocin-hydrolase (Sun et al., 2009; Nocek et al., 2012) to obtain resistance. In fact, the widely used polymyxin AMPs has caused serious resistance in clinically important bacterial species, in both human and veterinary medicine;
(6) *In vivo* studies about pharmacodynamics, pharmacokinetics, and stability of AMPs are few. It is unclear whether AMPs or their metabolites are harmful to the body, and the immune response and other issues still need to be verified;
(7) AMPs are unstable during transportation, and are easily hydrolyzed by proteases in the alimentary canal during use.

## PRO-, PRE-, AND SYNBIOTICS

### PROBIOTICS

Probiotics have been defined by the World Health Organization as "microorganisms which, administered live and in adequate amounts, confer a benefit to the health of the host." Probiotics are considered to be able to destroy pathogenic microorganisms by producing antimicrobial compounds such as bacteriocins and organic acids, improve gastrointestinal microbial environment by adherence to intestinal mucosa thereby preventing attachment of pathogens and competing with pathogens for nutrients, stimulate the intestinal immune responses and improve the digestion and absorption of nutrients. The commonly used probiotics include *Bacillus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Bifidobacterium*, *Bacteroides*, *Pseudomonas*, yeast, *Aspergillus*, and *Trichoderma*, etc. Microbiological feed additives used in EU mainly include *Bacillus* (*B. cereus* var. *toyoi*, *B. licheniformis*, *B. subtilis*), *Enterococcus* (*E. faecium*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus*), *Pediococcus* (*P. acidilactici*), *Streptococcus* (*S. infantarius*), and some fungi such as *Saccharomyces cerevisiae* and kluyveromyces (Anadon et al., 2006). Japan started using probiotics in 1960s, and China began the application of probiotics in 1980s. US-FDA approved 42 probiotics till 1989 (Gaggia et al., 2010). In 2000, the total sale of feed probiotics worldwide was $186 million.

However, the probiotic market is very less organized and the supervision and management of probiotic production are defective. For instance, China Ministry of Agriculture has approved 12 probiotics, but there are more than 50 probiotics being used in China. Due to lack of standards, animal poisoning, allergies, and diarrhea after using probiotics are reported from time to time. Despite years of experiences in the use of lactobacilli and bifidobacteria which are proved to be safe, the safety of other species still need to be examined (Borriello et al., 2003). Meanwhile, probiotics are potentially harmful to the congenitally immunodeficient animals (Balish and Wagner, 1998). Besides, previous studies have failed to consistently prove the beneficial effects of probiotics in animals. For example, microcin-producing *E. coli* could inhibit the growth of *Salmonella in vitro*, but the results *in vivo* were not satisfactory (Frana et al., 2004). Contradictory results with very heterogeneous studies are reported on the treatment and the prophylaxis of upper respiratory tract infections (Alexandre et al., 2014). Furthermore, probiotics have adverse effect on the normal gut flora. *Lactobacillus* and *Bacillus* can destroy the ecological balance of normal flora in the body, which may be related to the occurrence of urinary tract infections and other diseases. Dietary cider yeast can potentially alter the gut microbiota. However, such

changes depend on their endogenous microbiota that causes a divergence in relative response to that given diet (Upadrasta et al., 2013).

For putting probiotics into practice as feed additives, the followings are challenged: (1) the number of safe bacterial species is limited; (2) microbial preparations are easily inactivated in feed processing, transport, and storage processes; (3) they cannot withstand low pH in gastrointestinal tract and bile acids during use; (4) it is difficult to reach high enough number of viable cells to colonize in the intestine. In addition, because there are no adequate corresponding regulations and standards, probiotic products cannot be labeled with proper dose, indicated in suitable animal target as well as other factors that may affect the efficacy.

### PREBIOTICS

Prebiotics are non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract (Gibson et al., 2004). Prebiotics include oligosaccharides [such as fructooligosaccharide (FOS), mannan-oligosaccharide (MOS)], polysaccharides, natural plant extracts, protein hydrolysates, polyols, etc. Prebiotics can selectively proliferate intestinal bacteria, promote immune functions and show anti-viral activity. Some of them are able to promote mineral absorption and regulate metabolism. The applications of prebiotics as feed additives began in the late 1980s. China began to use them in the late 1990s. Currently, the most promising prebiotics are multifunctional oligosaccharides and acidifiers.

Prebiotics are stable compounds with no residue, no induced resistance, and wide variety of sources. However, nowadays in EU many prebiotic products are not authorized as feed additives under the commission regulation (EC) 1831/2003 (European Commission, 2003). This is due to some drawbacks of this kind of products. First, prebiotics themselves cannot inhibit and kill pathogens, thus they cannot prevent or treat bacterial infections as antibiotics do. Second, feeding with large quantity of prebiotics may cause bloating, diarrhea, and other adverse reactions due to the fermentation in the gastrointestinal tract (de Vrese and Schrezenmeir, 2008). Third, a study suggests that the prebiotic role of mannose is related to its oligosaccharide structure (Badia et al., 2013). Both β-galactomannan and MOS from yeast *Saccharomyces cerevisiae* attenuates *Salmonella*-induced secretion of IL6 and CXCL8, but cells treated with monosaccharide D-mannose show similar levels of these proinflammatory factors compared with the control of infection. Since the relationship between structure and physiological function of prebiotics is not clear, the efficacy of prebiotics is always variable with different animal species, ages, and physical conditions. Sometimes, mutual antagonism occurs. In addition, the high cost of prebiotic production limits their application in the animal husbandry industry.

### SYNBIOTICS

Synbiotics are the joint preparations of probiotics and prebiotics, and thus have the dual role of them (Andersson et al., 2001). There are some reports on the effect of synbiotics on the physiological and biochemical indexes of piglets including the enhancement of immune function in piglets, the improvement of average daily gain and digestibility, the reduction of diarrhea morbidity and mortality, the ease of weaning stress response, and the significant promotion of piglet performance (Gaggia et al., 2010). However, the reports of the beneficial effects of synbiotics on swine production are still limited (Modesto et al., 2009). The mixing proportions of probiotics/prebiotics for the majority of synbiotics are inadequate (Kolida and Gibson, 2011), thus resulting in a non-synergistic effect. So far, synergy mechanism of probiotics and prebiotics has not been thoroughly understood; hence, the extensive application of synbiotics has a long way to go.

## PLANT EXTRACTS

Plant materials are used widely in traditional systems of medicine (Savoia, 2012). Plant extracts, also known as phytobiotics, have been exploited in animal nutrition, particularly for their antimicrobial, anti-inflammatory, anti-oxidative, and anti-parasitic activities (Vondruskova et al., 2010; Hashemi and Davoodi, 2011). Many plants have beneficial multifunctional properties derived from their specific bioactive components. Biologically active constituents of plants are mostly secondary metabolites, such as terpenoids (mono- and sesquiterpenes, steroids, etc.), phenolics (tannins), glycosides, and alkaloids (present as alcohols, aldehydes, ketones, esters, ethers, lactones, etc.; Huyghebaert et al., 2011). Among 109 new antibacterial drugs, approved in the period of 1981∼2006, 69% originated from natural products, and 21% of the antifungal drugs were natural derivatives or compounds mimicking natural products (Newman, 2008).

Plant extracts are generally considered safe and effective against certain bacteria. They are extensively used in feed as growth promoters and health protectants (Hashemi and Davoodi, 2011; Abreu et al., 2012), particularly in Asian, African, and South American countries, and are gradually used in developed countries in recent years. In pig production, it is thought that oregano, cinnamon, Mexican pepper, thyme, oregano, and *Camellia sinensis* can decrease pathogenic microbial mass in the intestines (Manzanilla et al., 2004; Namkung et al., 2004; Zanchi et al., 2008); sangrovit, aged garlic extract, and allicin are able to increase body weight gain (Borovan, 2004; Tatara et al., 2008); thyme, clove, oregano, eugenol, and carvacrol are capable of improving pig performance (Oetting et al., 2006; Costa et al., 2007). Effects of phytogenic feed additives on the production performance of poultry are also reported (Hashemi and Davoodi, 2010).

It is considered that plant extracts at minimum inhibitory concentrations (MICs) of 100∼1000 μg/ml in the *in vitro* bacterial susceptibility tests possess antibacterial activities (Simoes et al., 2009). Useful antimicrobial phytochemicals can be divided into several categories, such as phenolics/polyphenols, terpenoids/essential oils, alkaloids, lectins/polypeptides (Windisch et al., 2008). Phytochemicals exert their antimicrobial activity through different mechanisms. For example, (1) tannins act by iron deprivation and interactions with vital proteins such as enzymes (Scalbert, 1991); (2) the main indoloquinoline alkaloid, cryptolepine, is a DNA intercalator and an inhibitor of topoisomerase (Karou et al., 2006); and (3) saponins form complexes with sterols presenting in the membrane of microorganisms, causing membrane damages and consequent collapse of cells (Morrissey and Osbourn, 1999). Essential oils have long been

recognized for their antimicrobial properties (Lee et al., 2004), but the exact antimicrobial mechanism is poorly understood. In fact, the antimicrobial activities of many plant extracts have not been elucidated clearly yet (Stavri et al., 2007). Some *in vivo* observations support the assumption that the general antimicrobial potential of phytogenic feed additives is contributed to a final reduction of intestinal pathogen pressure (Windisch et al., 2008). To the year of 2008, only two kinds of human-used antibacterial plant extracts have completed the clinical trials, and additional 13 plant extracts are under clinical trials (Harvey, 2008).

A common feature of phytobiotics is that they are a very complex blend of bioactive components. There is a lot of variations in the composition of phytobiotics due to the biological factors (plant species, growing location, and harvest conditions), manufacturing (extraction/distillation and stabilization) and the storage conditions (light, temperature, oxygen tension, and time; Huyghebaert et al., 2011). Only under certain circumstances, plant extracts could improve animal performance and control diseases. Bird growth responses to herbal plants are still controversial, since the exact quality as well as the quantity of the active chemicals in plant extract are required to determine the response for bird performance (Cross et al., 2007), which are often lacking. Some parameters that affect the efficacy of the phytobiotics mainly include the plant parts and their physical properties, the genetic variation of the plant, age of the plant, different dosage used, extraction method, harvest time, and compatibility with other ingredients (Yang et al., 2009). In addition, the beneficial effect of dietary phytobiotics can be influenced by the nutritional status of animals, the infection, the diet composition and the environment condition (Giannenas et al., 2003).

It is difficult to perform systematic and comprehensive toxicology studies and safety assessment on herbs and their extracts, due to their complex composition. The challenge is to identify and quantify the multitude of actions in order to claim improving feed utilization, animal physiology and health status. Currently, herbal feed additives on the market do not meet the "trace and efficient" principle for feed additives. They are commonly used at very large dose, generally at feed ratio of 1–2%, some up to 5%, and this may affect the nutrition of a feed. Another consideration when using phytogenic feed additives is the possible interactions with other feed additives. There are reports on the adverse interactions of phytogenics with enzyme preparations (Sarica et al., 2005) and with protein through partial denaturation (Anadon et al., 2005). In summary, although phytobiotics are a group of natural additives, researches on their mechanisms of action, compatibility with diet, toxicity and safety assessment need to be done before they can be applied more extensively in animal feeds.

## INHIBITORS TARGETING PATHOGENICITY

### QUORUM SENSING INHIBITORS

Bacterial pathogenicity is, in part, under the regulation and control of QS system (Swift et al., 2001). QS system consists of self-induced signaling molecules (autoinducers, AIs), receptors, and downstream regulatory proteins. AIs are *N*-acyl homoserine lactones (AHLs) secreted by Gram-negative bacteria, autoinducing peptide (AIPs) secreted by Gram-positive bacteria, autoinducer-2 (AI-2), and other signaling molecules such as quinolones, esters, and fatty acids.

Inhibitors targeting QS can block the functions of QS system and therefore prevent bacterial virulence regulated by QS system. QS inhibitors (QSIs) are classified into three groups including non-peptide small molecule, peptide (mainly AIPs homologs), and protein QSIs. Non-peptide QSIs mainly include AHLs analogs, such as ACP homologs, L/D-*S*-adenosylhomocysteine and butyryl-*S*-adenosyl-L-methionine (Parsek et al., 1999), which can interfere with the synthesis of QS signal molecules or the binding to the receptors. Mice treated with synthetic AIP-II had resistance to *S. aureus* infection (Mayville et al., 1999) and treated with furanone observed the decrease of virulence of *P. aeruginosa* (Hentzer et al., 2003). QS quenching enzymes and QS quenching antibodies are proteinaceous QSIs (Amara et al., 2011). The former, such as AHL-acylase, lactonase, oxidoreductases from *Rhodococcus* and paraoxonase from mammals, degrade signaling molecules. Human and murine paraoxonases 1 show the host modulators of *P. aeruginosa* QS (Ozer et al., 2005). In addition, competitive organisms are able to clear the signal molecule to quench QS (Kalia and Purohit, 2011). For instance, *E. coli* ingest AI-2s to influence the QS of *Vibrio harveyi* (Xavier and Bassler, 2005). Bacteria with AHL-degrading activity protect *Artemia* spp., rotifers and larvae of turbot or prawn from infection (Nhan et al., 2010). In animal serum, apolipoprotein B (ApoB) bind with AIP1 molecules of *S. aureus*, effectively reducing its QS (Peterson et al., 2008).

Importance of QSIs is inferred from a growing number of patents related to this field in the last few years and extensive researches in this field (Kalia and Purohit, 2011; Romero et al., 2012). QSI appears to be effective *in vitro* and in various animal models; however, all the structural classes of compounds that have been studied and patented have limitations when used *in vivo* (Bhardwaj et al., 2013):

(1) Except for LED209 that has entered pre-clinical trial, the vast majority of QSIs cannot be widely applied because of their toxicity to eukaryotic cells. Penicillic acid and patulin are toxic to human cells, and halogenated furanone has carcinogenic toxicity (Bjarnsholt and Givskov, 2007);
(2) Although QSI itself does not interfere with the growth of bacteria and thus dose not cause selective pressure on bacteria, it is still possible for bacteria to develop new resistance (Kalia and Purohit, 2011). For example, mutations of bacterial AHL synthases and QS signal receptors (e.g., LuxR) remain (Kalia and Purohit, 2011);
(3) Drugs based on AHL analogs could suffer from hydrolysis of lactone ring and drugs based on proteins could have stability problems. For example, lactonases produced in the human airway epithelia show to quench QS signals from *P. aeruginosa* (Chun et al., 2004). Therefore, special caution needs to be exercised in the development of synthetic anti-QS analogs based on the lactone core (Sintim et al., 2010). Unraveling the structures of molecules like AI-3 and deciphering the structural nuances of AI-2 receptors, LuxS and the myriad signal transduction cascades involved in QS processes are of utmost importance;

(4) Bacterium only use its specific signal molecules; therefore, it is difficult to find a broad-spectrum QSIs (Bjarnsholt and Givskov, 2008). Broad-spectrum QS quenching enzymes have good prospects, but the possibility that protein QSIs may cause host immune response should be considered;
(5) The degradation of QS signaling molecules will affect the normal activities of the host intestinal flora (Amara et al., 2011);
(6) Expression of virulence factors in biofilm-forming bacteria is much lower than that in the planktonic bacteria (Resch et al., 2005); hence, one has to consider the actual value to use QSIs for treating pathogens which have generated biofilms.

It is reported that bacteria are more sensitive to the antibiotics when the antibiotics are used in combination with QSIs. Therefore, combination usage serves a better strategy to enhance the antimicrobial effects and prevent the bacterial resistance.

## BIOFILM INHIBITORS

Biofilms are structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. Biofilm-forming bacteria may cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defense system (Hoiby et al., 2010). As for treating staphylococcal biofilm, protein synthesis inhibitors (e.g., oxazolidinones and tetracyclines), cell membrane and wall-active antibiotics (e.g., lipopeptides and glycopeptides) and inhibitors for DNA and RNA synthesis (e.g., rifampin) are often used (Kiedrowski and Horswill, 2011). Methane-thiosulfonate and mercurial *p*-hydroxymercuribenzoic acid could target sortases, a membrane enzyme catalyzing the covalent anchoring of surface proteins to peptidoglycans, which are involved in bacteria adhesion (Chen and Wen, 2011).

Biofilm formation involves bacterial cell adhesion, QS regulation, biofilm maturation, and bacteria spread; therefore, a single drug is difficult to completely remove pathogens in biofilms. Combination therapy is a wise choice, which generally uses antibiotics with antibiotics or antibiotics with biofilm inhibitor as the combination. Combination of both fluoroquinolones and macrolides or fosfomycin seems to be most effective regimen against biofilm infections in urinary tract (Kumon, 2000). Another promising strategy is the use of enzymes that can dissolve the biofilm matrix [e.g., polysaccharide hydrolases (Kaplan et al., 2004), DNases (Izano et al., 2008), proteases (Marti et al., 2010), and alginate lyases] as well as quorum-sensing inhibitors that increase biofilm susceptibility to antibiotics. It is shown that administration of DNase and alginate lyase enhances the activity of tobramycin against biofilms by dissolving the biofilm matrix of *Pseudomonas aeruginosa* (Alipour et al., 2009). In addition, monoclonal antibody of alginate shows certain damaging effects against *P. aeruginosa* biofilms (Mai et al., 1993), and enhances the ability of antibiotic to penetrate biofilms (Hatch and Schiller, 1998). Urokinase or lumbrokinase combining with fleroxacin significantly enhances the inhibition of *P. aeruginosa* biofilms (Selan et al., 1993). This combination not only degrades the polysaccharide protein complex in the bacterial biofilms, but also affects the bacterial DNA synthesis. Efflux pump inhibitor in combination with miconazole can effectively remove *Candida albicans* from the biofilm (Qi and Wang, 2009).

The way from molecular mechanisms of biofilm formation to anti-biofilm products is promising, but still a long one. Although biofilm inhibitors can inhibit biofilm formation, they do not inhibit bacterial growth or kill bacteria. Hence, when biofilm inhibitor use is discontinued, bacteria will produce biofilm again to protect themselves against the adverse environmental conditions.

## BACTERIAL VIRULENCE INHIBITORS

An important emerging strategy to combat bacteria seeks to block the ability of bacteria to harm the host by inhibiting bacterial virulence factors. Development of compounds inhibiting the function and transmission of bacterial toxins is a novel anti-infective strategy. The protein complex of anthrax toxin contains lethal factor (LF), edema factor (EF), PA, and other components. Single component is non-toxic, but the combination of LF or EF with PA will lead to a pathological effect (Young and Collier, 2007). A small molecular, hydroxamate (LFI), can bind to the active site of LF, inhibiting the activation of LF and preventing anthrax infection (Shoop et al., 2005). Cisplatin shows inhibitory effect to PA heptamer assembly, thus blocks the toxicity of LF and EF. However, only simultaneous feeding of cisplatin and a lethal amount of anthrax toxin has a protective effect on rodents, while delayed feeding of cisplatin would have resulted in a failure (Moayeri et al., 2006). Cholestyramine can bind with clostridial toxin to prevent its adsorption to intestinal epithelial cells, thus weakening the toxicity cause by the toxin.

The type three secretion system (T3SS) is found in over two dozens of Gram-negative bacteria and functions by injecting effector proteins directly into the cytosol of the host cells. One acyl salicylaldehyde compound targeting the T3SS of *Yersinia pseudotuberculosis* prevents the translocation of effector molecules, and thus attenuates the pathogens (Nordfelth et al., 2005). Different acyl salicylaldehydes inhibit two T3SS effector molecules to prevent *Salmonella* invasion of the intestinal epithelial cells (Hudson et al., 2007). Currently, studies on the acyl salicylaldehyde under bovine intestinal model are still limited.

Prevention of bacterial adhesion is equally important. Pilicides inhibit bacterial pili formation. Pyridone and its N-terminal amino acid derivatives competitively inhibit the chaperone protein binding to pilus protein. Pyridone is also able to inhibit blood clotting and biofilm formation, thus inhibiting bacterial adhesion (Pinkner et al., 2006). Regulating the expression of bacterial virulence factor is meaningful as well. For instance, virstatin inhibits the expression of *Vibrio cholerae* toxin and pilus, thereby prohibiting the colonization of *V. cholerae* in the gut (Hung et al., 2005). Virulence regulation can be achieved through different aspects, such as interfering bacterial QS (Clatworthy et al., 2007). Inhibition of the downstream effects of toxin is feasible. For example, $Cl^-$ secretion inhibitors can moderate diarrhea.

Toxin inhibitors carry out the function by disrupting the bacterial toxins, or modulate the host responses to the toxins. They do not directly inhibit bacteria, thus causing no bacteria resistance selection pressure. However, like the biofilm inhibitors, even

in the presence of toxin inhibitors, bacteria can still grow and reproduce. Therefore, in the absence of these substances, bacteria may produce toxins and exhibit virulence again.

## FEED ENZYMES

The nutrients for the multiplication and growth of bacteria in the intestinal tract are derived largely from dietary components, which are either not digested by digestive enzymes or absorbed so slowly that bacteria in host guts compete for them. Exogenous enzymes not only influence the absorption of nutrients but also produce nutrients for specific populations of bacteria through their action (Bedford and Cowieson, 2012). Therefore, their use has a direct impact on the microfloral populations (Apajalahti et al., 2004).

The most widely used feed enzymes are mixture of a variety of glycanases, and the single-using degrading enzyme is phytase (Ravindran and Son, 2011). Recombinant synthesized enzymes such as phytases and carbohydrases are commercially produced and sold as feed additives in monogastric food-animal production (Adeola and Cowieson, 2011). Phytase has significant effects on the digestibility of calcium, phosphorus, and minerals as well as the intestinal mucin production and the endogenous losses, all of which influence the nutrient supply and the intestinal environment which will alter the selection pressures on bacterial species (Bedford and Cowieson, 2012). Xylanase added to a wheat-based diet alleviates the pathological effects of *C. perfringens* in broiler chickens (Liu et al., 2012a). Dietary use of encapsulated lysozyme, as a feed additive in the diet of chickens significantly reduced the concentration of *C. perfringens* and gastrointestinal lesions due to the organism in the ilium (Liu et al., 2012b).

Despite the mature phytase market, other kinds of feed enzymes have different defects. Generally, the activity of the enzyme is low; the cost is high; and the production and quality control lack standards. The stability and activity of feed enzymes depend mainly on the production process (Slominski, 2011). It is well recognized that animal responses to feed enzyme additives are not entirely predictable, and these inconsistencies could be attributed *inter alia* to the enzyme type, the amount of enzyme applied, the presence of enzyme side activities, the diet composition and the animal variation (Ravindran and Son, 2011). The mechanisms by which feed enzymes influence the intestinal microbiota have been known for some time, e.g., through enhancing nutrient delivery to the host and by provision of fermentable oligosaccharides. It is still unknown, however, to which extent this effect contributes to the net benefit of the enzyme use, nor is it clear which are the major microbial species involved (Bedford and Cowieson, 2012). Recent work shows that if the use of enzymes is to replace prophylactic antibiotics, then the target must be to enrich the ileum and cecum with *Clostridium* cluster XIVa species and *E. coli* while depressing the numbers of *Lactobacillus* spp. (Smulikowska et al., 2010). Until an accurate description of a desirable flora can be documented, the feed enzymes can be appropriately used.

Since enzymes do not directly attack bacteria, but only reduce the substrates for the growth of bacteria, the antibacterial effect is not obvious. Glucose oxidase (GOD), mainly from *Aspergillus niger* and *Penicillium*, can specifically oxidize D-glucose to produce gluconic acid and hydrogen peroxide (Geisen, 1999). When hydrogen peroxide accumulates to a certain level, it can be harmful to intestinal bacteria and inhibits their propagation while consuming oxygen in the intestine, which may help the growth of *Bifidobacterium* and *Lactobacillus* and other beneficial anaerobic bacteria. An enzyme alginogel composed of GOD, lactoperoxidase, and guaiacol was able to prevent the formation of biofilms and inhibit the established biofilms (Cooper, 2013). Given that the mechanism of action of this enzyme is different from other feed enzymes, the related feeding effects of GOD are worthy of further study.

## PERSPECTIVES

Ideal alternatives to antibiotics should: (i) have non-toxic or no side effects on animals, (ii) be easy to eliminate from the body or consist of short term of residues, (iii) not induce bacterial resistance, (iv) be stable in the feed and animal gastrointestinal tract, (v) be easily decomposed and not affect the environment, (vi) not affect palatability, (vii) not destroy the normal intestinal flora of animals, (viii) kill or inhibit the growth of pathogenic bacteria, (ix) enhance the body resistance to the disease, (x) improve feed efficiency and promote animal growth, and (xi) have good compatibility. In fact, there are no alternatives to antibiotic that currently meet all the above mentioned requirements.

There is still a considerable gap between antibiotic alternatives and antibiotics concerning the effectiveness of disease prevention and growth promotion. Antibacterial vaccines are generally used for the prevention of bacterial infections, and currently only a small number of bacterial infective diseases can be controlled by vaccines. Immunomodulators and feed enzymes mainly preserve the health of animals, but do not directly kill or inhibit bacteria. Bacteriophages are currently only used in food, and the safety is still questionable. The composition of plant extracts and probiotics is complex and the quality in terms of stability is poor, resulting in varying effects and safety risks. Inhibitors targeting QS and virulence of bacteria are still in research with no approved products, and most QSIs are toxic to eukaryotic cells. Biofilm inhibitors show good results only when used in combination with antibiotics. Although AMPs can treat bacterial infections, the high cost and narrow antibacterial spectrum restrict their wide use, and they can still induce bacterial resistance. Meanwhile, proteinaceous compounds, for example, feed enzymes and AMPs that have been put into market as well as bacteriophage lysins, QS quenching enzymes and enzymatic biofilm inhibitors under development, are naturally unstable and easily degraded in the digestive tract. On the other hand, antibiotics can directly inhibit or kill bacteria with better antibacterial effect than all antibiotic alternatives. Moreover, antibiotics are made by single and relatively pure active ingredient with high stability, consistency, and quality ensured by good manufacturing practice. Considering clinical efficacy, humans have not yet found a more effective way than selecting appropriate antibiotics for the treatment of indicated bacteria.

Antimicrobial resistance is the major reason for EU to ban low dose of antibiotics as feed additive. In fact, the penicillin's producer, *Penicillium*, has coexisted with other bacteria for tens of thousands of years. Only after the extensive use of penicillin was

the emergence penicillin-resistance discovered. Drug pressure is proportional to the chance of development and dissemination of drug resistance; therefore, although antibiotic alternatives have not been associated with bacterial resistance yet, with a large number of these alternatives in use, bacteria might mutate eventually to develop resistance. Like the precedent use of antibiotics, the irregular and illegal use of antibiotic alternatives may also bring negative issues.

Meanwhile, we must not forget that "it's better to prevent than cure" (Bourlioux, 2013). For many developing countries, because of the poor farming environment and the high incidence of disease, antibiotics are still an effective tool in the prevention and control of animal diseases. It is shown that the ban of growth promoters in EU demands the improvement of the farm hygiene (Castanon, 2007). When the amount of "old" antibiotics used in feed reduces due to the ban, prevalence of bacterial infections in the target animals would likely increase without fundamental improvement of production environment. This may lead to the increase of therapeutic uses of advanced antibiotics and result in some unintended consequence that may cause new challenges for public health concern. Additionally, there are no scientific evidences to clearly separate the causal relationship between treatment use and prevention use of the antibiotics with respect to resistance development. The ultimate benefit and risk should be assessed before implementing such a policy as a result of political/social pressure, as bacteria may not necessarily "listen" to the policy-makers. Thus, the decision concerning the use of in-feed antibiotics should be made based on scientific approaches. The ban of antibiotics as growth promoters cannot be copied in every country of the world.

The efficacy of traditional antibiotics can still be improved. Some "old" antibiotics can find new bacterial targets and reinforce the anti-infectious therapy toward some MDR bacteria. It has been demonstrated that in many cases, there are non-carbapenem alternatives for the treatment of extended-spectrum-β-lactamase-producing *E. coli* (ESBL-Ec) infections (Fournier et al., 2013). Besides, new formulations can allow targeted drug delivery via nanoparticles and the association of molecules can reinforce the antimicrobial effect of antibiotics (Bourlioux, 2013). Furthermore, in empirical therapy, use of broad-spectrum bactericidal agents that will eradicate the presumed infective microorganism(s), which potentially could be MDR, should be preferred. Once an infection is under control and the culture and susceptibility results are reported, it is important to switch to the most suitable narrow-spectrum agent thus decreasing the potential of adverse drug effects and the risk of development of antibiotic-induced resistance (Lynch, 2012).

In summary, reasonable use of antibiotics and continuous development of alternatives to antibiotics are needed to ensure the long-term sustainable development of animal husbandry. We must strictly define the target animals, duration of the treatment and the withdrawal period, for prudent use of antibiotics as well as regulation/policy making regarding their use. At the same time, we must strengthen the supervision and enforcement of laws in order to control antibiotic resistance and residues from the food chain within established safe levels. Furthermore, we must improve the management of animal nutrition and production hygiene, since recent European developments showed a distinctly more positive outcome of the ban of antibiotic growth promoters than was anticipated during the first years after the ban due to the improvement of animal welfare (Cogliani et al., 2011). The research of antibiotics alternatives will be a long process. In addition to research and development of new efficient and safe alternatives, we should strengthen the study concerning the effects of combined use of antibiotics and their alternatives aimed at maintaining a healthy agricultural economy and preservation of potent antibiotics for efficacious therapy in humans.

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# Use of antibiotics as feed additives: a burning question

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## INTRODUCTION

Antibiotics are chemotherapeutic agents used for the clinical management of infectious diseases in humans, plants and animals. However a sizeable fraction of antibiotics produced every year all over the world is used for non-therapeutic purposes. In US alone, about 24.6 million pounds of antibiotics are used in animal agriculture annually and a substantial portion of this is used as growth promoters and not for the treatment of infections (Oliver et al., 2011). According to a recent report, out of 13 million kg of antibiotics administered to animals in 2010, the major portion was meant for promoting the growth of the livestock (Spellberg et al., 2013). The ability of low doses antibiotics to promote growth of animals and birds was discovered serendipitously in the 1940s (Gustafson and Bowen, 1997). Subsequently, it was widely exploited and by this time, addition of antibiotics to the animal feed to stimulate growth has turned into a global practice.

The basis of growth-promoting effect of antibiotics is not clearly known. It is postulated that microorganisms present in the animal feed consume a considerable portion of nutrients in the feed. They also inhibit absorption from the intestine and produce toxins having adverse effect on the health of the animals. The growth-promoting effect of antibiotics might stem from their ability to suppress these harmful organisms. It is also suggested that animals reared in the unhygienic environments always bear some latent infections, which trigger a cascade of events in their immune system. Cytokines produced in the process lead to the release of some catabolic hormones which cause wastage of muscles. Antibiotics relieve the animals of the need to produce cytokines by suppressing the causative agents of infections.

## BENEFITS ASSOCIATED WITH THE USE OF ANTIBIOTICS

Evidences available in the literature speak volumes on the beneficial effects obtained from antibiotics used as a feed additive. Pigs supplemented with antibiotics in their feed require 10–15% less feed to achieve a desired level of growth. Cost of feed constitutes a major portion of the expenses involved in rearing animals. Hence addition of antibiotics substantially cuts down the expenditure. Antibiotics added to the feed also ensure more efficient conversion of feed to animal product and improvement. The daily growth rate of animals subsisting on antibiotic-supplemented food is known to be improved by 1–10% compared to that of the animals provided feed without antibiotic. The meat obtained from antibiotic-fed animals is also of better quality with higher amount of protein and less amount of fat compared to that obtained from animals not supplemented with antibiotics (Hughes and Heritage, 2002). Use of tetracycline and penicillin in chicken feed led to a significant improvement in the production of eggs and hatchability besides feed efficiency (Gustafson and Bowen, 1997). Health of the livestock fed with antibiotic-mixed food is also markedly improved. Following addition of chlortetracycline and sulfamethazine to the feed, the rate of bovine respiratory disease morbidity, the rate of relapses and mortality and also the rate of animals diagnosed with chronic respiratory disease were found to be significantly decreased (Gallo and Berg, 1995). Benefits in terms of the rate and efficiency in the gain of body weight, decrease in mortality and morbidity and reduction in the occurrence of subclinical diseases, were observed using of antibiotics during all phases of growth of pigs (Cromwell, 2002). The adverse effects of inflammation and pro-inflammatory mediators in animals (e.g., reduction in growth, feed intake, reproduction, milk production, and metabolic health) are well-known. The anti-inflammatory potential of antibiotics (particularly macrolides) provides a rational basis of their beneficial effects which is independent of their antimicrobial effect (Buret, 2010). Hence there is no doubt about the important role of antibiotics in profitable and efficient production of livestock.

## RISKS ASSOCIATED WITH THE USE OF ANTIBIOTICS

On the other hand, use of antibiotics in animal feed as growth-promoters appears to promote emergence of antibiotic-resistant strains. The problem of bacterial resistance to antibiotics is a burning question throughout the world. According to an estimate of the World Health Organization, during the past decade number of deaths caused by some resistant strains exceeded the combined number of deaths caused by influenza, Human Immunodeficiency Virus and traffic accident (Yap, 2013). While emergence of antibiotic-resistant strains is most often correlated with the use of antibiotics, resistance is detected even in bacteria obtained from places which are uninhabited, thinly populated (Chattopadhyay and Grossart, 2010) and totally detached from human

intervention (Bhullar et al., 2012). In this backdrop, quite understandably the possibility that presence of antibiotics in the animal feed might contribute to the crisis, has triggered a vigorous controversy. It is widely believed that use of antibiotics as growth promoters promotes evolution and/or selection of antibiotic-resistant strains in animal farms. Reports published from time to time on the isolation of bacterial strains from animals, resistant to the antibiotics that are added to their feed, have fueled the debate further. It is also evident that the possibility of emergence of bacterial strains resistant to the therapeutically useful antibiotics for humans cannot be bypassed by substituting the antibiotics with their analogs in the animal feed. For example avoparcin is a glycopeptide antibiotic not used in humans. Use of this antibiotic as feed additive has been known to be associated with emergence of avoparcin- resistant strains, which are cross-tolerant to vancomycin, a glycopeptide antibiotic used in humans (Marshall and Levy, 2011) Transfer of resistance-conferring genes from the bacteria of animal origin to the bacteria of human origin has also been demonstrated in animal model (Moubareck et al., 2003). Dissemination of resistance is promoted even by sepiolite, a non-antibiotic feed additive, which facilitates horizontal gene transfer in the digestive tract of the animals (Rodríguez-Beltrán et al., 2013).

## THE CONTROVERSY

The proponents for the use of antibiotics in animal feed as growth- promoters however remain unconvinced about the potential of the practice to aggravate the problem of antibiotic resistance (Wallinga and Burch, 2013). They argue that the doses of antibiotics used for this purpose are small compared to their therapeutic doses and it is not definitely known whether such low doses really select for resistance or not. Even those, who accept that agricultural use of antibiotics promotes emergence of antibiotic-resistant strains, believe that evidence of this possibility having a major impact on human health is either non-existent or minimal (Turnidge, 2004). Notwithstanding the fact that bacterial isolates resistant to various antibiotics used in animals are found in humans, it is contended that such people might contract the infection from some other source and it is also possible that both the animals and the humans are infected with the same organism from a common source. Isolates from humans and animals in many cases are claimed to be genetically different (Phillips et al., 2004). The hypothesis on transmission of resistance through food chains is also not universally accepted. Those, who accept it, recommend good hygienic practices in the kitchen and use of vaccines in the birds and animals to reduce the incidence of transmission. Following a ban on the prophylactic use of antibiotics, an overall deterioration of animal health (in terms of diarrhea, weight loss and mortality) was observed in some cases. Hence a ban on the use of antibiotics in animals is believed to be associated with an increased incidence of food-borne diseases in humans as well as more frequent use of antibiotics for therapeutic purposes in animals (Casewell et al., 2003; Spellberg et al., 2013). Therefore restriction on the use of antibiotics as feed additive is considered unwarranted by the proponents. They firmly believe that advantages associated with use of antibiotics in animals outweigh the risks.

On the contrary, it has been demonstrated that exposure to sub-inhibitory concentration of some antibiotics can not only enrich resistant bacteria (Gullberg et al., 2011) but in some cases also stimulate the production of reactive oxygen species which might contribute to an increase in the rate of mutation and emergence of multidrug-resistant mutants (Kohanski et al., 2010). It was also shown that antibiotics in animal feed might facilitate phage-mediated gene transfer thus promoting dissemination of antibiotic resistance (Allen et al., 2011). Horizontal gene transfer, the major mechanism involved in dissemination of antibiotic resistance, is also fostered by sub-inhibitory concentration of some antibiotics (Couce and Blázquez, 2009). The number of food animals exceeds the number of humans by far. Hence use of antibiotics in animal farms poses a risk of creating a large reservoir of resistance genes, the far reaching consequence of which needs hardly to be over-emphasized (Turnidge, 2004). Adverse effects on the health and productivity of animals, observed following a ban on the use of antibiotics in animal feed by the European Union (EU), appeared to be diminished in course of time. Furthermore, the beneficial effects associated with the use of antibiotics were found to be waned in some particular cases (reviewed by Marshall and Levy, 2011). A systematic survey in Danish swine farms indicated improvement in the long-term productivity following decrease in the use of antibiotics in animal feed (Aarestrup et al., 2010). Besides being used as growth-promoters, antibiotics are also widely used for prevention and treatment of the infections of the livestock. Normal microbiota of the organism may be adversely affected by the antibiotics added to the feed. This phenomenon called dysbiosis may foster overgrowth of some already existing harmful microorganisms in the flora (e.g., *Clostridium difficile*), decreased production of short chain fatty acids and other beneficial compounds by the normal flora and increased susceptibility of the livestock to infections (Hawrelak and Myers, 2004). Thus the practice of addition of antibiotics to animal feed might be self-defeating.

## DILEMMA FACED BY THE POLICY MAKERS

Policy makers all over the world are in a quandary to formulate a guideline for the addition of antibiotics to the animal feed. The Guidelines for Industry issued by the Center for Veterinary Medicines of the Food and Drug Administration (FDA, 2012), USA recommend use of antibiotics only for the prevention, control and treatment of infections in animals but not for the promotion of growth, increased performance, and improved feed efficiency. Additionally, use of some antibiotics of critical importance (e.g., the third generation of cephalosporins) is restricted in animal agriculture and they are reserved only for use in humans. Development of suitable alternatives of antibiotics for the clinical management of the infections of the livestock appears to be the need of the hour (Allen et al., 2013). Search for prophylactic measures (e.g., vaccines) for the prevention of diseases is also of crucial importance. Unregulated sale and easy availability of antibiotics have significantly contributed to the problem in many developing countries, where antibiotics

are continued to be added to the animal feed as growth promoters. Moreover, unhygienic environment prevailing in the poultries and farm houses in these countries makes the animals more susceptible to infections and necessitates frequent use of antibiotics. Hence it is strongly advocated to do away with or minimize the use of antibiotics by improving the hygiene (Gulland, 2013). It might not be possible to impose a blanket ban (the approach based on precautionary principle adopted by the EU) on the use of antibiotics in animal farms in a global scale. But close monitoring of the situation is imperative everywhere to avert or restrain emergence of resistant strains. The "principle of proof" (gathering evidence before banning a particular compound), adopted by FDA seems to be a practicable approach to contain the conundrum. Recently, FDA has asked the antibiotic-manufacturers to relabel their products voluntarily in order to make people aware of its disapproval for the use of antibiotics as growth-promoters in animals. The label should also indicate that the use of antibiotics in animals must be supervised by a veterinarian. The proposal seeks to put an end to the use of medically useful antibiotics as growth-promoters and also to restrict the scope of prophylactic use of antibiotics in animals against pathogens. However it does not propose for any stricture on the use of non-human antibiotics (e.g., ionophores) in animals as growth-promoters. The initiative is highly appreciated by various organizations and eminent scientists though its success calls for the cooperation of the business lobby (Kuehn, 2014). Use of antibiotics in animal feed remains a highly-debated issue which calls for awareness among common people in the society.

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# Benefits and risks of antimicrobial use in food-producing animals

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Benefits and risks of antimicrobial drugs, used in food-producing animals, continue to be complex and controversial issues. This review comprehensively presents the benefits of antimicrobials drugs regarding control of animal diseases, protection of public health, enhancement of animal production, improvement of environment, and effects of the drugs on biogas production and public health associated with antimicrobial resistance. The positive and negative impacts, due to ban issue of antimicrobial agents used in food-producing animals, are also included in the discussion. As a double-edged sword, use of these drugs in food-animals persists as a great challenge.

**Keywords: antimicrobial drug, benefit, risk, animal food production, public health**

## INTRODUCTION

Over the decades, world population tremendously increased to 7 billion (Food and Agriculture Organization [FAO], 2012) and 870 million (12.5%) of them were estimated to be undernourished during 2010–2012 (Food and Agriculture Organization [FAO], 2012; Krehbiel, 2013). Demand for animal source food tends to be soaring day by day, especially in developing countries (Food and Agriculture Organization [FAO], 2009; Krehbiel, 2013). It is acknowledged that the antimicrobial use is one of the most successful chemotherapies. Various antimicrobials have made significant contribution for the prevention, control, and treatment of infectious diseases in animals since 1940s (Forman and Burch, 1947). Low and sub-therapeutic dose of antimicrobials plays very important role for the improvement of feed efficiency, promotion of animal growth, and prevention and control of the diseases (Dibner and Richards, 2005; Niewold, 2007). International market value of veterinary drugs (including antimicrobials) tremendously increased from $8.65 billion in 1992 to $20.1 billion in 2010 and in 2018, it is expected to increase to $42.9 billion (GIA, 2012; Reports, 2012).

It is undeniable that rational use of antimicrobials plays a vital role in the production of food animals and protecting public health, while irrational and irresponsible use may cause antimicrobial resistance. On the basis of "Swan Reports" in 1969, Great British took first action for the restriction of antibiotics, being used in animals or capable of cross-resisting with antibiotics used in human medicine. In 1973, the European Community (EC) commenced the withdrawal of some important antibiotic use as growth promoters in animal feed. After that, Sweden banned the use of all growth-promoting antibiotics in 1986. Avoparcin, bacitracin, spiramycin, tylosin, and virginiamycin were withdrawn as growth promoters in the European Union (EU) from 1995 to 1999, on the basis of precautionary principle. In 2006, all the uses of low-dose antibiotics (5∼40 ppm) in food animals, including flavomycin, avilamycin, salinomycin, monensin, and other animal-specific antibiotics, were banned in the EU with the intention to avoid their negative impact of resistant development (EPC, 2005; Marshall and Levy, 2011).

The benefits and risks of antimicrobials continue to be complex and controversial issues. The risks of antimicrobial drugs to public health associated with antimicrobial resistance raised great concern recently, while the benefits of antimicrobial drugs, such as prevention and treatment of animal diseases, protection of public health, enhancement of animal production, and improvement of environment, were disregarded most of the time. Many benefit-related claims have not yet been fully demonstrated in large-scale trials, and other trials revealed that the overall impact of the short-term benefits was poorly described. This article presents the benefits and risks of antimicrobials drugs used in food animals and discusses the positive and negative effects of the ban on antimicrobial growth promoters.

## BENEFITS OF ANTIMICROBIAL DRUGS

### PREVENTION AND TREATMENT OF ANIMAL DISEASES

With intensive animal production, bacterial and parasitic diseases became more and more frequent. According to an estimate, 80 types of bacteria, such as *Escherichia coli*, *Salmonella*, and *Clostridium welchii*, posed a serious threat to poultry industry. Mastitis, caused by *Staphylococcus aureus* in dairy animals, led to

a loss of $2 billion/year in the United States of America (USA) and an average cost of €485/dairy cow in the EU during 2012 (Heikkila et al., 2012). Due to infection caused by *Streptococcus pneumonia*, morbidity and mortality rates in calves increased to 40 and 20%, respectively (Akkermans and Vecht, 1994; Vogel et al., 2001). More than 50% of aquatic animals were infected by bacteria each year (Scarfe et al., 2011). *Vibrio vulnificus* became a potential health hazard for aquatic animals and human beings (Yano et al., 2004).

Approximately, 2169 parasites including 203 protozoa, 373 trematodes, 150 tapeworms, 404 nematodes, and 1030 arthropods have been found in livestock and poultry in China. About 4–20 billion dollars/year (8.3% annual output of animal husbandry) were lost due to parasitic diseases caused by coccidia, nematodes, ticks, and others in USA (Krausse and Schubert, 2010). Acute outbreaks of chicken coccidiosis paid a loss of 42 million pounds annually in the United Kingdom (Franklyn et al., 2010). In China, poultry industry had to face billions of dollars annual loss due to almost 100% chicken morbidity by coccidiosis (Zhang et al., 2013). Sheep helminthiasis led to a loss of 2.22 million dollars annually in Australia (Hosking et al., 2009; Larsen et al., 2009).

Over one hundred of antimicrobials, including β-lactams, aminoglycosides, tetracyclines, amphenicols, macrolides, sulfonamides, fluoroquinolones, lincosamides, polypeptides, and polyene, have been used in food-producing animals around the world. These antimicrobials have played an essential role in the prevention, treatment, and control of food animal diseases caused by pathogens, such as pathogenic *E. coli*, *S. aureus*, *S. pneumonia*, *Actinobacillus pleuropneumoniae,* mycoplasma, *Vibrio*, and others (Hoflack et al., 2001; Krausse and Schubert, 2010). It was reported that in USA, 52.1% of total antimicrobials were used for the treatment of infectious diseases in animals, where 90% of starter pigs, 75% of grower pigs, 50% of finisher pigs, and 25∼70% of cattle received the drugs through feed (Van Lunen, 2003; USFDA, 2009; GRACE, 2013). With a dose of 40 mg/kg, avilamycin in feed could remarkably decrease the incidence of diarrhea in post-weaning pigs (Partanen et al., 2007). When salinomycin was used in sows and pigs simultaneously, incidence of diarrhea in piglets was significantly reduced and the survival rate was increased by 13.95% (Nagaraja and Taylor, 1987). Sulfonamide and folic acid supplementation in diet increased live birth rate of piglets by 1% (Lindemann and Kornegay, 1989). Hence, it is concluded that the use of antimicrobials is a primary strategy for prevention and treatment of bacterial infections in food-producing animals.

Many antimicrobials have strong activity against parasites in animals. Use of sulfonamides in animals opened a new era of anti-parasitic drugs and made lots of parasitic diseases under control. Up till now, anti-parasitic drugs have shared about one-third sale of the global veterinary drug markets. Macrolides and benzimidazoles effectively controlled nematodes. Doramectin and ivermectin helped to prevent infection of *Argulus siamensis* in carp and *Labeo rohita* (Hemaprasanth et al., 2012). In rabbit, subcutaneous injection of ivermectin, at dose of 400 mg per kg, not only helped to clinical cure ear mite infection but also prevented loss of fur and thus, played a vital role for the improvement of fur production (McKellar et al., 1992).

Conclusively, due to unique advantages, such as exact targeting of pathogens, well-known mechanisms of activity and desired stability, antimicrobials justified their usage in livestock and poultry, and played important part for prevention and treatment of bacterial and parasite diseases.

## PROTECTION OF HUMANS AGAINST ZOONOSIS

Among animal infectious and parasitic diseases, more than 200 can affect human life. *Campylobacter* spp., *Salmonella* spp., *E. coli* O157, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila* from animals pose great health threat to both humans and animals (Altwegg and Geiss, 1989; Mellata, 2013). The United States Centers for Disease Control and Prevention (US-CDC) estimated that there were about 76 million annual cases of food-borne illness in USA, including 325,000 hospitalizations and 5000 deaths (Mead et al., 1999a). Annual cases of *Campylobacter* spp., *Salmonella* spp., *E. coli* O157, and *V. parahaemolyticus* were 1,963,000, 1,332,000, 62,500, and 5000, respectively (Mead et al., 1999a,b).

To some extent, antimicrobial agents guaranteed human food security and public health by controlling animal diseases and preventing transmission of zoonotic pathogens from animals to humans. When added to animal feed or drinking water, these drugs could significantly decrease the bacterial contamination in animal products. For examples, virginiamycin decreased the contamination of *Clostridium perfringens*, *Campylobacter* spp., and other food-borne pathogens in animal carcasses (Tice, 2001; Russell, 2003; Hurd et al., 2005). Salinomycin reduced infection of type C *Clostridium* in sows and weaning piglets by 43% (Nagaraja and Taylor, 1987). Neomycin in animal feed significantly reduced the number of *E. coli* O157: H7 in animal feces, and gentamycin reduced bacterial count in poultry eggs and meat (Elder et al., 2002; Doyle and Erickson, 2006). When cattle was fed with neomycin sulfate for 48 h and held for 24-h drug withdrawal period before slaughtering, it shed considerably less *E. coli* O157:H7 cells than those pen mates who did not receive the treatment (Elder et al., 2002). A farm-level study in 2008 by Ohio State University demonstrated that only 39% of hogs, raised on conventional antimicrobial operations, were infected with *Salmonella*, while those were 54% in case of antimicrobial-free operations (Nunes, 2008; AMI, 2010). Florfenicol (10 mg/kg) presented 100% efficiency for the treatment of *A. hydrophila* of *Piaractus mesopotamicus* (Carraschi et al., 2012). Oxytetracycline hydrochloride or norfloxacin in bait feed reduced the number of *A. hydrophila* in water by 46.86∼66.24%, indicating that the risk of the bacterial infection to humans be decreased.

## ENHANCEMENT OF ANIMAL PRODUCTION

In 1943, a few farmers in USA found that pigs fed with penicillin-fermented mixture grew faster (Wahlstrom et al., 1950; Hewes, 1955; Taylor and Gordon, 1955). In 1946, Moore found that low dose of streptomycin stimulated chick's growth (Moore and Evenson, 1946; Dibner and Richards, 2005). Subsequently, chlortetracycline, doxycycline, and sulfonamides helped growth promotion in calves, pigs, and chicken. Cunha from University of Florida and Stokstad from University of Washington

reported that penicillin in fermentation mixture functioned as a growth promoter for food-animals (Cunha et al., 1951). Legal use of the antimicrobials in feed has a history of over 60 years. Food and Drug Administration in USA (US FDA) approved these drugs as growth promoters for animals in 1951. Till 1978, 47.9% of the antimicrobials were used to be added in animal feed in USA. About 60% of poultry, 93% of chicken, 97% of growing pigs, and 80% of fattening pigs received antimicrobials through diet during the early 1990s. More than 40% of the drugs were added in animal feed at subtherapeutic level for improving animal production in USA during 1990s (Van Lunen, 2003). With a substantial contribution to the development of food-animal production at global level, veterinary antimicrobials tend to be necessities to cope with increasing food demand for humans.

Role of antimicrobials for the improvement of feed conversion ratio (FCR), animal growth, and reproductive performance has been well proven as given in **Table 1**, and discussed under following points (Cromwell, 2002). (1) Orally administered antimicrobials in pigs increased diet digestibility and improved feed utilization efficiency by 1.7∼5.1% and 6.9∼7%, respectively (Cromwell, 1999; Hardy, 1999; JETACAR, 1999; Van Lunen, 2003). (2) Addition of the drugs (e.g., chlortetracycline, sulfonamide, folic acid, carbadox, tilmicosin, tylosin, or sulfamethazine) in feed could remarkably improve the conception rate, farrowing rate, milk secretion, productive efficiency of sow, and live birth rate of piglet (Soma and Speer, 1975; Lindemann and Kornegay, 1989; Alexopoulos et al., 1998; Kantas et al., 1998; Weber et al., 2001; Partanen et al., 2007). (3) Feeding antimicrobials to pigs increased their weight gain by 1.9∼16.4% (Nagaraja and Taylor, 1987; Cromwell, 1999; JETACAR, 1999; Van Lunen, 2003; IFAH-EuroP, 2005). (4) Administration of antibiotics (bacitracin zinc, colistin sulfate, flavomycin, and florfenicol) in fish diet significantly improved the feed conversion and promoted their growth (He et al., 2011; Zhou et al., 2011). (5) Antimicrobial (tiamulin, nosiheptide,

**Table 1 | Part of evidences for the role of antimicrobials on feed utilization, growth promotion, reproductive performance, and carcass quality.**

| Example no | Reference | Drugs and animals | Parameters of animal production | Increase or decrease rate |
|---|---|---|---|---|
| 1 | Hardy (1999), Van Lunen (2003) | Antimicrobials to growing and fattening pigs | Digestion of energy | 5.10%↑ |
| | | | Digestion of nitrogen | 1.80%↑ |
| | | | Digestion of phosphorus | 3.40%↑ |
| 2 | Van Lunen (2003) | Antimicrobials to swine | Feed utilization | 7%↑ |
| | | | Average weight gain | 3.3–8.8%↑ |
| 3 | JETACAR (1999), Van Lunen (2003) | Antimicrobials to young pigs | Feed utilization | 4.60%↑ |
| | | | Average weight gain | 6.80%↑ |
| | | Antimicrobials to grower pigs | Feed utilization | 1.70%↑ |
| | | | Average weight gain | 1.90%↓ |
| 4 | Cromwell (1999), Van Lunen (2003) | Antimicrobials to piglet | Feed utilization | 6.90%↑ |
| 5 | IFAH-EuroP (2005) | Antimicrobials to food animal | Average weight gain | 4–5%↑ |
| 6 | Cromwell (1999), Van Lunen (2003) | Antimicrobials to piglet | Average weight gain | 16.40%↑ |
| 7 | Nagaraja and Taylor (1987) | Salinomycin to weanling piglets | Average weight gain | 15.82%↑ |
| 8 | He et al. (2011), Zhou et al. (2011) | (Bacitracin zinc, colistin sulfate, Flavomycin and florfenicol) to (Carassius, Carp or hybrid tilapia) | Average weight gain | 24.5∼40.87%↑ |
| 9 | Soma and Speer (1975) | Chlortetracycline to sow | Conception rate | 4.10%↑ |
| | | | Farrowing rate | 5.80%↑ |
| 10 | Lindemann and Kornegay (1989) | Folic acid to sow | Gestation gain | 18%↑ |
| 11 | Cromwell and Stahly (1985), Cromwell et al. (1984a,b), Lindemann et al. (1985) | Tiamulin, nosiheptide, and salinomycin to pig | Thickness of backfat | 9.7%↓ |
| | | | Thickness of total fat | 8%↓ |
| | | | Eye muscle area | 9.80%↑ |
| | | | Lean meat | 4.40%↑ |

↑ *Denotes increase, while* ↓ *denotes decrease.*

salinomycin, and tylosin) supplementation could also improve the carcass quality by decreasing the fat thickness and increasing the lean meat of food-producing animals (Cromwell et al., 1984a,b; Cromwell and Stahly, 1985; Lindemann et al., 1985; Van Lunen, 2003).

A lot of studies were carried out to find the mechanism involved in beneficial aspects of antimicrobials in animals. Jukes, Franti, and other scientists proved that the drugs attenuated intestinal wall and improved the digestibility of nutrients (Manson, 1968; Falkow, 1970; Jukes, 1970; Franti et al., 1971, 1972; Dibner and Richards, 2005). Midtvedt (1986) and Norin (1997) confirmed that oral doses of antimicrobials improved the structure of intestinal flora (Midtvedt, 1986; Norin, 1997). Salinomycin and avilamycin in feed improved the bioavailability of α-tocopheryl acetate in broilers by altering lipid absorption (Knarreborg et al., 2004). According to a previous review by Allen et al. (2013), antimicrobials have multifunctional role in animals, elaborated under following points: (1) these could reduce the colonization of intestinal bacteria and inhibit the growth of pathogenic microorganisms; (2) by decreasing the thickness of mucous membrane, led to more absorption of nutrients and reduced fermentation; (3) they directly neutralized the host immune response. In short, antimicrobials could affect the host intestinal flora, intestinal physiology, and immune system, and consequently, prevent disease, improve feed conversion, and enhance the growth of animals (Niewold, 2007). Till now, there are no appropriate alternatives which can replace antimicrobial growth promoters, in case those remain banned. Although numerous feed additives, mainly pre- and pro-biotic products, are commercially available now and seem to have potential to replace these growth promoters, but their true efficacy and mechanism of action in domestic animals remain unclear because of some inconsistent experimental results (Gaggia et al., 2010; Allen et al., 2013). Additionally, lack of safety evaluation and poor stability also limited the practical use of pre- and pro-biotic as feed additives.

## IMPROVEMENT OF ENVIRONMENT

According to a report of Center for Food Safety (CFS, 2013) and Food and Agriculture Organization (FAO, 2006), housing stress, due to over-crowding of animals, creates sweeping and devastating impacts on the natural and human environment leading to global warming, land degradation, air and water pollution, and loss of biodiversity. Livestock waste is one of the major sources of greenhouse gases, as the abnormal fermentation of gastrointestinal tract contents can produce lots of methane, ammonia, carbon dioxide, as well as stench gases (e.g., nitrate, ethylene acid, methyl mercaptan, hydrogen sulfide, methylamine, and trimethylamine). Fecal waste of animals generally contains 24% protein and 6.1∼17.96% amino acids. Nitrogen and phosphorus in the waste lead to environmental pollution, water eutrophication and ecological imbalance.

Some antimicrobials in feed could inhibit the abnormal fermentation and consequently, reduce the emission of greenhouse gases (mainly $CH_4$). For example, ionophores (monensin, lasalocid, and salinomycin), amoxicillin, ovoparcin, nigericin, or laidlomycin inhibited rumen microbial fermentation at different levels and thus, reduced the proportion of volatile fatty acids (VFA) and methane (Van Nevel and Demeyer, 1995; Fellner et al., 1997; Domescik and Martin, 1999). Since the mid-1970s, ionophorous antibiotics have been widely used as feed additives in ruminants due to their favorable effects on rumen fermentation and methane reduction (Kobayashi, 2010). Due to the efficacy and affordable price, ionophores have widely been used to reduce methane emission from livestock (Hook et al., 2010; Kobayashi, 2010). When ionophores (monensin and lasalocid) were mixed with rumen microorganisms *in vitro*, these inhibited methane by 50 and 44%, respectively and decreased $NH_3$ by more than 50% (Russell and Jeraci, 1984). In rumen models, ionophores (monensin, lasalocid, and salinomycin) inhibited 10∼20% lipolysis and biological hydrogenation (Van Nevel and Demeyer, 1977, 1995). The effects of the antibiotics on the abatement of methane production may be attributed to a selective antimicrobial action on rumen microbes (protozoa, ruminococci, streptococci, and lactobacilli). Addition of monensin and lasalocid in cow forage killed 82.5 and 76.8%, respectively, of the intestinal ciliated protozoa in rumen and hence, reduced the production of methane and VFAs (acetic acid and propionic acid) by ciliates (Guan et al., 2006). Generally, the gas production was reduced from 4 to 31% by monensin (Schelling, 1984; Rumpler et al., 1986; Kobayashi, 2010). A recent report has indicated that long-term administration of monensin in dairy cattle steadily reduced methane by 7% and this reduction persisted for 6 months with no adverse effect on milk yield (Odongo et al., 2007). However, previous studies also found that both, the methane level and protozoal number, returned to baseline after long-term administration of high concentration of the antibiotic (Guan et al., 2006; Odongo et al., 2007; Hook et al., 2010). The efficiency of monensin supplementation, for reducing methane output in ruminants, appeared to be different in the degree of abatement depending on the diet and animal used (Guan et al., 2006; Odongo et al., 2007; Hook et al., 2010). Effect of the drug on the methane levels in rumen was closely related to the ciliated, protozoal population. Microbial consortia, like protozoal population, in the ruminant gut may adapt to the antibiotics leading to the recovery of methane production yield, in case of a long-term usage. Therefore, the ciliates in the rumen may impact the outcome of antimicrobial supplementation, with adaptation being a possibility (Guan et al., 2006; Hook et al., 2010).

Through manure application, antibiotics got released into soil and could be absorbed by plants in arable land. Certain species of plants have the ability to bio-accumulate sulfamethoxine in their roots and stems, and this bioaccumulation was often higher in roots than in stems (Sarmah et al., 2006). Low concentrations of chlortetracycline and oxytetracycline in the soil media could markedly affect plant growth and development (Sarmah et al., 2006). However, there was a large variation in sensitivity among plant species to the soil used as the growth media (Sarmah et al., 2006).

When residues seeped into water, certain antimicrobials also played significant role in the prevention of water eutrophication for aquatic animals. For example, chlortetracycline, lomefloxacin hydrochloride, and sulfamethoxazole strengthened the absorption of nitrogen and phosphorus in water by aquatic plants, and chlortetracycline effectively removed 25% of water nitrate and

nitrogen (Dodds et al., 1991; Jones, 2010). On the other hand, the presence of antibiotic residues in environment may cause some adverse impacts, like acute and chronic toxicity, during early life stages of different aquatic organisms (Halling-Sorensen et al., 2003; Sarmah et al., 2006).

## NEGATIVE IMPACT OF ANTIMICROBIAL BAN

Ban on addition of sub-therapeutic antimicrobials in feed appeared to result in a certain extent of recovery of some bacteria and some unintended impacts on animal health and welfare (Drouin, 1999; Rose et al., 1999; Lovland and Kaldhusdal, 2001; Jensen et al., 2003). Emergence of *E. coli* and *Lawsonia intracellularis* infection in post-weaning pigs was significantly increased and consequently, the morbidity and mortality due to diarrhea were considerably increased (Casewell et al., 2003; Dibner and Richards, 2005). In Denmark, the mortality rate in weaning piglets increased from 2.7% (before the ban) to 3.5% (after the ban) and the morbidity rate of enteric infections in post-weaning pigs increased by 600% (Casewell et al., 2003; Dibner and Richards, 2005). In Sweden, chronic infections due to *E. coli* and *L. intracellularis* became more common and the mortality in weaning pigs increased by 1.5% (Wierup, 2001; Casewell et al., 2003).

To control animal diseases and to keep animals healthy, more therapeutic antimicrobials had to be used after the ban. It was reported that the usage amount of therapeutic antimicrobials in Denmark increased by 33.6%, from 48 tons/year in 2001 to 125.5 tons/year in 2010 (DANMAP, 2010). The increased amount of therapeutic antimicrobials was equal to or even more than the total quantity of antimicrobials being used before the ban (Phillips, 1999; Casewell et al., 2003; Phillips et al., 2004a,b; Turnidge, 2004; DANMAP, 2010). As shown in **Figure 1**, therapeutic use of tetracycline, penicillins, and macrolides markedly increased from 28.5, 16.4, and 13.4 tons in 2001 to 35.55, 27.1, and 16.8 tons in 2010, respectively (Phillips, 2007; DANMAP, 2010).

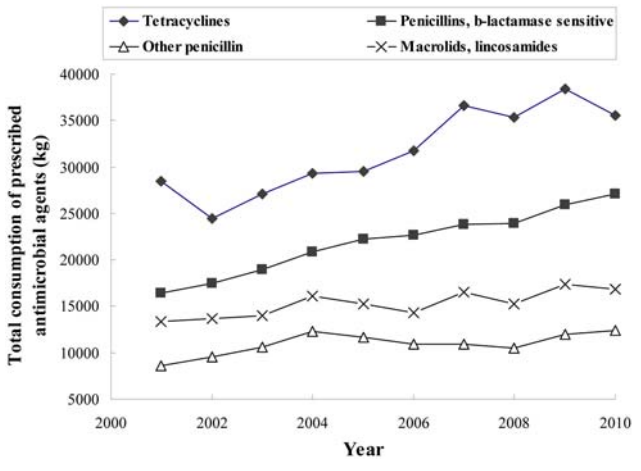


**FIGURE 1 | Estimated total consumption (kg) of prescribed antimicrobial agents for production animals in Denmark (data obtained from DANMAP, 2010).**

Withdrawal of low-dose antimicrobial use as feed additives may increase the level of pathogens such as *Salmonella* spp., *Campylobacter* spp., *Clostridium* and *E. coli* O157 in animal gut, boost the contamination of food and environment, and hence, enhance the opportunities for humans to be infected by these pathogens. Population of *Campylobacter* in broilers fed without antimicrobials was threefold higher than that in the broilers fed with any antimicrobials (Heuer et al., 2001). Incidence of food-borne *Campylobacter* in EU has been increased after the ban issue. Recovery of *Clostridium* from animal meat was also significantly increased in EU after its ban policy (Jones, 2000; Poduval et al., 2000). As a consequence, clostridial infections resulted in an outbreak affecting large human population in Demark and pointed out the high level of threat to public health (DANMAP, 2010). It is known that clostridial necrotic enteritis in animals is suppressed by some of the banned antimicrobials (e.g., virginiamycin). In the absence of these antibiotics, the bacterial population may increase in animal guts and colonization may lead to poor weight uniformity and fragile intestines in pigs and chicks. During food processing, infected animal carcasses could be the sources of contamination for food-borne pathogens and thereby, jeopardize food hygiene (Tice, 2001; Russell, 2003). Bacterial contamination of meat may, therefore, increase the risk of human infections. Contrary to EU, incidence of food-borne diseases in USA was declined by 23% in 1996 and among those, the infections due to *Campylobacter* and *Salmonella* were decreased by 30 and 17%, respectively. It was believed that ban of virginiamycin in USA might annually contribute to the death of 40,000 people, infected by *Campylobacter* (Cox, 2005).

The ban may lead to increased food-borne infections and elevated usage of therapeutic antimicrobials in both animals and humans. It is noteworthy that therapeutic use of antimicrobial agents in animals has a close relationship with the drugs used in humans with respect to the types of drugs used (Cook, 1999). The increased therapeutic use in animals may contribute to a worse, drug-resistance scenario both in animals and humans. It was noted that clinical isolates of vancomycin- or teicoplanin-resistant *Enterococci* from humans were very uncommon and the cases of quinupristin/dalfopristin-resistant *Enterococcus faecium* were very rare before the ban. Similarly, resistant *E. faecium* burden markedly increased and became a big challenge after the ban (Phillips, 2007).

After the European ban on growth-promoting antibiotics, it was found that FCR (total kg of feed used per grow out/total kg of live weight per grow out) in broiler was decreased by 0.016 kg/kg from November 1995 to May 1999 (1.78–1.796) in Denmark. Feed efficiency raised to a higher value of 1.83 immediately after the restrictions and more than 1.84 in late 1999 (Emborg et al., 2002; Dibner and Richards, 2005). Average daily gain of weaning piglets in Denmark was decreased from 422 g in 1995 to 415 g in 2001 (Casewell et al., 2003; Dibner and Richards, 2005). Production of broilers, cattle, and dairy cows was significantly decreased in 2006, as shown in **Figure 2**. In Sweden, weight gain of post-weaning piglets was reduced and feed costs were significantly increased after the abolishment of growth-promoting antimicrobial agents (Wierup, 2001; Casewell

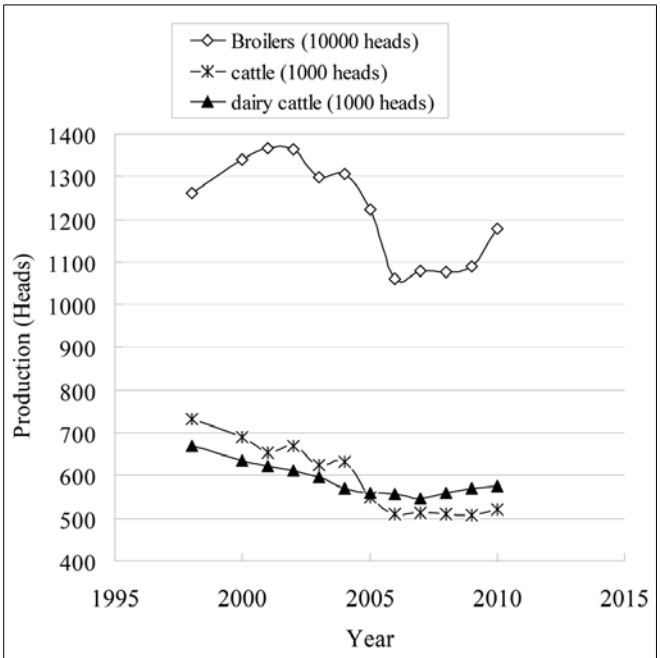


**FIGURE 2 | Production of food animals in Denmark from 1998 to 2010 (data obtained from DANMAP, 2010).**

et al., 2003). Even after 10 years, aquaculture production in Sweden was unable to return to the past level (Casewell et al., 2003).

If the use of antimicrobials is banned in USA, FCR may decline by 5% and more than 1100 $km^2$ area is required to plant corn and soybeans to meet the demand of feedstuff production. Consequently, required facilities for livestock and poultry production will be correspondingly increased by 100 million $m^2$, and farming area will also be increased by 500 million $m^2$ to obtain the current level of animal production (Casewell et al., 2003). It was estimated that withdrawal of antimicrobial growth promoters might lead to a loss of 5∼10 or even 40 dollars/pig in USA (Matthews, 2001). It does not seem to be a high cost for the developed countries but what will be the consequences if these antimicrobials are banned worldwide? It was also investigated that 25% of the current poultry industry and $3 billions would be additionally required to attain the current annual animal production (Rodehutscord et al., 2002; IFAH-EuroP, 2005). In conclusion, keeping in view the ever-increasing world population and its demands, more food animals should be raised to meet the food supply demand in the case of growth-promoting antibiotics remain prohibited and this increased number of animals will again lead to the increase in greenhouse gas emission and deeper environmental pollution.

## RISKS OF ANTIMICROBIALS

### INHIBITION OF BIOGAS PRODUCTION

Following the wide use of antimicrobial drugs in intensive animal production for growth promotion and prevention or treatment of disease, a large proportion of ingested drugs are excreted in manure and end up with livestock wastewater. Excreted antibiotics in the environment may partially inhibit methanogenesis in anaerobic waste-storage facilities, commonly used at Concentrated Animal Feeding Operation (CAFOs), and thus, decrease the rate at which bacteria metabolize animal waste products (Loftin et al., 2005; Sarmah et al., 2006).

During the anaerobic digestion of livestock waste, certain antimicrobials, including amoxicillin, aureomycin, oxytetracycline, thiamphenicol, florfenicol, sulfadimethoxine, and tylosin, had inhibitory effects on methane production (Lallai et al., 2002; Sun et al., 2009; Shi et al., 2011; Amin et al., 2012). However, no inhibitory effect but a stimulus for methane production was observed during anaerobic digestion of piggery wastewater in the presence of 10 mg/L florfenicol, amoxicillin, aureomycin, and sulfadimethoxine, while only the combination of high concentration of certain antimicrobials (130 mg/L florfenicol, 210mg/L amoxicillin, 10mg/L doxycycline, and 210 mg/L sulfadimethoxine) could decrease the methane production rate (Sun et al., 2009). Biogas volume, produced from per unit weight of biomass, was decreased with increasing concentrations of antibiotics, such as oxytetracycline, amoxicillin, and tylosin, and the inhibitory concentrations of oxytetracycline, amoxicillin, and tylosin were 8000, 9000, and 9000 mg/L, respectively (Amin et al., 2012). Only high concentration of thiamphenicol (160 mg/L), amoxicillin (120 mg/L), tetracycline (50 mg/L), and sulfamethoxydiazine (50 mg/L) had inhibitory effect on biogas production in the anaerobic digestion of pig waste slurry (Lallai et al., 2002; Shi et al., 2011). Actually, it is too difficult to attain those high concentrations of antibiotics in the excreta.

### ANTIMICROBIAL RESISTANCE CONCERNS

Misuse and overuse of antimicrobial may culminate in the development of drug-resistant pathogens resulting in poor response to treatment. Long-term and low-level exposure to antimicrobials may have greater selective potential than short-term and full-dose therapeutic use. A study observed that the percentage of tetracycline resistance genes in the fecal flora of conventionally raised feedlot steers was significantly higher than that in fecal samples from antimicrobial-free cattle (Harvey et al., 2009). Additionally, use of single antimicrobial may induce cross-resistance to antimicrobials used for animal and human medical therapy. For example, chlortetracycline use in growth rations was associated with ampicillin and tetracycline resistance in generic fecal *E. coli,* isolated from swine farms (Varga et al., 2009). Addition of chlortetracycline and sulfamethazine in cattle feed may be associated with higher prevalence (three to four fold greater than the control) of ampicillin- and tetracycline-resistant *E. coli,* isolated from the feces of treated animals (Alexander et al., 2010). Therefore, how to use antimicrobials, for effective treatment of bacterial and parasitic infections in food-producing animals, became the most important question for their use by avoiding the resistance development.

Regarding public health risk, more concern has been raised for the use of antibiotics in animals that may represent a potential threat to human health because the resistant pathogens in animals may transmit to humans and cause treatment failure of human medicines. A longitudinal study of the relationship

between antimicrobial-resistant *E. coli* from human wastewater and swine fecal samples reported that the use of injectable (e.g., ceftiofur sodium) or oral (e.g., chlortetracycline) antibiotics may contribute to the high levels of *E. coli* resistance in swine and human isolates. Thus, slaughter plant workers may be at higher risk of carrying multidrug-resistant *E. coli* as compared to workers dealing with other animals (Alali et al., 2008). A study analyzed the correlation between *E. coli* isolates from human blood stream and food-producing animals (poultry, pigs, and cattle) for the prevalence of antimicrobial resistance in 11 countries during 2005–2008. Results revealed that there were strong and significant correlations between the strains from animals (especially poultry and pigs) and humans for resistance to multiple drugs (especially ampicillin, aminoglycoside, and fluoroquinolone; Vieira et al., 2011). However, there is not enough and direct evidence to support the hypothesis that a large proportion of resistant isolates in humans be derived from animal source foods. Some recent studies suggested that some resistant isolates from humans were more easily transmitted from companion animals (having close contact) or birds (by bird droppings especially during their migration; Haenni et al., 2012; Loncaric et al., 2013). The resistant bacteria may also be released into the environment by humans and then transferred into new hosts in the environment (Hower et al., 2013; Verkade and Kluytmans, 2013). Based on the results of some current studies, Dr. Hurt pointed out that public health risk due to infected animals need more attention than antibiotic resistance concern (Hurd et al., 2008, 2012). Therefore, further efforts are required for the risk assessment of antimicrobial use in food animals to check their potential impact on public health.

### POSITIVE EFFECTS OF ANTIMICROBIAL BAN

Aim for the withdrawal of antimicrobial agents used was to prevent humans and animals from drug resistance. The ban on antimicrobial growth promoters led to decreased drug resistance in some bacteria. For example, according to report from the DANMAP, substantial reductions (from 80 to 2%) in the prevalence of vancomycin-resistant enterococci (VRE) were observed after ban of avoparcin as the growth promoter during 1995 and 2010 (DANMAP, 2010). *E. faecium* and *Enterococcus faecalis* are two of the most common *Enterococci* species and in Europe, only vancomycin-resistant *E. faecium* (VREF) is highly prevalent in poultry (Werner et al., 2008). VREF was still present as a threat in the food chain even after 15 years of the EU ban on avoparcin and could be detected in 47% of the broiler feces with a selective enrichment method (Garcia-Migura et al., 2007; DANMAP, 2010).

The ban on tylosin as the growth promoter had a remarkable effect on the level of macrolide (erythromycin) resistance in *Campylobacter coli* (most common *Campylobacter* species in pigs) from pigs as it decreased from 66 to 20% in Denmark between 1998 and 2005 (Hammerum et al., 2007). However, DANMAP data showed that during 2006–2010, macrolide resistance in *C. coli* varied within the range of 10–20% without significant reduction (DANMAP, 2010). In contrast to EU, macrolides (e.g., tylosin) in USA had been approved for usage in food-producing animals as growth promoter for decades. Macrolide resistance in *C. coli* isolated from poultry, although higher than that in *C. jejuni*, has no significant change (5–20%) between 2002 and 2010.

Use of enrofloxacin in poultry was withdrawn by US-FDA in 2005 because it was supposed to induce fluoroquinolone resistance in *Campylobacter* and *Salmonella* from poultry and contribute to the antibiotic treatment failure in humans [Food and Drug Administration (FDA), 1998, 2002; Nelson et al., 2007]. After withdrawal of enrofloxacin from poultry, the rate of fluoroquinolone resistance in *Campylobacter* and *Salmonella* had been reduced in chicken during 2005–2007 (NARMS, 2010). Human clinicians also observed a reduction in domestically acquired *Campylobacter* and *Salmonella* infections with decreased susceptibility to fluoroquinolones, and it was thought to be a great achievement regarding public health (Nelson et al., 2007). However, the incidence rate of fluoroquinolone-resistant *C. jejuni,* from chicken breast, again increased (15.2∼22.5%) in 2008–2010. Similarly, fluoroquinolone-resistant *Campylobacter* from broilers was also raised from 5.3 to 8.6% and from 0.8 to 7.7% in Denmark and Japan, respectively (CIPARS, 2008; JVARM, 2008; DANMAP, 2010; NARMS, 2010).

## CONCLUSION AND PERSPECTIVES

Definite targeting of pathogens, well-known mechanisms of activity, and preferable stability for administration are the unique advantages of antimicrobial use in food-animals for the prevention and treatment of bacterial and parasitic diseases, improvement of animal production performance, and protection of environment and public health. Withdrawal of antimicrobial use from food-producing animals may bring adverse effects on the production of food derived from animals and thus, on public health. EU banned only low-dose antibiotics (5∼40 ppm) for their use as growth promoters in food animals. Until now, without helping for the cause, it led to some negative effects on food animal production and public health. What will happen if the antimicrobial agents are banned worldwide, especially in some developing countries with rapid increase in human population and food demand?

As a double-edged sword, non-rational uses of veterinary antimicrobials may result in pressure selection of antimicrobial resistant pathogens which may endanger both the animal and public health. Additionally, the presence of antibiotic residues in the environment, associated with overuse of antimicrobial drugs, may adversely influence the manure treatment systems by inhibition of biogas production. An economic analysis about use and withdrawal of antimicrobial growth promoters in USA revealed that the withdrawal may cause increased cost ($10/person) for food consumption and antibiotic-resistant infections cost the US healthcare system an excess of $20 billion ($60/person) annually (APUA, 2010). However, it is unknown that how much of these $20 billion is due to antimicrobial resistance associated with their use in food-producing animals.

Recently, the US FDA has also proposed restrictions on the antimicrobial growth promoters because some available information and evidences suggested that the sub-therapeutic use may increase the risk of antimicrobial resistance. To make wise

strategies for controlling antimicrobial resistance and effectively respond to the public health concerns associated with drug resistance, FDA believes that it is very important and imperative to consider how antimicrobial drugs are being used and how to address their injudicious uses in nature. Only rational use and effective regulation can ensure a benefit–risk balance of the antimicrobial application in animal production. However, long-term policies will be required for the international regulation of antibiotic use in food-producing animals.

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# Carbadox has both temporary and lasting effects on the swine gut microbiota

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Antibiotics are used in livestock and poultry production to treat and prevent disease as well as to promote animal growth. Carbadox is an in-feed antibiotic that is widely used in swine production to prevent dysentery and to improve feed efficiency. The goal of this study was to characterize the effects of carbadox and its withdrawal on the swine gut microbiota. Six pigs (initially 3-weeks old) received feed containing carbadox and six received unamended feed. After 3-weeks of continuous carbadox administration, all pigs were switched to a maintenance diet without carbadox. DNA was extracted from feces ($n = 142$) taken before, during, and following (6-week withdrawal) carbadox treatment. Phylotype analysis using 16S rRNA sequences showed the gradual development of the non-medicated swine gut microbiota over the 8-week study, and that the carbadox-treated pigs had significant differences in bacterial membership relative to non-medicated pigs. Enumeration of fecal *Escherichia coli* showed that a diet change concurrent with carbadox withdrawal was associated with an increase in the *E. coli* in the non-medicated pigs, suggesting that carbadox pre-treatment prevented an increase of *E. coli* populations. In-feed carbadox caused striking effects within 4 days of administration, with significant alterations in both community structure and bacterial membership, notably a large relative increase in *Prevotella* populations in medicated pigs. Digital PCR was used to show that the absolute abundance of *Prevotella* was unchanged between the medicated and non-medicated pigs despite the relative increase shown in the phylotype analysis. Carbadox therefore caused a decrease in the abundance of other gut bacteria but did not affect the absolute abundance of *Prevotella*. The pending regulation on antibiotics used in animal production underscores the importance of understanding how they modulate the microbiota and impact animal health, which will inform the search for antibiotic alternatives.

**Keywords: carbadox, antibiotics, microbiome, phylotype, 16S rRNA, digital PCR**

## INTRODUCTION

Antibiotics are used in animal agriculture for both therapeutic and non-therapeutic applications (Animal Health Institute, 2012). Appropriately high doses of antibiotics are administered to treat or prevent disease (therapeutic use), and relatively low doses are typically used to improve feed efficiency (non-therapeutic use). These differing doses of antibiotics are important because they are at the core of regulatory efforts, with many countries banning or regulating non-therapeutic veterinary antibiotics that have human medical importance (European Union, 2003; FDA, 2012). This is because low-dose antibiotics can result in bodily concentrations of antibiotics that are subinhibitory to bacteria. Indeed, it is likely that both therapeutic and non-therapeutic doses of antibiotics can lead to subinhibitory antibiotic concentrations for some host-associated bacteria. Subinhibitory antibiotic concentrations are undesired because they can have adverse effects, in particular enhancing the selection for antibiotic resistance genes and their horizontal transfer (Barbosa and Levy, 2000; Smith et al., 2002; Barlow, 2009; Brewer et al., 2013), thus promoting the antibiotic resistance problem in animal and human pathogens.

Carbadox is a quinoxaline-di-N-oxide antibiotic compound that is fed to almost a third of nursery-age pigs in the US to control enteric diseases and improve feed efficiency (USDA, 2007). Medicated early weaning, including carbadox, is credited with nearly eradicating the enteric pathogen *Brachyspira hyodysenteriae* in domestic swine [cause of swine dysentery (Stanton et al., 1999)]. Carbadox inhibits bacteria by intercalating DNA and causing mutations, and this mutagenic property has led to its ban in many countries (Beutin et al., 1981; Chen et al., 2009). The current US regulation includes a 42-day withdrawal period prior to slaughter to prevent carbadox residues in the carcass (Joint FAO/WHO Expert Committee on Food Additives, 2003). It is unclear if it will be further regulated in the US because carbadox is not an antibiotic of human clinical importance (FDA, 2003).

We are interested in carbadox because of its importance to the US swine industry and its unknown effects on swine gut bacteria. One specific collateral effect of carbadox is the induction of prophages or prophage-like gene transfer agents, as has been shown *in vitro* in Shiga toxin-producing *Escherichia coli* (Kohler et al., 2000), *Salmonella enterica* serovar Typhimurium (Bearson et al., 2014), and *B. hyodysenteriae* (Stanton et al., 2008). In addition to these results, research in our lab on total swine fecal

phages suggested that prophages were induced in pigs that were fed either carbadox or ASP250 (penicillin, chlortetracycline, sulfamethazine) (Allen et al., 2011). Further identification of the effects of carbadox on the swine gut microbiome could lead to a greater understanding of its mechanism of growth promotion.

Here we analyzed the bacterial component of the swine fecal microbiota in samples taken prior to and during carbadox treatment, as well as periodically during the 6-week withdrawal period. We found that carbadox altered bacterial membership and community structure relative to non-medicated pigs, including a reduction in total bacteria. This study is an important step toward defining the effects of carbadox on the swine gut microbiome, which in turn will lead to informed alternatives to this antibiotic.

## MATERIALS AND METHODS

### SWINE

Piglets were acquired and managed in accordance with the National Animal Disease Center Animal Care and Use Committee guidelines, as previously described (Allen et al., 2011). At 3 weeks of age, 12 piglets from 2 litters were divided into two rooms of six pigs each, with equal representation of littermates and gender. All pigs were fed a standard starter diet (TechStart® 17-25, Kent Feeds, Muscatine, IA) *ad libitum* for 3 weeks, after which six control pigs continued to receive non-medicated feed while the other group received feed containing carbadox (50 g/ton). After 21 days of continuous feed with or without carbadox, all pigs (60 days old) were switched to a non-medicated maintenance diet (Pork Finisher diet, Kent Feeds). The age of pigs receiving carbadox and transitioning to maintenance diet are consistent with standard industry practices.

Feces were collected from each pig at multiple times before, during, and after antibiotic withdrawal (**Figure 1**), and DNAs were extracted with the PowerBiome DNA Isolation Kit using the manufacturer's protocol (Mo Bio Laboratories, Solana Beach, CA, USA).

### 16S rRNA GENE SEQUENCING

Amplification of the V1-V3 region of bacterial 16S rRNA genes from individual samples was carried out as previously described (Allen et al., 2011). Primers 8F (5′-AGAGTTTGATCCTGGC TCAG) (Weisburg et al., 1991) and 518R (5′-ATTACCGCGGCT GCTGG) (Muyzer et al., 1993) were designed with an eight-nucleotide unique sequence barcodes (Hamady et al., 2008; Allen et al., 2011). PCRs were performed for 22 cycles, and the products were separated by gel electrophoresis and purified using the MinElute kit (Qiagen Inc., Valencia, CA). Amplicons were sequenced on a 454 Genome Sequencer (GS) FLX using the manufacturer's protocol for Titanium chemistry (Roche Diagnostics, Branford, CT).

### SEQUENCE ANALYSIS

Sequence data that passed Roche's quality thresholds were processed by AmpliconNoise (Quince et al., 2011), mothur (Schloss et al., 2009, 2011), and Uchime (Edgar et al., 2011) to denoise sequencing data, remove barcodes, and reduce sequence artifacts produced during PCR. The Schloss analysis pipeline SOP was followed (Schloss et al., 2011). Briefly, mothur's implementation of the AmpliconNoise program reduced sequencing and PCR artifacts, then sequences were aligned to the Silva bacterial database (Quast et al., 2013). Screen.seqs, filter.seqs, and pre.cluster commands were performed in mothur to improve the quality of the dataset, and Uchime was used to remove chimeras (using the sequences of each sample as their own reference). OTU-based phylogenetic analysis (97% similarity cutoff) and hypothesis testing were performed with normalized data in mothur (Schloss et al., 2009). Data were normalized by subsampling each sample to 3454 reads and unless otherwise stated, and samples were analyzed by time and treatment (**Figure 1**). To visualize changes in community structure, the Bray-Curtis dissimilarity statistic (OTU data) and Pearson's correlations (vectors of environmental variables) were calculated and plotted by nonmetric multidimensional scaling (NMDS) in PAST (Hammer et al., 2001).

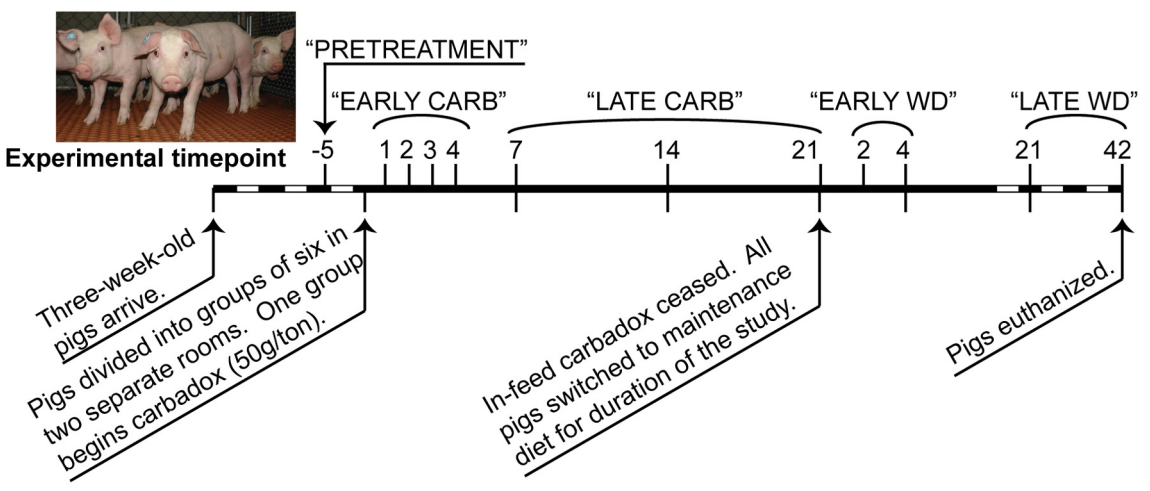


**FIGURE 1 | Timeline of carbadox feed-trial.** Sample days are noted above the thick line, and dates are noted below. Dashed region not to scale. Timepoints that were grouped for some statistical analyses are labeled along the top. CARB, carbadox; WD, withdrawal.

Community metrics such as diversity (Shannon index, inverse Simpson index), evenness (Heips index), and richness [best parametric model in CatchAll (Bunge, 2011; Bunge et al., 2012)] were calculated in mothur based on the OTU data. Multiple *t*-tests were performed on community metrics in GraphPad Prism v. 6.02 (La Jolla, CA). Statistical significance was determined using the Holm-Sidak correction for multiple comparisons when comparing medicated vs. non-medicated at a timepoint, or using the Kruskal-Wallis test with Dunn's multiple comparison correction when comparing the all days of the carbadox group to pre-treatment (alpha = 5%). The resilience index was calculated from Shannon, Heips evenness, and inverse Simpson diversity indices using the formula described by Shade et al. (2012) based on the diversity index of the microbiota of the carbadox-treated animals at pre-treatment, at the most significantly changed time (day 7), and at the first time that was not significant from pre-treatment (day 14). Samples were analyzed by treatment per timepoint, or by treatment per range of time (pre-treatment, early carbadox exposure [days 1–4], late carbadox exposure [days 7–21], early withdrawal [days 2 and 4], and late withdrawal [days 21 and 42]; **Figure 1**). For most statistics, samples were compared between pigs in the medicated and non-medicated groups at each time.

Taxonomic assignments of the 16S rRNA gene sequences were made using the Ribosomal Database project (RDP) web tools, with training set version 9.0 (Cole et al., 2009). The Metastats statistical software was used in mothur for making comparisons between samples and identifying trends (White et al., 2009). Analysis of similarities (ANOSIM) was also performed in mothur to test whether difference between groups were significant.

Linear discriminant analysis effect size (LEfSe) was performed using the LEfSe web tool on taxonomic assignments from RDP's sequence classifier (Cole et al., 2009; Segata et al., 2011). The LEfSe program was used to identify indicator organisms most likely to explain the differences between treatment groups with a logarithmic cutoff value of linear discriminant analysis (LDA) > 4.0.

### QUANTIFICATION OF PREVOTELLA (BY digPCR AND qPCR)

Digital PCR (digPCR) counts the number of target molecules in each DNA sample, enabling reliable estimates of the absolute number of target molecules in the original sample (feces), without the need for an internal standard. Digital PCR was performed to estimate the number of copies of *Prevotella* 16S rRNA genes in fecal DNA from carbadox-fed and non-medicated animals at day 4 of carbadox treatment (early exposure). The Quantstudio 3D digPCR system (Life Technologies) was used according to the manufacturer's recommendations and each sample was run in triplicate. *Prevotella*-specific digPCR primers were adapted from Mieszkin et al. (2009): Bac32 Fm, 5′AACGCTAG CTACAGGCTTAAC; Bac108R, 5′ CGGGCTATTCCTGACTATG GG; Bac82Probe, 5′ **6-FAM**-ACGGGTGAG/**ZEN**/TAACGCGT ATCCAAC-**IBFQ** (fluorophore and quenchers in bold). All reactions were performed using QuantStudio 3D Digital PCR Master Mix (Life Technologies, Carlsbad, California) following manufacturer's recommended cycling conditions. Each 15 μL reaction consisted of 1.0 μM of each primer, 0.2 μM probe, 1.0 ng or 0.1 ng DNA. *Prevotella* cells per gram of feces were calculated using the DNA quantity extracted from 0.5 grams of feces, and assuming a *Prevotella* spp. average of two 16S rRNA gene copies per cell [average of 58 *Prevotella* genomes available on the Integrated Microbial Genomes (IMG) website (Markowitz et al., 2012)].

Quantitative PCR (qPCR) was performed on the same samples as digPCR to evaluate the relative amount of *Prevotella* to total 16S rRNA gene copies (all bacteria). *Prevotella*-specific primers were used (F, 5′CGGGTTGTAAACTGCTTTTATGAAG; and R, 5′CGCTCCCTTTAAACCCAATAAA) as previously described (Okabe et al., 2007). Universal bacterial primers targeting the 16S rRNA gene (341F, 5′ CCTACGGGRSGCAGCAG; and 529R, 5′ ACCGCGGCKGCTGGC) (Baker et al., 2003) were used to amplify all bacterial 16S rRNA genes. All reactions were done using iTaq Universal SYBR Green Supermix following manufacturer's recommended conditions (Bio-Rad, Hercules, California). Each 20 μL reaction consisted of 0.01 ng fecal DNA and the *Prevotella* or universal primers at 0.2 μM or 0.5 μM, respectively. Relative quantification was calculated using the Pfaffl method (Pfaffl, 2001) and *t*-tests were performed in PAST (Hammer et al., 2001).

### *E. COLI* VIABLE CELL POPULATIONS

One gram of fresh feces was suspended in 10 ml LB broth. Samples were vortexed vigorously to make a slurry for 10-fold serial dilutions in phosphate buffered saline. Duplicate dilutions were plated on MacConkey agar medium (March and Ratnam, 1986) and incubated at 37°C. Lactose positive colonies (fecal coliforms, predominantly *E. coli*) were enumerated on the countable dilution the following day.

### DATA PRESENTATION

A *P*-value less than 0.05 with a *q*-value (false discovery rate) less than 0.05 was considered significant, and *R* between 0 and 0.3 was considered a slight correlation while *R* greater than 0.3 was considered a correlation. Data are deposited in NCBI's Short Read Archive (SRA) under accession numbers SAMN02645017-SAMN02645066 and are associated with BioProject PRJNA237795.

## RESULTS

### CARBADOX ALTERS BACTERIAL MEMBERSHIP EARLY IN THE TREATMENT

We first examined differences in bacterial membership at the phylum and genus taxonomic levels during the first week of carbadox treatment. The results showed that the *Firmicutes*, *Proteobacteria, Elusimicrobia*, *Planctomycetes*, and *Lentisphaerae* phyla were of lower relative abundance, while the *Bacteroidetes* phylum was of higher abundance in the medicated animals ($q < 0.03$) (**Figure 2**). Interestingly, *Bacteroidetes* populations increased in the medicated animals proportionately to the decrease in *Firmicutes* populations (∼17% change). This early carbadox-mediated transition to a *Bacteroidetes*-dominant microbiota was also seen when comparing early medicated animals to pre-treatment (∼25%). Genus-level taxonomic assignments were also analyzed, revealing significant differences between the medicated and non-medicated animal microbiotas during the early carbadox time points. Many genera showed a relative decrease

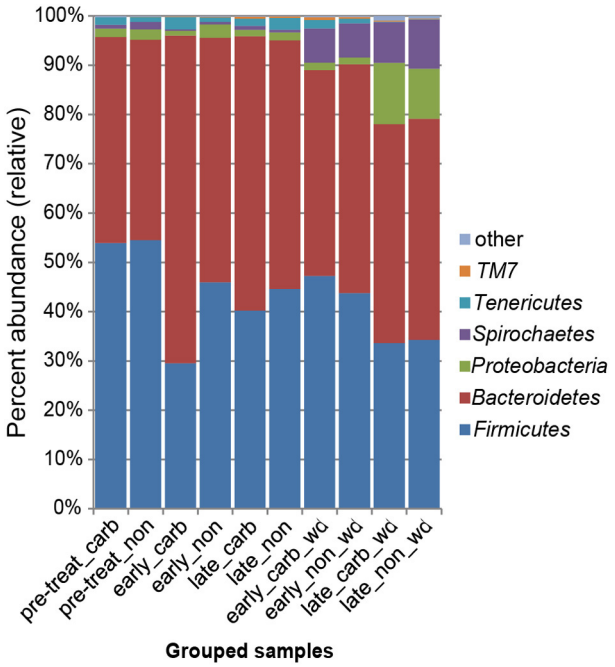


**FIGURE 2 | Bacterial phyla representation in the microbiota, based on taxonomic inference of bacteria (16S rRNA sequences).** Data are pooled by treatment and period of time. Carb, carbadox; wd, withdrawal; non, non-medicated.

with antibiotic treatment (Figure S1), most of which belonged to the *Firmicutes* phylum ($q < 0.05$). The relative increase in *Prevotella* spp. was reflected in the increase in *Bacteroidetes* in the carbadox-treated animals ($q < 0.05$).

The LEfSe analysis was used to resolve bacterial taxa associated with antibiotic treatment, which could define biomarkers for carbadox effects. This analysis explains why biological samples differ using tests of consistency and effect size estimation (Segata et al., 2011), with the results suggesting members of the community that benefit from or contribute to the community-wide effects described above. The results showed four genera to be enriched in early samples from carbadox-fed animals: *Prevotella*, *Roseburia*, *Faecalibacterium*, and *Asteroleplasma* (**Figure 3** and Figure S2). *Lactobacillus* was enriched in samples from the early non-medicated animals (**Figure 3** and Figure S2). This indicates that a few key members of the community could be the drivers of community dynamics.

## CARBADOX CAUSES RAPID CHANGES IN BACTERIAL COMMUNITY STRUCTURE

Comparisons of community structures apply broad measures of similarities among bacterial communities in their entirety, allowing inferences to be made about populations as a whole. The effects of dietary changes on bacterial community structure were first analyzed via OTUs binned at the 97% similarity level. Estimates of the total number of OTUs (bacterial richness) during the early treatment period were significantly lower for the communities in medicated animals (661 ± 55 vs. 962 ± 96, respectively; **Figure 4**). Additional measures of alpha diversity (Shannon diversity, Heips evenness, and inverse Simpson indices) of samples from medicated animals compared to non-medicated animals were significantly different at 2, 3, and 4 days after continuous carbadox, but not different in either late carbadox or at any time during the withdrawal period (Figure S3). Analysis of the community structure yielded further support, showing significant differences at days 3 and 4 of early carbadox treatment ($[R = 0.32, p = 0.015]$ and $[R = 0.54, p = 0.003]$, respectively), but not before starting antibiotic treatment ($p = 0.82$; **Figure 5**). Communities from medicated animals at the remaining time points did not clearly separate from those of the non-medicated animals. The alpha diversity indices of the microbiota from the carbadox-treated animals at each sample time were compared to their pre-treatment values to determine when the microbiota recovered. Significant differences were revealed from the pre-treatment diversity at days 2, 3, 4, and 7 but at no other times, including the withdrawal period ($p < 0.01$). The swine gut microbiota showed similar resilience in response to carbadox disturbance regardless of the diversity index used to calculate it (0.16, 0.17, 0.19 from the Heips evenness, inverse Simpson, and Shannon diversity indices, respectively). Together these data show that carbadox caused an initial decrease in both bacterial richness and evenness, and that the swine gut bacterial community structure recovered after 1 week of carbadox initiation despite the continued presence of carbadox.

## COMPARISON OF ABSOLUTE AND RELATIVE DIFFERENCES IN MICROBIOTA MEMBERSHIP

Because of the altered microbial diversity and reduction of richness early in the antibiotic treatment, digital PCR was used to evaluate whether statistically significant changes in the bacterial community of medicated animals was due to absolute changes in bacterial membership or due to reductions of carbadox-sensitive organisms. The relative abundance of *Prevotella* was most significantly impacted by carbadox and so was chosen for analysis by digPCR to determine if this was an absolute or relative change in the *Prevotella* population. Analysis of early carbadox samples (day 4) showed no significant differences in *Prevotella* counts ($LOG_{10}$) per gram of feces between medicated (10.0, $SE = 0.11$) and non-medicated animals (9.8, $SE = 0.26$). This suggests that observed differences in *Prevotella* sequence data are due to decreases of other bacterial species rather than to increases in absolute numbers of *Prevotella*. To verify the results of the phylotype analysis and to compare with the digPCR results, qPCR was performed on the same day 4 samples. Quantitative PCR of *Prevotella* abundances, relative to total 16S rRNA genes, confirmed a 1.8-fold increase in the relative abundance of *Prevotella* in the carbadox-fed animals ($p < 0.05$). These data demonstrate the value of using digPCR to resolve the absolute abundance of taxa that show relative changes in high-throughput phylotype analyses.

## CARBADOX ABROGATES *E. COLI* POPULATION SHIFTS DURING A DIETARY CHANGE

Culturing bacteria directly from feces also yields absolute counts, but selecting individual groups requires *a priori* knowledge and

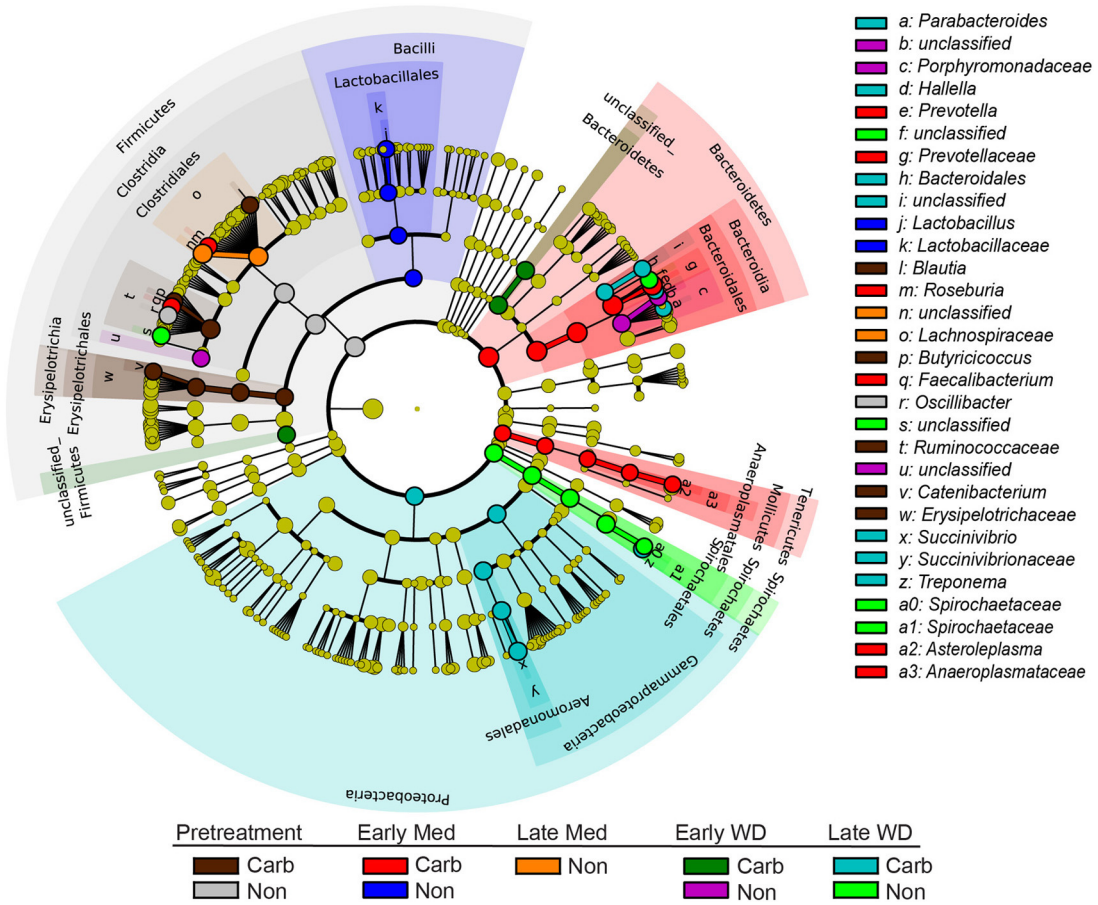


**FIGURE 3 | Taxonomic tree showing biomarkers that were significantly enriched with either antibiotic exposure or lack of exposure over time.** The LEfSe-generated tree shows bacterial nodes (diameter is relative to abundance in community) with a taxonomic hierarchy. Samples were grouped together as either carbadox-treated (Carb) or non-medicated (Non-med) over the period of the study as pre-treatment, early exposure, late exposure, early withdrawal, late withdrawal. No biomarkers were identified for the late carbadox samples. The cutoff value of linear discriminant analysis (LDA) > 4.0.

available selective media. Because carbadox is often used to prevent enteric diseases in young swine, we cultured *E. coli* from fecal samples at different experimental time points to monitor this species for potential enteric pathogens. No significant differences in *E. coli* colony forming units (CFUs) were observed during the carbadox-treatment portion of the study or late in the withdrawal period (Figure S4). Interestingly, *E. coli* CFUs were significantly different between the medicated and non-medicated groups on day 2 after the withdrawal of carbadox, with the difference being driven by increased *E. coli* CFUs in samples from non-medicated animals at that time (Figure S4). The diet for all animals was switched (consistent with industry practice) concomitant with the withdrawal of carbadox (see Methods), and this result suggests that the change in diet caused a brief increase in *E. coli* populations that was prevented in the pigs previously fed carbadox.

## CARBADOX MODIFIES THE DEVELOPMENT OF THE SWINE GUT MICROBIOTA

This study involved a time series that encompassed 21 days of antibiotic treatment and 42 days following its withdrawal, enabling the examination of the development of the swine gut microbiota. Members of the *Firmicutes* phylum were relatively more abundant at the beginning of the study (54% of total community) than at the end of the withdrawal period (34%) ($q < 0.01$; **Figure 2**). An increase in the relative abundance of other phyla [*Proteobacteria, Spirochaetes, Planctomycetes, Fibrobacteres, Synergistetes* ($q < 0.01$)] was observed over the 9-week study. Much of this maturation happened late in the experiment regardless of antibiotic treatment. However, carbadox treatment did exert some significant changes on the microbiota even after its withdrawal. Analysis of community structure revealed significant differences between the medicated and non-medicated groups late in the withdrawal period (21 days [$R = 0.49$, $p = 0.001$] and 42 days [$R = 0.54$, $p = 0.001$]), but not in the early withdrawal period (days 1, 2, 3, or 4) (**Figure 5**). This suggests significant changes in bacterial membership over time because there were no significant differences in comparisons of community metrics, such as richness and diversity, during the withdrawal period (**Figure 4** and Figure S3). The LEfSe analysis suggested bacterial members were relatively increased with these late-withdrawal differences, including *Succinivibrio*, *Hallella*, and *Treponema* in the

late-withdrawal medicated animals and *Spirochaetaceae* in the late non-medicated animals (**Figure 4**). Taken together, these results show that the swine gut bacterial community changes over time, and that carbadox influences these microbiotas even several weeks after its removal.

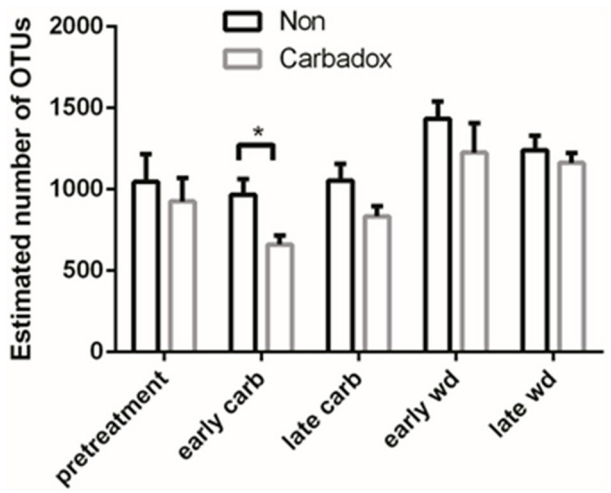


**FIGURE 4 | Operational Taxonomic Unit (OTU) estimates, averaged by treatment and time.** Bars indicate the standard error around the mean. Multiple comparisons star indicates significant difference at timepoint.

## DISCUSSION

Here we define short- and long-term effects of in-feed carbadox and its withdrawal on the swine intestinal microbiota. Carbadox is one of the most common antibiotics used in the US swine industry, with indications for both disease prevention and feed-efficiency improvement. We are interested in defining the effects of carbadox to inform both its mechanism-of-action and non-antibiotic alternatives (with similar effects on production performance). Carbadox was administered continuously for 3 weeks, constituting an ecological press rather than pulse disturbance to the microbiota (Shade et al., 2012). The results show that carbadox immediately and significantly altered the bacterial community, but it did not show the same effects from 1 to 3 weeks of continual administration. This demonstrates that the swine gut microbiota was initially disturbed by carbadox, but the microbial community structure recovered despite the continued presence of carbadox. Interestingly, the discontinuation of carbadox resulted in enduring effects at the species level but not on community metrics such as diversity, suggesting that carbadox altered the membership but not the structure of the community. Similar dynamics were observed in a study of a human undergoing press β-lactam therapy, which showed an immediate reduction in *Firmicutes* populations after 6 days, a reduction in overall species richness, and subsequent reestablishment of *Firmicutes* populations after 2 weeks (Perez-Cobas et al., 2013). Furthermore, analysis of physiochemical and microbial variables in a batch reactor has shown that microbial communities can

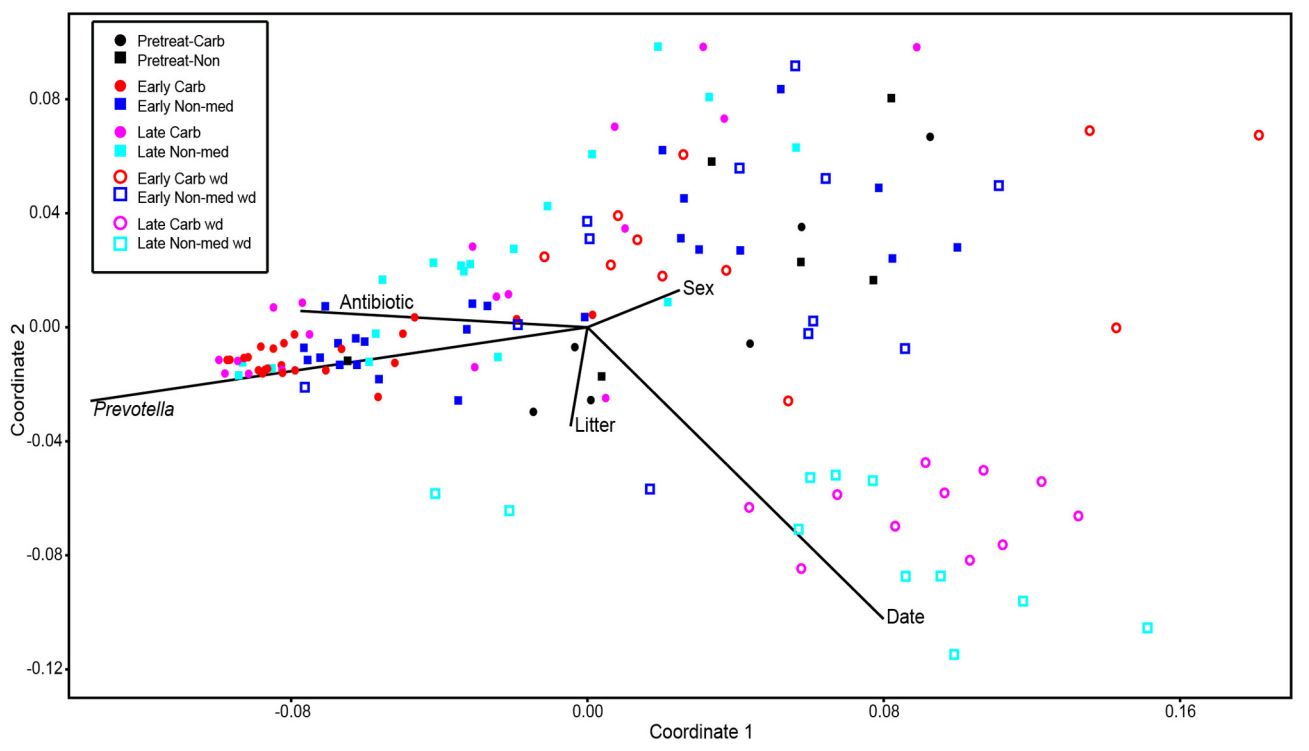


**FIGURE 5 | Non-metric multidimensional scaling analysis of OTU-based bacterial 16S rRNA gene sequence abundances in individual pig samples.** Environmental variables are plotted as vectors. The length of each vector is arbitrarily scaled, so only their directions and relative lengths should be considered. Stress proportion = 0.1656.

enter alternative stable states that have consequences on ecosystem processes (Burgmann et al., 2011). Further work is needed to determine how the carbadox-altered microbiota interacts with the host and how this relates to feed efficiency.

The most dramatic bacterial change was the relative increase in the *Prevotella* population during the first 4 days of carbadox exposure. However, based on the digPCR results, this was not due to an absolute increase in the *Prevotella* population, which was unchanged compared with samples from non-treated pigs, but rather to a reduction in other members of the microbiota. *Prevotella* is a well-studied swine commensal bacterium and has been identified as one of the most abundant genera in the pig intestine (Leser et al., 2002; Lamendella et al., 2011; Looft et al., 2012). The relative number of intestinal *Prevotella* has been shown to decrease after amoxicillin exposure (Mozes et al., 2013), suggesting that the antimicrobial effect on this genus is specific to the antibiotic being administered. *Prevotella* spp. metabolize recalcitrant food, such as hemicelluloses and pectin in the swine intestinal tract, which is important to animal health because fermentation end products, from these substrates, supply the host with a large portion of its energy supply. *Prevotella* was identified with *Roseburia* and *Faecalibacterium* as biomarkers that increase in relative abundance soon after carbadox administration. These bacteria are metabolically complementary since *Prevotella* produces acetate, and *Roseburia* and *Faecalibacterium* consume acetate to produce butyrate (Duncan et al., 2004a,b). Butyrate is a short chain fatty acid (SCFA) that has been shown to benefit host health (Flint et al., 2012; Furusawa et al., 2013). Additionally, links between bacterial fermentation products and host energy regulation suggest bacterial roles for improved feed efficiency. A recent study of fructo-oligosaccharides and other soluble fibers showed an intestinal microbiota shift in mice (increased *Bacteroidetes* populations and decreased *Firmicutes*) that resulted in increased SCFA production, specifically acetate, propionate, and butyrate (De Vadder et al., 2014). This led to increased concentrations of propionate in the blood, which induced intestinal gluconeogenesis to benefit glucose and energy homeostasis (De Vadder et al., 2014). Although the relative *Prevotella* increase in response to carbadox was relatively short-lived, and not an absolute change, studies of food-producing animals suggest that small health advantages at key production stages (e.g., weaning, transport, etc.) can convey significant performance improvements over time and at slaughter (Alexopoulos et al., 2004).

The health benefits of microbial SCFA production has been well documented, and certain diets and antibiotic alternatives have been shown to be particularly good modulators of SCFAs. Interestingly, studies on the host health- and microbiota-modulating effects of some prebiotics, such as dietary fiber, have shown microbiota shifts similar to what was shown in the present study with carbadox. The prebiotics arabinoxylan and inulin similarly caused a relative increase in *Prevotella*, *Roseburia*, and *Faecalibacterium* populations, thereby increasing propionate and butyrate production (Ramirez-Farias et al., 2009; Scott et al., 2014). *Prevotella* spp. also had relative increases after soluble fiber and non-starch polysaccharides were added to pig feed, and this diet was also associated with increased abundance of butyryl-coenzyme A (CoA) CoA transferase gene copies in feces (Metzler-Zebeli et al., 2010). It is as yet unclear if these microbiota shifts are related to the improved feed efficiency observed with agricultural antibiotics such as carbadox, but further studies are warranted.

One unexpected discovery was that carbadox abrogates a potential bloom in *E. coli* populations as a result of a diet change. Previous results from our lab and others have suggested that increased *E. coli* populations are a collateral effect of some ecosystem disturbances, including antibiotics (Janczyk et al., 2007; Looft and Allen, 2012). An abrupt change in diet has been shown to increase *E. coli* O157:H7 shedding in sheep (Kudva et al., 1997) and in pigs, diet change and weaning is associated with increase susceptibility to enterotoxigenic *E. coli* infection, a leading cause of post-weaning diarrhea (Wu et al., 2007). In the present study, we found that the initial carbadox disturbance caused no such *E. coli* increase. However, due to the pigs advancing age over the course of our extended experiment, the diet was changed from a standard nursery diet to a grower/maintenance diet (consistent with industry practice) at the same time that carbadox was withdrawn. This dietary change caused an increase in *E. coli* populations in the non-medicated animals, but not in the pigs that had previously been fed carbadox, demonstrating a protective effect of prior carbadox administration.

In contrast to the potential modulation of SCFA production described above, other commonly accepted mechanisms of how in-feed antibiotics improve feed efficiency are related to bacterial disease suppression, the reduction of the host's microbial load, or both (Dibner and Richards, 2005; Mathew et al., 2007). Our study showed a significant reduction of bacterial species richness after 4 days of continuous carbadox administration, confirming that the bacterial load is reduced albeit temporarily. Reduced bacterial richness has also been observed with the use of other in-feed antibiotics such as ASP250 (chlortetracycline, sulfamethazine, and penicillin) in swine (Allen et al., 2011). Regarding the potential mechanism of disease suppression, an intriguing result from our study is that the family *Spirochaetaceae* was lower in the carbadox-treated animals than the matched non-medicated animals 42 days after the withdrawal of carbadox. The *Spirochaetaceae* family includes *Brachyspira hyodysenteriae*, which is the causative agent of swine dysentery and one of the primary reasons carbadox is used in pig production in the US (Stanton et al., 1999). As efficacious alternatives to in-feed antibiotics continue to be explored, these data are a reminder of the need to diversify the antibiotic alternative armament with specific tools of targeting pathogens, such as vaccines, in addition to feed additives that are general modulators of bacterial community structure (Allen et al., 2013).

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## SUPPLEMENTARY MATERIAL

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# Antibiotics in Canadian poultry productions and anticipated alternatives

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The use of antibiotics in food-producing animals has significantly increased animal health by lowering mortality and the incidence of diseases. Antibiotics also have largely contributed to increase productivity of farms. However, antibiotic usage in general and relevance of non-therapeutic antibiotics (growth promoters) in feed need to be reevaluated especially because bacterial pathogens of humans and animals have developed and shared a variety of antibiotic resistance mechanisms that can easily be spread within microbial communities. In Canada, poultry production involves more than 2600 regulated chicken producers who have access to several antibiotics approved as feed additives for poultry. Feed recipes and mixtures vary greatly geographically and from one farm to another, making links between use of a specific antibiotic feed additive and production yields or selection of specific antibiotic-resistant bacteria difficult to establish. Many on-farm studies have revealed the widespread presence of antibiotic-resistant bacteria in broiler chickens. While some reports linked the presence of antibiotic-resistant organisms to the use of feed supplemented with antibiotics, no recent studies could clearly demonstrate the benefit of antimicrobial growth promoters on performance and production yields. With modern biosecurity and hygienic practices, there is a genuine concern that intensive utilization of antibiotics or use of antimicrobial growth promoters in feed might no longer be useful. Public pressure and concerns about food and environmental safety (antibiotic residues, antibiotic-resistant pathogens) have driven researchers to actively look for alternatives to antibiotics. Some of the alternatives include pre- and probiotics, organic acids and essential oils. We will describe here the properties of some bioactive molecules, like those found in cranberry, which have shown interesting polyvalent antibacterial and immuno-stimulatory activities.

**Keywords: growth promoters, non-therapeutic antibiotics, alternatives to antibiotics, cranberry, c-di-GMP, poultry production, broilers**

## INTRODUCTION

Since the discovery of penicillin by Fleming in 1928, several antibiotics which can be classified based on their molecular targets in bacteria (cell wall, protein synthesis, nucleic acids, folic acid metabolism) have been marketed for the treatment of infectious diseases both in animals and humans. The agents used in the treatment of animals and humans often belong to the same classes of antibiotics having similar modes of action and bacterial cell targets. This interface brings a variety of problems and worries. Bacteria developing resistance to these drugs in animals may be transmitted to humans or spread their mechanisms of resistance, which may eventually be found in human pathogens. Such a situation may lead to the loss of therapeutic efficacy in both veterinary and human medicine.

It is evident that antibiotics substantially improved public health. For example, since their discovery about 70 years ago, antibiotics have greatly reduced mortality and morbidity associated with infectious diseases and have increased life expectancy around the world. In addition to their therapeutic use, antibiotics also are deployed in animals for prophylaxis and growth promotion (improvement of animal zootechnical parameters). For example, antibiotics such as ceftiofur (a third generation cephalosporin), bacitracin (polypeptide) and virginiamycin (streptogramin) are used in poultry production to respectively prevent and control infections (respiratory diseases and necrotic enteritis) and to improve food conversion and body-weight gain. The use of antibiotics as growth promoters was adopted in the 1940s when animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues showed improved performances (Castanon, 2007). It has been estimated that antibiotic growth promoters in animals, through unspecific and not well defined mechanisms, improve bodyweight by 5–6% and feed efficiency by 3–4%, with the most pronounced effects observed in young animals (Butaye et al., 2003). However, the deployment of antimicrobial agents can change the bacterial environment by eliminating susceptible strains, and only allowing antibiotic resistant bacteria (i.e., those with higher fitness) to survive (O'Brien, 2002). Antimicrobial agents may thus modify

the intestinal microflora and create a favorable environment for establishment of resistant and pathogenic bacteria. Accordingly, positive associations were found between the presence of certain virulence genes and antibiotic resistance determinants (Aslam et al., 2012; Johnson et al., 2012). The impact of antimicrobial growth promoters on the development of antimicrobial resistant bacteria has been the subject of several reports and led to their ban in the European Union in 2006.

The poultry industry has grown and improved in recent years due to the continuous integration of various disciplines for production such as poultry health, nutrition, breeding, husbandry, and knowledge of poultry products (Anonymous, 2007). For example, in 1928, the average broiler required 112 days and 22 kg of feed to reach 1.7 kg. Since 1990, broilers required about 35–42 days and 4 kg of feed to reach 2 kg (National Research Council, 1999). Even though this improvement could be attributable in part to antibiotics, relevance of their use as growth promoters in feed needs to be re-evaluated. With modern broiler production practices, a broiler body weight of 1.8 kg can be reached by using 3.2 kg of feed in 35 days without addition of any antibiotic in feed (Diarra et al., 2007). In this chapter, we will review the use of antimicrobial agents in the Canadian poultry industry and discuss public health issues and concerns related to antibiotic resistant bacteria. We also will explore possible alternatives that could be developed in respect to food and environmental safety as well as to public and animal health and welfare.

## ANTIBIOTIC SELECTIVE PRESSURE

The use of antibiotics as growth promoters is negatively perceived because pathogenic bacteria of humans and animals have developed and shared a variety of antibiotic resistance mechanisms that can be easily spread within microbial communities. Nowadays, worldwide spread of antibiotic resistance mechanisms resulting from selective pressures (use of antibiotics) has undeniably reduced treatment options and therapeutic efficacy in human medicine. However, the relative responsibility of selective pressures occasioned by human medicine, veterinary or agricultural practices is still unclear. Furthermore, metagenomic studies have established some links between resistance mechanisms found in microorganisms from the environment and the clinic (Perry and Wright, 2013), making even more difficult the identification of the primary cause of selective pressure and support arguments for multiple sources of antibiotic resistance genes (Lupo et al., 2012).

Transformation and conjugation are mechanisms accommodating gene transfer among bacteria and are believed to play important roles in the rapid spread of antibiotic resistance (Chen et al., 2005). In addition, the horizontal transfer of mobile genetic elements also contributes to the evolution of emerging pathogens through dissemination of virulence genes. A variety of genetic materials, such as plasmids, can participate to this evolution (Carattoli, 2013). Moreover, integrative and conjugative elements (ICEs) can be disseminated through transferable elements like conjugative plasmids but can also integrate into the genome of new bacterial hosts (Burrus and Waldor, 2004). Transposons are also other mobile genetic elements that can contain antibiotic resistance gene cassettes such as resistance integrons (Hall, 2012). class 1 integrons, which can be disseminated through a wide variety of taxonomically divergent bacteria, are often found in bacteria associated with livestock and poultry (Mathew et al., 2007). Another mean for gene transfer across bacterial species of different taxa includes transduction (gene transfer mediated by bacteriophages) as evidenced by using a metagenomic approach for antibiotic resistance genes (Muniesa et al., 2013). Noteworthy, antibiotic resistance gene transfer can be insidious as phenotypic detection of inducible antibiotic resistance may be difficult and may account for the "silent" spread of such genes in bacterial communities (Chancey et al., 2012).

Hence, some bacterial isolates of animal origin might not be pathogenic to humans but they may carry and disseminate important antibiotic resistance genes. For example, the same *vanA* gene cluster involved in vancomycin resistance could be detected in enterococci of both human and animal origins, indicating horizontal transfer of gene clusters between enterococci of different origins (Conly, 2002; Hammerum, 2012). Similarly, multidrug-resistant commensal *E. coli* of animal origin represent an important reservoir of antibiotic resistance genes that can be transferred to other strains and bacterial species through contact with other animals or humans and through contaminated food (Szmolka and Nagy, 2013). Many food animals are now broadly recognized as carriers of livestock-associated pathogens that can in many occurrences cause diseases in the human host. For example, Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) have been transmitted from cows or pigs to humans and could cause diseases (Witte et al., 2007; Garcia-Alvarez et al., 2011; Laurent et al., 2012). Also recently, it was suggested that multiple cases of community-acquired urinary tract infections (UTI) caused by antibiotic-resistant bacteria could be considered outbreaks of foodborne origins (Nordstrom et al., 2013). In Canada, studies suggested that poultry meats could play a role in human infections (Manges et al., 2007) and that chicken represented the most probable reservoir of extraintestinal pathogenic *E. coli* causing UTI (Bergeron et al., 2012). Certainly, in view of the complexity of the antibiotic resistance spread allowed by various means (genes, resistant commensals, or resistant pathogens) from various reservoirs (food and environment), global coordinated actions are required (Marshall and Levy, 2011; Laxminarayan et al., 2013). Toward a global action in the Canadian poultry industry, at least two reasonable questions should arise. Are antibiotics acting as growth promoters still needed nowadays? What are possible alternatives to antibiotics that could be used in preserving poultry health while maintaining farm profitability, food safety and environmental health?

## POULTRY INDUSTRY IN CANADA

According to the Food and Agriculture Organization (FAO) of the United Nations, the world chicken production was estimated at 71,851,372 tons in 2005, up 3% from the previous year (Lacobucci et al., 2006). It is interesting to note that chicken production has been grown steadily worldwide since the early 1990s. From 1985 to 2005, 158% growth was recorded. The leading chicken-producing countries include the United States, China, the European Union and Brazil. In 2005, those four countries or group of countries accounted for about 61% of world chicken production. Canada was the thirteenth-largest chicken-producing country in 2005 with 981.2 million kilograms representing 1.4% of the world's production (Lacobucci et al., 2006).

Poultry production is an important industry in Canada. In 2012, the value of Canadian chicken products was estimated at $2.4 billion, involving 2645 regulated chicken producers and a large number of businesses associated with chicken farming http://www.agr.gc.ca/poultry/index_eng.htm). In the same year, Canada produced 1.02 billion kilograms of chicken (eviscerated weight), 60% of which was produced in the provinces of Quebec and Ontario. Canadian domestic consumption was 30 kilograms per person and retail purchases accounted for approximately 634 million kilograms representing 62% of Canada's total consumption. Canada exported over 5.9 million chicks to 13 countries; a commercial value estimated at over $14.5 million in 2012. The United States was the largest market (91%). Other countries included Mexico, Japan, the Philippines, and China (http://www.agr.gc.ca/poultry/index_eng.htm).

In Canada, several medical ingredients are approved as feed additives for poultry farmers. Among them, several classes of antimicrobial agents, such as glycolipids (bambermycin), polypeptides (bacitracin), ionophores (salinomycin), β-lactams (penicillin), streptogramins (virginiamycin), and tetracyclines (chlortetracycline) are used in broiler production for growth promotion and prevention of infectious diseases (**Table 1**). For broilers, salinomycin and bacitracin are widely used in starter, grower or finisher feeds while virginiamycin is used in the finisher. Many of the above antimicrobials are effective against Gram-positive bacteria such as *Clostridium perfringens*, the etiological cause of necrotic enteritis which is one of the main disease concerns for poultry producers worldwide (Stutz et al., 1983; Heredia and Labbé, 2001; Shojadoost et al., 2013). Since *C. perfringens* also is one of the foodborne pathogens associated with poultry, it is believed that antimicrobial agents targeting this pathogen also help to prevent any potential food safety problems.

The preventive use of antibiotics in poultry production may impact therapeutic efficacy in human medicine. Ceftiofur is a third-generation cephalosporin, marketed for use in turkey, cattle, swine, lambs, dogs, and horses. This antibiotic is often subcutaneously injected in day old chicks (0.17 mg/chick) or into eggs (0.08–0.20 mg) as a prophylactic measure in Canada to prevent the yolk-sac infection (omphalitis), a costly disease caused by *Escherichia coli* (Canadian Medical Association, 2009). This use is under Canadian provincial regulations which differ from province to province (Government of Canada, 2002). Overall data on antibiotic use in Canadian hatcheries are not available; however, it seems that about 30% of the chicks hatched in the province of Ontario would be treated mostly with ceftiofur followed by gentamicin (Rosengren et al., 2009). Ceftiofur is not used in humans, however, its analog ceftriaxone, another third-generation cephalosporin, is an important medical antibiotic used in humans. Resistance to these related antibiotics can be mediated by similar mechanisms involving genes such as $bla_{CMY-2}$, an AmpC-type β-lactamase that hydrolyzes third-generation cephalosporins.

In Canada as well as in several countries, various combinations of antimicrobial agents are used in feed depending on birds' ages, formulation and mixtures and such recipes greatly vary geographically and from one farm to another. Hence, despite the intuitive link between antibiotic usage in poultry and the emergence of antibiotic resistant bacteria, variations in antimicrobial usage make links between the use of specific feed additives and the selection of specific antibiotic resistant bacteria difficult to establish (Diarrassouba et al., 2007). Furthermore, the origins of antibiotic resistant bacteria remain uncertain and the sources are certainly numerous (Marshall and Levy, 2011). Consequently, antibiotic resistance in commensal enterococci can be found as early as in 1-day old chicks (**Table 2**).

**Table 1 | Agents approved as medicating ingredients in Canadian poultry feed.**

| Agents | Chicken | Turkey |
|---|---|---|
| Arsanilic acid | GP | GP |
| Bacitracin (zinc or methylene disalicylate) | GP, NE, EM | GP |
| Zinc bacitracin and procaine penicillin | EM | |
| Bambermycin | GP | GP |
| Chlortetracycline hydrochloride | GP, OT, ST | CRD, GP, HE, NE, OT, spE, ST |
| Oxytetracycline hydrochloride | OT, ST | CRD, OT, spE, ST, SI, SY |
| Virginiamycin | GP, NE | |
| 3-nitro-4-hydroxyphenylarsonic acid | GP | GP |
| Penicillin procaine | GP | |
| Amprolium | CO | CO |
| Clopidol | CO | |
| Decoquinate | CO | |
| Diclazuril | CO | CO |
| Halofuginone hydrobromide | CO | |
| Lasalocid sodium | CO | CO |
| Maduramicin ammonium | CO | CO |
| Monensin sodium | CO | CO |
| Narasin | CO, NE | |
| Narasin and nicarbazin | CO | |
| Nicarbazin | CO | |
| Robenidine hydrochloride | CO | CO |
| Salinomycin sodium | CO | |
| Semduramicin sodium | CO | |
| Zoalene | CO | CO |
| Hygromycin B | WO | |
| Piperazine | WO | WO |
| Tylosin phosphate | NE | |
| Nitarsone (4-Nitrophenylarsonic acid) | | BH |
| Novobiocin | | SY |

*Abbreviations: BH, blackhead; CO, coccidiosis; CRD, chronic respiratory disease; EM, early mortality; GP, growth promotion and/or feed efficiency; HE, hexamitiasis; NE, necrotic enteritis; OT, other nutritional uses; spE, non-specific enteritis, ST, stress; SY, synovitis; SI, sinusitis; WO, worms (Government of Canada, 2013a).*

## GROWTH PROMOTERS AND PERFORMANCE

Few studies have been performed to demonstrate the economic benefits of antimicrobial growth promoters in the Canadian poultry production system (**Table 3**). In controlled studies, the effects of diet supplementation with bambermycin, penicillin, salinomycin, bacitracin, salinomycin-bacitracin, virginiamycin,

**Table 2 | Antibiotic susceptibility phenotypes of some enterococci isolates from day-old chicks before placement and of some enterococci isolates found in freshly manufactured feed (starter, grower, and finisher).**

| Category[a] | Antibiotic | Antibiotic susceptibility phenotype[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day-old chick isolates | | | | | | | Feed isolates | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Starter | Grower | Finisher |
| I | Ciprofloxacin | R | I | S | S | S | S | S | I | S | I |
| | Daptomycin | S | S | S | S | S | S | S | S | S | S |
| | Linezolid | S | S | S | S | S | S | S | S | S | S |
| | Vancomycin | S | I | S | S | I | S | I | S | S | S |
| II | Erythromycin | R | S | R | R | I | R | S | S | S | I |
| | Gentamicin | S | S | S | S | S | S | S | S | S | S |
| | Kanamycin | R | S | R | R | S | R | S | S | S | S |
| | Lincomycin | R | R | R | R | R | R | R | R | R | R |
| | Penicillin | S | S | S | S | S | S | S | S | S | S |
| | Q/D[c] | R | S | R | R | S | S | S | R | S | S |
| | Streptomycin | R | S | R | R | S | R | S | S | S | S |
| | Tylosin | R | S | R | R | S | R | S | S | S | S |
| III | Chloramphenicol | S | S | S | S | S | S | S | S | S | S |
| | Nitrofurantoin | S | S | S | S | S | S | S | I | S | S |
| | Tetracycline | R | R | R | R | R | R | R | S | S | R |
| | Bacitrcin | R | R | R | R | R | R | R | R | R | R |
| IV | Flavomycin | R | S | R | R | R | R | S | R | R | R |

[a] *Category indicates antibiotic ranking based on importance in human medicine.*

[b] *The antibiotic susceptibility phenotypes are presented as S, sensitive; R, resistant; I, intermediary, using CIPARS susceptibility breakpoints (Government of Canada, 2013b).*

[c] *Quinupristin/Dalfopristin.*

chlortetracycline, monensin, and narasin on body weight, feed intake, feed efficiency, and mortality were evaluated (Diarra et al., 2007; Bonnet et al., 2009). No significant difference was noted between the treatment groups for the overall performance although virginiamycin and penicillin improved feed efficiency. The experiment conducted by Dumonceaux et al. (2006) found that dietary inclusion of virginiamycin increased body weight and improved feed efficiency from days 0 to 15 but that no difference was noted for bird's performance parameters for the remainder of the study. The used of chlortetracycline as a feed supplement at a rate permitted in Canada has been reported to induce no significant improvement in 21- and 42-day old live body weights or feed conversion efficiencies (Proudfoot et al., 1988). Avoparcin an analog of vancomycin, has not been approved in Canada, however, the growth promotion effect of this agent was reported in experimental turkeys by a Canadian study (Leeson and Summers, 1981). The economic effect of removing antibiotics used for growth promotion in commercial broiler chickens was evaluated in a non-randomized study in the USA (Graham et al., 2007). Positive production changes were associated with the use of antibiotic agents, but these benefits were insufficient to offset their cost (Graham et al., 2007). Well-designed on-farm studies should be encouraged in the Canadian poultry production system to support or not the use of growth promoting antimicrobial agents. With the improved hygienic and biosecurity practices currently observed in modern poultry production, there is a genuine concern that utilization of antibiotics as growth promoters in feed might no longer be useful.

## GROWTH PROMOTERS AND GUT MICROFLORA

The lives of human beings, livestock and poultry are closely associated with microorganisms and the microbiota of their gut plays an important role in their overall health, productivity and well-being (Callaway et al., 2008; Ley et al., 2008). The growth of normal intestinal bacteria varies with the gut environment, and there is an increasing interest in the commensal components of the gut microflora associated with food-producing animals (Yost et al., 2011). Due to public and possible food safety and environmental health concerns, the monitoring of the changes in the microbiome (microbial genomes) as a function of chicken production practices is imperative. Knowledge of the impacts of antimicrobial agents on the gut microbiome might lead to production practices that improve broiler intestinal health and growth performances.

The use of virginiamycin as a growth promoter was associated with an increased abundance of bacteria in the duodenal loop to proximal ileum, with fewer bacteria affected in the distal regions (ileocecal junction and cecum) indicating that virginiamycin modifies the composition of the chicken intestinal microbiota (Dumonceaux et al., 2006). Using the 16S rRNA

**Table 3 | Canadian studies evaluating growth promotion gains and health parameters of in-feed antibiotic supplementations.**

| References | Promoter | Route | Study objectives | Conclusions/Observations |
|---|---|---|---|---|
| Leeson and Summers, 1981 | Avoparcin (10 ppm) and robenz (33 ppm) alone or in combination | In-feed: Turkeys | Performance and carcass grades | Avoparcin improved weight gain irrespective of coccidiostat robenz inclusion. Feed utilization and carcass grades were not influenced by diet |
| Proudfoot et al., 1988 | Chlortetracycline (5.5 mg/kg) | In-feed: Broiler | Growth promotion | No further gain |
| Proudfoot et al., 1990 | Lincomycin (2.2 ppm) | In-feed or in water: Broiler | Growth promotion | No effect on mortality, efficiency of food utilization, final body weights or monetary indices |
| Dumonceaux et al., 2006 | Virginiamycin (20 ppm) | In-feed: Broiler | Performance; intestinal microbiota | Improved body weight and feed efficiency from 0 to 15 days. Increased abundance *Lactobacillus* spp. in the proximal digestive tract with fewer targets affected in the distal regions |
| Guban et al., 2006 | Bacitracin and monensin alone or in combination (0.5 g/kg) | In-feed: Broiler | Growth performance; population levels of *Lactobacillus salivarius*; bile salts deconjugation | Bacitracin increased feed intake and decreased conversion ratio while improving weight gain and concentrations of conjugated bile salts. Monensin increased fat digestibility. Antimicrobials reduced populations of *Lactobacillus salivarius* |
| Diarra et al., 2007 | Bambermycin (2 ppm), penicillin (2.2 ppm), salinomycin (60 ppm), and bacitracin (55 ppm) or a combination of salinomycin (60 ppm) + bacitracin (ppm) | In-feed: Broiler | Growth performances; pathogen counts; resistance phenotypes; resistance determinants | Except for penicillin (improvement of feed efficiency), no significant effect on performance; no effect on bacterial count in the intestine, ceca or litter. Significant effect on antimicrobial resistance phenotypes and genotypes |
| Brisbin et al., 2008 | Virginiamycin (11 or 22 ppm) | In-feed: Broiler | Antibody response | Enhancing systemic antibody responses to some antigens |
| Gong et al., 2008 | Bacitracin (50 ppm) | In-feed: Broiler | Ileum and caeca microbiota | Alteration of the microbiota composition in 3-day-old chicks but no effect on the microbial richness |
| Bonnet et al., 2009 | Bambermycin (2 ppm); penicillin (2.2 ppm); salinomycin (60 ppm); and bacitracin (55 ppm); a combination of salinomycin (60 ppm) + bacitracin (ppm); chlortetracycline (110 ppm), virginiamycin (11 or 22 ppm); monensin (99 ppm); narasin (70 ppm) | In-feed: Broiler | *E. coli* pathotypes and phylogenetic group | Affect the phylogenetic group and pathotypes distribution in the gut |
| Baurhoo et al., 2009 | Mannanoligosaccharide (0.2 or 0.5%); Virginiamycin (16.5 ppm); Bacitracin (55 ppm) | In-feed: Broiler | Performance; intestinal development; cecal and litter microbial populations; carcass parameters | No effect of antimicrobial on performance and carcass. Some effect on cecal and litter microbial population on day 34 |
| Salim et al., 2013 | Direct-fed microbial (DFM) such as *Lactobacillus reuteri* (0.1%) or a mixture of *L. reuteri*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (0.1%); Virginiamycin (0.1%) | In-feed: Broiler | Performance; immune response; cecal microbial population; ileal morphology | Increase performance from 0 to 21 days. DFM increases white blood cells, monocytes and the plasma immunoglobulin concentrations while decreases cecal *E. coli* population |

gene-based polymerase chain reaction followed by denaturing gradient gel electrophoresis profiling, dietary treatment with bacitracin (50 mg/kg) has been shown to alter the composition of the microbiota but did not change its richness (Gong et al., 2008). The authors demonstrated that the impact of bacitracin was particularly obvious in 3-day-old chicks. Lactobacilli were abundant in the cecal microbiota of 3-day-old chicks regardless of the dietary treatment with bacitracin (Gong et al., 2008). Recently, metagenomic sequencing approaches demonstrated that salinomycin-feeding (60 ppm) has a profound impact on the dynamics of the chicken ceca microbiome (Fung et al., 2013). These authors showed that the salinomycin fed group had an increased abundance of the Elusimicrobia, and a decreased abundance of Chloroflexi, Cyanobacteria, and Synergistetes. For example, the abundance of *Bifidiobacterium* spp. and *Lactobacillus* spp. increased significantly in the salinomycin-fed birds compared to the untreated control group. A functional analysis of environmental gene tags (EGTs) revealed that in the salinomycin-treated birds there was an increased abundance of the cell wall and capsule, iron acquisition, motility and β-lactamase gene categories while a decrease of multidrug efflux pump EGTs was detected (Fung et al., 2013). In addition to such Canadian studies, other authors demonstrated the impact of antimicrobial growth promoters on the chicken gut microflora (Knarreborg et al., 2002; Torok et al., 2011; Singh et al., 2013). For example, pyrosequencing followed by phylogenetic analyses indicated that diet supplementation with penicillin resulted in an elevated proportion of bacteria of the phylum Firmicutes from 58.1 to 91.5% and a decreased proportion of members of the phylum Bacteroidetes from 31.1 to 2.9% in the gut microflora of broilers compared to that observed in broilers fed with the control non-supplemented diet (Singh et al., 2013). Besides, the decrease of broiler ileal sucrase and maltase activities and increase of ileal mucosal immunoglobulin A (IgA) as well as the increase of *Lactobacillus* counts were suggested to be among the effects of bacitracin (55 ppm) and oxytetracycline (2.5 ppm) that could explain the improvement of feed efficiency in broilers from days 0 to 21 (Lee et al., 2011).

## GROWTH PROMOTERS AND RESISTANCE

The use of antibiotics in poultry production and the attendant selection of resistant bacteria has been the subject of numerous studies (Aarestrup, 2000; Angulo et al., 2000; O'Brien, 2002; Butaye et al., 2003; Asai et al., 2005; Anonymous, 2007; Castanon, 2007; Diarra et al., 2007; Diarrassouba et al., 2007). However, besides the simple principle that exposure to an antimicrobial agent can select for a resistant bacterium, the selection and dissemination of antimicrobial resistance is a complex phenomenon, which should be examined with ecological and population perspectives. Several studies have shown the presence of antibiotic resistant bacteria (*E. coli*, *Salmonella* serovars; *Enterococcus* spp., *C. perfringens*) in Canadian poultry (Diarrassouba et al., 2007; Diarra et al., 2010; Slavic et al., 2011; Agunos et al., 2012; St. Amand et al., 2013). Many antibiotic resistance genes in these bacteria have been identified on mobile genetic elements such as plasmids, transposons and integrons, allowing their dissemination among bacteria in the chicken gut or in extra-intestinal environments. However little is known about the selection, distribution and dissemination of antibiotic resistance genes in Canadian broiler chicken productions in relation to the use of specific therapeutic agents or antimicrobial growth promoters.

Recently, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) reported a possible association between ceftiofur-resistant *Salmonella enterica* serovar Heidelberg isolated from retail chicken meats and the incidence of ceftiofur-resistant *Salmonella* Heidelberg infections in humans across Canada (Dutil et al., 2010). In the province of Quebec, the prophylactic use of ceftiofur in broiler chickens coincided with the rise of the prevalence of ceftiofur resistance in *Salmonella* that significantly decreased following voluntary withdrawal of this antibiotic (Rosengren et al., 2009). In relation to this, it is noteworthy to mention that the presence of β-lactam resistant *Salmonella enterica* serovars Kentucky, Typhimurium, Enteritidis, and Heidelberg that harbored a variety of important β-lactamase genes (CMY, TEM, SHV) either alone or in combination with other resistance genes were reported in chickens (Diarra et al., 2014). This observation is of concern because the use of cephalosporins at therapeutic levels can decrease the susceptibility to other antibiotics such as tetracycline and amikacin which resistance genes can be co-located on CMY-2 plasmids (Hamilton et al., 2012).

Using antimicrobial agents in feed, it was demonstrated that multi-antibiotic-resistant *E. coli* can colonize and persist in the broiler gut. Of 256 *E. coli* isolates analyzed using DNA-microarray, 88% possessed at least one antimicrobial resistance gene with 42% showing multiple resistance genes (Diarra et al., 2007). The bacterial phenotypes and distribution of resistance determinants in *E. coli* were found to be modulated by feed supplementation with some of the antimicrobial agents used in broiler chicken production (Diarra et al., 2007; Thibodaux et al., 2008; Bonnet et al., 2009). In *E. coli*, class 1 intregron and the aminoglycosides resistance *aadA* gene were predominantly found in the isolates from bacitracin and salinomycin treatments (Diarra et al., 2007), while the streptogramin resistance *vatD* gene was more prevalent in enterococci isolated from virginiamycin-treated birds compared to that found in the control birds (Thibodaux et al., 2008). Detailed antibiotic resistance genotypes of a variety of enterococci isolated from the feces and ceca of Canadian commercial broiler chickens were reported (Diarra et al., 2010). Genes conferring resistance to aminoglycosides (*aac, aacA-aphD, aadB, aphA, sat4*), macrolides (*ermA*, *ermB, ermAM, msrC*), tetracycline (*tetL, tetM, tetO*), streptogramins (*satG_vatE8*), bacitracin (*bcrR*), and lincosamide (*linB*) were detected in corresponding resistant *E. faecium* and *E. faecalis* strains (Diarra et al., 2010). Although food-producing animals are not considered as a source of *Enterococcus* infection in humans, antibiotic-resistant enterococci from these animals may transfer their resistance genes to bacterial strains infecting humans. Thus, the prevalence of antibiotic-resistant enterococci, in poultry can constitute a serious public health problem.

Accurate estimates of the volume of antimicrobials specifically used as growth promoters in Canadian animal productions including poultry is lacking. According to the Canadian Institute of Animal Health estimates reported par CIPARS (Government of Canada, 2013b), a total of 1,766,126, 1,617,747, 1,615,571, and

1,632,364 kg of antimicrobials were distributed in Canada for use in animals in 2006, 2007, 2008, and 2009, respectively. During these years, tetracyclines which are broad spectrum agents, ranked first with 48.0, 46.6, 42.1, and 42.1% of all antimicrobials being used in 2006, 2007, 2008, and 2009, respectively. The total amount of tetracyclines used specifically in poultry production is unknown. However, a high prevalence of tetracycline resistance in both Gram negative and Gram positive bacteria has been reported in Canadian poultry farms and poultry meats which could be related to the extensive used of this antibiotic.

In Gram negative bacteria such as *E. coli*, tetracycline resistance is frequently mediated by several efflux genes. The *tetB*, one of such genes, seems to be the most prevalent in *E. coli* isolated from Canadian broilers (Diarrassouba et al., 2007; Bonnet et al., 2009). The tetracycline resistance genes can be associated with large plasmids, which often carry other antibiotic resistance genes, heavy metal resistance genes, and/or other pathogenic factors such as toxins (Forgetta et al., 2012). Hence, selection for any of these factors selects for these plasmids. Associations between the β-lactamase (*tem*), tetracycline (*tet*), sulfonamide (*sulI* or *sulII*), aminoglycoside [*ant(3″)-Ia* (*aadA*)] and phenicol resistance (*floR*) genes and class 1 integrons were reported in *E. coli* isolated from broilers (Diarra et al., 2007). These associations increase the risk of selection and dissemination of resistance.

In Gram positive bacteria, the *tetL* gene encodes a large protein which confers resistance to tetracycline by active efflux while *tetM* encodes a cytoplasmic ribosome protecting protein also leading to resistance. The *tetL* and *tetM* genes were the most frequently found in association with the *ermB* gene (encoding resistance to macrolide, lincosamide and streptogramin B quinupristin-dalfopristin) and the bacitracin resistance gene *bcrA* in enterococci isolated from broiler chickens (Diarra et al., 2010). As mentioned above, bacitracin is one of the antimicrobial agents used as a growth promoter and to prevent necrotic enteritis (**Table 1**). The use of this antibiotic can co-select for resistance to other unrelated antibiotics as well, which demonstrates that the spread of antimicrobial resistance is a complex phenomenon.

The origin of the antimicrobial resistant bacteria colonizing the broiler gut needs to be established. In our laboratory, examination of the gut contents of day-old chicks revealed the presence of about 1.6 Log CFU of enterococci spp. per gram (Diarra, unpublished data). Some of these isolates were multi-resistant to bacitracin, ciprofloxacin, erythromycin, tylosin, flavomycin, streptomycin, kanamycin, lincomycin, quinupristin-dalfopristin, and tetracycline (**Table 2**). In Canada and other countries where poultry production is intensive, high numbers of broilers are raised in confined and non-sterile environments. Broilers can be exposed to such environmental bacteria among which some could be resistant. For example, chicken feed has been shown to contain *E. coli*, *Klebsiella* and *Pseudomonas* spp. isolates resistant to four to nine antibiotics (Saleha et al., 2009). In another study, examination of 23 commercial broiler feed samples and of 66 samples of raw feeding materials revealed that feedstuffs and poultry feed are extensively contaminated with resistant enterococci in agreement with our observations (**Table 2**) and, to a lesser extent, by *E. coli* (da Costa et al., 2007). Note that other factors also contribute to bacterial gut colonization such as the age of the animals and the microflora may thus vary over time. This should be taken into account when assessing antimicrobial resistance prevalence.

## ENVIRONMENTAL PERSPECTIVES

Poultry litter, a mixture of materials including bedding, feces and feathers, is a valuable soil amendment that is rich in nutrients and can improve soil physical, chemical, and biological properties for agricultural crops (Brye et al., 2004). Most of the antimicrobial agents administrated through feed or water are not fully absorbed in the chicken gut and up to 90% of the administered dose of some of the antimicrobials can be excreted in the feces. Residues of chicken feed additives such as bacitracin, chlortetracycline, monensin, narasin, nicarbazin, penicillin, salinomycin, and virginiamycin can be detected in the litter at concentrations ranging from 0.07 to 66 mg/L depending on the compounds (Furtula et al., 2010). Such a litter, if not treated to remove these compounds, may be an important source of antimicrobial residues when used as fertilizer. These residues also could contribute in the selection of antibiotic resistant bacteria as demonstrated for ceftiofur residues by Call et al. (2013).

Litter can be a source of antimicrobial resistant bacteria as well. Various antibiotic resistant *E. coli* strains harboring genes conferring resistance to β-lactams ($bla_{CMY-2}$, $bla_{TEM}$), tetracycline (*tetAB*) and streptomycin (*strAB*) have been reported to survive for several months in soil following late summer litter application (Merchant et al., 2012). Estimating survival of antibiotic resistant and potential pathogenic bacteria in soil amended with raw untreated litter from broiler fed antimicrobial supplemented diets is essential for developing intervention strategies against resistant pathogens and toward pathogen control in agricultural soils.

Drinking water should be very low in bacterial counts and no pathogenic microorganism should be detected in it. From 2005 to 2006, a bacteriologic study on 353,388 drinking water samples from private wells in Alberta and Ontario found that 4.6% of these samples were contaminated with *E. coli.* Antibiotic susceptibility tests done on 7063 of these *E. coli* isolates showed that 10.5% were resistant mainly to tetracycline, sulfonamides, β-lactams or aminoglycosides (Coleman et al., 2013). These authors reported that such antibiotic resistant *E. coli* were more commonly isolated from farms housing chickens or turkeys than from properties without poultry.

Primary biological aerosols (airborne biological particles derived from, or which are composed of living microorganisms) are of special concern in poultry barns and slaughterhouses, where the high number of chickens handled in these facilities leads to the presence of substantial concentrations of bacteria and other microorganisms in air (Donham et al., 2000). Antibiotic-resistant bacteria have been reported in broiler chicken air (Brooks et al., 2010; Vela et al., 2012). Biofilm forming staphylococci harboring genes conferring resistance to tetracycline (*tetK*), lincomycin (*linA*), erythromycin (*ermB*), and β-lactams (*blaZ*) were isolated from air inside and outside broiler production facilities (Vela et al., 2012). The airborne dispersion of antimicrobial resistant bacteria should not be underestimated since the presence

of pathogenic bacteria in air represents a potential risk to poultry farm workers and to people working or living near these facilities.

## CONCERNS ABOUT FOOD SAFETY AND SPREAD OF ANTIBIOTIC RESISTANCE

Antibiotic-resistant bacteria constitute a major food safety issue. Antibiotic-resistant bacterial pathogens such as *Salmonella* or *E. coli* can infect humans through contact or consumption of contaminated food while non-pathogenic resistant isolates can transfer their resistant genes to human pathogens. Although multifactorial, practices contributing to the selection of antibiotic resistant bacteria include antibiotic use in livestock feed, and concerns about food safety and reduced efficacy of antibiotic treatment in human medicine have stimulated expert groups to action (Mathew et al., 2007; Laxminarayan et al., 2013).

Antibiotic resistance has become a worldwide threat to public health. For example in the United States of America (USA), according to a recent report from the Centers for Disease Control and Prevention (CDC), at least 2 million people become infected with "antibiotic resistant bacteria" among which at least 23,000 people die each year as a direct result of these infections (CDC, 2013). The USA National Antimicrobial Resistance Monitoring System (NARMS) assisted by the Food and Drug Administration (FDA) and the Department of Agriculture (USDA), monitor antimicrobial susceptibility of enteric bacteria from humans, retail meats and food-producing animals, in order to make decisions related to the approval of safe and effective antimicrobial drugs for animals (NARMS, 2012). In Canada, the Public Health Agency of Canada and the CIPARS track antimicrobial resistance to generate data helping to limit the spread of antibiotic resistant bacteria. More so, initiatives that collect data on commensal and environmental strains as reservoirs of antibiotic resistance genes are invaluable (Marshall and Levy, 2011). It is thought that the frequency of resistance genes in commensals may act as a marker of the emergence of resistance in pathogens (www.roarproject.org).

Concerns for safe food and effective medical antibiotics have pressured authorities for elimination of antibiotics as growth promoters as well as those of medical importance in animal production. Despite incomplete data, there were sufficient genuine and reasonable arguments for implementing such regulations in the European Union and similar policies and recommendations in North America were made based on the precautionary principle. For example, the CDC supports the strategy of the FDA to promote the judicious use of antibiotics that are important in treating humans. In Canada, there is a variety of efforts that follow this trend (Agunos et al., 2012). The Canadian Veterinary Medical Association is developing prudent and judicious antimicrobial use guidelines for veterinarians working with swine, beef or dairy herds and poultry flocks. The Veterinary Drugs Directorate (VDD) of Health Canada, which is responsible for the approval and registration of all antimicrobials for use in agriculture, is developing a risk management strategy to reduce the human health impact of antimicrobial resistance due to use of antimicrobials in animals.

Still, efficient control of foodborne pathogens remains a concern (Smadi and Sargeant, 2013) and removal of non-therapeutic antimicrobials from animal production may possibly increase the prevalence of pathogens in the animal gut and the frequency of foodborne illnesses. Alternatives to antibiotics are therefore required.

## ALTERNATIVES TO ANTIBIOTICS

Public pressure and concerns about food and environmental safety (antibiotic residues, spread of antibiotic genes and antibiotic-resistant pathogens) have driven researchers to actively look for alternative approaches that could eliminate or decrease the use of antibiotics while maintaining production yields and low mortality in poultry production. As discussed in previous sections, the biological basis for antibiotic effects on animal growth efficiency is most likely derived from effects on the intestinal microbiota, which in turn may reduce opportunistic subclinical infections, reduce the host response to the gut microflora, decrease competition for nutrients, and improve nutrient digestibility consequent to a reduction in some microbial fermentation by-products (Dibner and Richards, 2005). With such pleiotropic effects, it will be difficult to find alternatives to antimicrobials administered for prevention or provided as growth promoters in feed.

Several alternative strategies to antibiotics in poultry and livestock production are under investigation (Dahiya et al., 2006; Zakeri and Kashefi, 2011; Seal et al., 2013). Individual strategies examined included direct-fed microbial (probiotics) and live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance (Rajput et al., 2013; Salim et al., 2013); prebiotics, indigestible feed ingredients that beneficially affect the host by selectively stimulating the activity of beneficial bacteria resident in the animal tract (Patterson and Burkholder, 2003; Baurhoo et al., 2009; Samanta et al., 2013); vaccination (Desin et al., 2013) and immune-stimulation through cationic peptides and cytokines (Asif et al., 2004; Kogut et al., 2013); bacteriocins and antimicrobial peptides (Joerger, 2003; Svetoch and Stern, 2010); bacteriophages (Huff et al., 2005, 2013; Zhang et al., 2013); organic acids with antimicrobial activities; herbs, spices and other plant extracts (González-Lamothe et al., 2009); and controlled organic productions with emphasis on diet formulation and ingredient selection, cereal type and dietary protein source and level (Drew et al., 2004; O'Bryan et al., 2008). To date, none of these strategies have been systematically implemented. Consequently, exploration for new approaches to prevent poultry diseases and colonization of poultry by foodborne pathogens is continuing worldwide.

### BERRIES AS A GENERIC SOURCE OF BIOACTIVE MOLECULES

Natural products as tools for disease prevention and health maintenance have reached public acceptance leading to an accelerated research in this area. There are now abundant reports of plant products with bioactivities against a wide variety of pathogenic bacteria. Multiple classes of antibacterial products, including phenolic acids and polyphenols, phenanthrenes, flavonoids, and terpenoids have been described and reviewed (González-Lamothe et al., 2009).

Some products may have antibacterial activities of their own by significantly altering growth or bacterial cell structures. Others

which may be defined as "antibiotic potentiators or adjuvants" could allow reduction of antibiotic usage. Some may have anti-virulence effects or alter quorum-sensing necessary for efficient pathogenesis. Besides, others, defined as "immuno-stimulants" could assist the host immune system to adequately respond to the pathogen invasion, while others may positively affect the intestinal microbiota. Knowing that subclinical diseases caused by pathogens can impact productivity, this review presents some results on the potential of cranberry extracts to control pathogenic bacteria.

Cranberries, *Vaccinium macrocarpon* Aiton (Ericales: Ericaceae), are indigenous to wetlands of central and eastern North America (Eck, 1990). Canadian cranberry productions increased from 95,655 tons in 2009 to 134,575 tons in 2013. Most of the productions come from British Columbia, Quebec, New Brunswick, Nova Scotia and Prince Edward Island (Statistics Canada, 2013). Polyphenolic compounds are widely distributed in higher plants and are integral parts of the human diet. An important and often overlooked group of polyphenols is the proanthocyanidins (condensed tannins).

Particular interest is being shown in the proanthocyanidins from cranberry (Foo et al., 2000). Flavonoids in cranberry may reduce or prevent atherosclerosis by preventing oxidation of low density lipids (Reed, 2002). Cranberry proanthocyanidins at a concentration of 75 μg/mL were found to inhibit the adherence of *E. coli* to urinary epithelial cells, preventing or mitigating thus UTI (Foo et al., 2000; Howell and Foxman, 2002). Cranberry extracts were also reported to inhibit the sialyllactose-specific adhesion of *Helicobacter pylori* to immobilized human mucus, erythrocytes, and cultured gastric epithelial cells (Burger et al., 2002). Because the inhibitors of adhesion are not necessarily bactericidal, the selection of resistant strains is unlikely to occur and anti-adhesion agents represent an interesting therapeutic strategy (Sharon and Ofek, 2002). The potential of plant tannins, including proanthocyanidins, as alternatives to growth promoters in poultry has recently been reviewed by Redondo et al. (2014). It is expected that the value of cranberry-based food and nutraceutical products will remain high as health benefits of cranberry become more firmly established. Recent studies suggest that the potential health effects of cranberry are associated with its phytochemical constituents (Blumberg et al., 2013). Furthermore, studies have revealed that extracts from these sources can affect various bacterial functions including disruption of their cell envelope, which parallels that of some antibiotics widely used as growth promoters in the poultry industry.

It has however been difficult to isolate specific active components from plant extracts which often consist of a mixture of a large number of structurally related compounds (Puupponen-Pimiä et al., 2005). These compounds have varying degrees of bioactivity or even opposing effects (growth inhibitors vs. growth stimulants) and even some with cytotoxicity (Jaki et al., 2008). Also, the spectrum of activity or the mode of action of purified components is often very narrow or non-specific and the use of berry extracts or pomace containing mixtures of bioactive compounds has become an attractive alternative to create an added value to berry by-products.

The antimicrobial activities of cranberry extracts were evaluated against important pathogenic Gram negative bacteria such as *E. coli* and *Salmonella enterica* serovar Typhimurium, which is often associated with poultry (Wu et al., 2008; Harmidy et al., 2011). It has been reported that treatment with cranberry proanthocyanidins (CPACs) inhibited *Salmonella* invasion and enteropathogenic *E. coli* pedestal formation, likely by perturbing the host cell cytoskeleton by CPACs rather than by an effect on bacterial virulence itself (Harmidy et al., 2011). Dehydrated, crushed cranberries or purified CPACs were also shown to inhibit the expression of the flagellin gene (*fliC*) in uropathogenic *E. coli* (Hidalgo et al., 2011).

In order to study the pleiotropic effects of cranberry extracts on *E. coli*, we (Gattuso et al., 2008) and others (Lin et al., 2011) have used a DNA array-based approach in an attempt to correlate specific transcriptional signatures with modes of action. The effects observed on the transcriptome of *E. coli* exposed to cranberry extracts correlated with known characteristics of cranberry constituents such as condensed tannins (flavonoids) and phenolic acids that could possibly act as iron chelators. In view of these results, cranberry extracts could be used to perturb bacterial iron homeostasis and improve nutritional immunity in the gut (Hood and Skaar, 2012).

Based on our own experience, commercially available cranberry products like Nutricran®90 (NC90) and some of our own cranberry extracts yielded stronger growth inhibition effects against Gram positive pathogens such as *Staphylococcus aureus* (Diarra et al., 2013), *Listeria monocytogenes* (Block et al., 2012), and *Clostridium perfringens* (Delaquis et al., 2010), although the minimal inhibitory concentrations of the cranberry products were several times higher than that of conventional antibiotics such as penicillin. Similarly to work done with *E. coli*, transcriptional analyses by microarrays allowed determining the modes of action of the cranberry product NC90 against *S. aureus* (Diarra et al., 2013). The effect of cranberry on the *S. aureus* transcriptome yielded the identification of several bacterial genes known to be up-regulated by the presence of cell-wall acting antibiotics, such as oxacillin, vancomycin, and daptomycin (Singh et al., 2001; Utaida et al., 2003; Muthaiyan et al., 2008), as represented in **Figure 1**. More specifically, a group of genes known as the cell wall stress regulon was strongly up-regulated and clearly demonstrated an effect of cranberry on *S. aureus* cell wall biosynthesis. Ethanol extraction of pomaces (pressed cakes) from fresh fruits also produced a cranberry fraction (FC111) modulating the same marker genes as demonstrated by qPCR. *S. aureus* cell surface disruption by cranberry is also supported by work from Wu et al. (2008) and by cell wall biosynthesis assays (Diarra et al., 2013). Besides, it was noted that NC90 and FC111 also modulated the expression of some *S. aureus* genes (like *lytM*, **Figure 1**) that respond to membrane depolarization, as provoked by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and daptomycin (Muthaiyan et al., 2008). Interestingly, cranberry extracts also strongly down-regulated capsular biosynthesis genes, an effect that was corroborated by electron microscopy (**Figure 1**).

*Listeria* spp. are important foodborne pathogens that can be associated with various foods including fresh and frozen meat and poultry. Cranberry fraction FC111 also showed bactericidal

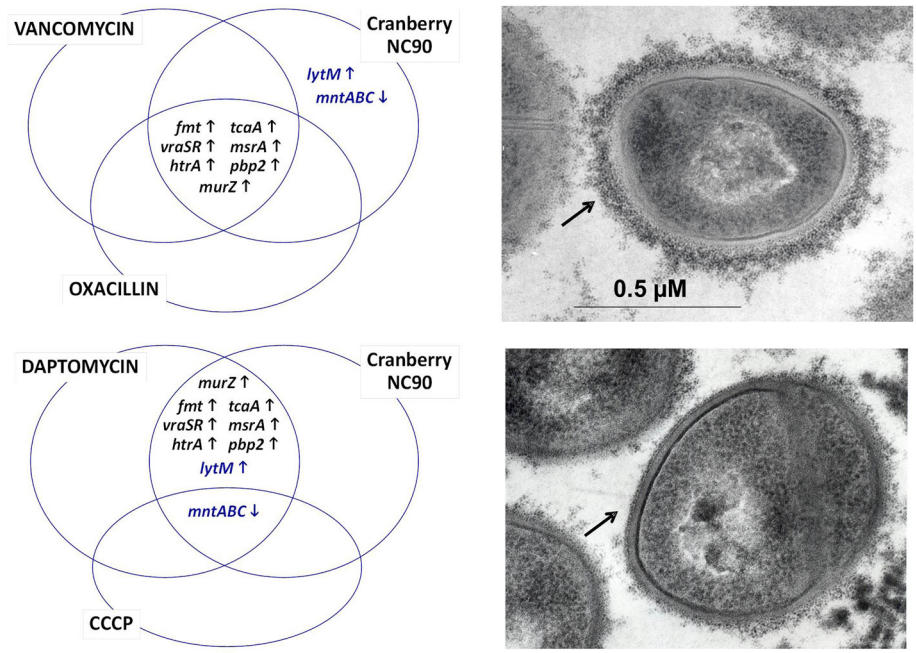


**FIGURE 1 | Venn diagrams showing some of the *S. aureus* genes up- and down-regulated following exposure to cranberry (left).** The transcriptional signature resembles to that of the cell wall stress stimulon provoked by peptidoglycan biosynthesis inhibitors such as vancomycin, oxacillin, and to some extent daptomycin. Compounds causing membrane depolarization like daptomycin and CCCP also share a common transcriptional signature with cranberry. Genes up- and down-regulated are represented by up and down arrows, respectively. Genes affected by cranberry also include those involved in capsular polysaccharide biosynthesis (Diarra et al., 2013), which correlates with the presence of a thinner capsule at the surface of *S. aureus* (lower right panel compared to the untreated control, top right panel). The capsule material (indicated by arrows) was labeled using polycationic ferritin as described before (Diarra et al., 2002).

effects as well as antibiofilm formation activities against *Listeria monocytogenes* (Block et al., 2012). Apostolidis et al. (2008) reported a proline dependent inhibition of *L. monocytogenes* by combinations of phenolic extracts of oregano and cranberry in both broth and cooked meat studies. These data indicate that further examination of the antimicrobial potential of cranberry extract is warranted (Wu et al., 2008).

The multiple biological effects of cranberry observed against *E. coli*, *Salmonella*, *S. aureus*, *C. perfringens*, and *Listeria*, certainly reflect the complexity of its composition and physical properties. The cranberry tannins include polyphenols and more specifically anthocyanins, flavonols and flavan-3-ols (Puupponen-Pimiä et al., 2005). Flavonoids, including anthocyanins and proanthocyanidins, are believed to be the major antimicrobial components (Puupponen-Pimiä et al., 2001). At this time, our mass spectrometry analysis of cranberry fraction FC111 could not determine if the observed antibacterial activity originates from iridoids, phenolics, or flavonoid components. Besides, we showed that the cranberry fraction FC111 obtained from pomace is an excellent natural polyphenolic product with potent antioxidant and vasorelaxant properties (Harrison et al., 2013), which combined with its antibacterial activities might represent an interesting alternative in poultry production. In this regard, a poultry feeding trial using a commercial whole cranberry fruit extract showed that a concentration of 40 mg of cranberry extracts per kg of feed induced low early mortality rates (improvement by 40% compared to the control) in birds. The mechanism of action leading to this improvement remains to be determined. However, diet supplementation with such extracts caused a shift of the intestinal tract bacterial population while not altering any broiler meat properties (Leusink et al., 2010).

## CYCLIC DIGUANOSINE MONOPHOSPHATE (C-DI-GMP)

c-di-GMP is a bacterial intracellular second messenger controlling diverse bacterial processes. This molecule is important for a wide range of pathogenic agents as it is involved in the modulation of the infection process through modulation of motility, cell adhesion and biofilm formation (Tamayo et al., 2007; Bordeleau et al., 2011). However, c-di-GMP is also a potent immunostimulatory agent that can modulate the host immune response and several reports demonstrated its adjuvant and therapeutic properties (Brouillette et al., 2005; Karaolis et al., 2007; Ogunniyi et al., 2008; Hu et al., 2009). Moreover, the ability of c-di-GMP as a mucosal adjuvant was also documented (Ebensen et al., 2007; Zhao et al., 2011). c-di-GMP might thus represent an interesting alternative to non-therapeutic antibiotics used in poultry production.

The infectious bursal disease virus (IBDV, Gumboro disease) is one of the major immuno-suppressive viruses affecting broilers. This virus is highly contagious and represents a major economic threat in poultry production worldwide (Bumstead et al., 1993). Since the effects of c-di-GMP on chicken immune responses had not yet been investigated, we evaluated the humoral immune response following oral administration or intramuscular

injection of c-di-GMP in conjunction with the IBDV vaccine S-706 in broiler chickens (Fatima et al., 2011). Results indicated that c-di-GMP stimulated IgA production in serum and confirmed the potential of this molecule as a mucosal adjuvant.

As mentioned above, an enteric pathogen of particular concern in poultry is *C. perfringens* Type A, the causative agent of necrotic enteritis (Timbermont et al., 2011). Hence, in an effort to explore strategies to control *C. perfringens*, we investigated the potential of c-di-GMP in a broiler challenge model (Fatima et al., 2013). We found that c-di-GMP can modulate *C. perfringens* colonization in the host ceca with no noticeable effect on the microbiota and the commensal bacterial community of the intestine. It will be interesting to investigate in more details the value of c-di-GMP as an in-feed additive in poultry production.

## CONCLUSION

Antibiotics are important tools for the treatment of old and emerging infectious diseases. Their efficacy for this purpose should be preserved as it is now well documented that their abusive and inappropriate use in humans, livestock and poultry selects for antibiotic resistant bacteria, compromising thus their therapeutic efficacy. One of questionable practices in animal agriculture is the use of non-therapeutic antimicrobials for growth promotion. Even if this practice was determinant in the past, its advantage in current modern agriculture including poultry production needs to be re-evaluated because of the actual prevalence of antibiotic resistant bacteria in livestock and poultry and their products worldwide. The presence of multi-drug resistant commensal bacteria (*Escherichia* spp., *Enterococcus* spp.) and foodborne pathogens such as non-typhoid *Salmonella* associated with poultry are some of the examples among others. It is imperative to determine the exact sources and ecology of these resistant bacteria in order to develop strategies to stop their spread. It is also urgent to develop alternatives to antimicrobial growth promoters that will not compromise livestock and poultry health as well as the actual industry productivity. Canadian studies in this area identified some promising sources of alternatives to antibiotics which have been discussed here.

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# Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry

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Antibiotics have been included in the formulation of feed for livestock production for more than 40 years as a strategy to improve feed conversion rates and to reduce costs. The use of antimicrobials as growth-promoting factors (AGP) in sub-therapeutic doses for long periods is particularly favorable for the selection of antimicrobial resistant microorganisms. In the last years, global concern about development of antimicrobial resistance and transference of resistance genes from animal to human strains has been rising. Removal of AGP from animal diets involves tremendous pressure on the livestock and poultry farmers, one of the main consequences being a substantial increase in the incidence of infectious diseases with the associated increase in the use of antibiotics for therapy, and concomitantly, economic cost. Therefore, alternatives to AGP are urgently needed. The challenge is to implement new alternatives without affecting the production performances of livestock and avoiding the increase of antimicrobial resistant microorganisms. Plant extracts and purified derived substances are showing promising results for animal nutrition, either from their efficacy as well as from an economical point of view. Tannins are plant derived compounds that are being successfully used as additives in poultry feed to control diseases and to improve animal performance. Successful use of any of these extracts as feed additives must ensure a product of consistent quality in enough quantity to fulfill the actual requirements of the poultry industry. Chestnut (hydrolysable) and Quebracho (condensed) tannins are probably the most readily available commercial products that are covering those needs. The present report intends to analyze the available data supporting their use.

**Keywords: tannins, antibiotics, poultry, growth promoting factors, necrotic enteritis, plant extracts, animal health**

## INTRODUCTION

Antimicrobial compounds were initially added to feed at therapeutic doses for treatment and prevention of infectious diseases but soon the growth promoting effect of antibiotics was observed. Therefore, since the beginning of the 1950s antibiotics have been added to feeds to improve feed utilization and growth of farm animals, reducing the cost of production (Moore and Evenson, 1946; Jukes et al., 1950). The use of antimicrobials as growth-promoting factors (AGP) should be distinguished from therapeutic and prophylactic use of antibiotics that are administrated at higher doses and for short periods of time.

The mode of action of AGPs is not yet fully understood. Different potential mechanisms have been proposed to explain AGP-mediated growth enhancement (Gaskins et al., 2002; Dibner and Richards, 2005; Page, 2006). The most accepted mechanism would be through modulation of the gut microbiota, which plays a critical role in maintaining the host health (Tuohy et al., 2005). Microbiota composition influences the intestinal environment and the development and responses of the host immune system against pathogenic and non-pathogenic antigens (Cebra, 1999; Kelly and Conway, 2005).

The poultry industry has massively adopted the use of AGPs, but comparatively, little research has been conducted in order to systematically evaluate the potential effects that antibiotics may have on the dynamics of the overall gut microbiota of chicken. Thus, studies are indispensable to elucidate the impact on bacterial community, including selection and distribution of antibiotic resistance genes among commensal bacteria in chickens fed with AGP (Diarra et al., 2007; da Costa et al., 2013).

The use of AGP in livestock and their role in selecting antibiotic resistant bacteria have been extensively reviewed (Butaye et al., 2003; Wegener, 2003; Kazimierczak et al., 2006; Landers et al., 2012). It is important to remark that AGP are used in sub-therapeutic doses for long periods, a situation that is particularly favorable to select antimicrobial-resistant microorganisms. During the last several years, global concern about development of antimicrobial resistance and transference of resistance genes from animal to human strains is rising (Salyers et al., 2004; Mathur and Singh, 2005; Devirgiliis et al., 2013). The potential risk of resistance generation and transmission led to the ban of the use of antibiotics as growth promoters in the European Union since year 2006. Although the relative contribution of foodborne transmission to antimicrobial resistance in humans remains unknown, it does exist and is likely to be more substantial than currently appreciated (Collignon and Angulo, 2006). Some studies suggest that the majority of antibiotic-resistant *Escherichia coli* strains carried by people may have been originated in food animals, particularly from poultry (Johnson et al., 2006).

In this context and to preserve the effectiveness of important human drugs (Casewell et al., 2003) the FDA prohibited the use of fluoroquinolones in chickens and turkeys in the United States, based on evidence that use of these antimicrobials in poultry caused development of resistance of thermofilic *Campylobacter* species. These resistant strains can be transmitted to humans with consequences to public health (Nelson et al., 2007). Selection of resistance in non-pathogenic bacteria is another potential risk. Some resistance genes may be present in non-pathogenic bacteria and then can be transferred to pathogenic microorganisms. Fairchild et al. (2005) showed that the oral administration of tetracycline did not induce significant changes in the chicken cecal bacterial community but they found that *Enterococcus* spp. susceptibility tests showed an increase on tetracycline MICs. These bacteria were positive for resistance genes, tet(M), tet(L), tet(K), and tet(O), which can be transferred to *Campylobacter jejuni*, conferring tetracycline resistance. The authors suggested that complex ecological and genetic factors could contribute to the prevalence and transfer of antibiotic resistance genes in the chicken production environment.

Despite the inconvenience of adding AGP to feed, it is generally accepted that intensification in modern poultry production and the increase in related stressors, e.g., feed changes or diet imbalances, may have different negative effects on animal health, e. g., reduced immune functions, high exposed susceptible population (Pinchasov and Noy, 1993). This may predispose broilers to colonization of the gastrointestinal tract by bacterial pathogens, producing a threat to bird's health and food safety. Removal of antibiotic AGP from animal diets implies a tremendous pressure on the livestock and poultry farmers, one of the main consequences being a substantial increase in the incidence of infectious diseases with the associated augment in the use of antibiotics for therapy (Inborr, 2001; Casewell et al., 2003; Grave et al., 2006). *Salmonella* spp., *Campylobacter jejuni* and *Clostridium perfringens* are considered to be the most important emerging and increasing threat for poultry and human health (Van Immerseel et al., 2004; Humphrey et al., 2007). The challenge is to implement new alternatives without affecting the production performances of livestock and also avoid the increasing of antimicrobial resistance.

Alternatives to AGPs had its origin in public health programs where nutritional interventions such as probiotics and prebiotics are used to ameliorate chronic human conditions such as inflammatory bowel disease (Guarner et al., 2002; Damaskos and Kolios, 2008) and irritable bowel syndrome (Fooks and Gibson, 2002). Formulation of diets focused on specific effects on gut health is becoming a reality in the monogastric animal industries because the maintenance or enhancement of gut health is essential for the welfare and productivity of animals when antibiotics are not allowed in feed. In this scenario raw plant extracts and derived tannins are showing promising results for food animal production (Huyghebaert et al., 2011).

## PLANT EXTRACTS AND TANNINS

Plants synthesize many aromatic substances, most of which are secondary metabolites. In many cases, these substances serve as plant defense mechanisms against predation. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment, others are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers). Tannins are water-soluble polyphenolic compounds of variable molecular weights abundantly found in nature which have the ability to precipitate proteins (Spencer et al., 1988; Cowan, 1999). Tannins can be classified into condensed and hydrolysable (Scalbert, 1991; Haslam, 1996). Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Current scientific evidence suggests that there is significant potential in the use of tannins to enhance nutrition and animal health, particularly for ruminants such as cattle (Frutos et al., 2004). Many studies of phenolic compounds (resveratrol, quercetin, rutin, catechin, proanthocyanidins) have been present in the last few years, most of these works were directed to improvements of human health and they demonstrate that tannins have multiple biological activities, including cardioprotective, anti-inflammatory, anti-carcinogenic, antiviral, and antibacterial properties attributed mainly to their antioxidant and antiradical activity (Frankel et al., 1993; Teissedre et al., 1996; Santos-Buelga and Scalbert, 2000). Recent studies in veterinary medicine mention that these effects are reflected in a better growth performance in different species of food producer animals. Tannins are also able to reduce the risk of livestock disease and transmission of zoonotic pathogens in a sustainable and environmentally friendly manner. Recent reports of the use of tannin in poultry show promising results (Van Parys et al., 2010; Anderson et al., 2012; Redondo et al., 2013b; Tosi et al., 2013).

## HISTORICAL CONSIDERATION OF TANNINS AS ANTI-NUTRITIONAL FACTORS

Traditional concepts in poultry nutrition consider tannins as anti-nutritional factors. In contrast with the effect on ruminant animals where tannins in the diet may have considerable nutritional benefits, tannins are generally considered undesirable in simple-stomached animals feed. In monogastric farm animals it is commonly accepted that dietary tannins reduce digestibility (in particular of crude protein) and consequently growth performance (Treviño et al., 1992; Smulikowska et al., 2001). In poultry, a considerable number of publications have shown the anti-nutritional effects of tannins in chicken feeding; these substances induce a worsening of productive performances as a consequence of decreasing voluntary feed intake and organic matter digestibility, especially the protein component (Barroga et al., 1985; Longstaff and McNab, 1991; Garcia et al., 2004; Longstaff and McNab, 2007).

Reports of anti-nutritional effects of tannins are mostly based on assays performed with relatively high concentrations of tannins in feed, mainly using purified condensed tannins or plant with excess of tannins as may be the tannic acid from sorghum grains. These experiments showed adverse effects such as decreased nutrient utilization, animal productivity, and death in certain animals. This limited experimental information and the fact that tannins act as a defense mechanism in plants against herbivores have been the origin of the widespread concept that tannins are negative for animals. However, it is now known that their beneficial or detrimental properties depend upon their chemical structure

(generally associated with the plant origin) and dosage, besides other factors such as animal species, the physiological state of the animal and composition of the diet. More recent evidence suggest that a moderate tannin level is able to improve both nutrition and health status in monogastric animals.

## IMPACT ON POULTRY PRODUCTIVE PERFORMANCE

Despite that tannins have been traditionally considered as anti-nutritional factors, is it now known that these substances can be beneficial to poultry. However, as it previously mentioned, several factors must be considered and evaluated such as the final concentration in feed, the structure of the compounds, the applied process during feed preparation, and plant factors, which may affect final tannin impact on birds digestive function and global health (Hagerman and Butler, 1980). Studies with different purified tannins confirm that chemical properties, like astringent taste and protein binding are variable among tannin extracts (Hofmann et al., 2006). Schiavone et al. (2008) showed that the use of chestnut extract in poultry feeding does not influence feed digestibility, carcass quality or nitrogen balance. In fact, it has a positive influence in growth performance if included in the diet up to 0.2% (on dry matter). Similarly Marzoni et al. (2005) studied the dietary effects of quebracho tannins in growing pheasants and demonstrated that the inclusion of 2% in feed did not affect growth performances. Furthermore, some authors mention that administration of chestnut tannins may change the droppings consistency, resulting in firmer droppings in treated groups which positively affect the litter status and thus improving the overall health status and welfare of chickens in intensive production systems. Moreover, the chestnut fruit content of phenolics (gallic and ellagic acid), which have been linked to various positive effects on human health such as antioxidant activity, a decrease in the risk of cardiovascular diseases, anticancer mechanisms, and anti-inflammatory properties (de Vasconcelos et al., 2010). Tannins also can be used in combination with other AGPs alternatives, as probiotics, showing a synergist effect in the promotion of gut health. A recent work reported that chestnut extracts exhibited a surprising effect in improving the tolerance to gastric transit of Lactobacilli, while chestnut fiber mainly improved the tolerance to bile juice (Blaiotta et al., 2013).

Although tannins can have beneficial effects on the digestion and therefore animal performance when incorporated into animal diets, their primary mode of action is often not sufficiently known to explain the final *in vivo* effects. Some authors suggest that low concentration of tannins can improve palatability of feed and raise performance of monogastrics by stimulating feed intake (Windisch and Kroismayr, 2006). Others suggest that stimulation of digestive secretions is often considered to be a core mode of action (e.g. Lee et al., 2003). Nevertheless, antimicrobial properties seem to be the most relevant mode of action, especially in young animals. In general terms, like AGPs, plant derived compounds would be involved in the modulation of the highly complex interaction between microbiota and the gastrointestinal tract. The resulting relief of the animal host from microbial activity and their undesired products might be responsible for the lower immune defense costs (Windisch and Kroismayr, 2006; Kroismayr et al., 2008). However, the complexity of the interactions and dynamics of the gut microbiota makes it very difficult to define such effects in quantitative terms.

## IMPACT ON POULTRY HEALTH

Over the last few years, the dietary role of tannins is receiving increasing interest as they may reduce the number of gastrointestinal parasites in mammals (Athanasiadou et al., 2000; Butter et al., 2002; Min et al., 2005) and birds (Marzoni et al., 2005). Tannins, such as condensed tannins from green tea or quebracho, have proven to have antimicrobial activity (Sakanaka et al., 2000; Elizondo et al., 2010) and affect gastrointestinal bacteria colonization in chickens and pigs (Hara et al., 1995; Hara, 1997). Multiple reports suggest the efficacy of tannins or plant extracts in the control of zoonotic pathogens like *Campylobacter* and *Salmonella*.

*Campylobacter* spp. is one of the leading sources of human bacterial diarrhea worldwide, with *Campylobacter jejuni* and *Campylobacter coli* representing the most frequently involved species (Adak et al., 1995; Kapperud et al., 2003). One of the main sources of infection is considered to be foods of poultry origin, intestinal carriage rate within individual flocks often exceed 80% (Anderson et al., 2012). Before AGP banning in determined countries an increase in the incidence of antimicrobial resistance was observed in this food borne pathogen (Desmonts et al., 2004). The antimicrobial activity of various hydrolysable and condensed tannin-rich extracts against *Campylobacter jejuni* reveals that both types of tannins inhibit the growth of this bacterium (Nohynek et al., 2006; Gutierrez-Banuelos et al., 2011; Anderson et al., 2012). It has been observed that condensed tannins may be less efficient than hydrolysable tannins in controlling *Campylobacter jejuni* when high concentrations of amino acids and soluble proteins are present (Anderson et al., 2012). The efficacy of adding selected tannins to poultry feed to diminish the *in vivo* incidence of *Campylobacter* spp., needs to be evaluated.

*Salmonella* serovar Enteritidis is one of the foodborne pathogens most commonly associated with the consumption of poultry products. Control strategies of the disease in humans are based on reducing contamination during slaughter and *Salmonella Enteritidis* load in birds. This was achieved with the use of AGP, which generates residues in meat and eggs and favors the selection of multi-resistant strains of *Salmonella* and other pathogens. Van Parys et al. (2010) found that chestnut (*Castanea sativa*) derived tannins were able to inhibit the *in vitro* growth of *Salmonella typhimurium*, but had no effect on the excretion of the bacteria in an infection model in pigs. Quebracho (*Schinopsis lorentzii*) raw extract shows bacteriostatic effect on *Salmonella Enteritidis in vitro*, and when used in an experimental infection model in broilers it was able to reduce the excretion of the bacteria (Redondo et al., 2013a). Similarly, Prosdócimo et al. (2010) found antibacterial activity of quebracho against *Salmonella Enteritidis* and *Salmonella* Gallinarum *in vitro*.

*Clostridium perfringens* is considered an important poultry pathogen that is the causative agent of necrotic enteritis and subclinical disease (Ficken and Wages, 1997). Both presentations of the disease have important economic impact on poultry production. This bacterium is an important example of antimicrobial banning consequences. AGPs have long been effective in prevention of necrotic enteritis in poultry flocks and after AGPs

withdrawal, the incidence of necrotic enteritis increased considerably (Van Immerseel et al., 2004). Inhibitory effects of tannins from different sources have been demonstrated. Previous report shows that tannins derived from chestnut and quebracho have *in vitro* antibacterial and antitoxin activities against *Clostridium perfringens* and its toxins and that mixtures of both tannins maintain individual activities (Elizondo et al., 2010). Subsequent results from this research group confirm the *in vivo* effects of chestnut and quebracho tannins in a broiler necrotic enteritis model reducing the incidence and severity of gross lesions and improving the productive performance of broiler chickens (Redondo et al., 2013b). This findings are reinforced by the results obtained from other authors with chestnut tannin added to diet in an *Eimeria* spp./*Clostridium perfringens* co-infection model (Tosi et al., 2013). Although chestnut tannins show strong bactericidal activity against *Clostridium perfringens*, most ingested tannin do not remain in the feces because it is hydrolyzed and degraded in the intestinal tract. In contrast, quebracho tannins are mainly condensed with lower antibacterial ability but most of the administered tannins remain in the fecal material. Therefore, those different abilities could be used to readily diminish the intestinal *Clostridium perfringens* load by chestnut and avoiding the reinfection by controlling the environmental contamination (i.e. feces and bedding) with quebracho tannins.

Different works reports the antiviral activity of some tannin against animal viruses. Ueda et al. (2013) test condensed and hydrolysable tannins from different sources against selected families of pathogenic animal virus and show that these compounds have an unspecific neutralizing effect on enveloped virus. The same group reports the induced aggregation of purified virions or BSA through association of tannins with proteins. Another potential mechanism was reported in works using human virus, like herpesvirus (Lin et al., 2011) and human immunodeficiency virus (HIV-1), in the same the authors suggest that reduce viral activity could be due to tannins binding to cell receptor like glycoproteins or CD4, respectively. Although they are few, works with avian viruses suggest that natural extracts containing specific tannins could contribute to control viral infections. Lupini et al. (2009) showed that both, chestnut and quebracho wood extracts, have inhibitory effect on avian reovirus (retrovirus) and avian metapneumovirus (paramyxovirus) before virus absorption to cells. In this work the author reports that chestnut and quebracho extracts reduce the extracellular viral activity, proposing that extracellular effect may be due to an interaction between tannins and viral proteins resulting in the inhibition of viral attachment and penetration of the cell membrane, as mention before for other virus. In the same work, they report a reduction in the intracellular viral activity only by quebracho extract, and propose that the main mechanism would be the inhibition of viral enzymes. The higher intracellular activity of quebracho extract could be due to the smaller size of tannins extracted from this plant that could penetrate the cells as suggested by Moreira et al. (2005).

Although tannins or plant derived extracts demonstrated activity against viral (Lupini et al., 2009), bacterial (Tosi et al., 2013), and protozoal diseases (Cejas et al., 2011), little is known about the mechanisms of these compounds on antimicrobial effects and growth promotion. Some of the explained modes of action for antimicrobials may help to define tannins main mechanism. Metabolism inhibition is one possible mechanism; Bae et al. (1993) showed that condensed tannins from birdfoot trefoil (*Lotus corniculatus* L.) were inhibitory to the endoglucanase activity of cellulose digesting *Fibrobacter succinogenes* S85 in the rumen. This may be applied to virulence factors as Elizondo suggests for *Clostridium perfringens* toxins (2010). On the other hand, iron deprivation has been suggested by some authors (Scalbert, 1991; Haslam, 1996; Mila et al., 1996). Tannic acid works like a siderophore to chelate iron from the medium, making it unavailable for the microorganisms. Iron is essential for most pathogenic bacteria and tannic acid shows three times more affinity for iron than *E. coli* siderophores (Chung et al., 1998).

One of the most accepted mechanisms of action of some plant tissues in animal diet is the shifts in intestinal microbiota composition. As reported for different groups, Gram positive bacteria seem to be more sensitive to plant extract with high tannins content (Nohynek et al., 2006; Engels et al., 2011). It is important to remark that microbiota changes have more impact on younger animals due to their constantly evolving microbiota. It is thought to take until the sixth week of age to achieve a mature microbiota (Barnes et al., 1972). Regardless of the mode of action, the chemical characteristics of the tannins are highly variable and different types of tannins can be present in one plant extract. Therefore, the origin of the plant extract added to the feed will be determinant in the final impact on microbiota and the animal performance.

## ECONOMICAL CONSIDERATION

Independently of the use of antimicrobials or any of the available alternatives to AGP, increase of the productive performance and animal welfare depends on the overall health status of broilers. A complete and continuous observation of the flock health status and performance must be considered. It requires regular necropsies, sampling, and identification of pathogens, together with periodic monitoring of productive parameters like feed intake and weight gain, flock uniformity and other conditions. This should provide an overview of the productive costs and allow measure the economic impact of a disease and choose cost-effective therapeutic or prophylactic strategies. The return-on-investment for alternatives to AGPs will depend on both the biological impact and the dynamics of the market price. Withdraw of AGP from the flock may cause a decreased growth rate, higher morbidity, and mortality; but the continuous use may led to increased condemnations due to residues in meat and derived products. In countries where the use of AGP is still allowed is imperative to consider the net economic effect of replacing them with alternative products that do not represent a threat to public health and leave no residues in meat and derived products. It will depend on several factors including impact effects on productive performance levels and the cost of any alternative potential technologies adopted to compensate for the termination of use of AGPs and may be offset by the benefits like access to more demanding markets or differential marketing, as in the case of organic foods. In countries where AGP are banned from poultry production, the negative impact may be temporarily compensated by the use of ionophore anticoccidials, which are excluded from regulation due to lack of

reports of relations between these substances and others antimicrobials. Taking into consideration that recent field investigations have demonstrated that animal husbandry use of antimicrobial agents increases the likelihood that domestic animal bacteria will develop resistance or cross-resistance to drugs approved for use in human medicine (Diarra et al., 2007; da Costa et al., 2013), experience with others groups of antimicrobials suggest that these chemicals are prompt to be removed from animal feed for the same reasons as AGPs, it is important to develop adequate alternatives to be used alone or combined with other control measures to improve the gut health. Further work is needed to define standards for the replacement of antibiotic compounds in poultry in terms of product type, identification of suppliers, poultry response criteria, regulatory status and veterinary definition (Rosen, 2003).

## CONCLUSION

The use of plant extracts appears as an attractive alternative to the use of antimicrobial growth promoter factors. These natural products do not leave residues in poultry-derived products. Also, plant extracts are complex substances with many bioactive principles that would have fewer chances to induce resistance in microorganisms.

Successful application of any of these extracts as feed additive must ensure a product of consistent quality in enough quantities to improve poultry production at AGPs levels and fulfill the actual requirements of poultry derived products consumers. If the products are effective and can be acquired in enough quantities to supply the poultry industry requirements, the decisive factor for the successful application will be the cost and it should be at least similar to those of the AGPs. Although numerous products available in market have been proved to be efficient in the field (Graziani et al., 2006; Lupini et al., 2009; Elizondo et al., 2010; Redondo et al., 2013b), many have less clear potential. Chestnut (hydrolizable) and Quebracho (condennsed) tannins are probably the most readily available commercial products that are being used and cover those needs as well as there is an important number of data supporting their usage.

The diversity of results presented in different papers show the complexity of elucidating effects of plant extracts over a determined microorganism or disease in different animal hosts. Further investigations needs to be done in order to describe the effects of plant extracts on pathogenic microorganism as well as in commensal microbiota and the impact of its use in animal production. This knowledge would allow the development of new and innovative products suitable to be incorporated in animal feed in order to improve animal production without compromising public health.

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# Antibiotic growth promoters enhance animal production by targeting intestinal bile salt hydrolase and its producers

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The growth-promoting effect of antibiotic growth promoters (AGPs) was correlated with the decreased activity of bile salt hydrolase (BSH), an intestinal bacteria-produced enzyme that exerts negative impact on host fat digestion and utilization. Consistent with this finding, independent chicken studies have demonstrated that AGP usage significantly reduced population of *Lactobacillus* species, the major BSH-producers in the intestine. Recent finding also demonstrated that some AGPs, such as tetracycline and roxarsone, display direct inhibitory effect on BSH activity. Therefore, BSH is a promising microbiome target for developing novel alternatives to AGPs. Specifically, dietary supplementation of BSH inhibitor may promote host lipid metabolism and energy harvest, consequently enhancing feed efficiency and body weight gain in food animals.

**Keywords: antibiotic growth promoters, bile salt hydrolase, *Lactobacilli***

## INTRODUCTION

Epidemiological studies have linked usage of antibiotic growth promoters (AGPs) to the emergence of antibiotic resistant bacteria (Wegener, 2003). Antibiotic-resistant bacteria as well as resistance determinants can therefore spread from animals to humans, compromising the effectiveness of antibiotics for treating human infections and posing a serious threat to public health (Wegener, 2003). For this reason, Denmark banned all AGPs in 1998 and European Union member nations banned all AGPs in 2006 (Wegener, 2003; Dibner and Richards, 2005). Therefore, there is a worldwide trend to limit AGP use in food animals (Turnidge, 2004; Dibner and Richards, 2005). Ending the use of AGPs creates challenges for the animal feed and feed additive industries. Several products, such as probiotics, prebiotics, and organic acids have been used to alter intestinal microbiota for improving animal health and production (Dibner and Richards, 2005). However, limited data is available to justify the choice of specific bacterial species or products for such microbiota manipulation. Examination of microbiota in response to AGP treatment would provide insights into the modes of action of AGPs and facilitate the development of more effective microbiota-based strategies for growth promotion. Recent studies on the relationship between AGP usage and gut microbiota strongly suggest that bile salt hydrolase (BSH) is an important target through which microbiome composition and function may impact host fat digestion and energy harvest.

### RESPONSE OF INTESTINAL BSH ACTIVITY TO AGP

Clearly a connection has been made between AGP usage, growth promotion, and intestinal bacterial populations, but the precise mechanism is yet to be delineated as to how these all coincide. Strides have been made in uncovering this mystery, however, and it has been shown that the growth-promoting effect of low-dose antibiotics does coincide with a decrease in BSH activity in the gut (Feighner and Dashkevicz, 1987; Knarreborg et al., 2004; Guban et al., 2006). BSH produced by gut bacteria catalyzes deconjugation of conjugated bile acids (CBAs, also referred to as conjugated bile salts) in the intestine (Begley et al., 2006). CBAs consist of a hydrophobic steroid core that is conjugated with either glycine or taurine. Thus, the CBAs are amphipathic and function as a more efficient "biological detergent" than unconjugated bile acids to emulsify and solubilize lipids for fat digestion (Begley et al., 2006). Consequently, BSH activity has significant impact on host physiology by disturbing CBA-mediated fat metabolism and endocrine functions (Begley et al., 2006; Jones et al., 2008).

Feighner and Dashkevicz (1987) provided early evidence that antibiotic feed additives affect the transformation potential and hydrolysis activity of BSHs from intestinal contents of poultry. By keying in on a more specific aspect of lipid metabolism, Knarreborg et al. (2004) demonstrated an enhanced bioavailability of α-tocopheryl (alpha-tocopherol) acetate in broilers given AGPs, and this was attributed to a reduced concentration of unconjugated bile salts. Furthermore, Guban et al. (2006) correlated dietary supplementation of AGPs to improved weight gain and fat digestibility in broilers, decreased population levels of *Lactobacillus salivarius*, and a reduced pool of deconjugated bile salts. Based on these discoveries, the growth-promoting effect of AGPs likely is partly attributed to the reduced BSH activity, and thus the improvement of host lipid metabolism.

### RESPONSE OF INTESTINAL MICROBIOTA TO AGP

With the aid of culture-independent molecular approaches, the investigations of the effect of AGPs on intestinal microbiota have been initiated in different food animals, including poultry and swine, which greatly improves our understanding of intestinal

**Abbreviations:** AGP, antibiotic growth promoter; BSH, bile salt hydrolase; CBA, conjugated bile acid.

microbiota changes in response to AGPs (Engberg et al., 2000; Knarreborg et al., 2002; Collier et al., 2003; Dumonceaux et al., 2006; Guban et al., 2006; Wise and Siragusa, 2007; Zhou et al., 2007; Danzeisen et al., 2011; Kim et al., 2012; Lin et al., 2013). As expected, oral administration of low-dose antibiotics affected diversity and relative abundance of gut microbiota in all these studies. However, based on the findings from these molecular ecology studies, it is challenging to definitively link specific bacterial populations to the enhanced growth performance due to AGP usage. Interestingly, independent studies (Engberg et al., 2000; Knarreborg et al., 2002; Dumonceaux et al., 2006; Guban et al., 2006; Zhou et al., 2007; Danzeisen et al., 2011; Lin et al., 2013) showed that AGP usage significantly reduced the population of *Lactobacillus* species, a major bacterial choice for probiotics development; in particular, population of *L. salivarius*, the dominant lactic acid bacterium present in the chicken intestine, was reduced in response to AGP treatment. Since *Lactobacillus* populations are major BSH-producers in small intestine (Begley et al., 2006), this interestingly bacterial shift due to AGP usage is consistent with the reduced BSH activity as described above. Taken together, these studies indicate that the AGP-mediated body weight gain in food animal is inversely related to the activity of BSH enzymes and the abundance of corresponding *Lactobacilli* producers.

## CHARACTERISTICS OF BSH

Bacterial BSH is a member of the choloylglycine hydrolase family of enzymes and is predominantly associated with gastrointestinal bacteria of both humans and animals. Additionally, it is classified as an N-terminal nucleophilic hydrolase and can recognize substrate at both the amino acid conjugate or steroid nucleus (Patel et al., 2010). BSH is particularly abundant in lactic acid fermenting probiotic strains like *Lactobacilli* and *Bifidobacteria* (Begley et al., 2006). BSH enzymes display either narrow or broad substrate specificity; most BSH enzymes from lactic acid bacteria showed higher catalytic abilities to hydrolyze glyco-CBAs than tauro-CBAs (Begley et al., 2006). Despite recent significant progress in the characterization of diverse BSH enzymes, research on BSH is still in its infancy (Patel et al., 2010). The natural functions of BSH enzymes for bacteria themselves are still not clear (Begley et al., 2006). One hypothesis is that BSH activity confers *Lactobacilli* tolerance to bile in the intestine (Begley et al., 2006). However, there are contradictory reports about the correlation between bile tolerance and BSH activity in intestinal bacteria (Begley et al., 2006). For example, production of BSH does not determine bile resistance level in *L. salivarius*, the dominant *Lactobacillus* species present in the chicken intestine (Fang et al., 2009). Regardless the natural function of BSH for its bacterial producer, it has been increasingly recognized that intestinal BSH plays an important role in host lipid metabolism and energy harvest (Begley et al., 2006; Jones et al., 2008). Recent probiotics studies have already shown that oral administration of BSH-producing *Lactobacilli* could affect lipid metabolism, consequently lowering cholesterol level in humans (Jones et al., 2012), rats (Kumar et al., 2011), and pigs (De et al., 1998), which is likely mediated through BSH activity. In the future, more functional, genomic, and microbiological studies are needed to better understand the role of BSH in the symbiosis relationship between gut microbiota and host.

## PRODUCTION OF BSH BY PROBIOTICS: A NEGATIVE TRAIT FROM ANIMAL PRODUCTION PERSPECTIVES?

It is no doubt that dietary probiotics, the normal commensal microorganisms, could exert various beneficial effects on food animals. However, probiotics do impose a variety of potential costs (or detrimental effects) to the animal host as well, which include the production of toxic metabolites, decreased fat digestibility due to production of BSH, and the increase of mucus secretion and gut epithelial cell turn-over (Gaskins et al., 2002; Dibner and Richards, 2004, 2005). Due to such opposite impact of probiotics on the host, it is not surprising that inconsistent results on growth performance of poultry have been observed following probiotic administration (Perumalla et al., 2011). In particular, results available from the literature on probiotic treatments often appear to be contradictory. *Lactobacilli* have been a major bacterial choice for probiotic development in poultry. In general, dietary probiotic supplementation in chicken increases intestinal populations of *Lactobacilli* (Smirnov et al., 2005). Since *Lactobacilli* are dominant BSH-producers in the intestine, dietary *Lactobacilli* treatment may negatively affect lipid metabolism and energy harvest, consequently imposing negative impact on body weight gain. Recently, two research groups (Mountzouris et al., 2010; Sharifi et al., 2012) have reported that probiotic supplementation to diets significantly reduced body weight gain, fat digestibility, and feed conversion in broilers; in these studies, two different commercially available probiotics with a mixture of various organisms (Protexin and PoultryStar) were used. Based on these findings, the authors have proposed that the detrimental effects of the probiotics on chicken growth are likely attributed to the production of intestinal BSH by *Lactobacilli* (Mountzouris et al., 2010; Sharifi et al., 2012). Therefore, overall beneficial effects associated with specific probiotics should be carefully evaluated. Understanding the science of potential negative traits of probiotics can help us develop "negative-traits-mitigation" strategy (e.g., BSH inhibitors) to optimize probiotic products for enhanced growth performance of food animals and profitability of feed additive industry.

## DISCOVERY OF BSH INHIBITORS

Based on the information reviewed above, BSH inhibitors are promising alternatives to AGP for enhanced production of food animals. This hypothesis has been partly supported by our recent study (Wang et al., 2012) in which a BSH enzyme with broad substrate specificity from a chicken *L. salivarius* strain was characterized. Examination of a panel of dietary compounds identified copper and zinc compounds as potent BSH inhibitor (Wang et al., 2012); notably, copper and/or zinc have been used at high concentrations to aid in feed efficiency and growth promotion in poultry (Ewing et al., 1998; Miles et al., 1998; Arias and Koutsos, 2006) and swine (Smith et al., 1997; Hill et al., 2000; Armstrong et al., 2004). However, long-term use of high doses of copper or zinc in animal feed has raised some serious concerns, such as copper/zinc toxicosis and environmental contamination. Therefore, discovery of potent, safe, and cost-effective BSH inhibitors is highly warranted.

In our recent study (Smith et al., 2014), a rapid and convenient high-throughput screening (HTS) system was developed and has been successfully used for identification of BSH inhibitors. This HTS strategy is based on the unique feature of BSH enzyme: hydrolysis of soluble unconjugated bile salts by BSH generates insoluble unconjugated bile salts that could form significant precipitations (Smith et al., 2014). After optimizing various screening conditions, a pilot HTS was performed using a small compound library comprised of 2,240 diverse compounds, leading to the identification of several promising BSH inhibitors with potential as alternatives to AGPs, such as riboflavin and phenethyl caffeate (Smith et al., 2014). In the future, larger scale HTS may reveal more novel BSH inhibitors. In addition, comprehensive animal trials are needed to determine the effect of "champion" BSH inhibitors on growth performance of food animals.

Interestingly, this HTS study also identified a panel of antibiotics as BSH inhibitor, such as various tetracycline antibiotics and roxarsone that have been widely used as AGPs in food animals (Smith et al., 2014). This unexpected finding suggests a new mode of action of low-dose antibiotics for promoting animal growth: some AGPs may exert their growth-promoting effect on food animals by direct inhibition of intestinal BSH enzymes for enhanced lipid metabolism and energy harvest. However, some AGPs, such as bacitracin that has been widely used in poultry industry (Chapman and Johnson, 2002), only displayed low or little inhibitory effect on BSH activity (Smith et al., 2014). The study by Smith et al. (2014) further showed the complexity of the modes of action of AGPs and provided new insights into the interactions between low-dose antibiotics and gut microbiome.

### CONCLUSIONS AND FUTURE DIRECTIONS

Independent studies have shown the link between usage of AGPs and reduced BSH activity as well as reduced population of some *Lactobacillus* species, the major BSH-producers, in the intestine of food animals. In light of these findings, BSH enzyme is a promising microbiome target for developing novel alternatives to AGPs for enhancing the productivity and sustainability of food animals. Recent characterization of a broad-spectrum BSH from *L. salivarius* (Wang et al., 2012) and development of an efficient HTS system for discovery of BSH inhibitors (Smith et al., 2014) have laid a solid foundation for us to develop BSH inhibitors-based feed additives to replace AGPs. In addition, the findings from screening of BSH inhibitors (Smith et al., 2014) suggest a new mode of action of low-dose antibiotics (direct inhibition of intestinal BSH) for promoting animal growth. In the future, large animal trials are needed to determine the effects of BSH inhibitors on growth performance of food animals. Given that research on BSH is still in its infancy, research on BSH ecology of BSH enzymes in the intestine and the role of BSH in host physiology is also highly warranted in the future.

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# Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion

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Antimicrobial resistance is a growing public health challenge worldwide, with agricultural use of antimicrobials being one major contributor to the emergence and dissemination of antimicrobial resistance (AMR). Globally, most antimicrobials are used in industrial food animal production, a major context for microbiomes encountering low-doses or subtherapeutic-levels of antimicrobial agents from all mechanistic classes. This modern practice exerts broad eco-evolutionary effects on the gut microbiome of food animals, which is subsequently transferred to animal waste. This waste contains complex constituents that are challenging to treat, including AMR determinants and low-dose antimicrobials. Unconfined storage or land deposition of a large volume of animal waste causes its wide contact with the environment and drives the expansion of the environmental resistome through mobilome facilitated horizontal genet transfer. The expanded environmental resistome, which encompasses both natural constituents and anthropogenic inputs, can persist under multiple stressors from agriculture and may re-enter humans, thus posing a public health risk to humans. For these reasons, this review focuses on agricultural antimicrobial use as a laboratory for understanding low-dose antimicrobials as drivers of resistome expansion, briefly summarizes current knowledge on this topic, highlights the importance of research specifically on environmental microbial ecosystems considering AMR as environmental pollution, and calls attention to the needs for longitudinal studies at the systems level.

**Keywords: agriculture, antimicrobials, metals, microbiome, resistome, mobilome, environmental pollution**

## INTRODUCTION

The intensive production of food animals is a major context for microbiomes encountering low-doses or subtherapeutic-levels of diverse classes of antimicrobial agents. For that reason, this review focuses on food animal production as an important laboratory for understanding the eco- evolutionary (interactions and intersection of ecology and evolutionary biology) mechanisms involved in bacterial responses to low-dose, sub-therapeutic pressures associated with antimicrobials. A majority of agricultural antimicrobials are used as feed additives for growth promotion in livestock and poultry (Silbergeld et al., 2008). This use began in the 1940s in the US, soon after the initiation of large-scale production of these drugs for clinical medicine. From the first approvals to the present, the concentrations of growth promoting antimicrobials (GPAs) in feeds have been stipulated by FDA regulation to deliver sub-therapeutic doses. Redefining GPA use as "therapeutic," "non-therapeutic," or "prophylactic" to comply with regulations in some countries and guidance by the US FDA does not change this condition from the microbial perspective.

This paper will not discuss the hypothesized mechanisms by which GPAs are asserted to increase growth and feed efficiency, since a recent large study by the Perdue Company reported that there were very small or non-significant differences in these outcomes among poultry flocks consuming feeds with or without GPAs (Engster et al., 2002; Graham et al., 2007). In contrast to earlier studies, including those at Lederle (Stokstad and Jukes, 1958-1959), this is the only study conducted under empirical conditions in poultry production over the lifespan of the animals.

GPA use employs agents from every mechanistic class and currently exceeds all clinical uses in terms of the proportion of total antimicrobial production in the US (Silbergeld et al., 2008; FDA, 2011) and, until recently, in the EU (Teuber, 2001). Information from other regions is not generally available, but given the global expansion of poultry and livestock production using methods similar to those first developed in the US (Graham et al., 2008), it is likely that global use of antimicrobials in animal feeds is also significant (see Arriola, 2011 for study of Peru). As a result, GPAs have had important impacts on selection and dissemination of antimicrobial resistance (AMR) worldwide through the food supply and environmental releases. Moreover, because GPAs are utilized most commonly as mixtures in animal feeds, the gut microbiome of poultry or livestock is exposed to multiple pressures acting on a range of molecular mechanisms associated with resistance development (Davis et al., 2011).

From early in the history of GPA use, it was recognized that the gut microbiome of poultry was responding to selection for resistance to drugs in feeds. Jukes acknowledged this as a truism,

but discounted any potential risks for human health (1972). With more concern, Starr and Reynolds (1951) reported that *Escherichia coli* isolated from the gut microflora of poultry flocks fed with streptomycin as a GPA were resistant to streptomycin, as compared to isolates from unexposed flocks. Since that time, numerous studies have documented associations between GPA use and temporal and geographic trends in AMR prevalence in animal wastes, food products, and human populations (documented most completely in studies in Denmark e.g., Aarestrup et al., 2001; Wegener, 2003, also see review by Silbergeld et al., 2008).

The use of molecular, genomic, and metagenomic methods to track AMR genes and AMR strains from food animal production has increased the strength of the evidence on this connection. These methods have also clarified implications of intensive food animal production for human health, demonstrating that the microbiomes of livestock and poultry are reservoirs for AMR pathogens and that resistance determinants can be transferred from these microbiomes to the environment and eventually to humans (Hammerum, 2012). This has been most recently demonstrated for livestock specific strains of methicillin resistant *Staphylococcus aureus* (van Loo et al., 2007; Waters et al., 2011; Price et al., 2012) and extraintestinal pathogenic *E. coli*, especially those phylogroups associated with urinary tract infection in humans (Jakobsen et al., 2010). Smet et al. (2011) reported that a plasmid carrying the $bla_{\mathrm{TEM-52}}$ gene encoding ESBL (extended-spectrum β-lactamase) could be transferred from a poultry strain of *E. coli* into human *E. coli* under simulated human caecal conditions. The same ESBL genes (including $bla_{\mathrm{TEM-52}}$), ESBL-encoding plasmids and ESBL-producing *E. coli* strains have been observed in poultry, chicken meat and humans (Leverstein-van Hall et al., 2011).

This paper briefly summarizes current knowledge on this topic and highlights the importance of research specifically on environmental microbial ecosystems for understanding the evolution and persistence of resistance associated with GPA use in agriculture. This reflects the fact that many events related to the emergence of AMR in agricultural settings occur in the context of interactions between the gut microbiome of food animals, such as chickens and pigs, and environmental microbiomes in those ecological niches impacted by disposal of animal wastes. These wastes are normally not treated prior to disposal and often contain AMR bacteria and transmissible genetic elements assembling resistance genes, as well as residual antimicrobials and their degradation products.

## CURRENT KNOWLEDGE

Over the past three decades, research has largely investigated the agricultural setting for AMR emergence primarily for the purpose of understanding the origin of AMR in food borne pathogens. Relatively recently, non-food pathways and the role of the environment have attracted increasing research attention. However, incorporating this knowledge into studies of microbial ecology and evolution as well as into studies of disease outbreaks and attributable risk of infection by drug resistant bacteria is still limited (Ashbolt et al., 2013).

## ECO-EVOLUTIONARY CONSEQUENCES OF INDUSTRIAL FOOD ANIMAL PRODUCTION ON THE GUT MICROBIOME OF FOOD ANIMALS

Current methods and conditions in industrial food animal production are quite different from traditional agronomy in terms of intensity and density as well as the use of GPAs (Silbergeld et al., 2008). These changes are likely to have affected animal gut microbiomes. Looft et al. (2012), using phylogenetic and metagenomic approaches, found that 14 days' exposure to subtherapeutic doses of chlortetracycline, sulfamethazine, and penicillin induced shifts in gut microbiota of pigs. These changes included an increase in the prevalence of *Proteobacteria* (primarily in *E. coli* species) and in the abundance and diversity of AMR genes specific to and beyond those used GPAs, as well as selection for other genes related to gene transfer, virulence, energy production, and energy conversion. Population shifts were also observed in the gut microbiome of pigs receiving the GPA tylosin (Kim et al., 2012). Using metagenomic pyrosequencing, Danzeisen et al. (2011) reported that the GPA mixtures of virginiamycin/monensin or tylosin/monensin enriched *E. coli* populations in the chicken cecal microbiome, along with genes encoding transport systems, type I fimbriae and type IV conjugative secretion systems. That study did not detect significant differences in AMR gene occurrence between GPA treatment and control groups, which may not represent the commercial poultry production conditions. While AMR genes are present in the gut microbiome of free-range chickens (Zhou et al., 2012), a diverse pool of AMR genes is found in conventionally raised chickens (Qu et al., 2008; Zhou et al., 2012).

The mobilome (Siefert, 2009), a collection of all mobile genetic elements (MGEs), is a functional component of the microbiome, and plays an essential role in microbial ecology and evolution by facilitating horizontal gene transfer among microorganisms (Frost et al., 2005; Gillings, 2013). There are some reports on impacts of GPAs on the animal gut mobilome (Danzeisen et al., 2011; Looft et al., 2012), but studies at the systems level are still rare. Antimicrobials, in addition to enriching preexisting AMR genotypes and phenotypes and providing pressure for evolutionary selection for *de novo* mutations that favor survival (Gullberg et al., 2011), also induce horizontal transfer of MGEs through mechanisms such as bacterial SOS response (Beaber et al., 2003) and translation attenuation (Wozniak and Waldor, 2010). Some recent studies have identified the contributions of GPAs to phage-mediated horizontal transfer of AMR genes in the animal gut microbiome. Allen et al. (2011) reported that the GPA mixture Aureomix 500 used in swine feed (containing chlortetracycline, sulfamethazine and penicillin) led to significant population shifts in both phage and bacterial communities, as well as induction of prophages (one type of MGEs) in swine fecal microbiomes. The altered phage metagenomes harbored multiple AMR genes including genes for multidrug resistance. Bearson et al. (2014) reported that the GPA carbadox induced phage mediated transfer of virulence and resistance genes in *Salmonella enterica* serovar Typhimurium, a human foodborne pathogen that frequently colonizes swine. More investigation is needed for other components of the animal gut mobilome, such as the plasmidome (Kav et al., 2012), before we can fully understand impacts of GPAs on the entire mobilome within the animal gut microbiome.

## ANIMAL WASTES: THE CONNECTOR BETWEEN THE GUT MICROBIOME OF FOOD ANIMALS AND THE ENVIRONMENT

The gut microbiome of poultry and livestock raised in confinement is transferred into animal excreta, which include the resistome and the mobilome. Also present in animal excreta are unmetabolized GPAs (Kumar et al., 2005; Sarmah et al., 2006) and active metabolites. Thus, the first nexus in the environmental pathway of the dissemination of animal husbandry originated AMR is poultry house litter or cesspits that collect wastes in swine barns (see **Figure 1**).

Within a confinement house, wastes (solid and liquid) accumulate inputs from animal excreta and other residues like spilled feed containing GPAs into a waste microbiome over the lifetime of a flock or a herd, and often for a longer period when poultry houses are not routinely cleaned between each flock or when septic impoundments are not emptied between herds (Volkova et al., 2009). During this period, the microbiomes of chickens and house litter interact and exchange organisms and resistance genes (Cressman et al., 2010; Shanmugasundaram et al., 2011). Eco-evolutionary events in the litter microbiome have not been carefully studied. But poultry litter is known to be a reservoir of AMR bacteria and resistance determinants, resistance-encoding MGEs and residual antimicrobials (Nandi et al., 2004; Furtula et al., 2009; Graham et al., 2009a; Cheng et al., 2013).


**FIGURE 1 | (A)** (left) Pigs in confinement house (photo USDA). Note slatted floor; wastes (including excreta and spilled feed) accumulate on this surface and are periodically washed down into a cesspit below the building. (right) A view of a swine production operation showing open cesspits that collect drainage from animal houses; disposal of these wastes involves spraying of liquids (photo S Wing). **(B)** (left) Chickens in a Maryland poultry house. Flocks are housed directly on litter, which contains excreta (as evident from the birds) as well as spilled feed. Litter is removed infrequently from poultry houses (photo J Graham). (right) The lower Pocomoke River, with poultry houses and land disposal of poultry waste (source: Integration and Application Network, University of Maryland Center for Environmental Sciences).

Environmental dissemination of the food-animal-associated microbiome and resistome occurs through waste holding and disposal. Food animal wastes are first released to the environment through on site storage (usually in open sheds for poultry or open impoundments for swine) and then discharged into the environment more broadly by land application as shown in **Figure 1**. The sites of land disposal may be close to or very distant from the sites of poultry or swine production (Leibler et al., 2009). Once released into the external environment either by unconfined storage or by deposition on land, the microbial and chemical constituents of animal wastes can be widely dispersed mainly through dust, air, and water movement, as well as by animal movement (flies, rodents, and wild birds) (Graham et al., 2009b).

Because of the intensity of food animal production in the US and other countries, this constitutes a major source of all gut microbiota being transferred to the environment (Silbergeld et al., 2008). Unlike human biosolids, no treatment is required prior to discharge of animal wastes, which are almost entirely (>90%) disposed of onto land (Graham and Nachman, 2010). Storage of poultry house litter or of swine waste, without specific composting procedures, does not reduce the burden of pathogens and AMR determinants (Gerba and Smith, 2005; Graham et al., 2009a). More intensive composting, though more effective in attenuating microbial loadings in wastes, does not significantly reduce loadings of AMR genes (Storteboom et al., 2007). Even multiple treatment lagoons are unable to completely remove AMR genes (McKinney et al., 2010).

For these characteristics of animal wastes—complex constituents, large volume, insufficient waste management, and broad contact with the environment—the appropriate focus for understanding the emergence and dissemination of AMR driven by use of subtherapeutic GPAs in agriculture is in the interactions between animal wastes and the environment. Intensive animal production operations and associated environmental compartments have been characterized as "genetic reactors" where new genotypes and phenotypes of resistance can evolve through genetic exchange and recombination, and re-enter humans and animals (Baquero et al., 2008). We conceive of a series of dynamic interactions between environmental microbiomes and the altered gut microbiome of poultry flocks that is represented within poultry house waste, which in turn integrates microbial loadings from multiple flocks within each house. This is shown in **Figure 2** (Davis et al., 2011).

## THE ENVIRONMENTAL MICROBIOME AND LOW-LEVEL ANTIMICROBIAL PRESSURE FROM AGRICULTURE

The environment is both the main receptor for animal wastes containing AMR determinants, particularly those on MGEs (Heuer et al., 2011), and the locus of the natural resistome (D'Costa et al., 2006, 2007) and mobilome (Siefert, 2009; Gillings, 2013) which together constitute the genetic resources available to microbial

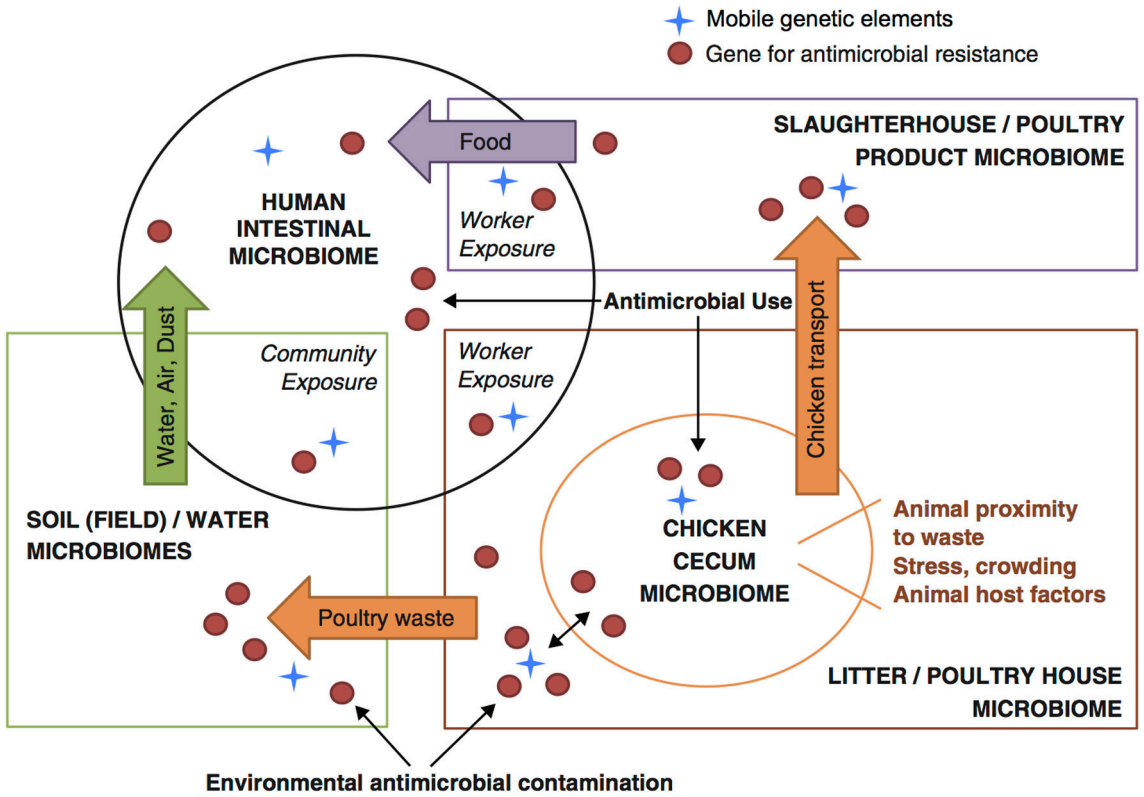


**FIGURE 2 | Conceptual framework for understanding flow of resistance genes and mobile genetic elements across microbiomes within food animals, the environment, and human populations (based on Davis et al., 2011).**

communities for surviving antimicrobial stress and the genetic machinery to transfer these genetic resources among bacteria within and among different microbial communities. The environmental resistome, which encompasses both natural constituents as well as inputs from anthropogenic activities like agriculture, is relevant to human health as it can be a source of resistance determinants found in human pathogens, particularly through the mobilome (Forsberg et al., 2012; Perry and Wright, 2013).

There is ample evidence in the literature on the impacts of intensive food animal production on the occurrence of AMR in directly or indirectly affected environmental compartments (including soils, sediments, and water) (reviewed by Joseph et al., 2001; D'Costa et al., 2007; Ghosh and LaPara, 2007; Stine et al., 2007; Silbergeld et al., 2008; Graham et al., 2009a; Martinez, 2009; Knapp et al., 2010; Heuer et al., 2011; You et al., 2012, 2013; Gaze et al., 2013; Jones et al., 2013; Wei et al., 2013). Using quantitative methods such as real-time PCR and LC-MS/MS, correlations have been demonstrated between the occurrence/abundance of AMR genes and the extent of antimicrobial use or drug concentrations in animal husbandry environments (Smith et al., 2004; Peak et al., 2007; McKinney et al., 2010; Wu et al., 2010; Zhu et al., 2013). These relationships may involve multiple mechanisms including the simultaneous loading of both genes and drugs into the ecosystem and/or *in situ* selection for AMR in the environment due to inputs of antimicrobials, metals, and other residues. Until recently it was thought that the concentrations of antimicrobial compounds or their degradation products in the environment, which usually range from μg/kg to mg/kg in sediment or soil samples (Kemper, 2008), were not high enough to select for resistance. However, laboratory studies have demonstrated that low concentrations of antimicrobials in the same range are sufficient to select for resistance through several mechanisms (Kohanski et al., 2010; Gullberg et al., 2011). A study involving both field and laboratory research and coupled with modeling reported a selective and persistent effect of sulfadiazine in pig manure on resistance genes in soil microbiota (Heuer et al., 2008). Interactions between drugs (and their degradation products) and the soil microbiome are influenced by the physicochemical properties of each drug, which affect their potential bioavailability in the environment such as soils (Tolls, 2001; Hamscher et al., 2005; Kemper, 2008). Also antimicrobials undergo biotic/abiotic degradation in the environment (Sarmah et al., 2006), and only some of the degradation products exhibit antimicrobial potency (Halling-Sørensen et al., 2002). Data on the bioavailability of agricultural antimicrobials *in situ* are still missing (Heuer et al., 2011).

There are demonstrated links between agricultural antimicrobial use and expansion of the environmental resistome and mobilome (reviewed by Nandi et al., 2004; Ghosh and LaPara, 2007; Heuer et al., 2008, 2011; Zhang et al., 2009; Allen et al., 2010; Gaze et al., 2013; Zhu et al., 2013). A historical analysis

of soil samples collected from 1940 to 2008 in the Netherlands found an exponential increase of AMR genes in agricultural soils (Knapp et al., 2010). Horizontal gene transfer largely contributes to the proliferation and persistence of AMR genes in the environment. After being introduced into the environment microbiome, animal-waste borne bacteria can transfer their AMR genes to indigenous bacteria through conjugation due to the frequent assembly of AMR genes on integrons, transposons and plasmids in the animal waste microbiome (Andrews et al., 2004; Nandi et al., 2004; Binh et al., 2008; Byrne-Bailey et al., 2011; Heuer et al., 2011; Zhu et al., 2013). This process can be stimulated by enhanced nutrient availability from animal wastes (van Elsas et al., 2003). Even after AMR bacteria of the animal gut microbiome died, their AMR genes can persist and be taken up by soil bacteria through transformation (Lorenz and Wackernagel, 1994) and thus proliferate in the environmental microbiome. Positive correlations have been observed between the concentration of antimicrobials and the abundance of MGEs carrying AMR genes in soil or water (Knapp et al., 2008; Zhu et al., 2013). But it is unclear whether this is due to enhanced horizontal gene transfer or selection on the recipient populations after transfer. Longitudinal and systemic studies on the environmental resistome and mobilome in response to agricultural stress are needed to assess its broad impacts on microbial ecosystems.

We have studied the presence of drugs, resistant bacteria, resistance genes, and resistance-encoding plasmids in soil microbiota near a waste storage site at a concentrated poultry production operation in the US in comparison to several sites within a protected state forest in the same region (You et al., 2012). Neither tetracycline nor chlortetracycline was detected in the forest soil samples, but measurable levels ($<10\,\mu g/kg$) were detected in the farm samples from near the storage site. Farm samples also contained a high proportion of tetracycline resistant bacteria as compared to forest samples. Resistance genes (*tetM, O* and *ermA, B, C*) as well as plasmids containing *tetL* were only detected in farm soils. While *tetL* was found in both farm and forest samples, its prevalence was much higher in the former. In all of the perspectives, soil from a less waste-affected site at the same concentrated poultry production operation showed no significant difference from forest soil. These results are similar to those published on soils impacted by swine wastes (Agerso et al., 2006; Ghosh and LaPara, 2007). The results of our study suggested that resistance could persist within the soil microbiome. A study by Sengelov et al. (2003) reported persistence of resistant isolates for 300 days after application of swine wastes to soil. Another study by Ghosh and LaPara (2007) reported that bacterial resistance levels in soil with excessive application of swine manure were sustained for at least 18 months.

In summary, poultry and livestock wastes are an important anthropogenic source of antimicrobial pressure, at low levels, for the microbiomes of hosts and environments. The ecological and evolutionary events in the environmental microbiome in response to animal waste inputs include interactions between antimicrobials (also other chemicals as exemplified in the next paragraph) and the environmental microbiome; interactions between the waste microbiome and the environmental microbiome; and interactions between the environmental microbiome and resistance genes in waste. These interactions are relevant to the expansion and persistence of the resistome within the environmental microbiome, which may in turn expose human microbiomes through multiple pathways. Because of the complex nature of these interactions and the diversity of environmental microbiomes, studies at a systems level using advanced "omics" methods are in particular need.

## INTERACTING STRESSORS IN THE GUT AND ENVIRONMENTAL MICROBIOMES–ANTIMICROBIALS AND METALS

Feeds for food animals are complex mixtures of natural products from both crop and animal sources, as well as recycled wastes and other additives (Sapkota et al., 2007). As a result, there are multiple potential stressors, in addition to GPAs, presented via feeds to food animal gut microbiomes. These include metals that are known to co-select for resistance in bacteria (Baker-Austin et al., 2006). For example, arsenicals are used as coccidiostats and growth promoters, copper and zinc are used as trace element supplements, mercury is present as a contaminant in fish meal (a major constituent of poultry feeds), as well as a range of metals in industrial waste byproducts permitted as additives to animal feeds (in the US). These metals are not metabolized (except in the case of arsenic, where chicken gut microbiota metabolize roxarsone, an organoarsenical, into the more toxic form of inorganic arsenic Stolz et al., 2007) and thus like antimicrobial drugs, they are excreted into wastes (Garbarino et al., 2003; Jackson et al., 2003) and transferred into soil through waste disposal (Gupta and Charles, 1999; Rutherford et al., 2003).

Previous microbiological research under both laboratory and field conditions have reported on interactions between these metals and antimicrobials in terms of co-selection for and co-transfer of resistance among bacteria via MGEs containing both metal and drug resistance genes (Bass et al., 1999; Baker-Austin et al., 2006; Singer et al., 2006; Stepanauskas et al., 2006; Wright, 2007; Tuckfield and McArthur, 2008; Novo et al., 2013). Significant positive correlations were observed between *tet* genes and several metals in a study of swine waste lagoons (McKinney et al., 2010), and between total AMR genes and copper in a study of manure, compost, and soils from swine farm (Zhu et al., 2013).

These interactions have extended to human health concerns. There is evidence that exposure of human hosts to mercury increases odds of their carrying antibiotic resistant *E. coli* (Skurnik et al., 2010). It has been known for some time that *tcrB*, a copper resistance gene, is transferrable and linked to genes encoding macrolide and glycopeptide resistance (Hasman and Aarestrup, 2002). This was recently confirmed independently by two research groups (Amachawadi et al., 2013; Silveira et al., 2014) who also showed that *tcrB* could be transferred by conjugation among *enterococci* from pigs, poultry, and cattle along with resistance genes for erythromycin, tetracycline, vancomycin, ampicillin, and gentamycin. Cavaco et al. (2011) reported that cadmium and zinc drive co-selection for methicillin resistance in *Staphylococcus aureus* through horizontal transfer of plasmids containing genes for both methicillin and metal resistance (*mec* and *czr*).

These findings indicate the need to consider interaction effects of antimicrobials and other stressors within agricultural settings in terms of driving AMR emergence and dissemination,

particularly in light of the complex nature of manufactured feeds utilized in food animal production.

## CONCLUSIONS: ANTIMICROBIAL RESISTANCE IS A FORM OF ENVIRONMENTAL POLLUTION

Resistance dissemination involves multiple microbiomes, each having a complex ensemble of microbes, particularly in the case of the environmental microbiome. These microbiomes are encountering diverse interacting stressors, and gene flow occurs through these microbiomes. Systems-biology approaches driven by "omics" methods (Raes and Bork, 2008) can improve our understanding of mechanisms of AMR development and persistence. Meanwhile, the "eco-health" perspective that takes into account linkages between ecosystems and health (Zinsstag et al., 2011) can improve our health practices, in particular those prior to human exposure.

An intriguing concept has been proposed, to consider AMR as environmental pollution, most comprehensively explained by Martinez (2009). There are some studies utilizing this concept to track AMR genes in landscapes, most frequently in studies of watersheds (Pei et al., 2006; Pruden et al., 2006). But this research, important as it is, still does not fully exploit the importance of the concept. If we think about "resistance" as a material thing, not just a behavior of bacteria, we can consider how this material behaves in environmental compartments such as water, soils, and sediments. Because of the importance of the environment as a locus for the emergence, persistence, and dissemination of AMR, particularly in relevance to low level AMR pressure derived from agriculture, this proposal merits further analysis at the theoretical level as well as more extensive field research in impacted environments.

In thinking about resistance as a pollutant, we can consider the approaches of environmental research in studying chemical pollutants. In many respects, resistance genes have the same properties that we consider in evaluating pollutants like pesticides.

Three characteristics are important in environmental health: hazard, persistence, and bioaccumulation. That is, does a substance in itself have properties that could seriously harm human health, does the substance remain unchanged in the environment without being broken down by natural processes, and finally is the substance taken up by organisms such that its levels increase over time in those species.

We can certainly say that an AMR gene is hazardous to human health because when it is present in a pathogenic bacterial cell or population it can result in failure of medical treatment of infection. Thus, resistance genes require incorporation into an organism to express their hazard, which is not so different from thinking the conversion of mercury into methyl mercury and its incorporation into fish. Low dose issues are important in understanding the hazards of agricultural use of antimicrobials since, similar to the hazards of some environmental chemicals, the pressure for selection for resistance may be more significant at lower doses (Vandenberg, 2014). This has been shown for failures of clinical antimicrobial treatment (Schentag et al., 2007).

The second characteristic of importance is persistence of resistance genes in the environment, about which we know relatively little. Resistance genes can survive in soils for long periods of time (Heuer et al., 2011). One recent study demonstrated that purified DNA from transplastomic plants encoding resistance to streptomycin could be detected as long as 4 years after being added into soils, and that these resistance genes could still be taken up by bacteria and incorporated into their chromosomal DNA for expression (Pontiroli et al., 2010). Persistence of resistant phenotypes is complex. There are both theoretical and empirical data to challenge the standard model of evolutionary selection for AMR or for susceptibility depending upon the presence or absence of antimicrobial stress (Andersson and Hughes, 2010). Theoretically, Levin and others have demonstrated that there are two evolutionary "choices" for resistant bacteria to adapt to the absence of stress when the expression of resistance exacts some cost to the bacterial populations in terms of physiological demands or reproductive rate (Levin et al., 2000; Rozen et al., 2007). One "choice" is reversion to susceptibility; the other choice is selection for a means to reduce this cost. Both choices involve mutation either back to the wild type gene or to a change in some other gene that is associated with increased Malthusian fitness. The second path may be a more adaptive strategy for microbial communities under continuous or periodic stress from antimicrobials, as in an environment with inputs of antimicrobial drugs from agriculture.

The third key characteristic of chemical pollutants in the environment in terms of raising alerts is bioaccumulation and biomagnification, or uptake and retention of chemicals in biota and increase in concentration within ecosystems through food chains. DNA (extracellular or intracellular) in the environment, like chemicals in the environment, can be accumulated by bacteria through transformation (Lorenz and Wackernagel, 1994) and on hierarchical compositions of MGEs through insertion (Frost et al., 2005). Biomagnification of a resistance gene occurs within microbiomes by the expansion of bacterial populations or MGEs carrying that gene. That is, (1) once a novel gene is taken up and incorporated into a bacterial genome, when that organism divides, its daughter cells each contain the new gene, which is a highly efficient process of increasing the total amount of that gene; or (2) once a novel gene is inserted in a MGE, when the MGE invades a bacterial community, diverse populations acquire the new gene through repeated infections.

## RESEARCH NEEDS

From this brief review, we can identify some important research needs relevant to increasing our understanding of low dose antimicrobial exposures (including but not limited to GPAs in agriculture) in the context of microbial ecology and evolution. These questions in many cases can best answered with longitudinal studies using state of the art methods to interrogate the microbiome and its constituent elements such as the resistome and the mobilome. As noted by others, there is a critical lack of such studies at the systems level that permit examining associations between changes in the environmental resistome and well annotated changes in the drivers related to agricultural land use (Singer et al., 2006; Shade et al., 2013).

(1) What does it mean to expose the microbiome to multiple antimicrobial and metal stressors at low doses?
(2) What does it mean to stress the gut microbiome continuously over the lifetime and generations of food animals in terms of resistance and other changes at the microbiome level?
(3) What events occur in food animal wastes in terms of expanding the resistome? What is the contribution of the continued presence of residual antimicrobials in wastes?
(4) What events occur in soils where food animal wastes are repeatedly applied and in sediments impacted by agricultural runoff? What is the contribution of the continued presence of antimicrobials at low concentrations in soils and sediments?
(5) How long is the persistence of resistant bacteria in animal wastes? How long is the persistence of resistance genes in the soil or sediment ecosystem?
(6) How can we quantify the contribution of food animal production using GPAs to the expansion of the environmental and human host resistome/mobilome?
(7) What are the major pathways from environmental compartments such as soils or sediments to humans; to what extent do these pathways involve passage through the microbiomes of wild or other domesticated animals?
(8) What are the most effective and efficient methods for studying these events at the system level?

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# Antibiotics promote aggregation within aquatic bacterial communities

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The release of antibiotics (AB) into the environment poses several threats for human health due to potential development of AB-resistant natural bacteria. Even though the use of low-dose antibiotics has been promoted in health care and farming, significant amounts of AB are observed in aquatic environments. Knowledge on the impact of AB on natural bacterial communities is missing both in terms of spread and evolution of resistance mechanisms, and of modifications of community composition and productivity. New approaches are required to study the response of microbial communities rather than individual resistance genes. In this study a chemostat-based experiment with 4 coexisting bacterial strains has been performed to mimicking the response of a freshwater bacterial community to the presence of antibiotics in low and high doses. Bacterial abundance rapidly decreased by 75% in the presence of AB, independently of their concentration, and remained constant until the end of the experiment. The bacterial community was mainly dominated by *Aeromonas hydrophila* and *Brevundimonas intermedia* while the other two strains, *Micrococcus luteus* and *Rhodococcus* sp. never exceed 10%. Interestingly, the bacterial strains, which were isolated at the end of the experiment, were not AB-resistant, while reassembled communities composed of the 4 strains, isolated from treatments under AB stress, significantly raised their performance (growth rate, abundance) in the presence of AB compared to the communities reassembled with strains isolated from the treatment without AB. By investigating the phenotypic adaptations of the communities subjected to the different treatments, we found that the presence of AB significantly increased co-aggregation by 5–6 fold. These results represent the first observation of co-aggregation as a successful strategy of AB resistance based on phenotype in aquatic bacterial communities, and can represent a fundamental step in the understanding of the effects of AB in aquatic ecosystems.

**Keywords: antibiotic resistance, experimental ecology, aquatic bacteria, ecological interactions, aggregation**

## INTRODUCTION

The occurrence of antibiotics (AB) in aquatic environments is of major concern because of potential spread of AB resistance and of ecosystem alteration (Levy, 1992).

Natural AB are constantly produced by microorganisms and their presence in the environment in very low concentrations was largely underestimate as a potential key factor in controlling ecological interactions (Gullberg et al., 2011). The increased use of AB, and the production of new generations of semi-synthetic AB raise even more concerns about their effect on the natural bacterial communities. Pharmaceuticals, including AB, are only partially eliminated in wastewater treatment plants, therefore residual amounts can reach surface waters or groundwater, where these bioactive compounds potentially impact on natural microbial communities (Hirsch et al., 1999; Czekalski et al., 2012). AB load has been quantified for a number of water bodies and water treatment plants all over the world, demonstrating a strong correlation between human activities (urbanization, farming) and the amount of AB released in the water (McArdell et al., 2003). Although most water treatment plants can reduce by an order of magnitude the concentrations of several AB by sorption transfer to sewage sludge (Giger et al., 2003), AB loads in the range of 5–10 $\mu g\ L^{-1}$ have been detected in a number of rivers in different highly anthropized areas around Europe in the last decades (Richardson and Bowron, 1985; Hirsch et al., 1999; Giger et al., 2003). The problem of AB in aquatic environments has been rather underestimated with the consequence of a poor policy on the control of the AB releases (Ternes, 1998; Sarmah et al., 2006) resulting in the discovery of a number new AB resistances developed in aquatic environments (Baquero et al., 2008; Kümmerer, 2009).

Currently used bactericidal or bacteriostatic AB, which are thus released in the environment, are grouped in three main modes of antimicrobial action: ABs acting against the bacterial

membrane or cell walls, (e.g., β-lactam), AB targeting protein synthesis through the ribosomal subunits (e.g., tetracycline), and ABs which interfere with the nucleic acid synthesis (e.g., fluoroquinolones) (Sengupta et al., 2013). These groups of AB are nowadays considered within the principal contributors to emergence and maintenance of new resistances within the natural bacterial communities, resulting in direct risk for human health (McArdell et al., 2003). A number of studies found large pools of genes involved in the development of resistances to clinically relevant AB within the complex bacterial communities of aquatic and terrestrial environments (D'Costa et al., 2006; Pruden et al., 2006), where horizontal gene transfer can promote their rapid spread and maintenance. Resistances generally covered the whole sets of AB tested, confirming the rapid adaptability of natural communities, not only to the natural AB but also to new synthetic ones: in 2005 the resistance of over 400 strains library isolated from soils to natural erythromycin (introduced in 1952) was of 27% while the resistance against the semisynthetic telithromycin (FDA approved in 2004) was already of 17% of the strains (D'Costa et al., 2006). This leads to the consideration of environmental bacterial communities exposed to anthropic impact as significant repository of AB resistances.

A number of possible factors can promote the development of a resistance to a specific AB in low dose, and bacterial communities usually exposed to high selection pressure in the environment, developed peculiar features (e.g., broad phylogenetic diversity, phenotypic plasticity, presence of peculiar strains within the rare species, competition pressure favoring rapid evolution) that makes them extremely feasible toward AB resistance spread.

Recently, ideal reservoirs of AB resistances in waters were identified by Drudge and coworkers (Drudge et al., 2012) in water flocs, microparticles composed by a number of tens to thousands of bacterial cells belonging to different species and eventually grown around an organic substrate, i.e., "marine snow" (Alldredge and Silver, 1988) and "freshwater snow" (Grossart and Simon, 1993). This can be attributed to the chemical richness of the flocs themselves coupled to the proximity of bacterial population that lives aggregated around the particle. The flocs can represent a barrier against AB penetration, reducing its concentration toward the center of the aggregate, and at the same time, because of proximity, can promote horizontal gene transfer and thus enhance AB resistance spread within the clustered bacterial community. These particular microenvironments are also well studied in oceanography and theoretical ecology, as they represent ideal hot-spots for bacterial production and organic matter degradation (Azam and Malfatti, 2007; Corno et al., 2013), with enhanced ecological interactions between organisms and complex food-webs in a spatially limited habitat.

It can be guessed that the presence of low doses of AB in waters, promotes not only resistance but, as more immediate response, other kinds of adaptations of the bacterial community, potentially involving alterations of the fitness and of species composition, with unpredictable effects on the stability of the system itself.

In order to deepen our knowledge on the ecological effects of AB in natural water bodies, we performed the first experimental study where different doses of AB triggered a natural bacterial community under controlled laboratory conditions. The bacterial community was designed in order to reproduce a very simplified non-AB resistant freshwater planktonic community. In continuous culture mimicking lake water conditions, bacteria were exposed to a cocktail of three antibiotics chosen from the most commonly used ones in Europe. We measured bacterial productivity and fitness, community composition, and phylogenetic distribution. Furthermore, we tested the degree of adaptation to AB of single strains and communities (i.e. acquisition of AB resistance) in order to assess the ecological impact of AB in concentrations comparable with anthropized waters in Central Europe on the bacterial community.

## MATERIALS AND METHODS

### SELECTION OF ISOLATES AND ANTIMICROBIAL SUSCEPTIBILITY TESTS

Four freshwater bacterial strains isolated from European lakes have been used for this study: *Aeromonas hydrophila* strain GC035, *Brevundimonas intermedia* strain GC044 and *Micrococcus luteus* strain GC037 have been isolated from Lake Zurich (Switzerland), while *Rhodococcus* sp. strain NO0007 has been isolated from Lake Maggiore (Italy). Partial 16SrDNA sequences of each strain are deposited in Genbank (accession nrs. KJ409640-43). The community composed by the mixture of these strains reproduces an extremely simplified bacterial community of a classical deep European oligo-mesotrophic lake: *A. hydrophila* (Gammaproteobacteria) and *B. intermedia* (Alphaproteobacteria) are common in freshwater and in particular conditions can dominate lakes communities (Farmer et al., 1992; Zwart et al., 2002), while *M. luteus* and *Rhodococcus* sp. (Actinobacteria) despite rather frequently found in freshwaters are always limited to very little numbers and non-significant relative abundances (Newton et al., 2011).

The four strains were preliminary subjected to antimicrobial susceptibility test to the AB further used in this study, namely levofloxacin, tetracycline, and imipenem (Oxoid, Milan, Italy) by disc diffusion method, according to the Clinical and Laboratory Standards Institute CLSI (2008). Because published breakpoints for the species used are missing, the interpretive standards of the diameter zone were applied as follow: non-Enterobacteriaceae or nonfastidious Gram-negative rods were used to interpret the diameter zone values for *B.intermedia* and *A. hydrophila; Staphylococcus* sp. for *M. luteus*, and *Corynebacterium* for *Rhodococcus* sp. (CLSI, 2008 and EUCAST, 2014). The same procedures and interpretative standards were used for the determination of antimicrobial susceptibility of the four strains isolated after the experiment in continuous culture.

### CONTINUOUS CULTURE DESIGN

Bacterial strains were pre-cultivated for 3 days in Artificial Lake Water medium (ALW, Zotina et al., 2003). Aliquots from each pre-culture were then used to prepare the experimental community, which was inoculated into the chemostat.

The main experiment was carried out in a one-stage continuous cultures system consisting of 9 reactors (**Figure 1**); three parallel chemostat vessels were run for each treatment (NO AB, AB+, AB++). The chemostat was assembled in a climate chamber (18 ± 1°C) with a night-day period of 12 h and ran for

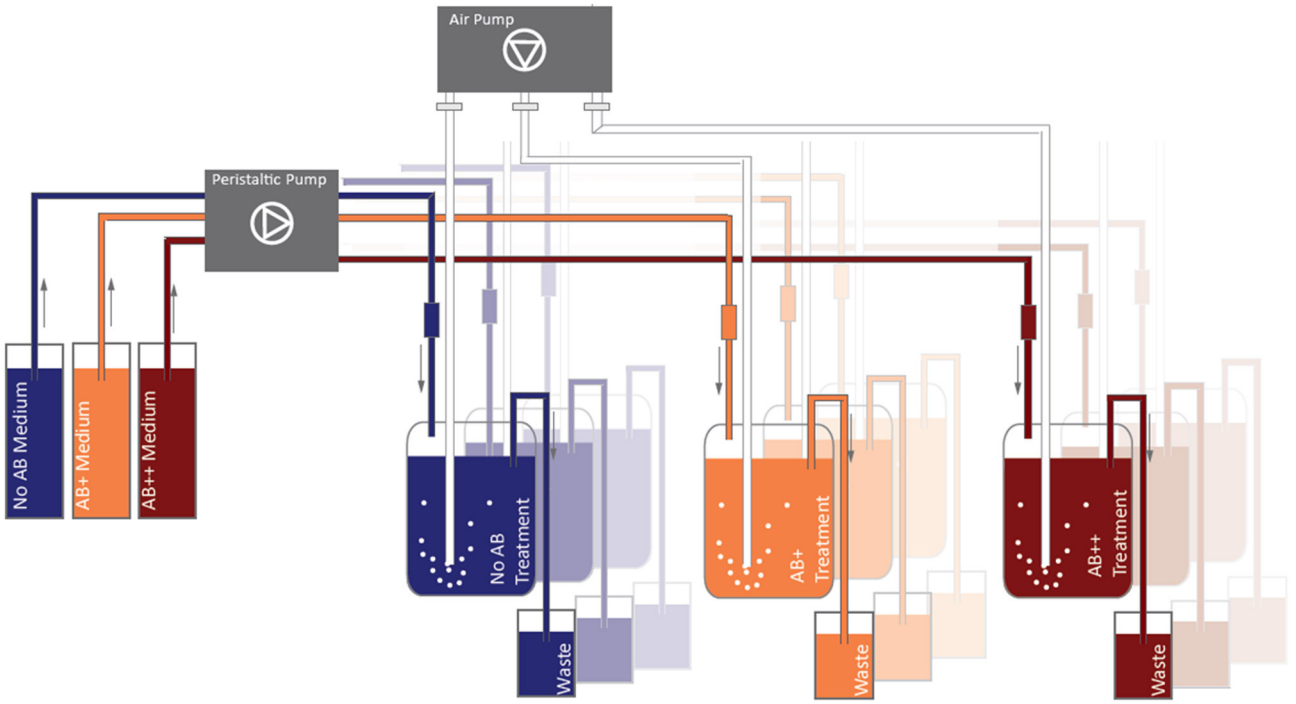


**FIGURE 1 | Continuous culture design.** Substrate was continuously renewed (0.2 d-1) through peristaltic pumps, aeration (fine bubbling) was performed in order to ensure a full mixing of the water in the vessel and to prevent the deposition of detritus.

25 days. The 9 vessels were filled with 750 ml of ALW medium enriched with 10 mg glucose $L^{-1}$ as the sole C-source. New medium was continuously pumped in the vessels by a multichannel peristaltic pump (Watson-Marlow 205S) from three 20 L reservoirs, in order to achieve a dilution rate of $D = 0.2$ $d^{-1}$. The vessels were aerated from the bottom by fine bubbling with sterile air (**Figure 1**).

AB impact on the bacteria was tested at different concentrations of the same AB cocktail, composed by an equal mixture of tetracycline (Sigma-Aldrich, CAS number 60548), imipenem (Sigma-Adlrich, CAS 74431-23-5; β-lactam, subgroup carbapenems), and levofloxacin (Sigma-Adlrich, CAS 100986854; fluoroquinolon) to a final concentration of 12.5 μg AB $L^{-1}$ (treatment AB+), and of 125 μg AB $L^{-1}$ (treatment AB++). Treatment NO AB, without antibiotics, was used as control. The AB mixtures were added directly to the AB+ and AB++ 20 L reservoirs. Concentration AB+ mimes the natural concentration of AB in polluted effluxes by waste water treatment plants in anthropized areas in Central Europe (Kümmerer, 2009), while concentration AB++ represent a concentration 10 times higher than the highest reported in nature. The selection of the three ABs used for the cocktail is consistent with the major AB groups nowadays consumed in Western countries (Food and Drug Administration report 2011—FDA Annual Report on Antimicrobials Sold or Distributed for Food-Producing Animals in 2011; UCM338170).

About 18 ml of the assembled freshwater community was inoculated into each of the 9 reactors to obtain a final concentration of $1.0 \times 10^6$ cells $ml^{-1}$ (composed by $0.25 \times 10^6$ cells $ml^{-1}$ of each strain). After an acclimation of 48 h without dilution, the system was switched on and the experiment started. Daily samples (5 ml/vessel) were collected for the analysis of bacterial abundance; weekly samples (25 ml/vessel) were taken for the analyses of community composition and phenotypical distribution.

## BACTERIAL ABUNDANCE AND PHENOTYPICAL DIVERSITY

Bacterial cell numbers were determined from 0.5 ml formalin-fixed (2% final concentration) samples stained with 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980), filtered onto 0.2 μm polycarbonate filters, and counted by epifluorescence microscopy (Axioplan, Zeiss). At least 400 bacterial cells were counted per sample. Different phenotypes were classified on the base of the bacterial social behaviors: free living single cells, microcolonies composed by few aggregated cells (generally belonging to the same strain), and larger aggregates composed by several clustered individuals belonging to different species (Corno et al., 2013). Images for the sizing of aggregates and microcolonies were captured with a DP72 high resolution camera (Olympus) and evaluated by image analysis (ImagePro Plus, Media Cybernetics).

Aggregates size was approximated by determining the maximal Feret dimension ($Fe_{max}$) of single cells cluster as detected on DAPI stained filters. Aggregates were grouped into size classes each 10 μm. Cell clusters composed by at least 5 cells, with $Fe_{max}$ <10 μm were considered as microcolonies. The relative importance of the aggregated cells within the bacterial community was obtain by the estimation of their average number for aggregate for each size class, then mediated to cover the aggregates size distribution for each sample (Corno et al., 2013). In detail, the

average cells number per aggregate was estimated by the median values for the corresponding aggregate size class and the average bacterial cell sizes, as obtained from epifluorescence microscopy. The reliability of the estimation was tested by comparing the organic carbon content of the bacterial community (estimated per bacterial cell per strain according to Loferer-Krössbacher et al. (1998) and the direct measurement of POC in the same sample. Briefly, the relative proportion of each strain in the sample, the specific amount of C per cell of each strain, and the difference between the total measured POC and the POC in single free living cells, were used to estimate the amount of C in the aggregates, then related to the different size classes (Corno et al., 2013). In order to reduce the potential approximation error in the different size classes we merged our results in the general group "Aggregates."

### BACTERIAL COMMUNITY COMPOSITION

The composition of the bacterial community was analyzed by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) coupled with fluorescence microscopy. Weekly collected samples were fixed with freshly prepared buffered PFA (1% final concentration) and then concentrated on 0.2 μm polycarbonate filters (GTTP, Millipore). Filters were rinsed twice with sterile phosphate buffered saline (PBS), air-dried and stored at −20°C until further processed. The following probes were used to determine the relative proportions of specific bacterial populations: ALF968 for Alphaproteobacteria (Glöckner et al., 1998), GAM42a (mixed with the corresponding competitor probe) for Gammaproteobacteria (Manz et al., 1992), and HGC69a for Actinobacteria (Roller et al., 1994). CARD-FISH was performed according to Sekar et al. (2003). The relative proportion of each strain was then counted by epifluorescence microscopy. Actinobacteria were additionally subdivided within the two strains used in this study, by visual recognition: while *Rhodococcus* sp. cells are rods of different length, *M. luteus* cell shape is always coccoid with constant dimensions, thus they are morphological easily distinguishable by epifluorescence microscope.

### POST CONTINUOUS-CULTURE RE-ISOLATION AND ANALYSIS

At the end of the experiment, each bacterial strain was isolated from each treatment on ALW agar plates in order to be tested for potential acquired AB resistance. *Rhodococcus* sp. strain could not be isolated from treatments AB+ and AB++; the clonal strain of the inoculum was used instead. Purity of the isolated strains was checked by CARD-FISH: only cultures with 100% of positive hybridization rate were then used in further experiments. Single cultures from the different isolates were tested for antimicrobial susceptibility as described above. Single cultures and re-assembled communities (composition is described below) were also tested for growth in 96 well plates: 200 μl culture in ALW medium enriched with 20 mg $L^{-1}$ of Glc, inoculum concentration 1 x $10^6$ bact $ml^{-1}$ in triplicate for each experimental treatment. For example, *A. hydrophila* isolated from NO AB treatment was inoculated in triplicate in NO AB, AB+, and AB++ wells. The disposition of the treatments and the distribution of the strains in the plates was randomized. Bacterial communities were re-assembled by mixing the strains in identical proportion to reproduce the same initial conditions as in the chemostats. Plates were incubated (same conditions than for the chemostat) for 48 h, radially shaken for 30 s every 30 min and growth was measured every 24 h as optical density (OD) of each well with a plate reader Glomax Multi-detection System (Promega) cleaned of the blank signal and other potential noises.

### STATISTICAL ANALYSES

In order to evaluate the significance of the difference in the median values between groups the Wilcoxon-Mann-Whitney test was applied. To test for statistical differences with the expected median value within a single time series a *t*-test was performed, after testing for normal distribution of the series. All analyses were performed by using software JMP10 (SAS Institute Inc.).

## RESULTS

### ANTIMICROBIAL SUSCEPTIBILITY TESTS IN PRE AND POST TREATED ISOLATES

The resistance antibiotypes of the four isolates—before and after the continuous culture experiments—are shown in **Table 1**. All strains resulted to be susceptible to the tested antibiotics with the exception of *B. intermedia* which showed intermediate resistance for levofloxacin. The correlation of the resistance antibiotypes

**Table 1 | Results of antimicrobial susceptibility tests by disk diffusion method.**

| Bacterial strains | Zone diameter values in mm (results) | | |
|---|---|---|---|
| | IMP | TET | LEV |
| *Aeromonas hydrophila* GC035 | 34 (S) | 28 (S) | 34 (S) |
| *A. hydrophila* (NO AB) | 33 (S) | 29 (S) | 33 (S) |
| *A. hydrophila* (AB+) | 34 (S) | 29 (S) | 34 (S) |
| *A. hydrophila* (AB++) | 34 (S) | 29 (S) | 34 (S) |
| *Brevundimonas intermedia* GC044 | 47 (S) | 38 (S) | 18 (I) |
| *B. intermedia* (NO AB) | 47 (S) | 38 (S) | 18 (I) |
| *B. intermedia* (AB+) | 47 (S) | 38 (S) | 18 (I) |
| *B. intermedia* (AB++) | 47 (S) | 38 (S) | 18 (I) |
| *Micrococcus luteus* GC037 | 33 (S) | 23 (S) | 22 (S) |
| *M. luteus* (NO AB) | 33 (S) | 23 (S) | 23 (S) |
| *M. luteus* (AB+) | 33 (S) | 23 (S) | 23 (S) |
| *M. luteus* (AB++) | 33 (S) | 23 (S) | 23 (S) |
| *Rhodococcus* sp. NO007 | 55 (S)* | 40 (S) | 26 (S) |
| *Rhodococcus* (NO) | 55 (S)* | 40 (S) | 26 (S) |

*Zone diameter values of the four isolates before the experiments /first row for each strain) and of those re-isolated from the 3 different treatments (NO AB, AB+, and AB++). The results of the interpretation are indicated in brackets (S, susceptible, R, Resistant, I, Intermediate; * values ≥ 55 are off scale). For disk zone diameter interpretation CLSI (2008) and EUCAST (2014) standards were used, considering the values for non-Enterobacteriaceae or nonfastidious P.aeruginosa, S.aureus and Corynebacterium* sp. *for A. hydrophila and B. intermedia, M. luteus and Rhodococcus* sp., *respectively.*

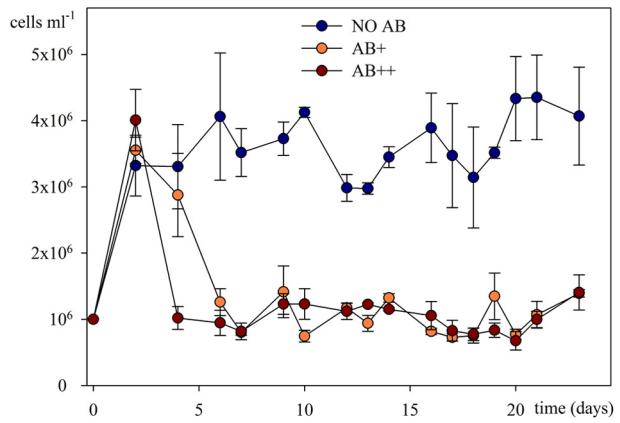


**FIGURE 2 | Bacterial abundances in continuous culture vs. time.** Overall bacterial numbers are given as mean of three replicates (± *SD*) for each of the three treatments.

allowed us to exclude potential pre-existing competitive advantages for single strains once exposed to AB.

### BACTERIAL ABUNDANCE AND COMMUNITY COMPOSITION

A steady community of about $4 \times 10^6$ bact ml$^{-1}$ characterized the treatment NO AB already from day 2, without significant fluctuations through the whole experiment ($P = 0.532$), while in treatments AB+ and AB++ the overall community abundance rapidly reduced by 75% (to about $1 \times 10^6$ bact ml$^{-1}$) independently by the AB concentration, and remaining then constant to the end of the experiment (**Figure 2**). No significant differences in numbers were detected between the treatments AB+ and AB++ considering the period 4–24 days ($P = 0.836$).

Community composition did not have important variations during the experiment after day 2 (**Figure 3**), either. The four strains could be detected in every sample on each date, thus extinction events were not detected for any AB concentration. Communities resulted either dominated by *A. hydrophila* (AB+), *B. intermedia* (AB++), or by an almost equal proportion of the two strains (NO AB), that together always achieved a relative abundance over 90% of the total bacterial number. The two Actinobacteria rapidly reduced from 50 to less than 10%, with a relative predominance of *Rhodococcus* sp. in all the treatments.

### PHENOTYPICAL DISTRIBUTION

The weekly assessment of the relative proportion of free living single cells, microcolonies composed by a few cells (in average up to 25), and larger cell aggregates (**Figure S1**) showed different aggregational behavior of the bacterial community once exposed to AB (**Figure 4**). Clustered cells (microcolonies and aggregates) at the beginning of the experiment never exceed 10% of the overall community. In treatment NO AB their proportion constantly decreased to less than 3%, while in treatments AB+ and AB++ they rapidly rose to 20-25%, keeping then constant up to the end of the experiment. Again, there was a significant effect of the AB ($P<0.001$ for both treatment comparison: NO AB vs. AB+ and vs. AB++), but no significant differences between the two treatments with AB ($P = 0.394$).

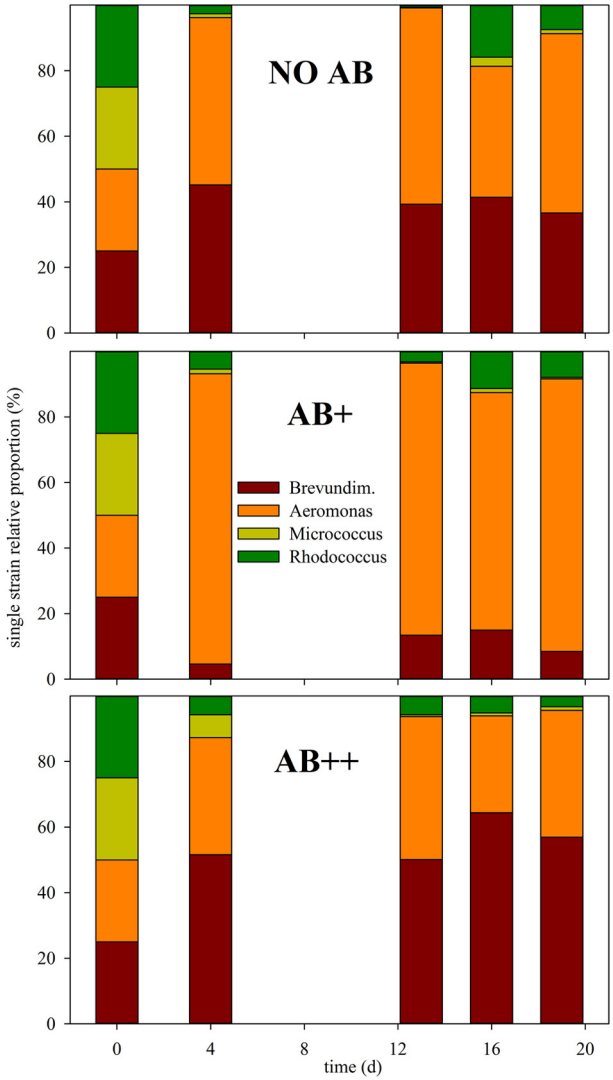


**FIGURE 3 | Relative proportion of each strain in continuous culture vs. time.** The relative proportion of each strain is given as mean of three replicates at day (d) 0, 4, 13, 16, and 20, for each of the three treatments (from top to bottom): NO AB, AB+, AB++.

### RE-GROWTH OF ADAPTED STRAINS

Bacterial strains isolated from the different vessels at the end of the continuous culture experiment were then tested for the development of AB resistance, potentially acquired while exposed to AB in the chemostat (**Table 2**). Zero or negative values between time 24 and 48 in the newly isolated single culture demonstrated that none of the three strains tested (*B. intermedia*, *A. hydrophila,* and *M. luteus*) could grow when re-inoculated in AB enriched media, independently by the AB concentration and by their treatment of origin. At this stage it resulted impossible to recover *Rhodococcus* sp. from the continuous culture treatments with AB, where *Rhodococcus* sp. was present only within co-aggregates. *Rhodococcus* sp. already demonstrated a very slow growth on agar, then, it is likely that in plates it suffered the competition by most growth-effective strains, and thus got outcompeted. For this

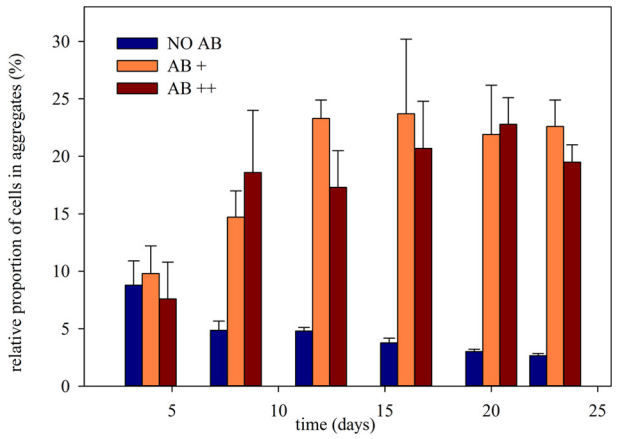


**FIGURE 4 | Relative proportion of cells clustered in continuous culture vs. time.** The relative proportion of aggregated cells is given as mean of three replicates (± *SD*) for each of the three treatments.

reason and for consistency, in the post-continuous cultures experiments we used the same *Rhodococcus* sp. strain we used at the beginning of the experiment for the inoculum in the chemostat.

These results are supported by the susceptibility confirmed with disk diffusion methods after the continuous culture experiment (**Table 1**). At the same time all strains responded positively to the growth in NO AB medium, without significant differences between the same strains isolated from different treatments.

The re-assembled communities, composed by an equal mixture of the strains isolated at the end of the continuous culture experiment, performed as expected in treatments NO AB, again without significant differences between communities isolated from different treatments with or without AB. In contrast, a significant growth of the communities composed by strains exposed to AB during the continuous culture experiment was detected when those were grown in presence of AB (**Figure 5**). The acquired adaptation observed in these communities is confirmed by the concomitant failure of communities from the NO AB treatment that, once exposed to AB, did not grow as the single strains that composed them. Also in this respect, the impact of AB caused a comparable response independently by the AB concentration in the medium.

## DISCUSSION

The assessment of the overall sub-lethal (or the sub-inhibitory) concentration of antibiotics for a natural bacterial community is basically impossible, as too many bacterial species and even subspecies can have different sensitivity to different AB. Moreover, the sensitivity of the different species composing the community can change in time and space with changing ecological parameters, community composition, and evolutionary history. At the same time, recent studies demonstrated that the response to AB in low concentrations by bacterial communities in anthropized open waters, or in waste water treatments, can significantly differ from the response of those in clinical environments (Gagneux et al., 2006).

**Table 2 | Growth of re-isolated strains and reassembled communities.**

| | Growth T24–T48 (Absolute OD values) | | |
|---|---|---|---|
| **Bacterial strains/communities** | **NO AB** | **AB+** | **AB++** |
| *Brevundimonas* (NO AB) | **0.083** | 0.023 | 0.009 |
| *Aeromonas* (NO AB) | **0.068** | −0.011 | 0.005 |
| *Micrococcus* (NO AB) | **0.040** | −0.019 | −0.003 |
| *Rhodococcus* (NO AB) | 0.020 | −0.007 | 0.008 |
| *Brevundimonas* (AB+) | **0.049** | 0.036 | 0.002 |
| *Aeromonas* (AB+) | **0.054** | −0.009 | 0.004 |
| *Micrococcus* (AB+) | **0.047** | −0.011 | −0.002 |
| *Brevundimonas* (AB++) | **0.056** | 0.011 | 0.016 |
| *Aeromonas* (AB++) | **0.050** | 0.013 | 0.005 |
| *Micrococcus* (AB++) | 0.022 | −0.003 | 0.002 |
| *Community* (NO AB) | **0.112** | −0.001 | 0.002 |
| *Community* (AB+) | **0.103** | **0.032** | **0.042** |
| *Community* (AB++) | **0.104** | **0.056** | **0.046** |

*Differences in optical density (OD) between growth time 24 and 48 h of bacterial cultures isolated from the different treatments at the end of the continuous cultures and of the relative mixed reassembled communities. In brackets it is mentioned the origin of the strain/community, while bold numbers indicate statistically significant growth ($P < 0.01$, student t-test).*

The design of a simplified bacterial community, tested under experimentally controlled conditions, reduces the complexity of the natural environment and allows speculations at the community level, excluding a number of ecological interactions (i.e., viral lysis, predation) that would exponentially increase the number of variables to be taken into account (Horňák and Corno, 2012), reducing our possibility to correctly evaluate the actual effect of the AB in the system. The four strains we selected for this study rather well represent a model community of a large Central European lake, with strains common in every water body and sometimes very abundant (*A. hydrophila* and *B. intermedia*) and others less successful in waters but anyway almost always represented within the "rare biosphere" (*sensu* Pedros-Alio, 2007).

The impact of AB was immediately clear in terms of productivity of the system (**Figure 2**) but, interestingly, the concentration of AB was not causing significant differences in this respect, whereas it resulted in a different selection in terms of bacterial community composition (BCC; **Figure 3**). In detail, the fitness of the bacterial community, measured in number of new cells produced (thus, for a chemostat, in cells abundance) was highly affected by the presence of AB, and the reduction in bacterial number (about 75% in treatments AB+ and AB++) was independent from the AB concentration. This result is not in accordance with the general theory, stating that being in low concentrations, the impact of AB in natural environment is negligible (Waksman, 1961). In this respect, a number of studies (reviewed by Sengupta et al., 2013) demonstrated a clear impact of low doses of AB in nature while our study demonstrated that AB can have an impact on the productivity of natural bacterial communities not only when in low doses, but that this impact can be comparable to higher dosages, raising our concerns for the release of AB into nature.

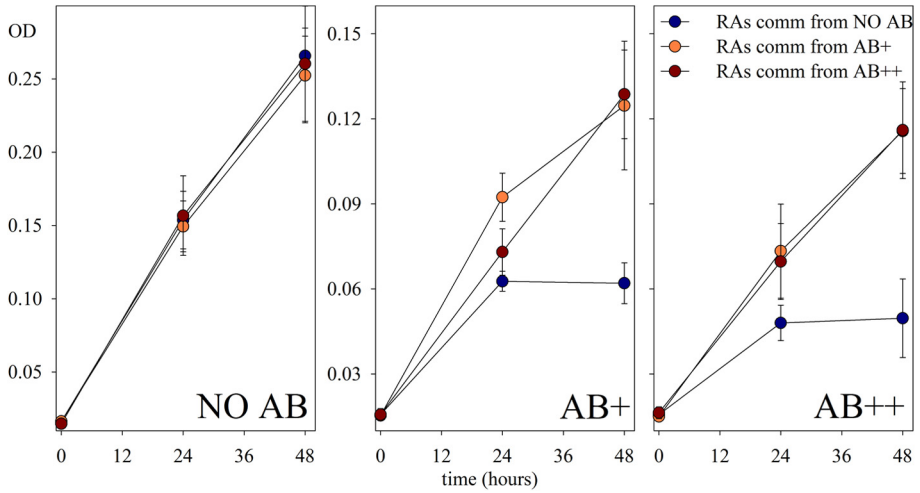


**FIGURE 5 | Growth of the reassembled communities.** Growth of communities reassembled (RAs comm) from the experimental strains at the end of the continuous culture treatments, and preferentially re-exposed to each experimental condition (NO AB, AB+, AB++). Values are in optical density (OD) of each sample, and are given as mean of three replicates (± *SD*) for each of the treatments.

Remarkably, the reduction of fitness had only a limited correspondence with alterations of the overall ecological success of the single strains (**Figure 3**). The two strains, *A. hydrophila* and *B. intermedia*, which dominated the community without AB, still kept having a clear supremacy also in presence of AB. Moreover the "rare" Actinobacteria did not gain any advantage by the reduction of potential competition for resources. *A. hydrophila* appeared to be the most successful strain in communities NO AB and AB+, while at higher AB dosage *B. intermedia* resulted to be the most abundant genotype. This can be related to a intermediate susceptibility of *B. intermedia* to levofloxacin in high dosages, or to a significant increment of the inhibitory activity of AB toward *A. hydrophila*. In any case, the modifications occurred in the BCC demonstrated that, despite the buffering capabilities of mixed bacterial communities can limit the impact of external factors, the presence of AB, even in low concentration, can modify the structure of the bacterial community. Reduced resistance and potential reduced resilience of the bacterial community can promote the success of allochtonous bacterial strains (e.g., pathogens, enhanced when AB are the disturbing factor) which would otherwise be eliminated by the means of competition.

Although modifications in the genotypic composition of the community were only partially affected by the presence of AB, the phenotypic distribution drastically changed. When exposed to AB the bacterial community switched from a free living single cells dominated community (treatment NO AB) to communities where almost 1 bacterial cell out of 3 clustered in aggregates with other cells (**Figure 4**). Since the experiment was conducted in a continuous culture system, with a constant efflux, this increment in proportion within the community has to be related to an effective clustering activity, thus in a significant competitive advantage for clustered cells in comparison to the free living ones. Large co-aggregates composed by up to 500 cells belonging to 2–4 different strains, as well as smaller microcolonies composed by only one strain rapidly appeared in presence of AB and, again, their proportion was comparable in treatments exposed to different AB concentrations. It can be speculated that the resistance strategy developed in our system by typical freshwater strains is based on the formation of aggregates, that ensure a AB free environment for the cells embedded into them, and that this strategy is thus equally efficient at AB+ and AB++ antibiotic concentrations. Co-aggregates and microcolonies represent a well-studied phenotype (or a specific "social behavior") in planktonic bacteria: aggregation can protect bacteria from predation (Corno and Jürgens, 2008), from starvation (Hahn et al., 2000), and can reduce competition and raise the productivity (Blom et al., 2010), thus preserves diversity and reduces the extinction risk in some peculiar cases (Horňák and Corno, 2012).

Comparable to biofilms on surfaces (Costerton et al., 1999), bacteria forming aggregates are surrounded by different forms of self-synthetized hydrated exopolymeric matrix effectively reducing the diffusion of the AB because of to the reduced permeability of the aggregate itself. Co-aggregation is thus a fast-developing winning strategy for planktonic bacteria once exposed to AB in low to intermediate concentrations, by forming particular niches where, possibly, the effect of AB is reduced by dilution, and the competition for resources can be less limiting (Corno et al., 2013). Similar observations have been recently published by Haaber et al. (2012) on pure cultures of *S. aureus*: under AB stress the formation of large clusters was accompanied by an increment in productivity. The authors found direct evidences of an increase in tolerance toward stressors like AB for *S. aureus* once aggregated. A very similar conclusion can be reached by our study on a more complex bacterial community, where treatments exposed to AB selected for bacteria with enhanced tendency toward aggregation, thus less sensitive to antimicrobials. It would be reasonable to consider the aggregational state, already present in reduced proportion at the beginning of the experiment mainly as microcolonies, not as an acquired feature, but simply a most competitive state in presence of AB. Nevertheless,

our results on the growth of recombined communities (**Figure 5**) show that communities reassembled with strains from treatments AB+ and AB++ perform significantly better than the one from NO AB treatment, once re-exposed to AB. The first reassembled communities somehow kept a "memory," possibly because they were isolated from cells belonging to aggregates. This observation implies that in nature, where clustering formation is common for many aquatic bacterial strains, aggregation can be a "ready to use" phenotypical adaptation for bacterial communities once exposed to AB in low concentrations, and that these microenvironments should deserve a deeper and more focused attention in the research of AB resistances in aquatic ecosystems. In the 96-wells plate we could test the short-term response in presence of AB of re-isolated strains and re-assembled communities from AB treatments: the difference in the growth curves of the re-assembled communities from treatments with AB in comparison with those of the "non-adapted" ones were already highly significant after 48 h from the beginning of the incubation. While the latter dropped in abundance, the "adapted" communities were still in exponential growth. We do not consider the enhanced OD values recorded for the "adapted" communities as an evidence for potential resistance but as a confirmation that the adaptation observed was due to coexistence of the different strains, and that it is an acquired feature. We can speculate that the observed differences in OD are given by aggregate formation, as we did not measured the phenotypical composition of the communities within the wells. Our opinion is that after the initial spin of the bacterial growth due to the fresh amount of organic C available, the AB had its impact on the less adapted communities, and the difference become evident after 48 h. A similar trend in growth has been detected in chemostat, too. In comparison to the analyses of communities in chemostat, with OD in wells there is a loss in the power of the analysis, excluding the possibility for a discrimination between active and inactive cells: the missed growth of many cultures after 48 h could be accounted to a stable community, but also to a stable number of inactive cells, thus to a dead community.

Finally, the composition of almost the totality of the aggregates we observed in this study was multispecific. Not only, single strains isolated from treatments under AB pressure, did not perform better than the control strains once exposed to AB, while the same strains in the reassembled communities did. We can thus suggest that only through multispecific co-aggregation the community gains an advantage against the antimicrobial action. The complexity of the interactions within the clustered cells raised enormously, resulting in the formation of a specific microenvironment where, through proximity and species interactions, the resistance of the single bacterial cells against AB rose.

We can thus suggest co-aggregates as ideal environments for fast adaptations to AB presence in aquatic systems. Our results, following the observations of Costerton et al. (1999) of biofilms as ideal environments for the development of AB resistances and pathogen preservation in clinical environments, and of Drudge et al. (2012) on the enhanced ability for aquatic bacteria to share AB resistance genes in flocs compared open waters, highlight the importance of aggregational states in the understanding of the ecology of aquatic ecosystems exposed to AB.

AB contamination of global water supplies is raising, increasing the risk of the development of clinically important AB resistance in these environments in the close future, with this study we offer one of the first attempts to get deeper into the ecology of bacterial communities exposed antimicrobials in low doses.

## ACKNOWLEDGMENT

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00297/abstract

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# Evolution in action: dissemination of *tet*(X) into pathogenic microbiota

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In a recent publication by Leski et al. (2013), the authors reported the occurrence of multidrug-resistant *tet*(X)-containing bacterial strains in a hospital in Sierra Leone. Among 52 clinical isolates, 11 (21%) have been confirmed *tet*(X)-positive. All the positive strains have been isolated from urinary tract infections and identified as *Enterobacter cloacae, Comamonas testosteroni, Escherichia coli, Klebsiella pneumoniae, Delftia acidovorans, Enterobacter* sp., and other members of Enterobacteriaceae and Pseudomonadaceae (Leski et al., 2013).

The need for careful monitoring of *tet*(X) dissemination is dictated by the fact that the enzyme encoded by the gene, a flavin-dependent monooxygenase, is capable of degrading almost all tetracyclines, including the third-generation tetracycline, tigecycline (the minocycline derivative 9-tert-butyl-glycylamido-minocycline) (Yang et al., 2004; Moore et al., 2005). The US FDA approved tigecycline in 2005, and its use in the EU was authorized in 2006. Its use is approved for complicated skin and intra-abdominal infections as well as community-acquired pneumonia (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021821s021lbl.pdf). The antibiotic is very efficient in treatment of a number of infections, including those resistant to the first- and second-generation tetracyclines (Bertrand and Dowzicky, 2012). Despite being considered as a drug of last resort, its use is steadily increasing, at least in the US (Huttner et al., 2012).

Although tigecycline resistance has not been tested at the time of isolation (Leski et al., 2013), the high frequency of *tet*(X) encountered in clinical samples signifies a worrying trend. In the previous analysis of the occurrence and phylogeny of the *tet*(X) genes it has been established that these genes can be detected in environmental DNAs and isolates as well as commensal bacteria (Aminov, 2009). Further studies have not spotted any expansion beyond these ecological niches. The presence of *tet*(X) has been detected in the human gut bacteria (de Vries et al., 2011), intestinal *Bacteroides* strains (Bartha et al., 2011), sewage treatment plants (Zhang and Zhang, 2011), and an oxytetracycline production wastewater treatment system (Liu et al., 2012). But now *tet*(X) is detected in a variety of clinical isolates and accepted human pathogens (Leski et al., 2013). The *tet*(X) sequences from this study have been added to the previous dataset (Aminov, 2009), and the phylogenetic tree has been recomputed (**Figure 1**). It is not surprising to see a tight clustering, with a 100% bootstrap support, of the *tet*(X) sequences from Enterobacteriaceae bacterium SL1 and *Delftia* sp. SL20 with the known *tet*(X) genes, given the high similarity of sequences within the cluster that exceeds 99%.

It is important to note here that there is no access to tigecycline (Tygacil®, Pfizer Inc.) in the hospital where *tet*(X)-positive samples were collected nor it is available through the independent pharmacies and hospital dispensaries operating in the area (Leski et al., 2013). Still, 87% of pharmacies dispense the "older" tetracyclines without prescription. As the authors suggest, this selective pressure of continuous application of tetracyclines may serve to maintain and spread *tet*(X) and other tetracycline resistance genes into pathogenic microbiota. Also, the probability of co-selection cannot be ruled out. The authors indicated the presence of mobile genetic elements in some isolates, and 10 out of 11 isolates appeared to be harboring multidrug resistance determinants.

In animal production systems, the penetration of *tet*(X) into the pathogens happened earlier. This can be demonstrated with the example of *Riemerella anatipestifer*, a causative agent of septicaemia anserum exsudativa (Segers et al., 1993). Septicaemia leads to major economic losses in duck production (Ryll et al., 2001; Sarver et al., 2005) but it also affects other bird species (Sandhu and Rimler, 1997; Hess et al., 2013). The *R. anatipestifer* strain, resistant to ampicillin, chloramphenicol, gentamicin, amikacin, tetracycline, nalidixic acid, and trimethoprim/sulfamethoxazole, was isolated in 2005 from waterfowl in Taiwan (Chen et al., 2010). It carries pRA0511 plasmid, which, in addition to two chloramphenicol acetyltransferases and a multi-drug ABC transporter permease/ATPase, also encodes TetX. The gene sequence has been incorporated into the existing dataset (Aminov, 2009) and recomputed (**Figure 1**). Similar to the genes from human pathogens, the gene from the poultry pathogen is confidently grouped into the *tet*(X) cluster. Three genomic sequences of *R. anatipestifer*, published (Yuan et al., 2011) or available as database entries (GenBank accession numbers CP003787 and CP004020), also carry chromosomally encoded genes similar to *tet*(X) (**Figure 1**). Interestingly, four other strains of *R. anatipestifer*, for which genome sequences are available (Mavromatis et al., 2011; Zhou et al., 2011; Wang et al., 2012; Yuan et al., 2013), have not yet acquired *tet*(X). No information regarding antibiotic use practices at sampling sites where *R. anatipestifer* strains have been isolated is available in the cited publications.

It seems that the use of even 'older' antibiotics may contribute to the resistance to newer antibiotics. There is no access to the third-generation tetracycline, tigecycline (Tygacil®, Pfizer Inc.), in the areas sampled in Sierra Leone (Leski et al.,

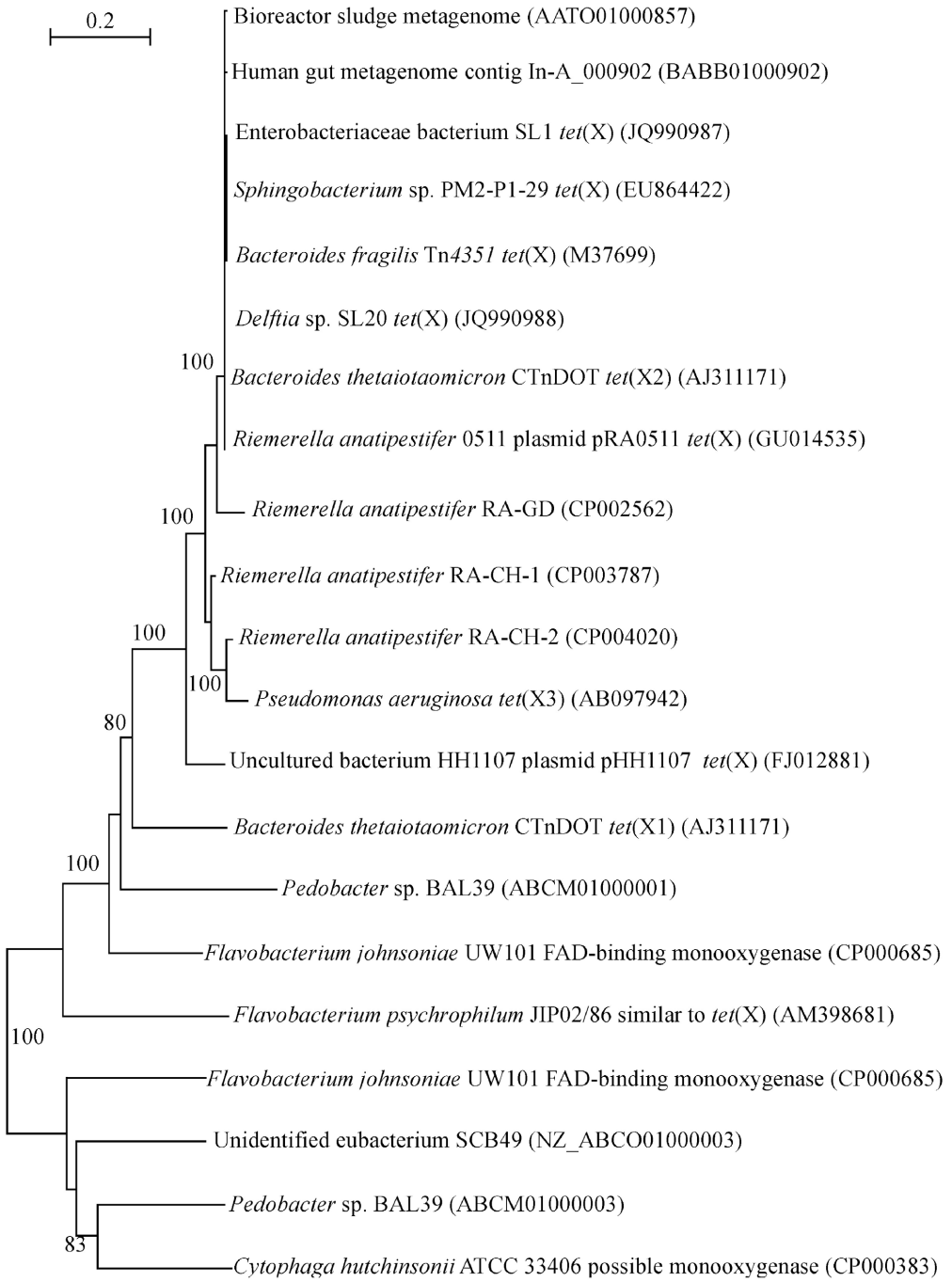


**FIGURE 1 | Neighbor-joining tree of the *tet*(X) and flavin monooxygenase-encoding genes.** Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of nucleotide sequences used in this analysis are given in parenthesis.

2013). It is also highly unlikely that this expensive new antibiotic is used in duck production, most likely these are the first-generation tetracyclines. Thus the conclusion is that the selective pressure by older antibiotics drives the resistance to a newer antibiotic and contributes to the dissemination of this resistance to pathogens.

The flavoprotein monooxygenase group of enzymes is found in many metabolic pathways involved in the region-specific hydroxylation of organic substrates in all three domains of life (Harayama et al., 1992). Based on sequence similarity and 3D structural data, the enzymes are divided into six classes (van Berkel et al., 2006). Class A enzymes, to which TetX belongs, are

generally involved in the degradation of phenolic compounds by *ortho*- or *para*-hydroxylation of the aromatic ring (Moonen et al., 2002).

Bacteria that carry these genes are omnipresent and can be encountered in a variety of ecosystems, including soil, aquatic ecosystems, and intestinal tract; some are opportunistic pathogens. Accordingly, the range of biochemical reactions performed by this class of enzymes is quite broad, and they may play an important role in the global carbon and nitrogen cycles (Chen et al., 2011; Wang and Shao, 2012). Interestingly, the range of metabolic activities expressed by these enzymes also includes the modification of many antibiotics. Besides the tetracylines discussed here, this range is extended to such structurally different antibiotics as rifampin (Andersen et al., 1997), mithramycin (Prado et al., 1999), griseorhodin (Li and Piel, 2002), chromomycin (Menendez et al., 2004), and auricin (Novakova et al., 2005).

The genetic context of flavin monooxygenase genes has been discussed earlier (Aminov, 2009). In brief, the majority of the genes analysed is almost uniformly associated with mobile genetic elements, including the plasmid-encoded *tet*(X) discussed here (Chen et al., 2010). The genes in this class are also highly incongruent with taxonomic positioning suggesting horizontal gene transfer events. They are also subject to frequent duplication events, which are partially illustrated here with the paralogous genes from *Flavobacterium johnsoniae* UW101 and *Pedobacter* sp. BAL39 (**Figure 1**).

The case of flavin monooxygenases is a vivid example demonstrating enormous adaptability of bacteria: they can freely move their protective armours amongst a variety of ecological compartments in response to yet another challenge, this time inflicted by humans in the form of antibiotic selective pressure. The global microbiota has been dealing with environmental challenges for billions of years to become sophisticated genetic engineers moving genes around with ease (Aminov, 2011). Combined with the readily available massive metabolic resources of the environmental metagenome, the microbiota seem capable of countering any kind of environmental or anthropogenic assault.

We are living in a fascinating era with technological advancements that allow us to see almost instantaneously the evolutionary events leading to the emergence of novel pathogens armed with resistance mechanisms against the most advanced antibiotics that we have been able to design. We should not underestimate the enormous genetic flexibility and the vast metabolic capabilities of the environmental microbiota. Based on our technical capabilities and knowledge acquired during the antibiotic era (Aminov, 2010), we have to make every effort, at every level possible, to preserve the power of antibiotics. Taking a bystander position in this situation is not acceptable.

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# Genomic interplay in bacterial communities: implications for growth promoting practices in animal husbandry

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The discovery of antibiotics heralded the start of a "Golden Age" in the history of medicine. Over the years, the use of antibiotics extended beyond medical practice into animal husbandry, aquaculture and agriculture. Now, however, we face the worldwide threat of diseases caused by pathogenic bacteria that are resistant to all existing major classes of antibiotic, reflecting the possibility of an end to the antibiotic era. The seriousness of the threat is underscored by the severely limited production of new classes of antibiotics. Evolution of bacteria resistant to multiple antibiotics results from the inherent genetic capability that bacteria have to adapt rapidly to changing environmental conditions. Consequently, under antibiotic selection pressures, bacteria have acquired resistance to all classes of antibiotics, sometimes very shortly after their introduction. Arguably, the evolution and rapid dissemination of multiple drug resistant genes *en-masse* across microbial pathogens is one of the most serious threats to human health. In this context, effective surveillance strategies to track the development of resistance to multiple antibiotics are vital to managing global infection control. These surveillance strategies are necessary for not only human health but also for animal health, aquaculture and plant production. Shortfalls in the present surveillance strategies need to be identified. Raising awareness of the genetic events that promote co-selection of resistance to multiple antimicrobials is an important prerequisite to the design and implementation of molecular surveillance strategies. In this review we will discuss how lateral gene transfer (LGT), driven by the use of low-dose antibiotics in animal husbandry, has likely played a significant role in the evolution of multiple drug resistance (MDR) in Gram-negative bacteria and has complicated molecular surveillance strategies adopted for predicting imminent resistance threats.

**Keywords: multi drug resistance, lateral gene transfer, bacterial genomes, complex resistance loci, Antimicrobial growth promotion**

## INTRODUCTION

Antibiotics are central to modern medical and veterinary practice. However, the effectiveness of existing antibiotics in both medical and veterinary practice is under threat due to the rapid rise of multiple drug resistance (MDR) (Gootz, 2010). The 2014 World Health Organisation (WHO) Global report on surveillance of antimicrobial resistance makes this point clear—"A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century." (WHO, 2014). This possibility was illustrated by antibiotic treatment failure in the 2011 epidemic in Germany in which enteroaggregative *Escherichia coli* (EAHEC) O104:H4 caused a large outbreak of acute gastroenteritis and haemolytic uremic syndrome. EAHEC O104:H4 is an example of a newly emerged pathogen that possesses novel combinations of acquired antibiotic resistance genes and virulence factors. In the case of EAHEC O104:H4 an enteroaggregative *E. coli* had acquired a $stx_2$ shiga toxin gene, a characteristic feature of enterohaemorrhagic *E. coli* genomes, via a bacteriophage (Grad et al., 2013); a plasmid encoded CTX-M-15 gene (encoding the antibiotic resistance factor, extended-spectrum beta-lactamase) (Kunne et al., 2012); and a chromosomally-located complex antibiotic resistance gene cluster, identified recently as the region of divergence 1 (Frank et al., 2011; Januszkiewicz et al., 2011; Ahmed et al., 2012; Radosavljevic et al., 2014). The extensively antibiotic resistant *E. coli* clonal group ST131 is similarly a globally emergent pathogen with novel combinations of acquired antibiotic resistance genes and virulence factors (Johnson et al., 2010; Andersen et al., 2013; Banerjee and Johnson, 2013; Price et al., 2013). Since 2011 there has been an increase in the number of reports describing hybrid *E. coli* genomes with a repertoire of virulence and resistance genes, complicating precise pathotype allocations (Bielaszewska et al., 2014; Prager et al., 2014; Toval et al., 2014). However, it is difficult to determine whether the increased number of reports equates to an increasing rate in the emergence of these novel pathogens. It could simply mean that

the increase in reports of novel pathogens is due to the growing affordability of, and improvements in, whole genome sequencing technologies and molecular diagnostics that enable rapid and accurate identification of emerging clones. However, increasing emergence of novel strains is also likely- given the rapid rise in human population, increased demands on food production and the speed by which people and commodities move globally. Either way, the suite of acquired resistance genes these novel pathogens harbor clearly indicates the significant role played by Lateral Gene Transfer (LGT) in shaping these genomes. Antibiotic resistance genes have a propensity to cluster on mobile elements and consequently a single LGT event can result in resistance to multiple antibiotic classes (Toleman and Walsh, 2011). Notably also, these trends are not restricted to Gram-negative pathogens but apply additionally to Gram-positive genomes.

Antibiotic resistance existed in nature long before the discovery of antibiotics 70 years ago. For example, genes encoding resistance to β–lactam(s), tetracycline and glycopeptide antibiotics have been identified from ancient metagenomic samples of 30,000 years old Beringian permafrost sediments (D'Costa et al., 2011). Controlling the spread of antibiotic resistance into diverse pathogens, and particularly multidrug resistance, requires better use of currently available antibiotics. It is insufficient to rely on the development of new antibiotics or antibiotic adjuncts. More effective use of existing antibiotics can be guided by molecular surveillance of microbiomes.

It is now clear that multiple distinct antibiotic resistance genes forming complex resistance gene loci (CRL) in MDR bacterial genomes were likely acquired from disparate backgrounds. Many MDR bacteria resulting in hospital infections carry genes encoding resistance to antibiotics used frequently in food animal production or resistance genes that have been acquired from aquatic and soil environments (Szczepanowski et al., 2009; Popowska and Krawczyk-Balska, 2013; Wibberg et al., 2013). This is not surprising given that New Generation DNA sequencing has revealed a vast repertoire of drug resistance determinants across diverse microbiomes (Forsberg et al., 2012). It has, however, largely gone unnoticed, because of the way in which molecular surveillance is carried out differentially in hospital and veterinary microbiology laboratories.

Screening procedures in hospital and veterinary laboratories are quite justifiably influenced by the urgent need to deal with an infectious threat; thus screening is carried out on the basis of resistance to the set of antibiotics that are of relevance to the respective unit. This means, for example, that although mercury resistance transposons are one of the largest carriers of multiple antibiotic resistance genes, veterinary and clinical hospital microbiology laboratories do not screen for mercury resistance. Focused surveillance approaches have thus resulted in an underestimated view of the real problem of antibiotic resistance. In recognition of the importance of fully understanding the problem of antibiotic resistance, recent WHO and UK government reports have advocated a "one health" approach that uses genomic surveillance of both clinical and food animal isolates (Department of Heath and Department for Environment Food and Rural Affairs, 2013; WHO, 2014).

The disparate reservoirs of drug resistance determinants raises important questions regarding the extent of transmissibility of the pool of resistance genes and the range of bacteria that can acquire them as a consequence of LGT. This review will give an overview of antibiotic resistance gene reservoirs with particular focus on the reservoirs in food animals, soil and water resulting from the in-feed use of large quantities of sub-therapeutic doses of antibiotics as growth promoting agents in food animal husbandry. It will highlight a significant gap in our knowledge regarding the evolution of MDR in bacteria: the range of factors that trigger the selection and mobilization of antibiotic-resistance genes from environmental reservoirs into clinically-relevant organisms. The complexity of the evolution of MDR in bacteria has myriad implications, including, for example, for the choice of alternative food animal growth promoters other than in-feed antibiotics. This review will point to considerations relevant to this choice.

## AN ASSESSMENT OF THE REPERTOIRE OF RESISTANCE GENES AND EFFECTS OF LOW DOSE ANTIBIOTICS

### ANTIBIOTIC RESISTANCE GENES PRESENT IN DISPARATE RESERVOIRS

The antibiotic resistance genes that accumulate in the genomes of bacteria are likely to have their origins in disparate reservoirs, a conclusion arrived at in several independent recent studies on the evolution of MDR (Aminov, 2011; Amos et al., 2014; Guerra et al., 2014; Hsu et al., 2014; Ojer-Usoz et al., 2014) and evidenced also by the widespread dissemination of various antibiotic resistance genes, including those encoding resistance to extended spectrum β-lactamases in birds and other wildlife species (Costa et al., 2008; Poeta et al., 2008; Simoes et al., 2010). Aquatic and soil environments serve as sinks of older generation antibiotics including sulphonamides, tetracycline, amoxicillin, ampicillin and trimethoprim (Boxall, 2004; Monteiro and Boxall, 2009). The pools of antibiotic resistance genes available for dissemination (known as the "resistome") are enriched by veterinary and agricultural practices, human sewage and hospital waste (Tennstedt et al., 2005; Galvin et al., 2010; Heuer et al., 2011; Wellington et al., 2013; Hsu et al., 2014; Ojer-Usoz et al., 2014).

Genes imparting antibiotic resistance move within bacterial population(s) through LGT, independent of the linear "parent to progeny" descent (Tennstedt et al., 2005; Stokes and Gillings, 2011). The genes can be from a wide "gene pool" and can be transmitted between closely and distantly related species. Examples in which LGT plays a significant role in the evolution of important multi-resistant pathogenic bacterial genomes include, but are not restricted to, *E. coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*. Notably, these organisms can survive in a range of environmental niches outside of the human body, including fomites in hospitals, soil and aquatic environments. Consequently, there exists diverse niche-adapted pools of microbial communities that can provide opportunities for the sampling and exchange of genetic information. Further, these opportunities are likely to be influenced significantly by the ecology of the niche-adapted microbial communities (Heuer et al., 2009, 2011; Galvin et al., 2010; Forsberg et al., 2012; Wellington et al., 2013).

### FOOD ANIMALS, SOIL, AND WATER ARE IMPORTANT RESERVOIRS OF ANTIBIOTIC RESISTANCE GENES

Reservoirs of antibiotic resistance genes that are increasingly recognized as important are the gastrointestinal tract of food animals, soil and water. This is because of the practice of using sub-therapeutic doses of antibiotics to promote the growth of food animals and the prophylactic use of antibiotics to prevent disease in food animals. A significant percentage of administered antibiotics are excreted in animal waste. Un-metabolized antibiotics concentrate in animal waste ponds and, depending on their chemical composition, partition to different fractions during the wastewater treatment processes (Kolpin et al., 2002; Ghosh and Lapara, 2007; Watanabe et al., 2010; Cheng et al., 2013; Li et al., 2013). Thus, antibiotics enter into environments/ecosystems where bacteria pathogenic to humans may also survive (Sarmah et al., 2006). The antibiotics used with food animals for growth promotion and prophylactically include a set of FDA (FDA, 2011) approved drugs, recognized by the World Health Organization as important to treat human diseases (**Table 1**) (Aarestrup et al., 2008). Further, the quantities of antibiotics administered to food animals are very large. In Australia, in the period 2005 to 2010, 98% of veterinary antibiotics sold were for use in food animals. Only approximately 43% were sold for therapeutic or prophylactic purposes (no distinction could be drawn between "therapeutic" and "prophylactic" uses as it was difficult for those surveyed to estimate the proportions of products used for these purposes). This means that, on average, for the surveyed period, sales of antimicrobials for growth promotion averaged 35.3 tons; in 2005–2006 this was as much as 47.2 tons (APVMA, 2014). Importantly, a large proportion of the antimicrobials sold (almost 77%), were administered in feed.

The practice of using sub-therapeutic doses of antibiotics to promote growth of food animals became widespread following initial studies of the effect of antibiotics on the growth of broiler chickens (Elam et al., 1953; Jacobs et al., 1953; Izat et al., 1990; Dibner and Richards, 2005; Castanon, 2007). Antibiotics are used for growth promotion in doses lower than the recommended minimum effective concentrations (MEC) for therapeutic purposes but often at concentrations greater than the minimum inhibitory concentration (MIC) of specific drug/microbe combinations (Berrang et al., 2007; Alexander et al., 2008; Mirzaagha et al., 2011; Holman and Chenier, 2013). The practice of using antibiotics to promote growth of food animals has been banned in several countries in Europe due to concerns over the development and spread of antimicrobial resistance (Gilbert, 2011; Maron et al., 2013).

### IMPACT OF SUB-INHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON BACTERIA

The practice raises concerns over the development and spread of antimicrobial resistance because sub-inhibitory concentrations of antibiotics can enhance LGT (Barr et al., 1986; Torres et al., 1991; Stevens et al., 1993); potentially act as signal transduction molecules (Romero et al., 2011) in the transition from planktonic to biofilm phase during the process of monomicrobial and polymicrobial biofilm formations,(Bagge et al., 2004; Hoffman et al., 2005); and globally modulate transcriptional activity. Sub-therapeutic doses of β-lactam antibiotics has been shown to enhance the rate of conjugative plasmid transfer (Barr et al., 1986) and tetracycline has been implicated in driving the lateral transfer of integrative conjugative elements (Torres et al., 1991; Stevens et al., 1993). Conjugative transfer of single-stranded (ss) DNA (the mechanism by which plasmids are transferred from donor to recipient cells) induces the bacterial SOS response, which in turn up-regulates expression of the integron integrase resulting in the capture or exchange of resistance genes in Gram-negative pathogens (Baharoglu et al., 2010). Integron-mediated gene shuffling events in *V. cholerae* and *E. coli* were elevated significantly (4.5-fold and 37-fold increase respectively) in response to antibiotic mediated stress, which in turn triggered the SOS response. In addition, the study also reported up-regulation of the frequency of integron-associated excision/shuffling of gene-cassettes by 141-fold in *V. cholera* and 340-fold in *E. coli*, in a SOS response mediated event. In a recent study, Zhu et al. (2013) demonstrated the presence and relative abundance (an increase of 198-fold on average) of 149 antibiotic resistance genes (of 244 included in the study) conferring resistance to common in-feed and veterinary antibiotics used in the swine farming industry in China within 36 metagenomic samples extracted from compost, manure and soil. The study also provided evidence of abundance and enrichment of common transposase genes [identified in the course of a previous study (Aziz et al., 2010)] known to be frequently associated with antibiotic resistance genes. The conclusions of the study included a statement of the likely roles played by the subset of mobile elements in LGT of resistance genes in China (Zhu et al., 2013).

In a pioneering transcriptome analyses study, Goh et al. provided direct evidence of the global modulation of transcriptional activity of genes in *Salmonella enterica* serovar Typhimurium in the presence of sub-inhibitory concentrations of antibiotics like erythromycin (used as Antimicrobial growth promoters or AGP for poultry and swine) and rifampicin (Goh et al., 2002). Further gene-expression studies have now presented data on the influence of sub-inhibitory concentrations of antibiotics on the global transcriptome of genes related to virulence (Subrt et al., 2011), colonization (Bagge et al., 2004), motility, SOS stress response (inducible DNA repair system) and biofilm formation (Kaplan et al., 2012) for many important human pathogens (Davies et al., 2006). Notably, stress responses have been shown to enhance bacterial adaptation through increased mutation and LGT (Aertsen and Michiels, 2006; Baharoglu and Mazel, 2011).

## LGT OF RESISTANCE GENES AND CO-SELECTION OF OTHER RESISTANCE DETERMINANTS BY THE FORMATION OF COMPLEX RESISTANCE LOCI

### COMPLEX RESISTANCE GENE LOCI—MULTIPLE ANTIBIOTIC RESISTANCE GENES AND MOBILE ELEMENTS

Genes that encode resistance to antibiotics often appear clustered in the genomes of many bacteria, forming CRL (Parkhill et al., 2001; Szczepanowski et al., 2005, 2009; Tennstedt et al., 2005; Roy Chowdhury et al., 2009, 2011; Venturini et al., 2010; Toleman and Walsh, 2011). In addition to antibiotic resistance genes, the CRL possess diverse mobile genetic elements (predominantly insertion

**Table 1 | List of FDA approved Antibiotic classes and countries still using them in growth promotion, associated resistance genes and genetic scaffolds that laterally co-mobilize them with other antibiotic and metal resistance genes.**

| Antibiotic classes: specific examples | Countries still using them | Associated resistance genes | Association of genes with mobile elements | Examples of co-resistances |
|---|---|---|---|---|
| Penicillins: amoxicillin, ampicillin | United States (Mathers et al., 2011), Sudan (Eltayb et al., 2012) | blaTEM genes | blaTEM genes transposons (Bailey et al., 2011) and plasmids (Cain et al., 2010) | Lead, cadmium, zinc and chromium (Yamina et al., 2012)<br>*mer* genes; mercury (Mcintosh et al., 2008) |
| Glycopeptides: avoparcin, vancomycin | Mexico (Maron et al., 2013) | *van* genes | *van genes* [transposons (Jensen et al., 1999; Leavis et al., 2003) and plasmids (Zhu et al., 2010, 2013)] | *tcrB* gene; copper (Hasman and Aarestrup, 2002) |
| Macrolides: erythromycin, tylosin, tilmicosin, kitasamycin, oleandomycin | United States (Kim et al., 2012), Australia (Hughes and Heritage, 2004), Mexico (Maron et al., 2013) | *erm* gene cluster<br>CmeABC multi-drug efflux pump (Lin et al., 2007) | *erm* gene cluster [transposons (Li et al., 2011; Ramos et al., 2012) and plasmids (Wendlandt et al., 2014)] | *erm* genes; tetracycline and streptogramin (Ramos et al., 2012)<br>*tcrB* gene; copper (Hasman and Aarestrup, 2002) |
| Streptogramins: virginiamycin, quinupristin-dalfopristin | United States (Kieke et al., 2006), Australia (Hughes and Heritage, 2004), Mexico (Maron et al., 2013) | *vatD* and *vatE*<br>*erm* gene cluster<br>*satA* (Hammerum et al., 1998)<br>*varS* (Lee et al., 1999; Kieke et al., 2006) | *vatD* and *vatE* plasmids (Allignet and El Solh, 1999)<br>*erm* gene cluster [transposons (Li et al., 2011; Ramos et al., 2012) and plasmids (Wendlandt et al., 2014)] | *erm* genes; macrolide and tetracycline (Ramos et al., 2012) |
| Sulfonamides: sulfisoxazole, sulfadimethoxine, sulfamethazine | Sudan (Eltayb et al., 2012), United States (APUA, 2010) | *sul* genes | *sul* genes [transposons (Cain et al., 2010), plasmids (Wu et al., 2010) and clinical class 1 integrons (Stokes and Hall, 1989; Wu et al., 2010)] | *czcA* gene; zinc, cadmium and cobalt (Stokes et al., 2006; Gillings et al., 2008)<br>*mer* genes; mercury (Mcintosh et al., 2008) |
| Tetracyclines: chlortetracycline, oxytetracycline, doxycycline | United States (Cox and Popken, 2010; Mathers et al., 2011), China (Wu et al., 2010), Sudan (Eltayb et al., 2012) | *tet* genes | *tet* genes [transposons (Schmitt et al., 1979; Ramos et al., 2012) and plasmids (Han et al., 2012; Wendlandt et al., 2014)] | *erm* genes; macrolide and streptogramin (Ramos et al., 2012)<br>*mer* genes; mercury (Mcintosh et al., 2008) |
| Polypeptides: bacitracin | Mexico (Maron et al., 2013) | *rgpA-F*, *mbrA-D* (Tsuda et al., 2002)<br>*bcr* | *bcr* [plasmids (Tremblay and Archambault, 2013)] | |
| Amphenicols: chloramphenicol | China (Li et al., 2013) | *cmlA*<br>*floR*<br>*fexA* and *fexB*<br>*cfr*<br>*cat* gene | *cat* gene [transposons, (Cain and Hall, 2012), plasmids (Mcintosh et al., 2008) and integrons (Bunny et al., 1995)] | *mer* genes; mercury (Mcintosh et al., 2008) |

elements, transposons and integrons) clustered either in independently replicating units, like plasmids, or in genomic islands within the bacterial chromosome. Mobile elements facilitate dissemination of CRL within bacteria. Acquisition of a CRL by any bacterium thus provides a survival mechanism to it when exposed to an entire range of antimicrobial agents. CRL evolve rapidly, especially within Gram-negative bacteria, and they generally contain hotspots where additional resistance genes can accumulate. Lateral transfer of genes within the gastrointestinal tracts of humans and food animals, and in reservoirs where environmental bacteria mix with bacteria derived from anthropogenic activities (sewage, hospital waste and food production animal waste) significantly contribute to these evolutionary processes (Forsberg et al., 2012). Although mobile genetic elements are a key component of CRL, there is currently limited understanding of the different types of mobile genetic element(s) that may contribute to: (1)

the spread of CRL; (2) the diversity of resistance gene reservoirs that can be utilized by the mobile elements to form CRL and (3) the mechanisms by which resistance genes assemble on laterally mobile segments of DNA.

## INTEGRONS ARE MOBILE ELEMENTS ASSOCIATED WITH CRL AND FOUND IN DIVERSE BACTERIA

Integrons represent a group of genetic elements that are most commonly found in association with CRL and have been implicated in the rapid evolution of multi-drug resistance within Gram-negative pathogens (Martinez et al., 2013). Based on the amino acid sequences of the integrase/recombinase gene *intI* (Recchia and Hall, 1995) integrons can be differentiated into several classes, all of which are found in diverse microbial communities (Marquez et al., 2008). Classes 1–3 are most frequently associated with the dissemination of MDR within Gram-negative bacteria, although recently class 1 integrons have also been identified in Gram-positive bacteria (Nandi et al., 2004; Shi et al., 2006; Xu et al., 2010, 2011). Exchange of resistance genes within different classes of integrons (from diverse bacteria) has been reported in a range of microbial communities (Labbate et al., 2008; Roy Chowdhury et al., 2009). The role of class 1 integrons in the evolution of MDR within hospitals is most comprehensively documented (Djordjevic et al., 2013; Martinez et al., 2013), although they are also frequently reported from environmental bacteria (Holmes et al., 2003; Stokes et al., 2006). Functionally, a class 1 integron is a two-component site-specific gene recombination system. Structurally a class 1 integron comprises an integrase gene and a tandem array of a variable number of independently acquired mobile genes. The independently mobile units of a class 1 integron are called "gene cassettes" and are made up of a promoter-less open reading frame and a recombination site. Class 1 integrons most frequently contain multiple gene cassettes, in structures called "cassette arrays," that can be expressed from a single promoter (Stokes and Hall, 1989) in an operon-like manner. Exchange/insertion/deletion of gene cassettes (by the process of site-specific recombination) and expression of these genes enables their host to exhibit multiple "acquired" resistance phenotypes simultaneously and relatively quickly (on an evolutionary time scale).

A key component of the integron integrase is the integrase promoter ($P_{int}$) (Stokes and Hall, 1989). The regulation and expression of the integron integrase is an adaptive response to stress, including that induced by sub-lethal dosages of antibiotics in any environment (Guerin et al., 2009). Hocquet et al. described a metronidazole induced cassette rearrangement in a class 1 integron that resulted in the formation of a fused *gcuF1-bla*$_{OXA-28}$ cassette, which subsequently led to the expression of the fused β-lactamase gene and ceftazidime resistance in a *P. aeruginosa* isolate (Hocquet et al., 2012). A LexA binding site is found conserved across the vast majority of integrase genes, indicating the likelihood of bacterial SOS mediated up-regulation of most integron integrases (Guerin et al., 2009). Importance of the LexA binding site has been experimentally demonstrated in *Vibrio* spp. and *E. coli* (Guerin et al., 2009).

Class 1 integrons isolated from clinical samples, are predominantly linked to the Tn*3* family of transposons, particularly Tn*21* (Liebert et al., 1999) and Tn*1696* (Partridge et al., 2001), which in turn harbor clustered mercury resistance genes. Class 1 integrons also have a propensity of targeting resolution sites of plasmids or other transposons on plasmid backbones (Kholodii et al., 1995; Minakhina et al., 1999). Thus, in addition to carrying genes conferring MDR, class 1 integrons also associate with other mobile elements, which independently carry other combinations of resistance genes to form laterally mobile resistance scaffolds (Parkhill et al., 2001; Szczepanowski et al., 2005; Tennstedt et al., 2005; Wibberg et al., 2013).

## CO-SELECTION AND TRANSFER OF ANTIBIOTIC AND OTHER RESISTANCE GENES—THE ROLE OF PLASMIDS

Plasmids play a central role in the lateral transfer of CRL within both closely and distantly related Gram-negative bacteria. Identical CRL are frequently seen piggy-backing on different plasmid backbones isolated from different hosts, supporting the role of plasmids in the transfer of drug resistance loci. Such events are best exemplified by plasmids carrying identical complex Tn*21*-associated MDR regions (Tennstedt et al., 2005; Szczepanowski et al., 2009; Venturini et al., 2013). Recent examples of such plasmids with complex resistance loci isolated from waste water treatment plants include IncF plasmids pRSB225 (Wibberg et al., 2013) and pRSB107 (Szczepanowski et al., 2005) and the series of IncP-1 plasmids reviewed recently by Popowska and Krawczyk-Balska (2013). Although isolated from waste water treatment plants, all these plasmids and a set of 140 plasmids described in a recent plasmid metagenomic study by Szczepanowski et al. clearly indicate that the resistance gene pool within the CRL on the plasmids are an admixture of genes that have originated from different bacterial communities (Szczepanowski et al., 2009).

The co-selection of antibiotic resistance genes by plasmids containing transposons with genes encoding resistance to heavy metals (such as mercury, cadmium, copper, and zinc) adds to the complexity of the evolution of laterally mobile clustered resistance regions (Seiler and Berendonk, 2012). This is particularly so when considering food animals and their environments (e.g. soil and water) as reservoirs of antibiotic resistance determinants; metals like copper and zinc, all of which are often used in animal husbandry for growth promotion (Jacob et al., 2010). This phenomenon of co-selection of resistance genes on plasmids increase the opportunity for microbial populations, that proliferate both in the gastrointestinal tracts of mammals and that survive in soil and aquatic environments, to act as major conduits in the flow of resistance genes between these reservoirs.

Plasmids pO26-CRL, pO26-$CRL_{125}$ (isolated from an enterohemorrhagic *E. coli* specimen from a human with bloody diarrhea) and pO111-$CRL_{115}$ (isolated from a bovine source in Australia) are examples of plasmids with identical clustered antibiotic and metal resistance genes that have been identified recently by our group (Venturini et al., 2010, 2013). The CRL in these plasmids are characterized by a Tn*21*-associated class 1 integron truncated by the presence of a composite IS*26* transposon, Tn*6026* (Cain et al., 2010). Composite transposons, another type of mobile element frequently associated with CRL, are defined as segments of DNA bounded by two related insertion elements (or IS elements). DNA segments between two IS elements mobilize

intervening genes as a single unit. Composite transposons therefore have the ability to capture and mobilize random pieces of DNA from many diverse genetic locations, promoting genetic diversity. In relation to evolution of MDR, composite transposons can allow the movement of clustered antibiotic resistance genes from one bacterial genome to another through the capture of such genes into bacteriophages or conjugative plasmids that can then move readily between bacteria. Tn*6026* is an example of a composite transposon, which consists of two independently mobile composite transposons, Tn*6029* and Tn*4352* (Cain et al., 2010; Martinez et al., 2013). Collectively the CRL (described above as Tn*6026*) confers resistance to ampicillin, kanamycin, neomycin, streptomycin, sulfathiazole and trimethoprim. Similar structures, consisting of Tn*6029* derivatives and essentially consisting of the same group of resistance genes have been described in plasmids such as pASL01a, which circulates in commensal *E. coli* in the human population in West Africa (Labar et al., 2012), and pHCM1, from a human *E. coli* isolate from a patient living in the Mekong Delta in Vietnam (Parkhill et al., 2001). However, the group of plasmids described by us, pO26-CRL, pO26-$CRL_{125}$,and pO111-$CRL_{115,}$ is a clear example of plasmid mediated LGT of CRL between animal and human reservoirs in recent times.

IncA/C plasmids can provide conjugative functions in trans to mobilize integrative elements such as *Salmonella* Genomic Island 1 (SGI1) (Douard et al., 2010). SGI1 harbors genes encoding resistance to seven antibiotics (ampicillin, chloramphenicol, florphenicol, streptomycin, spectinomycin, tetracycline and sulphonamides). The resistance genes are clustered on a 13-kb class 1 integron, In104, embedded within a 42.4-kb genomic island embedded between *thdF* and *yidY* genes (Boyd et al., 2001; Levings et al., 2005). SGI1 is widely dispersed among a range of *S. enterica* serovars and *Proteus mirabilus* (Levings et al., 2005, 2006, 2007; Djordjevic et al., 2009; Le Hello et al., 2011, 2012), clearly indicating the lateral transfer of the element within bacteria occupying disparate hosts. A structurally similar CRL is also found in SGI2 described initially from a *S. enterica* serovar Emek strain in Australia. Major differences between SGI1 and SGI2 are in the physical location of the class 1 integron on the genomic backbone and in the different set of resistance gene cassettes seen associated with the integron found in SGI2. The integron, known as InEmek is a close variant of In104 seen in SGI1 (Levings et al., 2008) and the entire island shows characteristic features that mobilize SGI1.

## NOVEL STRATEGIES AND ALTERNATIVES TO IN-FEED ANTIBIOTICS

The growing knowledge of how LGT drives the evolution of MDR in bacteria and the importance of disparate pools of resistance genes has implications for the use of antibiotics as growth promoters in food animals, suggesting a need for novel alternatives to in-feed antibiotics. These alternatives should, in development, pay regard to: (1) the potential dangers for increasing evolution of MDR in bacteria when altering the microbial ecology of the gut (as most currently used growth promoters appear to do) (Dibner and Richards, 2005); and (2) consideration of questioning the effectiveness of in-feed antibiotics as growth promoters (Bengtsson and Wierup, 2006). Kim et al. conducted a comparative fecal microbiome study on two separate porcine populations, one of which was given a diet supplemented with the macrolide tylosin (a commonly used AGP), while the other group represented the control (Kim et al., 2012), i.e., did not receive tylosin, and reported the flux of microbial communities within them over a period of time. The fecal microbiome of each group was determined, which initially showed a prominent shift in microbial content for all species detected, proving that AGPs such as tylosin alter the native intestinal flora, resulting in less competition for nutrients and ultimately leading to the desired growth promoting effects. However, this study also showed that fecal microbiomes from the control group eventually became indistinguishable to those of the tylosin-fed group, offering an air of redundancy to the use of AGPs with respect to health and welfare of the animals. An important aspect of this study was that it was conducted in two separate farm settings, instead of the common choice among similar studies of an infectious disease isolation facility, making the results more comparable and applicable to the pig-farming industry.

Any alternative strategy for growth promotion in food animals over antibiotics needs to consider the growing understanding of the triggers for lateral transfer of CRL and the warning provided by evidenced movement of CRL between disparate bacterial hosts as consequences of administration of sub-inhibitory dosages of antibiotics. Controlling the spread of antibiotic resistance is not so simple as banning non-therapeutic in-feed antibiotics and using anything else as a growth promoter. This is clearly demonstrated by the problems associated with the use of heavy metals as growth promoters in relation to lateral co-transfer of associated antibiotic resistance genes. Further, an ideal alternative to in-feed AGPs should ideally meet the definition for feed additives set by The Official Journal of the European Union (Council Directive 89/107/EEC). The derivative clearly states "any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods" (Council Directive 89/107/EEC). With respect to farming and animal husbandry, the novel additive should ideally improve the characteristics of the feed itself as well as dairy and deli products derived from the receiving animal. They should increase the efficiency of animal performance and welfare via the gastrointestinal microbiota and satisfy the nutritional requirements of the animal. Additionally, the additive should not adversely impact the environment or human/animal health. From this perspective, it is questionable whether the environmental accumulation of minerals, such as zinc oxide (often used as an alternative to in-feed antibiotics), is worth the positive effects of growth promotion and prophylaxis for conditions such as *E. coli* mediated post-weaning diarrhea in piglets (Pluske, 2013).

A diverse range of options, which could possibly minimize or ideally alleviate forces that drive lateral transfer of resistance genes within the gut microbiome, have been proposed as alternatives of AGPs including, but not limited to, enzymes, nutraceuticals,

amino acids and plant extracts (Askbrant et al., 1994; Hill et al., 2000; Barrerra et al., 2004; Schone et al., 2006; Muhl and Liebert, 2007; Nortey et al., 2007; Ragland et al., 2007; Jacela et al., 2008). These have shown varying outcomes in the promotion of growth in different animals (Supplementary Table 1). Therefore, it is most likely that a combination of these substitutes may serve as a better alternative that could account for all benefits and functions of in-feed antibiotics than any individual substance tested for the purpose. This idea was investigated within poultry by Ohimain and Ofongo (2012). They reported the effects of using a mixture of probiotics, prebiotics and enzymes as a dietary supplement in poultry. The probiotics and prebiotics had a synergistic effect, while the enzymes enhanced digestibility of the feed, increasing the amount of nutrients available for absorption. The combined effect improved digestive health in the chickens and consequently protected them against common microbial diseases such as enteritis (Ohimain and Ofongo, 2012). Probiotics, on the other hand, have been documented to prevent inflammation in the gut, reduce the incidence of meat contamination and promote growth, proving a propitious alternative to AGPs (Patterson and Burkholder, 2003). Use of non-pathogenic *E. coli* probiotics in combination with a low-protein diet in a porcine model was shown to reduce mucosal populations of pathogenic *E. coli* K88, subsequently decreasing the incidence of post-weaning diarrhea. These effects were attributed to the production of colicin by some bacteria introduced via the probiotic product (Bhandari et al., 2010) as well as the unavailability of protein normally utilized by the pathogenic *E. coli* for energy production. A similar study used a low-protein diet with the addition of essential amino acids (Heo et al., 2008) and described similar results. Further development of such strategies would assist in controlling the spread of antibiotic resistance, as well as reduce appearance of medical conditions such as post-weaning diarrhea in piglets, a major cause of economic hardship in swine production (Pluske, 2013).

One of the main problems of developing alternatives to in-feed AGPs is that the mechanism of action of AGPs is still largely unknown. No general consensus for the mechanism of action of AGP exists, however, several hypotheses have been proposed and evaluated, including: (1) reduction in total bacterial load as a consequence of administrated antibiotics; (2) reduction in the number of pathogenic bacteria resulting in better animal health; and (3) manipulation of the gut microflora in a way that natural immune and metabolic responses balance resulting in healthy animals (Dibner and Richards, 2005). Like existing alternatives to in-feed antibiotics, proposed novel alternatives tend to have a mechanism of action which directly affects the composition and quantity of bacteria within the animal's gastrointestinal system, suggesting a link between commensal bacteria and growth performance. The effects of AGP on porcine and broiler chicken intestinal microbiomes have been reported (Collier et al., 2003; Dumonceaux et al., 2006), as well as the positive effects of the change in composition of these microbiomes in response to in-feed antibiotics. These studies mirror those reported by Kim et al., as well as provide evidence to the hypothesis of this mode of action. Collier et al. and Dumonceaux et al. also identified bacterial composition within specific regions of the gastro-intestinal tract, and in doing so created a link between the alterations of bacterial communities most affected by the antibiotics and areas of the intestine most involved in nutrient absorption.

Several reports have linked the use of AGPs (Feighner and Dashkevicz, 1987; Knarreborg et al., 2004; Guban et al., 2006) to the reduction in activity of intestinal bile salt hydrolase (BSH). This enzyme directly affects lipid metabolism within the host and is produced by bacteria that occur naturally in the gut. The observation led to a new avenue of research into factors that target bacterial products rather than the bacteria themselves. Based strictly on an *in vitro* study, Wang et al. (2012) proposed the use of bile salt inhibitors as growth performance enhancers (Wang et al., 2012). Whether BSH inhibitors would adequately function in an *in vivo* model is still unclear. Zinc and copper, (common feed additives for increasing feed efficiency and promoting growth), were shown to cause inhibition of BSH and improve lipid metabolism. There are likely to be a number of non-metal BSH inhibitors, so this research path, with further studies into non-environmentally contaminating compounds, is a promising replacement for in-feed antibiotics.

## CONCLUDING REMARKS

Laterally acquired DNA is a major driver of genome plasticity and poses problems for the reliable identification and classification of emerging pathogens, a necessary pre-requisite for using molecular surveillance as a tool for guiding effective use of existing antibiotics. For many years microbiological and molecular epidemiological surveillance studies within the veterinary and hospital environments have focused on characterizing their own targeted bacteria and antibiotic resistance profiles rather than taking a larger "one health" view of microbial resistance. Consequently, the molecular structures and CRL scaffolds that laterally mobilize drug resistance determinants between communities have been relatively under-studied. The sequences of plasmids; transposons; integrons insertion elements; genomic islands and other mobile elements need to be comprehended fully to unravel the real complexity of the problem. MDR evolves where antibiotics are used heavily, irrespective of whether it is in human clinical environments, food animal production settings, aquaculture or horticulture. Genetic phenomena like LGT inextricably links genes from disparate reservoirs to form CRL. These concepts, although increasingly recognized by the scientific community are not clearly communicated to the general public. Raised awareness of the complexity and far reaching implications associated with antibiotic resistance and a "one health" approach are critical for a global, sustained effort needed to alleviate the serious threat posed by multiple antibiotic resistant infectious agents. Molecular surveillance strategies embracing a "one health" approach are expected to provide a better understanding of how the genes flow through microbial communities and therefore provide a platform to more accurately predict and contain imminent threats posed by MDR bacteria.

One of the major challenges going forward is to stem the use of antibiotics and seek effective alternatives in our food production industries because antibiotic resistance gene reservoirs associated with food animals contribute to the evolution of MDR bacteria. One starting point for this is the development of alternatives to in-feed AGPs. This development should include studies that

incorporate a full range of animals within the farming industry, as intestinal microbiomes are likely to differ between species. It should further include new approaches to gauge the effects of probiotics and alternate in-feed growth promoters.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00394/abstract

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