Alternatives to antibiotics in poultry feed: molecular perspectives

Gayatri Suresh, Ratul Kumar Das, Satinder Kaur Brar, Tarek Rouissi, Antonio Avalos Ramirez, Younes Chorfi & Stephane Godbout

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Alternatives to antibiotics in poultry feed: molecular perspectives

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Abstract

The discovery of the growth promoting property of antibiotics led to their use as antibiotic feed additives (AFAs) in animal feed at sub-therapeutic doses. Although this has been beneficial for animal health and productivity, it has been, essentially, a double-edged sword. The continued and non-judicious use of AFAs has led to the selection and dissemination of antibiotic-resistant strains of poultry pathogens such as Salmonella, Campylobacter and Escherichia coli. The rapid spread of drug-resistant pathogens as well as emergence of antibiotic-related environmental pollutants is of global concern. Hence, the identification and development of new and effective alternatives to antibiotics that do not hinder productivity is imperative. For this, it is essential to understand not only the molecular basis of development of resistance to AFAs but also the mechanisms of action of AFA alternatives and how they differ from AFAs. This review provides a molecular perspective on the alternatives to antibiotics that have been proposed till date and their current trends, as well as novel approaches such as development of improved delivery systems.

Introduction

For almost eight decades, antibiotic feed additives (AFAs) have been used in poultry for increased productivity and efficiency. It has been estimated that by 2030, a total of 105,596 (±3605) tons of antimicrobials will be consumed in food animal production globally (Van Boeckel et al. 2015). Several studies indicate that the use of antimicrobials has resulted in increased productivity and decreased cost for consumers (Ricke et al. 2012). The primary function of AFAs in poultry is as growth promoters. The administration of AFAs at sub-therapeutic dosages has been shown to increase growth rate, feed conversion and consequently, broiler performance. Additionally, AFAs are also used for the treatment of infections caused by common poultry pathogens such as Salmonella, Campylobacter, Escherichia coli, Listeria and Clostridium (Jorgensen et al. 2002; Bohaychuk et al. 2006; Hafez 2011). The antimicrobial activity of these drugs is due to the different targets they attack, such as cell wall, cell membrane, protein synthesis and DNA replication.

Some of the antimicrobials used as feed additives in poultry are penicillin, neomycin, erythromycin, chlorotetracycline, oxytetracycline, streptomycin, chloramphenicol and fluoroquinolones (McEwen and Fedorka-Cray 2002).

Despite their substantial contribution to the poultry industry, AFAs are under surveillance due to an increase in the incidence of drug resistance, caused majorly by the use of these drugs by livestock farmers without veterinary consultation or proper directions for dosage (Bbosa and Mwebaza 2013). The prolonged and unregulated use of these antimicrobials has led to the selection of drug resistant strains of pathogens. For example, a primary cause of salmonellosis in humans – an MDR (multidrug resistant) strain of Salmonella typhimurium was first identified in livestock (Woc-Colburn and Bobak 2009). According to studies done by Lovine and Blaser (2004) and Tambur et al. (2010), emergence of drug resistant strains of C. jejuni causing disease in humans can be associated with the use of antimicrobials in poultry farms. Cross resistance to antibiotics used in...
humans can also develop due to their chemical similarities with veterinary antibiotics.

The Swann committee report released in United Kingdom in 1960 raised serious concern worldwide over the safety of the use of AFAs in animal industry. The oxy tetracycline resistance of humans was found to be transferred from animal meat and this had led to restriction or ban in uses of AFAs. As more cases of bacterial antibiotic resistance (through transfer from animal to human) were reported from different parts of the world, the controversy over the use of AFAs became a public concern by the end of 1990. Due to the long term ramifications of AFAs, they were banned in Sweden in 1986. From 1997 to 2006, the European Union (EU) phased out the use of AFAs in animal feed and veterinary prescriptions (Cogliani et al. 2011). The Netherlands government set a target of 70% reduction in the use of AFAs by 2015. Food companies such as McDonalds and Tyson Foods have announced their future policies on sourcing of chickens from antibiotic free poultry farms. The restriction or ban on the use of AFAs in Europe has primarily resulted in the reduction in actual exposure to antibiotics. Scientifically, this trend has been evaluated by calculating the Animal Level of Exposure to Antimicrobials index (ALEAI). The estimation of ALEAI is done from different factors such as Population Correction Unit and dose (concentration x time) of antibiotics. At the first Poultry Summit Europe (17–18 May 2016, Utrecht, The Netherlands), the participants suggested different strategies such as biosecurity, data in breeding and hygiene in the feed mill for antibiotic reduction in poultry industry (Koeleman 2016).

However, this ban has been at the cost of productivity, animal welfare and subsequently, an increase in the veterinary use of therapeutic antibiotics has been documented (Casewell et al. 2003). This was seen in Denmark, where, following the ban of AFAs, there was almost a 30% reduction in drug resistant strains from livestock; however, a 33% increase in the therapeutic use of antibiotics by farmers was reported subsequently (Ferber 2003). In North America, antibiotics continue to be used as feed additives. However, in 2005, enrofloxacine and sarfloxacin – both fluoroquinolones – were banned from use in poultry in the United States, followed by a ban on the extra-label usage of cefotiofur (a cephalosporin) (Prescott and Dowling 2013). Unlike the EU and the United States, Canada has no formal restraints on the use of AFAs, and veterinarians in Canada can prescribe antibiotics for extra-label use (Maron et al. 2013; Prescott and Dowling 2013). With the global consumption of poultry increasing at an annual rate of more than 3.6% (Revell 2015), it has become imperative to develop feasible alternatives to feed antibiotics without compromising on productivity.

Drug resistance versus efficacy of antibiotic feed additives: molecular domain

Resistance to antimicrobials can be facilitated through mechanisms such as reducing outer membrane permeability by downregulation of porins to decreased drug uptake, genetic modification of target by mutation, methylation of target to prevent antibiotic binding, enzymatic inactivation of drug, bypassing pathways affected by the drug or overexpression of efflux pumps for active transport of drugs out of cell (Atterbury et al. 2011). The field of molecular basis of antibiotic resistance has been garnering interest. In recent times, several genes have been found to be associated with the development of drug resistance. Bacterial resistance to AFAs can be intrinsic or acquired. Intrinsic resistance occurs due to random mutations in the bacterial chromosome, and is transmitted vertically to progeny cells. Extrinsic resistance can be attributed to bacterial mechanisms of horizontal gene transfer (HGT) which can transfer resistance genes to other bacteria (e.g. known human pathogens) (Diarra and Malouin 2014; Toutain et al. 2016). The methyl-directed mismatch repair (MMR) pathway (coded for by the “Mut” genes) is a post-replication DNA repair system that specifically targets mismatched bases, thus ensuring fidelity of DNA replication, and preventing recombination between dissimilar bacterial species. Therefore, bacteria with an inactivated MMR system are prone to both spontaneous mutation and recombination with diverged species (LeClerc et al. 1996). The spread of antibiotic resistance genes can majorly be attributed to HGT. There are three mechanisms for HGT: (i) transformation, in which extracellular DNA is taken up by competent cells; (ii) transduction, in which gene transfer is facilitated through bacteriophages; and (iii) conjugation, in which DNA is transferred via a physical contact between a donor and recipient cell (von Wintersdorff et al. 2016). The plasmid-mediated dissemination of drug resistance genes via conjugation is the major cause of the current magnitude of dissemination of drug resistance. Plasmids are autonomously replicating extrachromosomal DNA molecules which either inherently carry genes coding for drug resistance or mobilize these genes from bacterial chromosomes by recombination. Additionally, clustering of several resistance genes on a single plasmid can lead to selection of multidrug resistant strains via a single “horizontal transfer event” (Barlow 2009). Conjugal plasmids have genes which code for formation of a pilus between the donor and the
lipopolysaccharide and outer membrane proteins of the recipient cell. A junction is formed between the two cells for transport of the DNA and a pore formed in the recipient cell facilitates the entry of the DNA into it (Thomas and Nielsen 2005). Transposons are also mobile genetic elements which can be transferred via conjugation and are implicated in spread of antimicrobial resistance (von Wintersdorff et al. 2016). Thus, HGT mechanisms play a very important role in creating new resistant strains of pathogens.

Additionally, antibiotic-related contaminants (antimicrobial resistant pathogens, antimicrobial resistance genes and antibiotic drugs) are classified as emerging environmental pollutants (Keen and Patrick 2013). Soil and water resources are the main reservoirs of antibiotic resistance. Antibiotics added in animal feed or drinking water are not completely metabolized in the gut of poultry and about 70% is the drug is excreted in its unmetabolized form (Kümmerer 2009). Animal wastes containing residual AFAs, when used as manure, can introduce antibiotics, resistant bacteria and antibiotic resistance genes in the soil ecosystem (Furtula et al. 2010; Franklin et al. 2016). Recently, Ho et al. (2014) reported the high concentration of flumequine (1.3 mg/kg dry weight) in soil samples that were treated with broiler contaminated with flumequine, doxycycline and trimethoprim among others. Antibiotics in soil can either get degraded within a few hours or days, or may persist in soil for several months to years (Jechalke et al. 2014), depending on soil parameters, temperature, pH and adsorption of antibiotics to organic matter in manure (Tasho and Cho 2016). Therefore, an excessive use of AFAs can lead to their accumulation in the soil and cause significant alteration of soil microbiota. Qingxiang et al. (2009) showed that exposure to oxytetracycline had an adverse effect on the structure and activity of the rhizosphere soil microbial community. Studies have also documented the accumulation of antibiotics in plants for which poultry litter was used as manure (Kumar et al. 2005; Tasho and Cho 2016).

Residual AFAs in soil promote selective proliferation of resistant strains and increase the diversity of drug resistance genes which can be subsequently be transferred vertically or horizontally (Ding and He 2010; Jechalke et al. 2014). These pollutants can also enter ground and surface water resources from contaminated soils by leaching or water run-off through livestock wastewater, and affect the microbial community composition (Barra Caracciolo et al. 2015). In aquatic environments, antibiotics may get degraded (fully or partially), or may accumulate in tissues of aquatic organisms (Gaw et al. 2014), that could subsequently be consumed by humans. The dissemination of antibiotic resistance in the environment (from “farm-to-fork”) has been depicted in Figure 1.

The mechanisms of the growth promotion by AFAs are not completely understood and are thought to be by inhibition of subclinical infections, reduction of metabolites which can affect growth such as end products of bile degradation, reduction of nutrient availability to pathogens, thinning of intestinal epithelium and exhibition of anti-inflammatory action on macrophages and granulocytes (Brüssow 2015). The poultry gut is a complex and dynamic microbiome, with the ratio of microbes to host cells being approximately 10:1. Gaskins et al. (2002) proposed that the growth promotion by AFAs is primarily due to inhibition of the normal gut microbiota which would increase the host nutrient availability as well as reduce maintenance costs, i.e. a “bacteria-centric” effect. Studies have also shown that decrease in the gut microbiota improves fat utilization as these bacteria can degrade bile salts leading to decreased digestibility of fat. Gut microbiota are also known to increase thickness of the gut leading to reduced nutrient absorption, as well as, elicit host immune response due to their antigenic determinants which is an energy utilizing process (Milanov et al. 2016). Another school of thought states that growth promotion by the sub therapeutic use of AFAs due to antibiotic activity is unlikely, and rather, can be attributed to their “host-centric” effect, i.e. immunomodulatory effect in the host. Niewold et al. stated that the effect of AFAs is “growth-permitting” rather than “growth promoting”, and is due to their ability to inhibit intestinal inflammation (thus preventing thickening of the intestine wall, as well as waste of energy rather than an antimicrobial mechanism (Niewold 2007).

Hence, an ideal alternative to AFAs would interact with immune cells that modulate inflammatory response, and/or would target a site different from that of conventionally used antibiotics for antimicrobial activity, thus evading the selection of drug resistant strains. Several alternatives to AFAs, having similar effects in animals, have been proposed (Table 1), and their effects in broiler performance have been evaluated (Table 2).

Most of the proposed AFA alternatives primarily have a “host centric” mechanism of action, which makes it less likely for the development of resistance against them. It is essential to study the molecular basis of the mechanism of the growth promotion activity of the AFAs as well as the potential alternatives (Table 3) for the identification of suitable alternatives that differ from the default modalities either in their target or their mechanism of action to evade the development of resistance, but would have effects similar to the antibiotics in the host, whether it is reduction of pathogen
load or immunomodulation (Allen et al. 2013; Rios et al. 2016). Although there is extensive literature on molecular mechanisms of bacterial resistance and proposed mechanisms of AFAs, there is no comprehensive review of the molecular basis of the mode of action of the alternatives to AFAs. This review attempts to bridge this gap in knowledge, for the development of an effective alternative to feed antibiotics (Figure 2).

**Phytogenic feed additives (PFAs)**

Plant secondary metabolites or phytochemicals are organic bioactive compounds produced by plants during their normal metabolism and may or may not have nutritional value (Hashemi and Davoodi 2010, 2011). They are known to have antioxidative and immunomodulatory properties, and are thought to increase nutrient digestion and absorption in the gut (Ahmed et al. 2013). Their antimicrobial action is suggested to be due to mechanisms such as disruption of pathogen membranes, affecting virulence by modification of cell surface, activation of cells of the immune system, promotion of beneficial bacteria in the gut (Diaz-Sanchez et al. 2015). A study by Abudabos et al. (2016) reported that in broilers challenged with *S. typhimurium*, phytogenic feed additives were found to have an effect similar to avilamycin on the growth and blood biochemical profile. Plant secondary metabolites have also been studied for their immunomodulation effects, which are thought to be due to (i) induction of heat shock proteins which increase the efficiency of protein translation, (ii) induction of Toll-like receptors (TLRs) which recognize conserved microbial molecules and activate immune response in the host and (iii) inducing the proliferation of Th-1 and Th-2 cells (subtypes of T-Helper cells) to maintain the balance between cellular and humoral branches of immunity (Hashemi and Davoodi 2012). Phytogenic feed additives have also been shown to increase livestock productivity by increasing feed efficiency by the stimulation secretion of intestinal mucus, saliva and bile, as well as by inducing morphological changes such as increase in villi and crypt size in the

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**Figure 1.** Spread of antibiotic resistance in environment.
Phytobiotics are a complex blend of organic molecules, and the multiple active components could each have a different mode of antimicrobial action. Due to this, it would be more difficult for bacteria to develop resistance to them.

Essential oils (EO) have been studied extensively as an alternative to AFAs. EOs are volatile, aromatic, lipophilic compounds, composed primarily of terpenes and phenylpropenes (Hengl et al. 2011). Antibacterial property of EOs could be attributed to their lipolytic property, and their hydrophobic components, that compromise the bacterial cell membrane structure by altering its permeability to ions (Helander et al. 1998), leading to disruption of bacterial enzyme system, decrease in bacterial growth and “cell leakage”. A study by Kollanoor et al. (2012) showed the efficiency of eugenol and trans-cinnamaldehyde to reduce *Salmonella enteritidis* colonization in chicken ceca by downregulation of the motility and virulence genes.

Apart from EOs, PFAs also include tannins, and saponins herbs, spices, oleoresins, flavonoids and alkaloids (Patra and Saxena 2009), with different mechanisms of antimicrobial action. Tannins can cause iron deprivation or interact with specific cellular enzymes, saponins form complexes with membrane sterols leading to membrane damage, and alkaloids inhibit topoisomerase and interrupt DNA synthesis (Hashemi and Davoodi 2011).

Dietary inclusion of PFAs is also known to be beneficial due to their antioxidant property (which is thought to protect lipids in feed from oxidation), reduction of oxidative stress, immune cell proliferation, increased production of intestinal and pancreatic enzymes, and increased cytokines production, and anti-inflammatory properties (Alloui et al. 2014; Gadde et al. 2017). A study by Khadem et al. (2014) made use of extracts from *Macleaya cordata* (which is rich in anti-inflammatory alkaloids, such as sanguinarine and chelerythrine), as a feed additive and found that the plant extract downregulated the expression of inducible NO synthase, thus explaining the anti-inflammatory effect.

Some components of EOs can stimulate the secretion of digestive enzymes like trypsin, amylase and lipase by interacting with cellular receptors in the pancreas, which can increase feed intake and feed conversion ratio (Valientes 2014). A blend of essential oils of peppermint, anise, clove and caraway has been evaluated for its anti-inflammatory and antioxidative properties. It was shown to inhibit factors responsible for inflammation (interleukin 8, monocyte chemoattractant protein and intracellular adhesion molecule) by the downregulation of a transcription factor responsible for expression of these genes, and also activate the cellular pathways which induced genes for defence against reactive oxygen species (Steiner and Syed 2015). In September 2015, a phytonutrient formulation, CCC (carvacrol + cinnamaldehyde + capsicum oleoresin) was...

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**Table 1. Comparison of Antibiotic Feed Additives and proposed alternatives to AFAs.**

<table>
<thead>
<tr>
<th>Alternatives to AFAs</th>
<th>Antibiotic feed additives</th>
<th>Phytogenic feed additives</th>
<th>Probiotics</th>
<th>Prebiotics</th>
<th>Feed acidifiers</th>
<th>AMPs</th>
<th>Bacteriophages</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct antibacterial activity</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Immuno-modulation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Possible</td>
</tr>
<tr>
<td>Proliferation of beneficial bacteria</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Nutrient absorption</td>
<td>Increased due to thinning of epithelial wall</td>
<td>Increased due to induction of digestive enzymes</td>
<td>Increased due to induction of digestive enzymes</td>
<td>Unknown</td>
<td>Increased due to gut acidification and increased protease activity</td>
<td>Thought to increase</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Development of resistance</td>
<td>Very common</td>
<td>Could be difficult due to multiple targets of action</td>
<td>No, due to indirect antimicrobial activity</td>
<td>No, due to indirect antimicrobial activity</td>
<td>Not very common</td>
<td>Not very common</td>
<td>Yes, but more than one phage available for single host</td>
<td>No</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
<td>Vulnerable to proteolysis</td>
<td>Vulnerable to heat and proteolysis</td>
</tr>
</tbody>
</table>
Table 2. Evaluation of effects of AFA alternatives in broiler performance.

<table>
<thead>
<tr>
<th>AFA alternative</th>
<th>Effect in poultry</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phytogenic feed additives</strong></td>
<td>(i) Increase in body weight</td>
<td>(Bernard et al. 2016; Peng et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(ii) Improvement in feed conversion ratio and carcass yield</td>
<td>(Jahan et al. 2016; Sadeghi et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(iii) Decrease in pathogen counts</td>
<td>(Chang et al. 2016; Lan et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(iv) Improvement of fatty acid profile in egg yolk</td>
<td>(Raza et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(v) Increased serum proteins and antioxidant status</td>
<td>(Alizawqari et al. 2016)</td>
</tr>
<tr>
<td>Probiotics</td>
<td>(i) Increase in body weight and feed conversion</td>
<td>(Gheisar et al. 2016; Hatab et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(ii) Decrease in pathogen count, increase of beneficial bacteria in gut</td>
<td>(Olnood et al. 2015; Li et al. 2016)</td>
</tr>
<tr>
<td>Prebiotics</td>
<td>(i) Increase in disease resistance, broiler efficiency and nutrient availability</td>
<td>(Ganguly 2015)</td>
</tr>
<tr>
<td></td>
<td>(ii) Increase in weight and population of beneficial bacteria</td>
<td>(Arsi et al. 2015; Pourabedin and Zhao 2015)</td>
</tr>
<tr>
<td></td>
<td>(iii) Decrease in pathogen count</td>
<td>(Kim et al. 2011; Shang et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>(iv) Reversal of coccidial lesions</td>
<td>(Chand et al. 2016)</td>
</tr>
<tr>
<td>Feed acidifiers</td>
<td>(i) Decrease in pathogen count</td>
<td>(Koyuncu et al. 2013; Sultan et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>(ii) Improvement in body weight gain ad feed conversion ratio</td>
<td>(Sohail et al. 2015; Reda et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(iii) Improvement of phytate phosphorus utilization</td>
<td>(Rafacz-Livingston et al. 2005)</td>
</tr>
<tr>
<td>AMPs</td>
<td>(i) Decrease in pathogen counts</td>
<td>(Khans et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(ii) Increase in beneficial bacteria, nutrient absorption, weight gain and feed conversion ratio</td>
<td>(Wang et al. 2011; Aguirre et al. 2015)</td>
</tr>
<tr>
<td>Bacteriophages</td>
<td>(i) Prevention of diseases in birds</td>
<td>(Kim et al. 2013; El-Gohary et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>(ii) Decrease in pathogen count</td>
<td>(Hunger et al. 2015; Kötter et al. 2013)</td>
</tr>
<tr>
<td>Antibodies</td>
<td>(i) Lowering fecal shedding of and cecal colonization by pathogen</td>
<td>(Al-Adwani et al. 2013; Hermans et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>(ii) Improving feed efficacy and intestinal health of birds</td>
<td>(Mahdavi et al. 2010)</td>
</tr>
</tbody>
</table>

Table 3. Mechanisms for growth promotion of proposed alternatives to AFAs.

<table>
<thead>
<tr>
<th>Feed additives</th>
<th>Antibiotic activity</th>
<th>Immunomodulatory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytogenic feed additives</td>
<td>(i) Disruption of membranes and enzyme systems due to lipolytic activity</td>
<td>(i) Induction of heat shock proteins to increase protein translocation</td>
</tr>
<tr>
<td></td>
<td>(ii) Modification of cell surface</td>
<td>(ii) Activation of Toll-like receptors and T₃₃ cell proliferation</td>
</tr>
<tr>
<td></td>
<td>(iii) Inhibition of DNA synthesis</td>
<td>(iii) Stimulation of mucus, bile etc by intestinal epithelium</td>
</tr>
<tr>
<td></td>
<td>(iv) Downregulation of virulence genes</td>
<td></td>
</tr>
<tr>
<td>Probiotics</td>
<td>(i) Competitive exclusion by decrease of pH, and competing for nutrients and attachment sites</td>
<td>(i) Proliferation of immune cells such as macrophages, monocytes, NK cells, T cells, etc.</td>
</tr>
<tr>
<td></td>
<td>(ii) Induce production of antimicrobial peptides by epithelium</td>
<td>(ii) Increase production of immunoglobulins, cytokines, and reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Enhance mucin production by intestinal epithelium to prevent bacterial translocation</td>
</tr>
<tr>
<td>Prebiotics</td>
<td>(i) Production of antimicrobial compounds on fermentation</td>
<td>(i) Activation of macrophages/dendritic cells by interaction with specific receptors on cell surface</td>
</tr>
<tr>
<td></td>
<td>(ii) Binding to bacterial surface receptors to prevent adhesion to intestinal epithelium</td>
<td>(ii) Increase mucin and goblet cell production by gut epithelium</td>
</tr>
<tr>
<td></td>
<td>(iii) Production of host antimicrobial peptides</td>
<td>(iii) Downregulation of proinflammatory cytokines, oxidative stress</td>
</tr>
<tr>
<td>Feed acidifiers</td>
<td>(i) Inhibition of enzyme system and disruption of membrane structure and cell turgidity by decreasing cytoplasmic pH</td>
<td>(i) Elicit faster immune response by increase in CD4 and lymphocyte cell counts</td>
</tr>
<tr>
<td></td>
<td>(ii) Disruption of DNA, transcription and translation</td>
<td>(ii) Inhibition of proinflammatory pathways</td>
</tr>
<tr>
<td>AMPs</td>
<td>(i) Altering membrane permeability, leading to cell lysis by transmembrane pore formation</td>
<td>(i) Proliferation of immune cells and production of cytokines</td>
</tr>
<tr>
<td></td>
<td>(ii) Inhibition of cell cycle, activation of lytic enzymes, production of free radicals</td>
<td>(ii) Mast cells stimulation leading to vasodilation</td>
</tr>
<tr>
<td>Bacteriophages</td>
<td>(i) Lysis of bacterial cells by specific lytic phages</td>
<td>(iii) Induction of wound repair mechanisms</td>
</tr>
<tr>
<td></td>
<td>(ii) Lysis of peptidoglycan-cell wall by phage encoded enzymes</td>
<td></td>
</tr>
<tr>
<td>Antibodies</td>
<td>(i) Binding to cell surface receptors to prevent gut adherence and colonization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ii) Agglutination, increased phagocytosis by surface modification</td>
<td></td>
</tr>
</tbody>
</table>
officially approved as the first 100% botanical zoo technical additive to increase growth in broilers in Europe. Data gathered from 20 years of field trials conducted in different parts of the world confirmed the efficacy of CCC as potential alternative to default modalities such as avilamycin, bacitracin, flavophospholipol, or enramycin (Finding alternatives to antibiotics 2016). The immunomodulation effect of CCC was shown by Lillehoj et al. (2011), who reported the downregulation of expression of oxidative stress, and the upregulation of inflammatory cytokines and genes associated with metabolic and endocrine systems in chicken.

Probiotics

The molecular mechanisms of the probiotic–host interaction of immunomodulation are not completely understood. Proposed mechanisms include increase in cell-mediated immune response, TLR signalling and antibody production and decrease of cellular apoptosis, etc. (Khan et al. 2016). Host intestinal epithelial cells and dendritic cells have certain receptors (e.g. TLRs, nucleotide-binding oligomerization domain (NOD) proteins) which are activated by probiotic MAMPs (microorganism-associated molecular patterns) such as fimbriae, flagella, lipopolysaccharide, lipoteichoic acid and peptidoglycan. Activation of these receptors leads to induction of signal transduction pathways in the host cell for transcription of genes coding for chemokines and cytokines, which can subsequently stimulate host systemic and mucosal immunity (Ajithdoss et al. 2012; Hardy et al. 2013). IL-12, a pro-inflammatory cytokine, and IL-10, an anti-inflammatory cytokine, are of particular interest with respect to probiotics. Immunostimulatory probiotics induce IL-12 proliferation, which in turn increases potency of Natural Killer (NK) cells and induces T helper pathways. Immunoregulatory probiotics induce IL-10 proliferation which then induces T regulatory pathway (Yaqoob 2014).

Probiotics have also been known to alter the gut epithelial architecture. The mucus layer (composed of a class of glycoproteins known as mucins) along with the gut epithelium forms the first line of host defence. Studies have shown that certain probiotic species increase the expression of MUC-2 and MUC-3 genes which code for synthesis of mucins by goblet cells.

Figure 2. Overview of mechanisms of alternatives to antibiotic feed additives.
Increased mucus production in the gut prevents the adherence and subsequent colonization of the intestinal epithelium by pathogenic bacteria (Smirnov et al. 2005; Hardy et al. 2013). Additionally, probiotics can also maintain the integrity of epithelial tight junctions by upregulating genes that code for junction proteins which are responsible for tight junction signalling, as well as restoration of mucosal integrity (Syngai et al. 2016). Seth et al. (2008) reported that probiotic produced proteins protect tight junctions from H₂O₂ induced disruption by the activation of Protein kinase C isoforms and mitogen-activated protein (MAP) kinase. Several mechanisms have been reported for toxin inhibition by probiotic species, such as production of a protease to degrade the toxin and its receptor, reduction of cyclic Adenosine MonoPhosphate (cAMP) production which eventually prevents diarrhea and binding of the toxin to receptors on the probiotic surface (Khan and Naz 2013).

Probiotics do not exert a direct antimicrobial effect on pathogenic bacteria in the gut, rather they employ competitive exclusion (CE) to prevent pathogen colonization. This along with the complex, multiple mechanisms of action of probiotics makes it difficult to develop bacterial resistance to probiotics.

**Prebiotics**

The proposed mechanisms of action for prebiotics include blocking receptor sites for pathogen adhesion, immunomodulation, production of antimicrobial compounds on fermentation and modifying gut morphology (Pourabedin and Zhao 2015). Immunomodulation by prebiotic is thought to be due to activation of innate immunity by the interaction of the sugars with certain receptors present on surface of dendritic cells and macrophages, which can then stimulate production of cytokines, proliferation of lymphocytes and activity of NK cells (Hashim 2012; Saad et al. 2013).

Extensive studies have been carried out on mannan oligosaccharide (MOS), obtained from the outer cell wall of *S. cerevisiae*. Mannans, present in MOS bind to mannose-specific type-I fimbriae on Gram-negative pathogens (such as *E. coli* and *Clostridium*), thus preventing them from adhering to, and colonizing the gut (Sinovec and Marković 2005). Xiao et al. reported the alteration of expression of several genes – *APOA1* and *LUM* – in the bird jejunum by dietary supplementation of MOS. They also reported the association of MOS with increased expression of genes responsible for oxidative phosphorylation, mitochondrial electron transport chain and antioxidant enzymes (Xiao et al. 2012). MOS have also been shown to affect goblet cell number and mucin production in host gut, as well as host mRNA expression, although the mechanism for this is not clear (Cheled-Shoval et al. 2014). Avian immunity can also be improved by MOS by the activation of macrophages through mannose-specific receptors (Hashim 2012). Shao et al. (2016) reported that supplementation of β-glucans in broiler diets stimulated the synthesis of host antimicrobial peptides against *Salmonella* infection. Dietary supplementation of inulin in broilers has been shown to upregulate transcription of genes for immune system, cholesterol synthesis and bile production in chicken (Tsurumaki et al. 2015). In another study, Babu et al. (2012) reported that inulin treatment can decrease the expression of IL-1β, a pro-inflammatory cytokine, thus preventing IL-1β associated cell death of macrophages, and aiding in antibacterial effect. In a microarray-based gene expression study, Sevane et al. observed a modification of the liver transcriptome profile of chicken supplementation of feed with inulin, such as upregulation of genes for transcription, translation and protein metabolism, which could be correlated to increased growth performance. The same study also reported that inulin supplementation caused the upregulation of three genes – *TNFRSF1B*, *ACSL6* and *PPARA* – responsible for anti-apoptotic activity, fatty acid metabolism and energy metabolism, respectively, as well as downregulation of three genes which contribute to oxidative stress, and consequently disease pathogenesis, thus showing the immunomodulatory effect of the prebiotic (Sevane et al. 2014). The antibacterial activity of prebiotics is due to CE and selective promotion of proliferation of beneficial bacteria, which makes it difficult for pathogens to develop resistance against them.

**Feed acidifiers**

Organic acids and their salts have been used for decades as feed additives and are considered as Generally Recognized as Safe (GRAS) for meat products. Undissociated acids can diffuse through its lipid membrane into the bacterial cell, where they dissociate, thus decreasing the cytoplasmic pH, consequently inhibiting normal enzyme activity and causing cell leakage (Ricke 2003). Other proposed mechanisms include disruption of DNA (by altering purine structure), RNA and protein synthesis as well as interfering with cytoplasmic membrane structure and cell turgidity (Mani-Lopez et al. 2012). Antibacterial activity of organic acids on *Campylobacter* spp, *E. coli*, *Salmonella*, *Campylobacter perfringens* and *Listeria monocytogenes* has been reported (Chaveerach et al. 2002; Skrivanova et al. 2006; Over et al. 2009). Organic acids have also been studied
for their effect on gut mucosa and their immunomodulatory action. Dietary supplementation of organic acids have shown to increase the counts of CD4 cells and T-Cell Receptor II lymphocytes, which corresponds to a faster immune response (Khan and Iqbal 2016). Additionally, organic acids are also being studied for their role in improvement of phytate phosphorus utilization in chickens (Rafacz-Livingston et al. 2005). Short chain fatty acids (SCFA) have been reported to upregulate genes involved in epithelial cell growth, division, differentiation, proliferation and apoptosis (Hashemi and Davoodi 2011). SCFAs such as acetic acid, citric acid, lactic acid, propionic acid, butyric acid and their N, K or Ca salts are commonly included in feed as they have been shown to improve performance, feed quality and modulate disease resistance of broiler (Abdel-Fattah et al. 2008; Sohail et al. 2015; Reda et al. 2016). For example, several studies have been carried out on potential of butyric acid as feed supplement. Butyric acid has been reported to downregulate the expression of the Salmonella pathogenicity island 1 genes responsible for virulence and invasion of epithelial cells (Van Immerseel et al. 2006). Butyrate has been shown to increase production of tight junction proteins in the cell, thus decreasing permeability of intestinal epithelium to invasion by pathogens (Van Deun et al. 2008; Andreopoulou et al. 2014).

Butyrate has been studied extensively for its anti-inflammatory properties. Two pathways have been proposed for this: (i) inhibition of IFN-γ induced STAT 1 activation and (ii) inhibition of histone deacetylase activity leading to hyper acetylation of Fas promoter, upregulation of Fas promoter and consequently of T cell apoptosis mediated by Fas (Zimmerman et al. 2012). Another mechanism for the reduced production of proinflammatory cytokines by butyrate is thought to be inhibition of the activation of kappa B, a nuclear factor (Onrust et al. 2015). Sunkara et al. (2011) reported that butyrate could induce the synthesis of host defence peptides in chicken, as well as increase the activity of chicken monocytes against S. enteritidis with minimum production of inflammatory cytokines. Zhou et al. reported that sodium butyrate could modulate macrophage activity by suppressing the production of matrix metalloproteinase (MMP), which causes inflammation. They also found that in chicken macrophage, cell lines challenged with S. typhimurium LPS, butyrate inhibited the production of IL-1β, IL-6 and IFN-γ (pro inflammatory cytokines) (Zhou et al. 2014).

Although organic acids are to be considered to be food-grade, studies have shown acid tolerance by S. typhimurium via the production of several acid shock proteins and the maintenance of internal pH by amino acid decarboxylases (Mani-Lopez et al. 2012).

**Antimicrobial peptides (AMPs)**

These small cationic oligopeptides are effector molecules of innate immunity, and have shown activity against bacteria, fungi and enveloped viruses (Thacker 2013). It is suggested that the bactericidal activity of AMPs is due to the formation of pores in cytoplasmic membrane of pathogens, thus altering membrane permeability, and, disruption of DNA and protein synthesis (Shryock and Richwine 2010). Positively charged AMPs get attracted by electrostatic interactions to structures on bacterial surfaces (e.g. lipopolysaccharide on Gram-negative walls and teichoic acids on Gram-positive walls), and reach the cytoplasmic membrane, where they bind to and aggregate in the lipid bilayer, thus forming transmembrane pores leading to cell leakage, loss of osmotic regulation and cell lysis. Other possible mechanisms of the antimicrobial activity of AMPs (after internalization by the cell) are activation of phospholipases and autolysins, inhibition of cell division by inducing filamentation in target cells, disruption of cell cycle and generation of reactive oxygen species (Brogden 2005). AMPs have also been studied for their immunomodulatory properties such as induction of cytokines, proliferation of cells of immune system, modulation of gene expression, vasodilation caused by histamine release due to stimulation of mast cells, inhibition of fibrin clot lysis to prevent pathogen dissemination, and induction of wound and tissue repair (Finlay and Hancock 2004). AMPs have also been shown to downregulate the lipopolysaccharide (LPS)-stimulated production of proinflammatory cytokines by suppression of TLR signalling responses (Hancock and Sahil 2006). Studies have evaluated the antibacterial activity of AMPs against Salmonella, Campylobacter, Listeria and Escherichia spp (Evans et al. 1995; van Dijk et al. 2007; Ebbensgaard et al. 2015), as well as the immunomodulatory and growth promotion effects of AMPs in broilers (Yurong et al. 2006; Liu et al. 2008; Aguirre et al. 2015; Józefiak et al. 2016). Several AMPs produced by bacteria – called bacteriocins – have also been identified as potential feed additives (Diez-Gonzalez 2007; Svetoch et al. 2008; Xie et al. 2009; Wang et al. 2011; Messaoudi et al. 2012; Kogut et al. 2013; Perumal et al. 2016). Although there have been studies documenting development of resistance against AMPs, it is a difficult process, as AMPs target the bacterial membrane, and altering the composition and organization of membrane lipids would be not be a feasible solution for the bacteria (Zasluff 2002).
**Bacteriophages**

One of the earliest documented uses of phage therapy was by Felix d’Herelle in 1917, when he used phage preparations for the treatment of dysentery and cholera. However, the extensive study on the therapeutic use of phages gained momentum only after the emergence and spread of antibiotic resistance. Phage therapy exploits the therapeutic potential of lytic phages. These phages bind to specific receptors on bacterial cell surface, release their genetic material into the cell and use the host cell machinery to synthesize multiple virion particles. Once the viruses have matured, cell wall is lysed, thus releasing progeny phages (Johnson et al. 2008). Several studies have reported the antibacterial potential of bacteriophages against poultry pathogens such as *E. coli*, *S. enteritidis* and *C. jejuni* (Huff et al. 2002; Atterbury et al. 2003; Carrillo et al. 2005; Huff et al. 2005; Wagenaar et al. 2005; Atterbury et al. 2007; Lim et al. 2012; Hungaro et al. 2013; Seo et al. 2016). Not just whole phages, even phage-encoded enzymes have been investigated for their antibacterial properties. These enzymes can be classified into virion associated peptidoglycan hydrolases (VAPGH) and endolysins. VAPGHs disrupt the peptidoglycan layer in bacterial cell wall after phage absorption; therefore, they are associated with “lysis from without”. They produce a small hole in the cell wall to facilitate transfer of viral DNA into the cytoplasm. It is believed that VAPGHs have conserved, rarely modified bonds and multiple lytic domains in their structure which development of resistance to these difficult (Rodriguez-Rubio et al. 2013). However, limited research has been done on VAPGHs so far. Endolysins act with holins and are responsible for cell wall lysis (“lysis from within”) at the end of the lytic cycle. They hydrolyze the peptidoglycan layer in the bacterial cell wall, causing osmotic lysis and cell death, and release of the mature virions. Endolysins have a cell-wall binding domain, for binding to the substrate (and cell wall debris after lysis), and enzymatically active domains which cleave specific bonds in the peptidoglycan (Schmelcher et al. 2012). Endolysins consist of glycosidases, amidases, carboxypeptidases and endopeptidases (Keary et al. 2013). Genes coding for these endolysins can be expressed in suitable vector systems. Lysins Ply 118 and Ply 511 (both amidases), when expressed in *Lactococcus lactis*, were shown to induce lysis of *L. monocytogenes* (Coffey et al. 2010). Another amidase, Ply 3626, could be a possible candidate for control of *C. perfringens* (Fenton et al. 2010). The antibacterial activity of recombinant lysins against *C. perfringens* and *L. monocytogenes* was also reported by Seal (2013). A chimeric, thermostable endolysin constructed from ΦCP26F and ΦGVE2 was shown to have antibacterial activity against *C. perfringens* (Swift et al. 2015).

The main drawback for the use of bacteriophages is the development of bacterial resistance, and several mechanisms have been proposed to explain this, such as blocking of viral adsorption on surface receptors, degradation of viral genome by restriction-modification systems and phage superinfection exclusion, but this can be prevented by using a bacteriophage “cocktail” (Borie et al. 2014). Bacteriophages are thought to have evolved along with the specific bacterial host; therefore, if the host develops resistance, the phages can also undergo mutation to overcome the resistance mechanism. Additionally, unlike antibiotics, the number of bacteriophages specific for a host are not limited, hence resistance to bacteriophages is not considered as a serious threat (Bragg et al. 2014).

**Antibodies**

Oral administration of antibodies for the development of passive immunity is an upcoming approach for the treatment of pathogens in humans as well as animals. With respect to poultry, egg yolk antibodies (IgY) are of importance as a replacement of AFAs. IgYs are maternal antibodies that the laying hens transfer to their offspring via the egg yolk, a phenomenon that was first described by Klemperer in 1893 (Berghman et al. 2005; Schade et al. 2005). Antibodies specific to an antigen are produced by inducing the immune system of hens by exposure to the antigen, and these antibodies are transferred to the egg yolk. Eventually, the egg yolk is separated from the white, and antibodies are extracted from it, purified and can be utilized as a feed additive (Chalghoumi et al. 2009a; Yegani and Korver 2010). Primarily, the antibacterial activity of IgY is thought to be due to binding of the IgY to bacterial structures such as LPS, flagella and pili, and thus preventing the adhesion to and colonization of the intestinal epithelium by the bacteria. This binding could also reduce bacterial toxin production by alteration of cellular signalling cascades. Other proposed mechanisms include pathogen agglutination, structural changes on cell surface on binding leading to increased phagocytic activity and toxin neutralization (Xu et al. 2011). Passive immunization with IgY has also been documented to increase growth and broiler performance. Interleukin-1 (IL-1) is a cytokine that is produced during inflammation. IL-1 further induces production of Cholecystokinin (CCK), a neuropeptide that has been found to cause anorexia growth suppression. IgYs specific to CCK have been shown to improve broiler performance (Cook 2004; Gadde et al. 2015). Several studies have reported the efficacy of IgY against *E. coli*,
Clostridium, Campylobacter and Salmonella (Tamilzarasan et al. 2009). Lee et al. (2002) and Chalghoumi et al. (2009b) demonstrated that IgY against S. enteritidis and S. typhimurium could inhibit bacterial growth in vitro. Rahimi et al. (2007) showed that chicks supplemented with S. enteritidis-specific IgY had lower faecal shedding and cecal colonization by the pathogen. The efficacy of IgYs developed against C. jejuni colonization-associated-proteins (CAPs) has been demonstrated in vitro (Ali-Adwani et al. 2013). Also, immunization of chickens with IgYs against whole cell lysate or hydrophobic protein of C. jejuni has been shown to reduce C. jejuni counts in the ceca after pathogen challenged. Additionally, antibodies may also improve feed efficacy, as reported by Mahdavi et al., that the oral administration of IgY against E. coli O78:K80 via egg yolk powder enhanced intestinal health and broiler performance (Mahdavi et al. 2010). Compared with antibiotics, antigen-specific IgYs are safer, more efficient and less toxic. Since they are polyclonal antibodies, it is easy to develop an effective molecule without identification of specific epitopes on the antigen. Although bacteria cannot develop resistance to antibodies, their biggest disadvantages are their susceptibility to proteolytic degradation in the gut as well as their expensive large-scale production (Mine and Kovacs-Nolan 2002; Gadde et al. 2015). Therefore, studies are required to develop a simple and cost effective method of IgY production that gives high yield and purity.

**Approaches for development of novel alternatives**

Several properties deemed desirable for an optimal alternative to AFA have been cited in the literature, primarily, having a well-defined mode of action, exhibiting toxicity only to the pathogen and not the host, being non-virulent, not causing induction of drug resistance, cost efficacy, maintaining stability in feed and after industrial treatment, improving feed efficacy and promoting growth, not altering palatable and being environmentally safe (Shane 2005; Cheng et al. 2014; Caly et al. 2015). However, none of the existing alternatives individually meet the requisites to replace the efficiency and cost efficacy of AFAs. Phytogenic additives, prebiotics probiotics, synbiotics, organic acids and feed enzymes have been reported to give inconsistent results. However, some studies have reported that a combinational approach may be more beneficial. Synbiotics are feed additives that combine both prebiotics and probiotics, which together have a synergistic effect. The growth and activity of the probiotic is stimulated by the prebiotic, thus benefitting the host health and growth (Falaki et al. 2011; Mousavi et al. 2015; Saiyed et al. 2015; Tang et al. 2015; Hamasalim 2016) However, supplementation of synbiotics does not always have a beneficial effect, and these inconsistencies can be addressed by proper selection of the probiotic strain (based on its fermentation profile) and dosage of the prebiotic (Methner et al. 1999; Geier et al. 2010; Hahn-Didde and Purdum 2015; Murshed and Abudabos 2015; Abudabos et al. 2015, Flores et al. 2016). There is also documentation of recent studies of effect of other combinations on broiler performance, such as phytogenics and organic acids (Hassan et al. 2015; Aristimuňa et al. 2016; Tanzin et al. 2016), probiotics and organic acids (Abudabos et al. 2017), and prebiotic and enzymes (Rebolé et al. 2010). A clearer understanding of the molecular mechanism of action of phytogenic additives, prebiotics and probiotics would help to design more efficient combinations of alternatives.

Another area that holds promise is the designing of better delivery systems such as microencapsulation, in which “droplets of the bioactive compound can be surrounded by a coating or embedded in a homogenous or heterogeneous matrix” (Ganesh and Hettiarachchy 2015). Encapsulation is associated with increased stability, and controlled release of the bioactive compound in the host, and in poultry encapsulation of the bioactive compound has been shown reduce pathogen load, and increase counts of beneficial bacteria (Gheisari et al. 2007; Lee et al. 2015; Rajasekaran and Santra 2015). It could be an effective delivery system where the AFA alternative is vulnerable to proteolytic lysis and/or low pH, such as is the case for AMPs, phages, vaccines and antibodies. Nanoparticle delivery systems (Schlesinger et al. 2013; Adamu Ahmad et al. 2016) and gel vaccine delivery systems (Jenkins et al. 2014) are more recent developments that have been patented, and can be used in animal feed. Another delivery system that is of particular interest is in ovo injection, in which the bioactive substances are injected into the air chamber of eggs or directly into the growing embryo, giving the advantages of precision, control on amount of substance administered and reduced labour costs (Dankowiakowska et al. 2013). For poultry, in ovo injections have been documented for vaccines against Eimeria (Barbour et al. 2015), subunit vaccine against Campylobacter (Kobierecka et al. 2016), probiotics, prebiotic and synbiotics (Maiorano et al. 2012; Yamawaki et al. 2013; Pruszynska-Oszmalska et al. 2015; Płowiec et al. 2015), essential oil and organic acid blend (Toosi et al. 2016), etc.

**Concluding remarks and future perspectives**

To evade intracellular resistance mechanisms that bacteria have developed against conventional antibiotics, it is essential to develop alternatives with different targets.
If the proposed alternative has multiple targets for antibiotic activity (such as in the case of phytobiotics or AMPs), it is much harder for bacteria to develop an effective resistance mechanism against it. Alternatives such as probiotics and prebiotics have a host-centric mechanism of antibacterial action, i.e. they interact directly with the host gut epithelium, which in turn prevents colonization by pathogen. Though organic acids have been used as feed additives for many years, resistance to these is not very common. This could be due to the fact that organic acids do not bind to any specific receptors on cell surface and their mode of entry into the cell is by simple diffusion. Similarly for AMPs, they bind to the cell surface by electrostatic attraction rather than to a specific receptor. Bacteriophages and antibodies are emerging technologies and have their limitations; however, they have high specificity for a particular host and, therefore, are superior to conventional antimicrobials which have an indiscriminate mode of action (i.e. against both pathogens and beneficial gut bacteria).

Current research suggests that for an effective AFA alternative, the focus should be on its potential immunomodulatory efficacy, i.e. towards a host-centric approach. Management of inflammatory and immune responses in the host facilitates the diversion of nutrients and energy towards increased production. Also, since these mechanisms are not directly antimicrobial, there is a lesser chance of development of resistance.

In the face of the growing population and consecutive increase in poultry consumption, the need of the hour is to develop a suitable alternative to AFAs. Till such an alternative is developed, the judicious use of AFAs along with improved conditions of poultry rearing is an absolute necessity.

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