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PREVALENCE OF ANTIBIOTIC-RESISTANT *E. COLI* IN POULTRY AND EFFECTS OF AN ORGANIC ACIDS BASED FEED ADDITIVE AND AN SYNBIOTIC PREPARATION ON THE LEVEL OF ANTIBIOTIC-RESISTANT *E. COLI*

Dissertation

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submitted by

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DECLARATION OF ORIGINALITY

I hereby declare to the best of my knowledge, that the content of this doctoral thesis at the University of Natural Resources and Life Sciences Vienna (BOKU) is based on my own original research. All assistance received and references cited have been acknowledged. Furthermore, I declare that this thesis has not been previously or concurrently submitted to any other educational institution for achieving any other academic degree.

Signature:

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Vienna, February 2018

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ABSTRACT

In addition to being used for the treatment of human infections, antibiotics are also widely used for the treatment of infection, disease prevention and the growth promotion of food-producing animals worldwide. The use of antibiotics has resulted in the emergence of antibiotic resistance, which is a cause of global concern for human and animal health. Among meat-producing animals, poultry is an increasing source of meat. The amount of antibiotics used as well as the prevalence of resistance is partly known for few countries; however, a global overview is missing.

The first section of this thesis identifies the antibiotic agents legalized and the levels of antibiotic resistance reported in *Escherichia coli* isolated from broilers originating from large poultry-producing regions, including the US, China, Brazil and countries of the EU (Poland, United Kingdom, Germany, France and Spain), which produce more than half of the global poultry meat supply.

The data search provided interesting results and showed that fluoroquinolones, 3rd generation cephalosporins and macrolides ("highest priority, critically important" antibiotics for human medicine according to the WHO) are approved for use in large poultry-producing regions, with the exception of fluoroquinolones in the US and cephalosporins in the EU. Tetracyclines, aminoglycosides, sulfonamides and penicillins are registered for use in poultry in all evaluated countries. The resistance rates in *E. coli* to representatives of these antibiotic classes are high in all countries. The resistance rates to fluoroquinolones and quinolones in the US, where fluoroquinolones are not registered for use, are below 5%, while the average of resistant *E. coli* is above 40% in Brazil, China, and the EU, where use of fluoroquinolones is legal. These findings demonstrate that the production of poultry meat without fluoroquinolones is possible and that the ban of fluoroquinolones has led to low resistance rates in *E. coli*.

In general, it is assumed that the occurrence of antibiotic resistance stems from the use of antibiotics in animal production. However, the extent to which antibiotic resistance is associated with the use of chemical and biological agents used for the expressed purpose to control, deter, inhibit or kill harmful microorganisms is poorly understood according to the

FAO (2018). The aim of the second part of this thesis was to evaluate the effect of enrofloxacin as well as an acid-based feed additive (FA) on the prevalence of antibiotic-resistant *E. coli* in broilers. Treatment with enrofloxacin increased the number of *E. coli* resistant to ciprofloxacin, streptomycin, sulfamethoxazole and tetracycline; it also decreased the number of *E. coli* resistant to cefotaxime and extended-spectrum beta-lactamase-(ESBL)-producing *E. coli* in the ceca of broilers. Supplementation with the FA contributed to a significant decrease in the number of *E. coli* resistant to ampicillin and tetracycline compared to the those in control and enrofloxacin-treated groups, as well as to a decrease in sulfamethoxazole- and ciprofloxacin-resistant *E. coli* compared to that in the enrofloxacin-treated group.

In the third part of the thesis, we investigated the impact of ampicillin, a FA as well as a synbiotic preparation on the prevalence of antibiotic-resistant *E. coli* in the ceca of broilers receiving oral challenge with avian pathogenic *E. coli* multiresistant to ampicillin, cephalexin and nalidixic acid. The administration of ampicillin for five days led to a significant increase in the number of *E. coli* resistant to ampicillin, amoxicillin-clavulanic acid, cefoxitin and ceftriaxone, which all belong to the ß-lactam antibiotic family. Tested feed additives did not increase the prevalence of resistant determinants in the gut of broilers. Moreover, the effect of the tested feed additives on the prevalence of resistant *E. coli* was demonstrated by lower ceftriaxone MIC values than to those in the antibiotic group. Additionally, the synbiotic fed group showed lower ceftriaxone MIC values when compared to the antibiotic group.

It may be concluded, that a high prevalence of resistant *E. coli* in all experimental groups was observed in both studies. The treatment of broilers with antibiotics led to an increase in resistant *E. coli*, but this effect was not observed for FA and synbiotics. Moreover, the number of *E. coli* resistant to some antibiotics was lower in the group of broilers supplemented with FA or a synbiotic than that in the other control groups.

ZUSAMMENFASSUNG

Neben der Verwendung für die Bekämpfung von Infektionen beim Menschen werden Antibiotika auch weltweit zur Behandlung von Infektionen, zur Prophylaxe und zur Wachstumsförderung von Lebensmittel-produzierenden Tieren eingesetzt. Die breite Verwendung von Antibiotika führt zur Entstehung und Verbreitung von Antibiotikaresistenzen, welche ein zunehmendes Risiko für die Gesundheit von Mensch und Tier darstellen. Unter den Fleisch-produzierenden Tieren nimmt Geflügel eine zunehmend dominierende Rolle ein. In diesem Zusammenhang sind die dabei eingesetzten Antibiotikamengen sowie die Prävalenz der Antibiotikaresistenzen allerdings nur für wenige Länder bekannt und ein globaler Überblick fehlt bislang.

Im ersten Abschnitt dieser Arbeit wurden die jeweils für die Geflügelmast zugelassenen antibiotischen Substanzen identifiziert und dem Niveau der Antibiotikaresistenzen in aus Broilern isolierten *E. coli* gegenübergestellt. Dabei wurden die Daten der größten Geflügelfleisch-produzierenden Länger/Regionen verglichen (entsprechen >50% der globalen Geflügelfleischproduktion): USA, China, Brasilien und die EU-Mitgliedsländer (Polen, Vereinigtes Königreich, Deutschland, Frankreich und Spanien).

Die Datenrecherche lieferte wichtige Erkenntnisse und zeigte, dass die Fluorchinolone, Cephalosporine und Makrolide der 3. Generation ("Critically important antimicrobials for human medicine according to WHO“) für den Einsatz in großen Geflügel produzierenden Regionen zugelassen sind. Ausnahmen dabei sind die Fluorchinolone in den USA und die Cephalosporine in der EU. Tetracycline, Aminoglykoside, Sulfonamide und Penicilline sind in allen untersuchten Ländern für die Verwendung bei Geflügel registriert d.h. zur Verwendung zugelassen. Die Resistenzraten in *E. coli* Isolaten gegenüber Vertretern dieser Antibiotikaklassen sind in allen untersuchten Ländern hoch. Die Resistenzraten gegenüber Fluorchinolonen und Chinolonen in den USA (Fluorchinolone sind hier nicht zur Verwendung zugelassen) liegen unter 5%, während die Durchschnittsrate resistenter *E. coli* Isolate in Brasilien, China und der EU über 40% liegt (der Einsatz von Fluorchinolonen ist in diesen Ländern legal). Diese Ergebnisse zeigen, dass die wirtschaftliche Produktion von Geflügelfleisch ohne den Einsatz von Fluorchinolonen möglich ist und das Verbot des Einsatzes von Fluorchinolonen zu niedrigen Resistenzraten in *E. coli* geführt hat.

Im Allgemeinen wird angenommen, dass das Auftreten und die Verbreitung von Antibiotikaresistenzen unter anderem auf den Einsatz von Antibiotika in der Tierproduktion zurückzuführen sind. Dabei ist jedoch der Umfang und die Dynamik, in der Antibiotikaresistenzen mit dem Einsatz nicht-antibiotischer antimikrobieller Substanzen zur Hemmung und Abtötung unerwünschter Mikroorganismen in Zusammenhang stehen, nach FAO (2018) noch wenig verstanden.

Im zweiten Teil dieser Arbeit wurde die Wirkung von Enrofloxacin und die eines auf organischen Säuren basierenden Futtermittelzusatzes (FA) auf die Prävalenz antibiotikaresistenter *E. coli* bei Broilern untersucht. Der Einsatz von Enrofloxacin führte zu einer Erhöhung der Anzahl an gegen Ciprofloxacin, Streptomycin, Sulfamethoxazol und Tetracyclin resistenten *E. coli* Isolate. Parallel wurde die Rate an *E. coli* Isolaten mit Resistenz gegen Cefotaxim sowie Extended-Spectrum-Beta-Lactamase-(ESBL)-produzierende *E. coli* in der Ceca von Broilern verringert. Die Supplementierung des Futters mit FA trug zu einer signifikanten Abnahme von Ampicillin- und Tetracyclin-resistenten *E. coli* im Vergleich zur Kontroll- und Enrofloxacin-behandelten Gruppe, sowie zu einer Abnahme von Sulfamethoxazol- und Ciprofloxacin-resistenten *E. coli* im Vergleich zu der mit Enrofloxacin-behandelten Gruppe bei.

Im dritten Teil der Arbeit wurde der Einfluss von Ampicillin, FA und einer synbiotischen Präparation auf die Prävalenz von antibiotikaresistenten *E. coli* im Blinddarm von Masthühnern untersucht. Zuvor hatten die Tiere eine orale Gabe mit einem geflügelpathogenen *E. coli* Stamm mit Resistenzen gegen Ampicillin, Cephalexin und Nalidixinsäure bekommen. Die Verabreichung von Ampicillin für fünf Tage führte zu einem signifikanten Anstieg von *E. coli* mit Resistenzen gegen folgende ß-Lactam-Antibiotika: Ampicillin, Amoxicillin-Clavulansäure, Cefoxitin und Ceftriaxon. Die beiden eingesetzten Futterzusatzstoffe (FA, synbiotisches Präparat) erhöhten die Prävalenz resistenter *E. coli* im Darm von Broilern nicht. Darüber hinaus wurden in den Gruppen mit den Futtermittelzusatzstoffen niedrigere Ceftriaxon-MHK Werte im Vergleich zur Antibiotikagruppe festgestellt.

Zusammenfassend wurde eine hohe Prävalenz von resistenten *E. coli* in allen Gruppen der beiden Fütterungsstudien beobachtet. Der Einsatz von Antibiotika bei den Broilern führte jeweils zu einem Anstieg an antibiotika-resistenten *E. coli.* Dieser Effekt wurde für die beiden geprüften Alternativen (FA und Synbiotikapräparat) nicht beobachtet. Im Gegenteil, es konnte eine reduzierte Anzahl an resistenten *E. coli* in den mit FA oder dem Synbiotikapräparat behandelten Gruppen im Vergleich zu den Kontrollgruppen festgestellt werden.

LIST OF PUBLICATIONS

Publications related to this thesis

I. Roth, N., A. Kaesbohrer, S. Mayrhofer, U. Zitz, C. Hofacre, and K. Domig. 2018. The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: A global overview. Poultry Science, https://doi.org/10.3382/ps/pey539

II. Roth, N., S. Mayrhofer, M. Gierus, C. Weingut, C. Schwarz, B. Doupovec, R. Berrios, and K. Domig. 2017. Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in cecum of broilers. Poultry Science 96:4053-4060.

III. Roth, N., C. Hofacre, U. Zitz, G.F. Mathis, K. Moder, B. Doupovec, R. Berghouse, and K. Domig. 2019. Prevalence of antibiotic resistant *E. coli* in broilers challenged with a multiresistant *E. coli* strain and receiving ampicillin, an organic acids based feed additive or a synbiotic preparation. Poultry Science, https://doi.org/10.3382/ps/pez004

Peer reviewed conference proceedings (oral presentations)

1. Roth N. Effect of the Feed Additives on the Prevalence of Resistant Bacteria. [World Nutrition Forum 2018, Cape Town, South Africa, 3-5 Okt. 2018]

2. Roth N., Mayrhofer S., Gierus M., Weingut C., Schwarz C., Doupovec B., Berrios R., Domig K. Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in cecum of broilers. [The American Association of Avian Pathologist (AAAP) Annual Meeting 2018, Denver, USA, 13-17 Jul. 2018]

3. Roth N., S. Mayrhofer, M. Gierus, C. Weingut, C. Schwarz, B. Doupovec, R. Berrios, and K. Domig. Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in broilers. [V-International Conference on Antimicrobial Reserch – ICAR 2018, Torremolinos, SPAIN, 24-25 May 2018]

4. Roth N., Mayrhofer S., Gierus M., Weingut C., Schwarz C., Doupovec B., Berrios R., Domig K. Role of feed additives in the strategy to reduce the prevalence of antimicrobial resistance in broilers. [4th International Conference on Antimicrobials, Multiple Drug Resistance & Antibiotics Resistance, Las Vegas, USA, Apr. 20-24, 2018]

5. Roth N., A. Kaesbohrer, S. Mayrhofer, U. Zitz, C. Hofacre, and K. Domig. Prevalence of antibiotic resistant bacteria in animals – A global perspective. [4th International Conference on Antimicrobials, Multiple Drug Resistance & Antibiotics Resistance, Las Vegas, USA, Apr. 20-24, 2018]

6. Roth N. Strategies to overcome antibiotic resistance in turkey production. [Turkey science and production conference, Chester, UNITED KINGDOM, 21-23 March, 2018]

7. Roth N. Strategies to overcome antibiotic resistance in broiler production. [Nordic Poultry Conference, Malmö, SWEDEN, 7-8 Nov, 2017]

8. Roth N., Mayrhofer S., Doupovec B., Berrios R., Domig K. J. The effect of an acid based feed additive on the intestinal level of ESBL-producing *E. coli* in feces of swine. [European Symposium of Porcine Health Management. Prague, CZECH REPUBLIC, 3-5 May, 2017]

9. Roth N., Mayrhofer S., Doupovec B., Berrios R., Domig K. J. The effect of an acid based feed additive on the intestinal level of ESBL-producing *E. coli* in feces of swine.

10. Roth N., Mayrhofer S., Domig K.J. Monitoring of antibiotic resistant bacteria in food animals. [Livestock production in the post antibiotic era - Global Challenges University

Alliance (GCUA) workshop - Swedish University of Agricultural Sciences (SLU), Uppsala, SWEDEN, Nov. 30 - Dec 3, 2015]

11. Domig K.J., Mayrhofer S., Roth N., Kneifel W. Monitoring the livestock resistome. [Livestock production in the post antibiotic era - Global Challenges University Alliance (GCUA) workshop - Swedish University of Agricultural Sciences (SLU), Uppsala, SWEDEN, Nov 30 - Dec 3, 2015]

Peer reviewed conference proceedings (poster presentations)

1. Roth N., Xiao Y., Qu L., Kovacs A., Tacconi A., Wie-dong Sun. (2017): Effect of natural antimicrobial substances on Gram-negative bacteria and their efficacy in broilers. [21st European Symposium on Poultry Nutrition, Salou, SPAIN, 8-11 May 2017]

2. Roth N., Mayrhofer S., Doupovec B., Berrios R., Domig K. The Effect of an Acid-Based Feed Additive on the Intestinal Level of ESBL-Producing *E. coli* in Swine. [16th BOKU Symposium, University of Natural Resources and Life Sciences, Vienna, AUSTRIA, 27 Apr. 2017]

3. Roth N., Mayrhofer S., Doupovec B., Berrios R., Waxenecker F., Sucher P., Domig K. Detection of antibiotic resistant *E. coli* in weaned piglets. [24th International Pig Veterinary Society Congress, Dublin, IRELAND, 7 - 10 Jun, 2016]

4. Roth N., Kovacs A., Pruckner S. (2016) The effect of permeabilizing substances on the outer membrane of gram-negative bacteria and their efficacy in weaning pigs. [4th International Conference on Antimicrobial Research, Torremolinos, SPAIN, 29 Jun-1Jul 2016]

5. Roth N., Breitsma R., Kovacs A., Berrios R., Doupovec B., Goelss F., Wegl G., Klose V. (2014) Influence of the acidifier compound on counts of fecal resistant *E.coli* and growth performance of weaning pigs. [13th BOKU Symposium, University of Natural Resources and Life Sciences, Vienna, AUSTRIA, 29 Apr. 2014]

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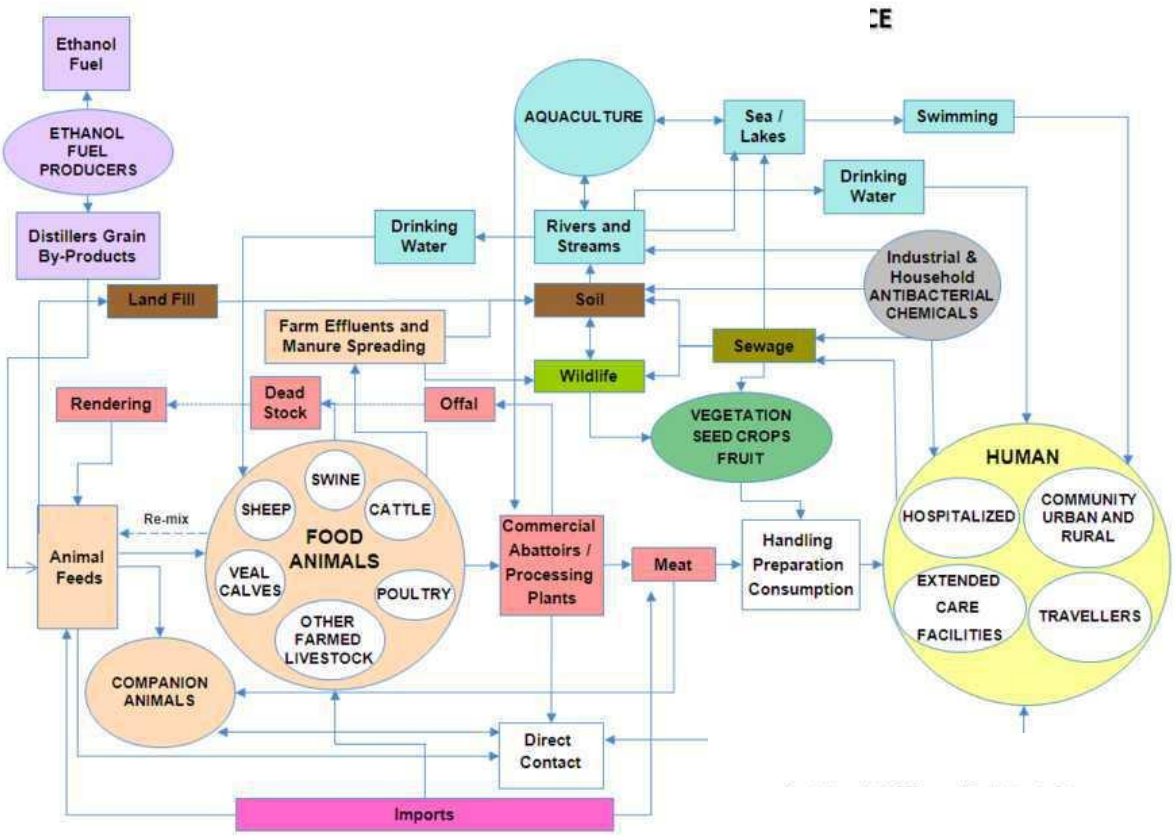
I. INTRODUCTION

Problem of antibiotic resistance and antibiotic use in animal production

The antibiotic is an antibacterial class of antimicrobials, and of greatest interest for public health (Pagel and Gautier, 2012). Antibiotic is a substance produced by one microorganism that selectively inhibits the growth of another; synthetic antibiotics, usually chemically related to natural antibiotics, have since been produced that accomplish comparable tasks (MedicineNet, 2018). Antibiotics are essential for the treatment and prophylaxis of human infections as well as for the treatment of animal and plant infections (Marshall and Levy, 2011; Milillo and Ricke, 2010). Although antibiotics were introduced into the clinical practice only in the middle of the last century, they have been present in the nature since the existence of microorganisms. The process of antibiotic resistance is a natural evolutionary phenomenon for microorganisms that are constantly adapting to survive (WHO, 2018). Due to the high amount of antibiotics in the ecosystem since their use for treatment and prevention of diseases, antibiotic resistance is an increasingly serious threat to global public health. All use of antibiotics including appropriate, inappropriate, over- and under-use drives the development and spread of antibiotic resistance (Interagency Coordination Group on Antimicrobial Resistance, 2018). Antibiotic resistance is a complex and multifactorial problem. There are many potential pathways by which resistant bacteria may transfer between populations of humans, animals, fish, water sources and plants, as shown in Figure 1. Therefore, reduction of antibiotic use each sector of use is reasinable in order to achieve reduction of the prevalence of antibiotic resistance.

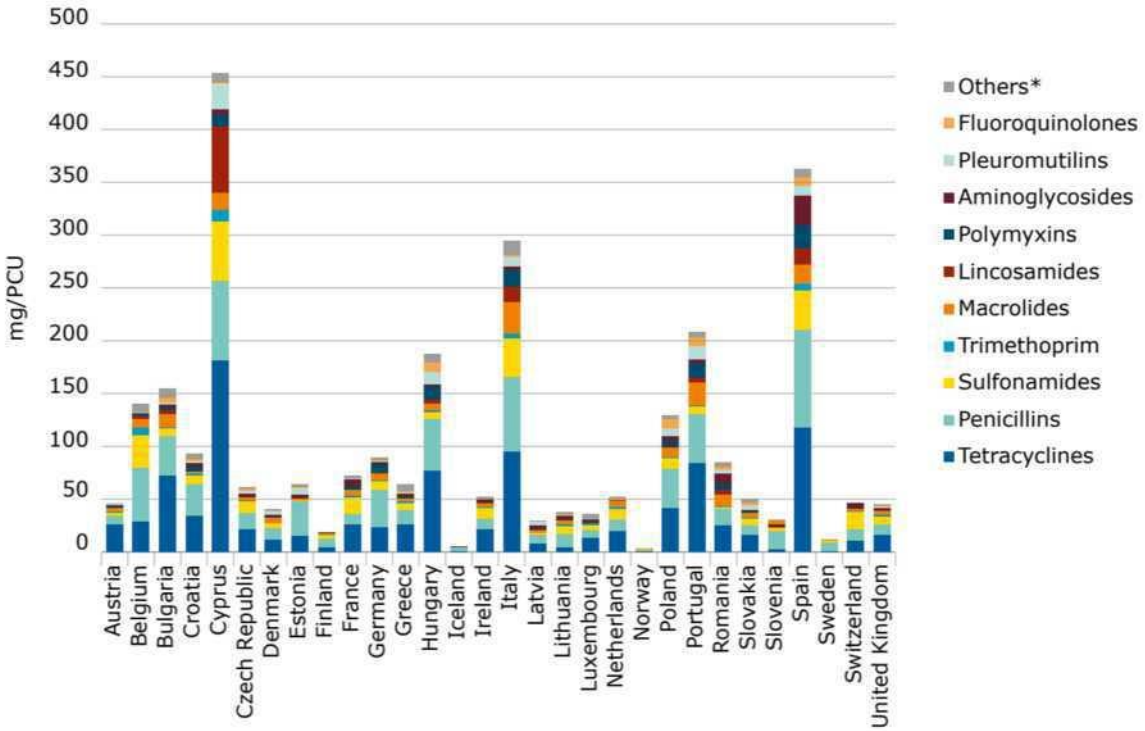
Figure 1: Epidemiology of Antimicrobial Resistance in the environment.



Adopted and modified from Linton (1977) by Rebecca Irwin, Health Canada (Prescott, 2000) and IFT.

In order to reduce antibiotic use, it is important to measure it. There is a national monitoring of antimicrobial use in some countries (European countries, USA, Japan). Figures from the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) Report show that a total of 7 787 tonnes of active ingredients of veterinary medicinal products were sold for use in livestock in the 30 reporting countries (European Medicines Agency, 2018). The use of antibiotics to produce the same ammont of meat is very different depending on the country as shown in Figure 2.

Figure 2. Sales of antibiotics for food producing animals in 30 European countries in 2016.



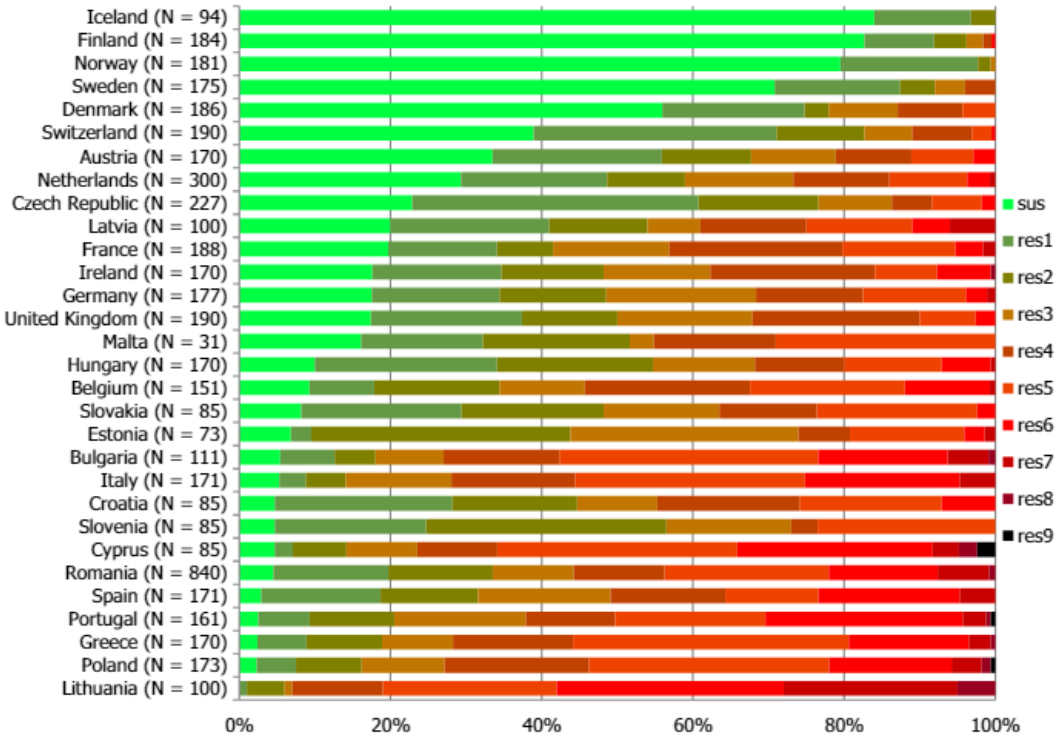
*Amphenicols, cephalosporins, other quinolones and other antibacterials (classified as such in the ATCvet system). Source: European Medicines Agency, 2018

Sales of antibiotics for animal species is needed in oreder to understand and reduce the antibiotic use. These data are available for few European countris, the US and Japan. There is no data available on global use of antibiotics. Poultry is one of the world´s fastest growing sources of meat production. It is known that antibiotic in poultry are used for the treatment of disease, disease prevention and growth promotion (Poole and Sheffield, 2013). The work of this thesis is the identification of antibiotic substances and there amounts, which are used in the large poultry producing countries: the US, China, Brazil and countries of the EU - Poland, United Kingdom, Germany, France and Spain. National listings of all medical products that are approved for use in poultry were screened for active antibiotic substances that may be used in feed, water or administered parenterally.

Antibiotic resistance in poultry

Harmonized integrated surveilance of antibiotic resistance in food producing animals and food is implemented in only a limited number of countries (WHO, 2014). Similarly to antibiotic use monitoring in Europe, it is recognizable that antibiotic resistance is different depending on the country. Figure 3 presents the distribution of multiresistant *E. coli* in different European countries. Exact comparison of antibiotic use in poultry and resistance in *E. coli* from broilers in Europe is not possible, as data on antibiotic use in poultry are nor available. However, we observe that contries with low prevalence of antibiotic resistance in poultry like Iceland, Finnland, Norway, Sweden, Denmark are also using low ammounts of antibiotics in livestock production, which can be observed comparing Figure 2 and Figure 3.

Figure 3: Frequency distribution of *E. coli* isolates completely susceptible and resistant to 1 – 11 antibiotic classes in European countries in 2016.



N: total number of isolates tested for susceptibility against the whole harmonised set of antimicrobials for *E. coli*; sus: susceptible to all antimicrobial classes of the harmonised set for *E. coli*; res1–res9: resistance to 1 up to 11 antimicrobial classes of the harmonised set for *E.*

coli. Source: EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018.

The evaluation of resistance rates in *E. coli* from poultry originating from large poultry producing countries combined with data about antibiotic use in poultry should provide global comparison of data. We hypothesize that the application of antibiotics leads to high and consistent resistance levels in *E. coli* isolates, whereas ban of some classes of antibiotics results in low resistance levels. This review is the first comprehensive evaluation of data recording the authorized antibiotics for poultry production combined with AR data in *E. coli* isolates in large poultry producing regions.

Influence of biocidal feed additives on antibiotic resistance

The European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) were reviewing the measures to reduce the use of antimicrobials in animal husbandry in the EU as well as reviewing the recent scientific developments in the area of possible alternatives to the use of antimicrobials in animal husbandry in the EU. The outcome is presented in the comprehensive scientific optinion and shows that implemented animal husbandry and disease prevention measures improve animal health and welfare, and therefore reduce the need to use antibiotics (Murphy, et al., 2017). This measures include external biosecurity, compartmentalisation, production groupings, housing design, building, maintenance, nutrition, stress reduction, vaccination and genetic selection. Same report provides a list of the alternatives to antibiotics, althouth gaps in knowledge that limit the use of alternatives to antimicrobials in animal husbandry were identified. To the identified alternatives belong organic acids, probiotics, competitive exclusion, synbiotics, passive immunisation, bacteriophages, immunomodulators, Zinc oxide, clay minerals and teat sealants. Some of the named alternatives to antibiotics have biocidal activity. For some biocides, results from laboratory experiments show that exposure to particular active ingredients or biocidal products can result in increased tolerance of certain microorganisms to the active ingredient and also other antibiotics (Food and Agriculture Organization of the United Nations, 2018).

In general, the extent to which AR is associated with the use of biocides and disinfectants – chemicals and biological agents used for the expressed purpose to control, deter, inhibit or kill harmful microorganisms –is poorly understood. The aim of the second and third parts of the thesis was the evaluation of the effect an acid-based feed additive (FA) as well as synbiotic on the prevalence of antibiotic-resistant *E. coli* in broilers in comparison to antibiotics enrofloxacin and ampicillin.

Organic acid based feed additives are frequently used in poultry production due to their bactericidal activity, both in feed and in the gastrointestinal tract (Ricke, 2003). The effect of non-antibiotic antimicrobial compounds like organic acids and cinnamaldehyde on resistant *E. coli* is not clear. There is an indication that exposure to non-antibiotic antimicrobial agents can induce or select for bacterial adaptations that results in decreased susceptibility to one or more antibiotics (Wales and Davies, 2015). In contrary, the reduction of extended-spectrum cephalosporin producing *E. coli* has been associated with the use of acidified drinking water in a risk factor study performed in Belgian broiler farms (Persoons, et al., 2010).

Synbiotics are defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (Gibson and Roberfroid, 1995). The application of the synbiotic preparation may reduce *E. coli* and total coliform populations in the intestines of broiler chickens (Dibaji, et al., 2014). The influence of synbiotic preparation on *E. coli* and AR *E. coli* counts was evaluated with investigation of this thesis.

E. coli were used in the studies as indicator bacteria to determine development of resistance in broilers. *E. coli* may frequently be exposed to selective pressure caused by antibiotic treatments and may contribute considerably to the spread of antibiotic resistance (Simoneit, et al., 2015). Moreover, avian pathogenic *E. coli* (APEC) cause various diseases collectively termed as colibacillosis in chickens, which are responsible for significant economic losses in the chicken industry (Hammerum and Heuer, 2009; Mohamed, et al., 2014). Therefore, oral challenge with multiresistant *E. coli* was conducted to see the effect of multiresistant *E. coli* on the susceptibility profile of commensal *E. coli*, and compare it with antibiotic ampicillin.

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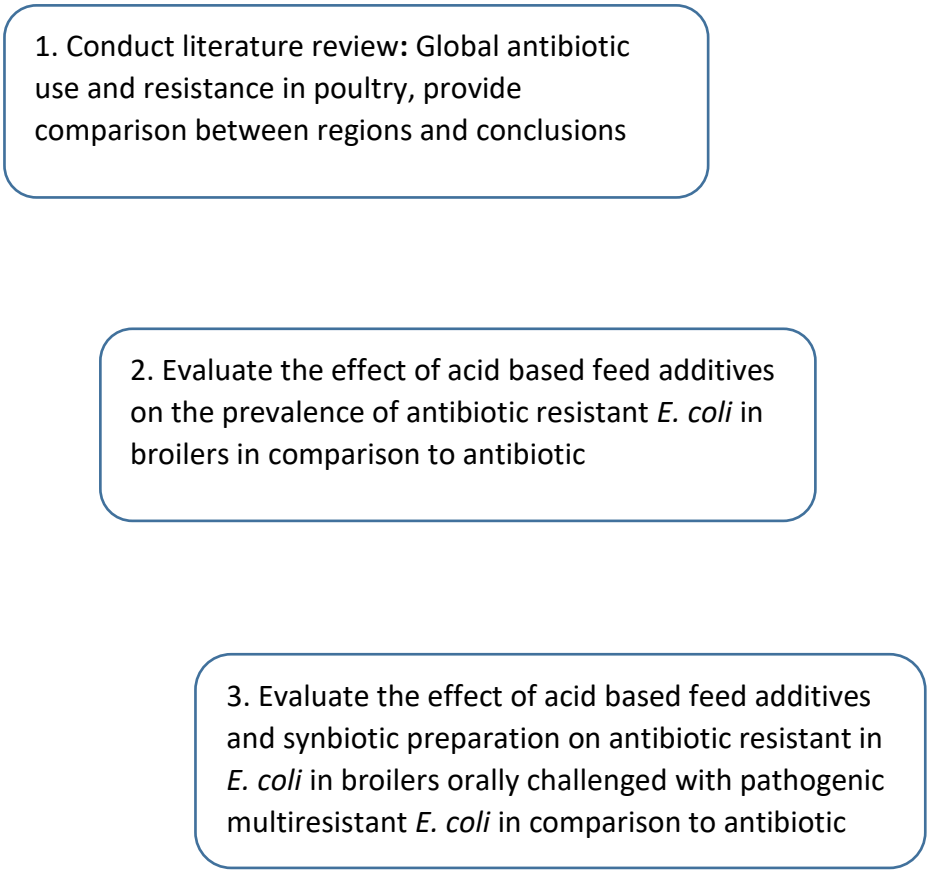
II. AIMS AND OBJECTIVES

The main aim of this research was to provide an overview about antibiotic use and resistance in poultry on a global perspective and to evaluate the effect of feed additives on the prevalence of resistant *E. coli* in broilers. Figure 4 illustrates the three key steps of research process throughout this thesis. Subsequently, the individual steps are described in more details.

Figure 4: Key steps of the research process identifing aims and objectives of the thesis.



1. Conduct literature review

The objective of this study was to identify the legalized antibiotics, the amounts thereof administered and the level of AR monitored in *E. coli* isolated from broilers originating from the large poultry producing regions US, China, Brazil, Poland, United Kingdom, Germany, France and Spain. We hypothesize that the application of antibiotics leads to high and

consistent resistance levels in *E. coli* isolates, whereas ban of some classes of antibiotics results in low resistance levels.

2. Evaluate the effect of acid based feed additives on the prevalence of antibiotic resistant *E. coli* in broilers and compare it to the effect of enrofloxacin

Trial was planned to be conducted in the EU (Austria). Outcome of the literature review should provide a base for used for the choice of antibiotic as a positive control group in the trial. Antibiotic, that shows high resistance level in *E. coli* from poultry in the EU, which use is causing concerns and needs to be replaced and is used to combat *E. coli* problems in poultry should be used in the trial. Accordingly, it was decided to use enrofloxacin. Due to the antimicrobial activity of organic acids, the level of antibiotic resistant bacteria in the gastrointestinal tract of broilers may increase or decrease. The present study therefore evaluates the effect of enrofloxacin and feed additives based on organic acids on the prevalence of resistant *E. coli* in the gastrointestinal tract of broilers.

3. Evaluate the effect of acid based feed additives and synbiotic preparation on antibiotic resistant in E. coli in broilers orally challenged with pathogenic multiresistant *E. coli* and compare it to ampicillin

It was planned to conduct this trial in the US. Concerns about the development of fluoroquinolone-resistant *Campylobacter* species in poultry led to a withdrawal of enrofloxacin in poultry in the United States in 2005. The resistance to enrofloxacin in the US is very low, therefore another antibiotic, that is used for the treatment of *E. coli* problems and causing resistance in the US should be used in the trial. It was decided to use ampicillin. The antimicrobial activity of an organic acids based feed additive, as well as the application of a synbiotic preparation may influence the level of AR bacteria in the gastrointestinal tract of broilers. The present study therefore evaluates the effect of these feed additives as well as ampicillin on the prevalence of resistant *E. coli* in the gastrointestinal tract of broilers under oral challenge with a multiresistant pathogenic *E. coli* strain. Method of an oral challenge with ampicillin needed to be developed prior the study start.

III. PUBLICATIONS

1. LITERATURE REVIEW - **The application of antibiotics in broiler production and the resulting antibiotic resistance in *E. coli*: A global overview**

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The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: A global overview

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ABSTRACT The increase in antibiotic resistance is a global concern for human and animal health. Resistant microorganisms can spread between food-producing animals and humans. The objective of this review was to identify the type and amount of antibiotics used in poultry production and the level of antibiotic resistance in *Escherichia coli* isolated from broilers. Isolate information was obtained from national monitoring programs and research studies conducted in large poultry-producing regions: US, China, Brazil, and countries of EU—Poland, United Kingdom, Germany, France, and Spain.

The survey results clearly display the absence of a harmonized approach in the monitoring of antibiotics per animal species and the evaluation of resistances using the same methodology. There is no public long-term quantitative data available targeting the amount of antibiotics used in poultry, with the exception of France. Data on antibiotic-resistant *E. coli* are available for most regions but detection of resistance and number of isolates in each study differs among regions; therefore, statistical evaluation was not possible. Data from France indicate that the decreased use of tetracyclines leads to a reduction in the detected resistance rates. The fluoroquinolones, third-generation cephalosporins, macrolides, and polymyxins ("highest priority critically important" antibiotics for human medicine according to WHO) are approved for use in large poultry-producing regions, with the exception of fluoroquinolones in the US and cephalosporins in the EU. Tetracyclines, aminoglycosides, sulfonamides, and penicillins are registered for use in poultry in all evaluated countries. The approval of cephalosporins in China could not be evaluated. The average resistance rates in *E. coli* to representatives of these antibiotic classes are higher than 40% in all countries, with the exception of ampicillin in the US. The resistance rates to fluoroquinolones and quinolones in the US, where fluoroquinolones are not registered for use, are below 5%, while the average of resistant *E. coli* is above 40% in Brazil, China, and EU, where use of fluoroquinolones is legalized. However, banning of fluoroquinolones and quinolones has not totally eliminated the occurrence of resistant populations.

Key words: antimicrobial, avian, *E. coli*, resistance, poultry

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INTRODUCTION

The application of antimicrobials results in the emergence and spread of antimicrobial resistance, which is a cause of worldwide concern (Garcia-Migura et al., 2014). An antimicrobial agent is defined as a "naturally occurring, semi-synthetic or synthetic substance that exhibits antimicrobial activity (kills or inhibits the growth of microorganisms) at concentrations attainable in vivo. Anthelmintics and substances classed as disinfectants or antiseptics are excluded from this definition" (World Organisation for Animal Health, 2016). Although antimicrobial agents are active against bacteria, protozoa, viruses, and fungi, it is the antibacterial class that is of greatest interest for public health (Page and Gautier, 2012). Thus, the present review will exclusively focus on the antibacterial class of antimicrobial agents. The term antibiotic will be applied throughout this paper, as this term is widely used. This paper provides information on the antibiotic usage (**AU**) and

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antibiotic resistance (**AR**) in broilers. The term poultry is used in the paper if the sources cited do not clearly distinguish between the poultry and broilers. However, 87% of poultry production is broiler production (FAO, 2010).

Accelerated evolutionary trends toward AR are a major threat to human and animal health (Harbarth et al., 2015; World Health Organization, 2015; European Food Safety Authority and European Centre for Disease Prevention and Control, 2016). In addition to being essential for the treatment and prophylaxis of human infections, antibiotics are also widely applied in food-producing animals, which can serve as a reservoir of antibiotic-resistant bacteria and AR determinants that may be transferred to humans (Marshall and Levy, 2011). Subsequently, the effectiveness of antibiotics in humans decreases, resulting in treatment failures (Aarestrup et al., 2008; Mellata, 2013; European Food Safety Authority and European Centre for Disease Prevention and Control, 2016).

In recent decades, broilers have increased in relevance as a meat source. Data about AU and AR in *Escherichia coli* in broilers are shown for the largest broiler meat producers worldwide: the United States, Brazil, China, and countries of European Union—Poland, United Kingdom, Germany, France, and Spain. These regions count for approximately 60% of the total worldwide broiler production, as shown in Figure 1 (Association of Poultry Processors and Poultry Trade in the EU Countries, 2015; United States Department of Agriculture, 2016).

Broiler meat produced by these countries is exported globally. For example, broiler meat from Brazil reaches 142 countries (Ministry of Agriculture Livestock and Farming in Brazil, 2016a). The amount of exported broiler meat per country in 1,000 metric ton is as follows: Brazil, 4,090; US, 3,057; EU, 1,180; China 375 (United States Department of Agriculture, 2016). This review lists the governmental authorization and monitoring of antibiotics in use in poultry and the available data of AR in *E. coli* of broiler origin. *Escherichia coli* is regarded as indicator organism of AR for a wide range of bacteria (EFSA, 2008; Kaesbohrer et al., 2012). Data from the monitoring programs and available scientific literature about AR in *E. coli* from the US, Brazil, China, and the large poultry producers in the EU from 2000 to 2017 were considered. All sources were obtained through online database searches including the Web of Science, PubMed, Scopus, and Google using translations from Chinese, Portuguese, Polish, German, French, and Spanish.

The objective of this study was to identify the legalized antibiotics, the amounts thereof administered, and the level of AR monitored in *E. coli* isolated from broilers originating from the large poultry-producing regions such as US, China, Brazil, Poland, United Kingdom, Germany, France, and Spain. We hypothesize that the application of antibiotics leads to high and consistent resistance levels in *E. coli* isolates, whereas ban of some classes of antibiotics results in low resistance levels. This review is the first comprehensive evaluation of data recording the authorized antibiotics for poultry production combined with AR data in *E. coli* isolates in large poultry-producing regions.

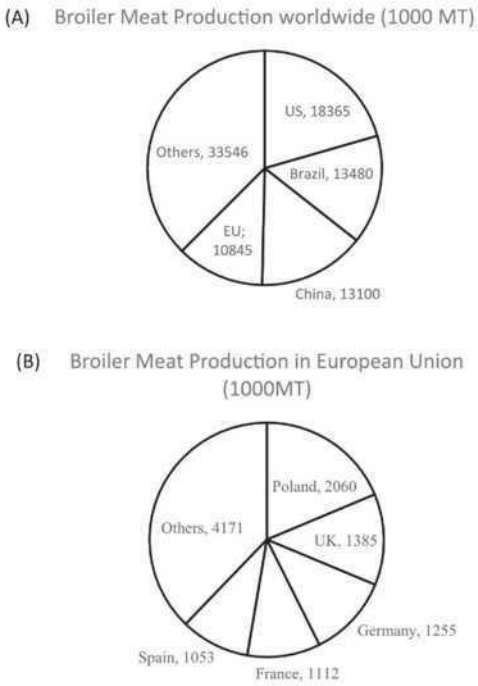

Figure 1. Broiler meat production in 1,000 metric tons (MT) by country worldwide (A) and in the European Union (Association of Poultry Processors and Poultry Trade in the EU Countries 2015; United States Department of Agriculture, 2016).

USE OF ANTIBIOTICS

Antibiotics in poultry are generally administered to the entire flock and are used for the treatment of disease (therapy), disease prevention (methaphylaxis), and growth promotion (Poole and Sheffield, 2013). Antibiotic growth promoters were banned in the EU in 2006, in the US in 2017 and are currently allowed in Brazil and China (European Commission, 2005, AccessScience Editors, 2017). Antibiotic usage for disease prevention is permitted in all large poultry-producing countries. Antibiotics are applied for the treatment of intestinal infections such as colibacillosis, necrotic enteritis, and other diseases generally caused by *Salmonella*, *E. coli*, or *Clostridium* spp. These infections are a major concern among poultry leading to enormous economic losses (United States Department of Agriculture, 2015). The type and extent of AU differ from country to country based on the country's economy, and its level of development, animal husbandry, and the animal species (Archawakulathep et al., 2014). The method of administration and the volume of antibiotic used vary

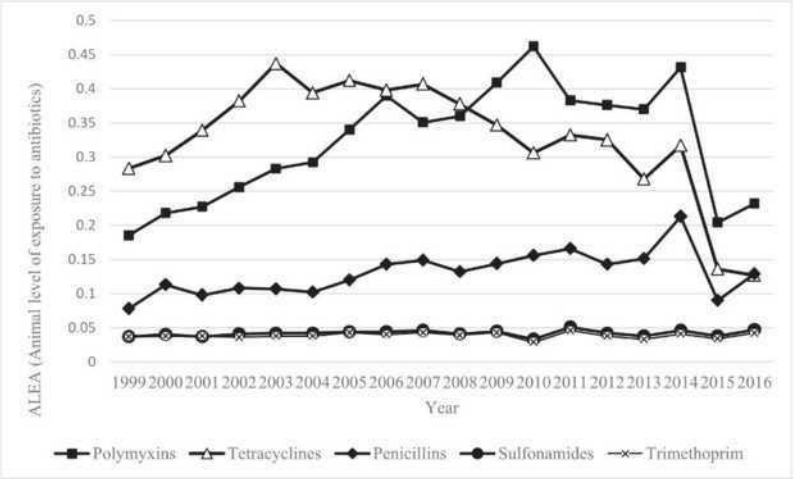

Figure 2. Sales of antibiotics for use in poultry by class between 1999 and 2013 in ALEA (France), modified from French Agency for Food Environmental and Occupational Health & Safety and French Agency for Veterinary Medicinal Products (2017). ALEA = [Live weight treated]/[Total number of animals] × [Weight of adult animals or at slaughter].

depending on the stage of production and the risk of disease (Rosengren et al., 2010). In general, there are different methods of monitoring AU: following the sales of antibiotics is one method, although long-term data for individual animal species are unavailable (with the exception of France); the detection of prescribed antibiotics per animal species; and the detection of AU in animals on farm level.

The French Agency for Veterinary Medicinal Products has monitored the sale of active antibiotic substances applied in poultry production since 1999. In 2016, 106 metric ton of active antibiotic substances were sold for use in poultry. This number represents 20% of all veterinary antibiotics and an average annual consumption of 47 mg of active ingredients per kilogram of chicken produced (French Agency for Food Environmental and Occupational Health & Safety and French Agency for Veterinary Medicinal Products, 2017). Figure 2 shows the changes in the sales of mainly applied antibiotic classes, expressed using the Animal Level of Exposure to Antimicrobials indicator for poultry between 1999 and 2016. In 2016, poultry herds were basically treated with polymyxins, penicillins, and tetracyclines, then with sulfonamides and trimethoprim (French Agency for Food Environmental and Occupational Health & Safety and French Agency for Veterinary Medicinal Products, 2017).

No quantitative monitoring data of AU in broilers are currently available in most large poultry-producing countries. Only in the US, data on AU of medically important antibiotics per animal species are available for 2016, which do not provide the complete picture on AU in broilers as it does not include non-medically important antibiotics (US Food and Drug Administration, 2017). However, the list of antibiotics that are approved by regulatory agencies may provide an indication of the use of antibiotics in poultry production in every country considered in this report. National listings of all medical products that are approved for use for poultry in the US, Brazil, China, Poland, United Kingdom, Germany, France, and Spain were analyzed for active antibiotic substances that may be used in feed, water, or administered parenterally.

The WHO categorizes fluoroquinolones, third- and fourth-generation cephalosporins, macrolides, glycopeptides, and polymyxins as "highest priority critically important" antibiotics for human medicine due to the limited availability of alternatives for the treatment of bacterial infections. These antibiotics are the preferred option for the treatment of serious human infections (World Health Organization, 2011). The data in Table 1 show that fluoroquinolones, third-generation cephalosporins, macrolides, and polymyxins are approved for use in poultry in the largest poultry-producing countries, with the exception of fluoroquinolones in the US and cephalosporins in the EU. The FDA banned the use of enrofloxacin in poultry in the US in 2005 (US Food and Drug Administration, 2005).

ANTIBIOTIC RESISTANCE IN *E. COLI* FROM BROILERS

The use of antibiotics in poultry production increases the selection pressure for antibiotic-resistant bacteria (Diarra and Malouin, 2014). *Escherichia coli* are commensal bacteria that are ubiquitous in animals and humans. Because of their widespread availability, monitoring of commensal bacteria allows the comparison of

Table 1. Antibiotic substances approved for use in poultry by national regulative authorities in the US, Brazil, China, Poland, United Kingdom, Germany, France, and Spain based on national reports.[1]

Antimicrobial class	Compound	US	BR	CN[2]	PL	GB	DE	FR	ES
Aminoglycosides	Apramycin		x	x		x			
	Gentamicin	x	x						
	Hygromycin	x							
	Kanamycin		x						
	Neomycin	x	x	x	x		x	x	x
	Spectinomycin	x	x	x	x		x	x	
	Streptomycin	x	x						
Arsenical	Arsanilic acid	o		x					
	Nitarsone	o							
	Roxarsone	o	x						
ß-lactams—penicillins	Amoxicillin		x	x	x	x	x	x	x
	Ampicillin		x	x			x	x	
	Benzlypenicillin	x	x				x		x
	Phenoxymethyl-penicillin		x		x		x		
ß-lactams—1 g cephalosporins	Cefalexin		x						
ß-lactams—3 g cephalosporins	Ceftiofur	x	x						
Diaminopyrimidines	Ormethoprim	o							
	Trimethoprim		x	x	x	x	x	x	x
Fenicols	Florfenicol			x	x				
	Thiamphenicol			x	x				
Fluoroquinolones	Ciprofloxacin			x					
	Difloxacin			x	x				
	Enrofloxacin		x	x	x	x	x		x
	Flumequine				x			x	x
	Norfloxacin		x						
Glycophospholipid	Bambermycin	x		x					
Ionophores	Hainanmycin			x					
	Lasalocid	x		x					
	Maduramicin	x	x	x					
	Monensin	x	x	x					
	Narasin	x	x						
	Salinomycin	x	x	x					
	Semduramicin	x	x						
Lincosamides	Lincomycin	x	x	x	x		x	x	x
Macrolides	Erythromycin	x		x	x			x	
	Tylosin	x	x	x	x	x	x	x	x
	Tilmicosin		x	x	x	x	x		x
	Spiramycin				x			x	
	Tylvalosin				x		x		
	Kitasamycin		x	x					
Orthosomycins	Avilamycin	x		x					
Phosphonic acids	Fosfomycin		x						
Pleuromutilins	Tiamulin		x		x	x	x		x
Polypeptides	Enramycin		x	x					
	Bacitracin	x	x	x					
Polymyxmins	Colistin	x	x	x	x	x	x	x	x
Quinolone	Halquinol		x						
	Oxolinic acid							x	
Streptogramins	Virginiamycin	x		x					
Sulfonamides	Phalysysulfathiazole		x						
	Sulfachlorpyrazine				x		x		
	Sulfachlorpyridazine	x		x					
	Sulfadiazine		x			x		x	
	Sulfaguanidine		x						
	Sulfadimethoxine	x	x		x		x	x	x
	Sulfadimidine		x				x	x	
	Sulfamerazine	x							
	Sulfamethazine	x	x						
	Sulfamethoxazole		x	x	x		x		x
	Sulfamethoxypyridazine		x					x	
	Sulfanilamide		x						
	Sulfaquinoxaline	x	x	x			x	x	
	Sulfisoxazole		x						
	Sulfomyxin	x							
Tetracyclines	Chlortetracycline	x	x	x	x	x	x		x
	Doxycycline		x	x	x	x	x		x
	Oxytetracycline	x	x	x	x		x	x	x

Table 1. *continued*

Antimicrobial class	Compound	US	BR	CN[2]	PL	GB	DE	FR	ES
	Tetracycline	x		x	x	x		x	x
Thiostrepton 50S	Nosiheptide			x					

[1]Following national reports were used: US—US Food and Drug Administration 2016; Brazil—Ministry of Agriculture Livestock and Farming in Brazil 2008, 2014, 2016b; China—Ministry of Agriculture of People's Republic of China, 2001, 2013; Poland—The office for registration of medicinal products medical devices and biocidal products in Poland 2016; the United Kingdom—Veterinary Medicine Directorate UK 2016; Germany—German Federal Ministry of Health 2016; France—French Agency for Food Environmental and Occupational Health & Safety 2016; Spain—Spanish Agency of Medicines and Sanitary Products 2016.

[2]CN—the list of licensed antibiotics in China does not include parenterally administered antibiotics.

US—USA, BR—Brazil, CN—China, PL—Poland, GB—United Kingdom, DE—Germany, FR—France, ES—Spain, o—antibiotic was voluntary withdrawn by producers.

the selective pressure effects in all relevant populations and is considered useful as an early alert system, for tracking emerging resistance in livestock and possible spread to animal-derived food (EFSA, 2008). Due to this prevalence, they are widely accepted as indicator bacteria for AR in Gram-negative bacteria populations and serve as a model for studying the emergence of AR (Kaesbohrer et al., 2012). Additionally, *E. coli* as well as other bacteria of the commensal flora can form a reservoir of AR genes that may be transferred between bacterial species, including organisms capable of causing disease in both humans and animals. The effects of antibiotics used and the trends in the prevalence of AR in food-producing animals can be more accurately investigated in this indicator bacterium, than in foodborne pathogens (European Food Safety Authority, 2008). Reflecting the awareness of the AR problem and the need for research on the triggers that cause AR development and spreading, some countries established strategies for surveillance and monitoring programs that concern AR and its determinants. Usually, national monitoring studies publish data yearly and use the same criteria for the determination of antibiotic resistances (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018; US Food and Drug Administration, 2018). However, there is no harmonized evaluation of AR in different monitoring programs, which makes the comparison between regions impossible.

An overview of the prevalence of AR in *E. coli* is given for the US, Brazil, China, Poland, the United Kingdom, Germany, France, and Spain by using the results of their national monitoring programs as well as the scientific literature. Each scientific study evaluates AR using different methodology (and number of isolates); therefore, the statistical comparison is not possible. However, descriptive presentation of available data is possible. The source of AR data from each country is provided below in the description of each country. Additionally resistance rates and their evaluation from national monitoring systems of Poland, the United Kingdom, Germany, France, and Spain are presented in the section Supplementary material, due to the large volume of data. All cited resistance rates from 2000 to 2017 are presented with the support of GraphPad PRISM (2016). The outcome of the evaluation is the plot diagram (Figures 4 and 5), which is presented in the section overarching view.

Because of the harmonized sampling and detection of AR at the national levels, the AR results are shown in the form of figures, allowing the comparison of AR in *E. coli* over time. The data regarding AR in *E. coli* that is reported in scientific publications are presented in tabular format and includes the number of *E. coli* isolates that were tested and the average percentages of detected AR (Tables 2 and 3). If more than one set of data were indicated, data on the minimal and maximal AR rates in *E. coli* are additionally presented in

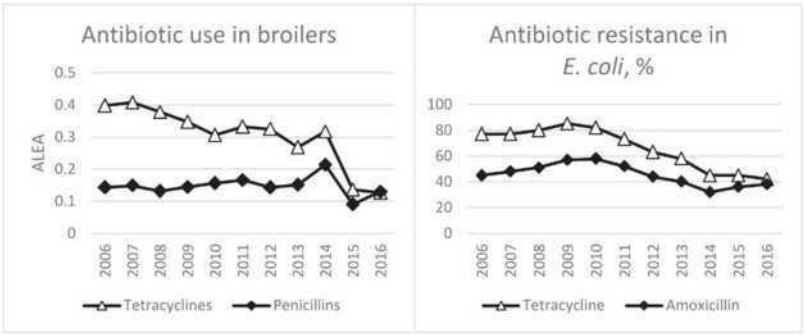

Figure 3. Tetracycline and penicillins use in poultry and resistance in *E. coli* isolates from broilers in France. ALEA (Animal level of exposure to antibiotics) = Animal level of exposure to antibiotics.

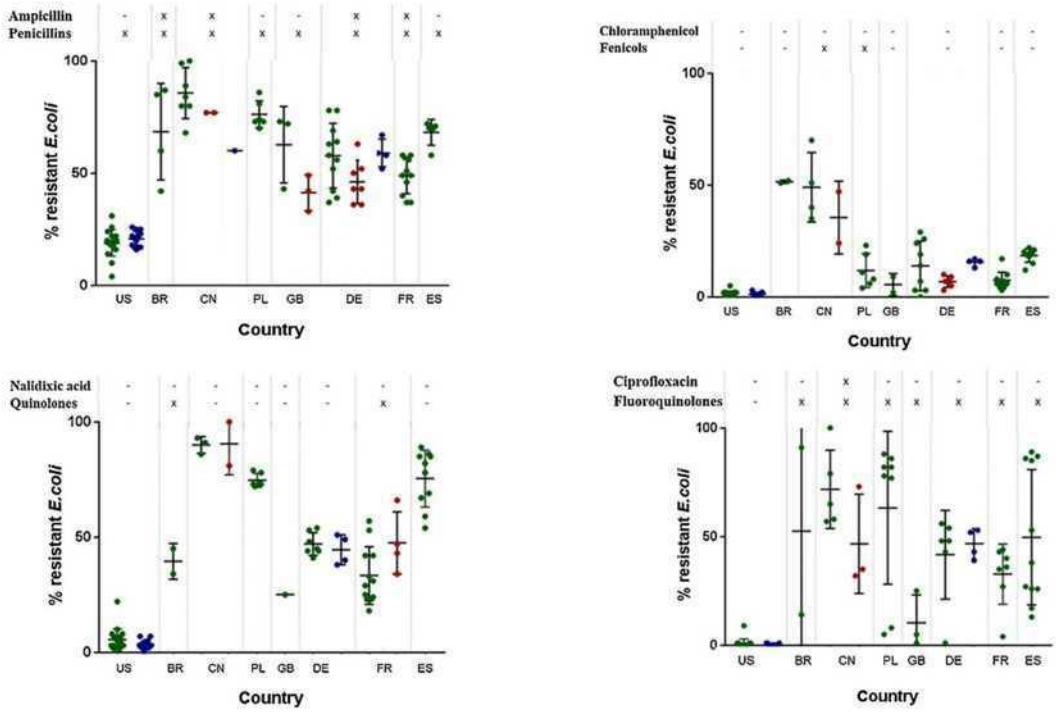

Figure 4. Resistance rates in *E. coli* to antibiotics from healthy animals (green dots), chicken retail meat (blue dots), and diseased chickens (red dots) detected within scientific studies or national monitoring programs. Each dot represents 1 study or data set in 1 yr. On the top of the figure, status of approval for the specific antibiotic tested for resistance (first line), the antimicrobial class (second line).

the tables. Throughout the paper, the percentage of AR is defined as the percentage of resistant *E. coli* as proportion of the number of tested *E. coli* isolates.

The literature analyzed in this review uses different breakpoints to determine AR; additionally, resistance levels of *E. coli* were determined in the different time points. Therefore, any interpretation of these results should consider this issue/fact. Nevertheless, surveillance systems, together with scientific literature, provide valuable contribution for the overview of the occurrence of AR in the indicator organism *E. coli* combining this data with possible AU in large poultry-producing regions. The evaluation of data is included in the description of each country and it shows the need for global harmonized approach in the detection of AU and AR.

United States of America

Established in 1996, the National Antibiotic Resistance Monitoring System (**NARMS**) is a national public health surveillance system in the US. The United States Department of Agriculture (USDA) reports each year AR in *E. coli* isolates from retail raw chicken meat, caecal *E. coli* isolates from slaughtered animals, and isolates from processing plants collected as part of the Hazard Analysis Critical Control Point(HACCP). All data are available and easy to compare on NARMS interactive database (US Food and Drug Administration, 2018). NARMS uses similar methods and defined protocols, which supports the analysis. The monitoring of AR in the US shows similar resistance rates in *E. coli* from retail meat, slaughterhouse, and intestinal samples. The testing of AR from different sources to the same antibiotics makes this comparison possible. Antibiotic resistance rates in *E. coli* remain on the same level from 2000 to 2015, only resistance to streptomycin decreases from 78 to 46%. Independent from the source of the isolates, resistance rates of approximately 45% were detected for streptomycin, tetracycline, and sulfamethoxazole-sulfisoxazole. Antibiotic resistance percentages of *E. coli* from broilers in the US obtained by scientific publications show similar resistance rates for streptomycin and tetracycline as detected in the monitoring programs (Johnson et al., 2007; Smith et al., 2007; Zhang et al., 2011; Millman et al., 2013; Rothrock et al., 2016). However, this comparison cannot be statistically evaluated due to the different methodologies of AR determination in scientific studies and national monitoring. Streptomycin and tetracycline are approved for use in poultry. In contrast, sulfamethoxazole and sulfisoxazole are not licensed, but there are other sulfonamides that are approved and therefore may influence the

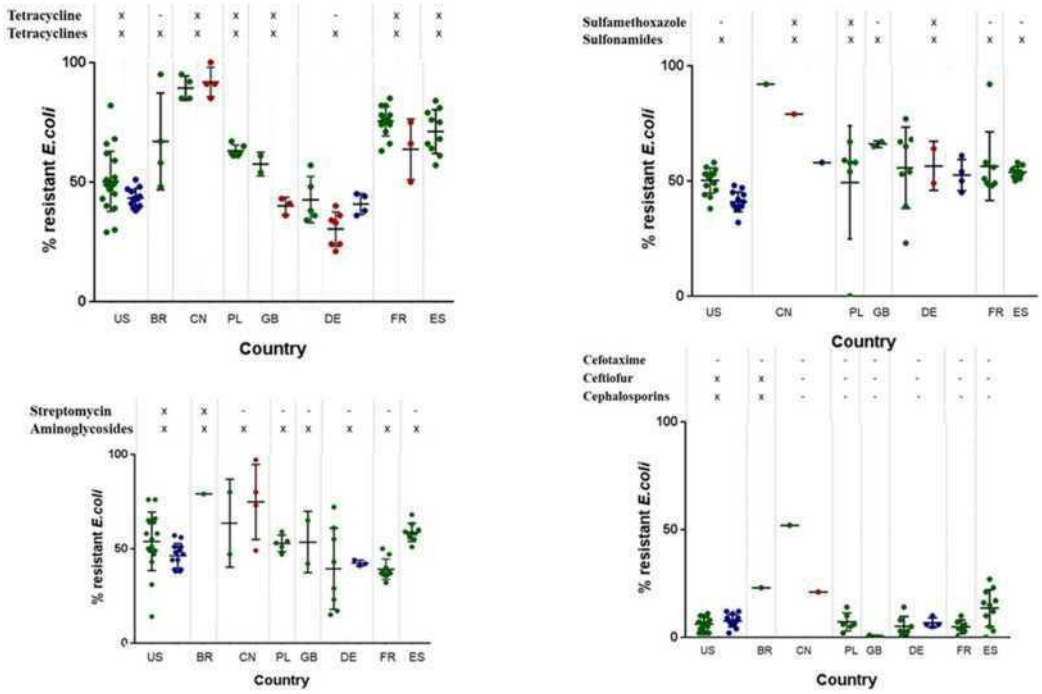

Figure 5. Resistance rates in *E. coli* to antibiotics from healthy animals (green dots), chicken retail meat (blue dots), and diseased chickens (red dots) detected within scientific studies or national monitoring programs. Each dot represents 1 study or data set in 1 yr. On the top of the figure, status of approval for the specific antibiotic tested for resistance (first line), the antimicrobial class (second line).

resistance rates. Resistance rates to gentamicin (approved for use) and ampicillin (not approved, but penicillins approved) are approximately 40 and 20%, respectively. Consequently, detected higher resistance rates in *E. coli* may be driven by the use of antibiotics, but quantitative data on AU would be needed in order to confirm this hypothesis.

Brazil

Brazil does not have any central microbiology reference laboratory. Therefore, no regular AR-monitoring data are available (Rossi, 2011). An overview of the scientific publications that presents the percentages of AR in *E. coli* isolates from broilers in Brazil since 2001 is shown in Table 2.

In general, the low number of studies as well as use of different numbers of isolates in each study does not allow any proper conclusions about resistance rates, which shows the necessity of national monitoring. According to Table 2, the highest detected resistance rates of *E. coli* from broilers were identified in the study of Barros et al. (2012) for lincomycin, erythromycin, and oxolinic acid, with 100, 97, and 88% of resistant isolates, respectively. Lincomycin is registered for use in Brazil, while erythromycin and oxolinic acid are not allowed, but other representatives of macrolides (tylosin and tilmicosin) and quinolones (halquinol) are registered for use. Resistance rates to penicillins and ampicillins are around 75 and 65%, respectively. Representatives of both antibiotic classes may be used for poultry in Brazil. More studies found variations in the AR rates of *E. coli* from broilers to the antibiotic combination of sulfamethoxazole and trimethoprim. Hence, 1 study reports that 100% of the 174 isolates that were tested were resistant (Bezerra et al., 2016), while 3 other studies detected resistance rates of 66 and 68% in 66 and 91 isolates, respectively (Cardoso et al., 2002; Stella et al., 2013). Moreover, 2 additional studies present resistance rates of 27 and 28% in 70 and 120 tested isolates, respectively (Pessanha and Filho, 2001; Korb et al., 2015). The variation of resistance rated may be explained by different locations of studies as well as different times of detections.

China

There is no national monitoring of AR in *E. coli* isolates from poultry in China. Table 3 presents an overview of the percentages of AR in *E. coli* isolates from healthy and diseased broilers in China from available scientific literature from 2004 to 2017.

The results presented in Table 4 indicate that the AR rates in *E. coli* to sulfonamides and tetracyclines

Table 2. Percentages of *E. coli* isolates from broilers in Brazil exhibiting resistance to antibiotics published in scientific literature based on Cardoso et al. (2002), Barros et al. (2012), Bezerra et al. (2016), Pessanha and Filho (2001), Stella et al. (2013), and Korb et al. (2015).

Class	Compound	% res average	% res min	% res max	No. of studies	No. of isolates
Aminoglycosides	Gentamycin	27	26	28	2	244
	Streptomycin	79			1	91
Cephalosporines	Cefalexin	31	0	61	1	35
	Cefepime	10			1	120
	Cefotaxime	23			1	120
	Ceftazidime	3			1	120
	Ceftiofur	43			1	174
	Ceftriaxone	24			1	120
	Cephalothin	65	51	78	2	161
Fosfomycins	Fosfomycin	29	10	45	3	360
Lincosamides	Lincomycin	100	100	100	1	35
Macrolides	Azithromycin	49			1	174
	Erytromycin	97			1	91
Nitrofurans	Nitrofurantoins	13			1	120
Penicillines	Amoxicillin	65	50	84	2	101
	Ampicillin	69	42	87	4	455
Phenicols	Chloramphenicol	52	51	52	2	244
	Thiamphenicol	51	25	77	1	35
Polymyxins	Polymyxin B	1			1	174
Quinolone	Ciprofloxacin	53	14	91	2	294
	Enrofloxacin	40	13	76	2	155
	Nalidixic acid	40	34	45	2	199
	Norfloxacin	59	38	76	2	101
	Oxolinic acid	88			1	66
Tetracyclines	Chlortetracycline	74	63	84	1	35
	Oxitetracycline	81	62	100	1	35
	Tetracycline	67	48	95	4	455
Combination of compounds	Trimethoprim-Sulfamethoxazole	60	27	100	6	556

% res—average value of percentages of antibiotic resistant *E. coli* found in referenced studies.
% res min—minimal value of percentages of antibiotic resistant *E. coli* found in referenced studies.
% res max—maximal value of percentages of antibiotic resistant *E. coli* found in referenced studies.

are around 80%, and 40% to phenicols. Representatives of all 3 antibiotic classes are approved for the use in poultry. The resistances vary in the ranges of 50 to 100% for quinolones, 30 to 80% for penicillins, 20 to 70% for aminoglycosides, and 4 to 45% for cephalosporins. Quinolones and penicillins are registered for the use in poultry, while cephalpsporins are not. However, it has to be taken into account that listed licensed antibiotics in China do not include parenterally administered antibiotics.

Antibiotic susceptibility testing of 326 *E. coli* isolates from food animals collected in China over the last 4 decades showed that AR in *E. coli* in the country has increased since the 1970s (Song et al., 2010). Furthermore, an evaluation of 540 *E. coli* isolates from broilers showed that resistance to amikacin, ampicillin, aztreonam, ceftazidime, cefotaxime, cefalothin, chloramphenicol, ciprofloxacin, fosfomycin, levofloxacin, norfloxacin, nalidixic acid, piperacillin, and trimethoprim-sulfamethoxazole increased significantly from 1993 to 2013 (Chen et al., 2014). Liu et al. (2016) observed a major increase of the relevance of colistin resistance in *E. coli* in China due to the detection of the plasmid-mediated colistin resistance mechanism MCR-1.

European Union

The European Food Safety Authority (**EFSA**) and the European Centre for Disease Prevention and Control (**ECDC**) jointly analyzed data submitted by EU Member States regarding AR in *E. coli* from broilers. The organizations presented the results in the annual "European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food". Last report evaluates AR in bacteria isolated in 2016 (EFSA/ECDC, 2018). Antibiotics were selected based on their relevance to public health and/or their epidemiological relevance. Epidemiological cut-off values were used for the AR interpretation (European Food Safety Authority, 2008). France, Germany, Poland, and Spain have participated in the monitoring since 2008. An overview of the prevalence of resistance for the largest poultry-producing countries in the EU for 2015 is shown in Table 4.

In addition to this EU monitoring, Poland, the UK, Germany, and France have national AR-monitoring systems. Some data from national monitoring are used for EU monitoring. Thus, they correspond to each other. There is a variation in resistance rates in

Table 3. Percentages of *E. coli* isolates from broilers exhibiting resistance to antibiotics in China, based on Dai et al. (2008), Chen et al. (2014), Ho et al. (2011), Gai et al. (2015), Lei et al. (2010), Jiang et al. (2011), Lu et al. (2010), Wu et al. (2015), Yang et al. (2004), Yu et al. (2012), and Zhang et al. (2012a,b, 2014, 2016).

Class	Compound	% res	% res min	% res max	No. of studies	No. of isolates
Aminoglycosides	Amikacin	18	3	46	10	2,784
	Apramycin	68			1	45
	Gentamycin	50	9	82	11	2,302
	Kanamycin	59	24	97	5	951
	Neomycin	26	7	50	3	705
	Spectinomycin	26	15	42	3	403
	Streptomycin	71	47	97	6	1,480
	Tobramycin	14			1	389
Carbapenems	Meropenem	0			1	540
Cephalosporines	Cefalexin	8	5	11	2	553
	Cefazoline	33	9	92	4	1,157
	Cefotaxime	37	21	52	2	627
	Ceftazidime	18			1	540
	Ceftiofur	45	0	90	2	116
	Ceftriaxone	4	2	8	3	647
	Cefalothin	32	27	41	4	1,206
Fluoroquinolones	Difloxacin	91			1	71
	Ciprofloxacin	62	32	100	8	2,272
	Enrofloxacin	71	38	100	7	1,479
	Gatifloxacin	67			1	71
	Levofloxacin	44	21	63	4	1,187
	Norfloxacin	53	21	100	5	1,322
	Ofloxacin	37	24	50	2	476
	Orbifloxacin	76			1	71
	Sarafloxacin	100			1	71
	Sulfadimidine	100			1	45
Fosfomycins	Fosfomycin	16			1	540
Monobactams	Aztreonam	10			1	540
Nitrofurans	Nitrofurantoins	3			1	540
Penicillines	Amoxicillin	54			1	389
	Ampicillin	81	60	100	10	2,581
	Piperacillin	30			1	540
Phenicols	Chloramphenicol	44	24	69	6	1,854
	Florfenicol	41	15	78	4	682
Quinolone	Nalidixic acid	91	81	94	5	1,465
Polymyxins	Colistin	9	5	13	2	251
	Polymyxin B	1			1	389
Sulfonamides	Sulfamethoxazole	76	58	92	3	294
	Sulfisoxazole	83			1	87
	Doxycycline	79	48	93	6	1,594
Tetracyclines	Tetracycline	87	85	100	9	2,164
	Amoxicillin/clavulanate	50	0	100	5	1,156
Combination of antimicrobials	Cefoperazone-Sulbactam	51			1	373
	Trimethoprim-Sulfamethoxazole	78	66	93	8	2,314

% res—average value of percentages of antibiotic resistant *E. coli* found in referenced studies.
% res min—minimal value of percentages of antibiotic resistant *E. coli* found in referenced studies.
% res max—maximal value of percentages of antibiotic resistant *E. coli* found in referenced studies.

E. coli among the mentioned EU countries. Nevertheless, comparing the obtained averages of resistance, that of ampicillin (70%) was the highest one. Approximately 60% resistance rates were detected for ciprofloxacin, sulfamethoxazole, tetracycline, and nalidixic acid(European Food Safety Authority and European Centre for Disease Prevention and Control, 2018).

Poland Poland began to implement a national monitoring program for AR in commensal *E. coli* isolates from broilers in 2009 (Wasyl et al., 2012). Antibiotic resistance levels to several antibiotics in *E. coli* isolates from broilers at slaughterhouse level from 2009 to 2014 are available (Wasyl et al., 2013; European Food Safety Authority and European Centre for Disease Prevention and Control, 2015, 2016). Data show high AR rates (from 70 to 90%) to ciprofloxacin, nalidixic acid, and ampicillin, and 50 to 70% to tetracycline, sulfamethoxazole, and streptomycin. Wasyl et al. (2013) detected increasing trends of ampicillin and cefotaxime resistance in the observed *E. coli* isolates.

United Kingdom Antibiotic resistance data for *E. coli* isolated from broilers in the United Kingdom are available from 2 distinct AR-monitoring programs: the EU-monitoring and the clinical monitoring programs (Veterinary Medicines Directorate, 2015). The

Table 4. Percentage of antibiotic resistant *E. coli* isolated from broilers in selected European countries in 2016 (EFSA/ECDC, 2018).

Antibiotic class	Compound	PL	GB	DE	FR	ES	Average
Number of isolates		173	190	177	188	171	
Aminoglycosides	Gentamicin	10	7	7	3	36	13
ß-lactam cephalosporines	Cefotaxime	3	0	1	4	9	3
	Ceftazidime	3	0	1	2	8	3
ß-lactam penicillines	Ampicillin	91	67	56	56	63	67
Diaminopyrimidines	Trimethoprim	62	43	38	47	37	45
Fenicols	Chloramphenicol	25	4	10	7	17	13
Fluoroquinolone	Ciprofloxacin	90	22	60	36	91	60
Macrolides	Azithromycin	5	0	2	0	11	4
Polimyxmins	Colistin	3	0	4	3	1	2
Quinolone	Nalidixic acid	78	21	45	34	88	53
Sulfonamides	Sulfamethoxazole	71	53	47	55	50	55
Tetracyclines	Tetracycline	73	44	28	62	61	54
	Tigecycline	2	0	0	0	0	0

PL—Poland, GB—United Kingdom, DE—Germany, FR—France, ES- Spain.

EU-monitoring program isolated *E. coli* from healthy broilers across the United Kingdom. The clinical monitoring program is a passive monitoring program. Its aim is the evaluation of AR in bacteria that are isolated from clinical samples of diseased animals to antibiotics of veterinary relevance. Both monitoring programs show high AR rates of *E. coli* to ampicillin and tetracycline. Bywater et al. (2004) and Randall et al. (2011) confirm the higher AR rates to ampicillin and tetracycline in *E. coli* in UK. An analysis of fluoroquinolone resistance in *E. coli* from feces samples of 68 broiler farms detected resistance to ciprofloxacin in 50% of these farms (Taylor et al., 2008).

Germany The Federal Office of Consumer Protection and Food Safety reports on AR monitoring in Germany. Similar to the monitoring programs of the UK, 2 different surveillance systems exist in Germany. The first one (Reports on food safety-zoonoses monitoring) monitors healthy animals and the products thereof. The second system is the GERM Vet Report that contains data about the resistance of animal pathogens. Germany monitors the AR in *E. coli* isolates from intestinal samples of broilers, chicken meat, and diseased animals. Data from all systems show higher resistance rates to ampicillin and sulfamethoxazole than to other antibiotics (German Federal Office of Consumer Protection and Food Safety, 2012a, 2012b, 2014, 2015, 2016). For *E. coli* that were isolated from intestinal samples and retail meat, the resistance rates to ciprofloxacin, nalidixic acid, streptomycin, tetracycline, and trimethoprim are between 40 and 60%. Except for trimethoprim, these antibiotics are not allowed for use in broilers in Germany. However, other representatives of the corresponding antibiotic classes may be used. The resistance rates of *E. coli* from diseased chickens to ciprofloxacin are approximately 7%. It seems that the resistance to ciprofloxacin is lower in diseased animals than in healthy animals, and resistance to cephalosporins is higher in diseased animals than in healthy animals. However, the resistance rates must be measured over longer periods of time to confirm these differences.

France France participates in the EU monitoring of AR in animals and presents data since 2004. The French Agency for Veterinary Medicinal Products (ANSES-ANMV) provides reports on the French surveillance network for AR in pathogenic bacteria of animal origin (RESAPATH). The RESAPATH presents the results of the monitoring of AR in *E. coli* from diseased hens and broilers that are treated by veterinarians as part of their regular clinical services. Additionally, AR data are available from the EU Summary reports (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011, 2012, 2013, 2014, 2015, 2016, 2018).

Tetracyclines and penicillins are approved for use in poultry in France. Due to the availability of the quantitative use of antibiotics in poultry, a comparison between antibiotic use and resistance is possible, as can be seen in Figure 3. The use of penicillins in France was stable over time and resistance level to amoxicillin (40%) was the same in 2006 and 2016, although there was an increase and decrease of resistance rates between 2006 and 2016. The decrease in the use of tetracycline between 2006 and 2016 was accompanied by a decrease of tetracycline-resistance rates in *E. coli* between 2006 and 2016. Thus, less use of tetracyclines may result in less resistance to those antibiotics. This hypothesis needs to be confirmed in other countries, and the availability of quantitative data of antibiotic use allows this comparison. In contrast, the use of polymyxins does not correspond with the resistance rates of colistin. The use of polymyxins is high in France, as it can be seen in Figure 2, but the resistance level to colistin is around 3%. Low colistin resistance levels in *E. coli* can be observed in large poultry-producing European countries. However, it needs to be taken into account that in vitro antimicrobial susceptibility testing for polymyxins is challenging and may be associated with high major errors (Bakthavatchalam et. al, 2018). Therefore, no conclusion can be made to the correlation of antibiotic use and resistance to colistin.

Spain The Spanish AR surveillance network "Red de Vigilancia de Resistencias Antibioticas en Bacterias de Origen Veterinario" (**VAV**) was formed in 1996 (Moreno, 2000). The VAV reported data on AR in *E. coli* that were taken from healthy broilers during the period from 1999 to 2005 (Ministry of Agriculture Fisheries and Food in Spain 2005, 2006). Additionally, AR data are available from the EU Summary reports (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011, 2013, 2015, 2016, 2018).

The fluoroquinolones enrofloxacin and flumequine are allowed for use in poultry in Spain. The monitoring data show that AR to ciprofloxacin in *E. coli* increased from 17% in 2001 to 91% in 2016, and that of nalidixic acid from 60% in 2001 to 88% in 2014. Whether an increased use of the fluoroquinolones may have influenced this increase cannot be evaluated without the presence of quantitative AU data. Tetracycline and the penicillins are also registered for use in Spain. The AR resistance rates to tetracycline and ampicillin are approximately 70%. A decrease of resistance to tetracycline was observed between 1999 and 2016.

OVERARCHING VIEW AND RECOMMENDATIONS

Quantitative AU data for poultry are not available for most large poultry-producing countries. Availability of data for AR in *E. coli* is limited for Brazil. Scientific publications from all regions as well as national monitoring of USA and Europe use different methods for the determination of AR. Harmonized approach in detection of AR is of special importance to provide the global evaluation of data. However, the list of all approved antibiotics for poultry provides valuable qualitative data for all countries, which was combined with AR rates in *E. coli* and presented with the mean of a plot diagram. Data for AR rates from the US were used from the surveillance systems of *E. coli* isolates from retail raw chicken meat, caecal *E. coli* isolates from slaughtered animals, and isolates from processing plants collected as part of HACCP (US Food and Drug Administration, 2018). Additionally, data from the scientific publications on AR in *E. coli* from the US were also included. Data for AR rates in China and Brazil are included in Tables 2 and 3. Data for AR from large European poultry producers were used from European monitoring as well as available data from national resistance monitoring systems in Poland, the UK, Germany, France, and Spain from 2000 to 2017. All used data from national monitoring systems are available in section Supplementary material of this manuscript.

There is a representative amount of AR data from *E. coli* for some antibiotic classes such as aminoglycosides, penicillins, cephalosporins, fenicols, quinolones, fluoroquinolones, sulfonamides, and tetracyclines for all large poultry-producing countries. The resistance rates of these antibiotics are represented in plot diagrams by dots, as shown in Figures 4 and 5. Each dot on the diagram represents the resistance rate detected during 1 scientific study or a national monitoring program from 2000 to 2017.

Tetracyclines, aminoglycosides, sulfonamides, and penicillins are registered for use in poultry in all countries. The resistance rates in *E. coli* of broiler origin to representatives of these antibiotic classes, e.g., tetracycline, sulfamethoxazole, streptomycin, and ampicillin, are higher than 40% in all countries, with the exception of ampicillin resistance in the US. This outcome indicates that the use of these antibiotics in poultry results in high resistance rates; however, quantitative data on antibiotic use in poultry would be essentially needed for the confirmation. The resistance rates to fluoroquinolones and quinolones are lower in the US in comparison to other large poultry producers where the use of fluoroquinolones is allowed. These findings demonstrate the possibility to produce broilers without fluoroquinolones, which may result out in low resistance rates, but there was no elimination of the occurrence of resistant population.

Colistin, as a representative of the polymyxins, and tylosin, as representative of the macrolides, are both allowed for poultry use in all countries for oral treatment or injection solution, but there is only a limited amount of resistance data available. Due to the detection of plasmid-located colistin resistance genes in some countries, the assessment of resistance rates to this antibiotic would be essential.

There are several classes of antibiotics that are approved for use in poultry, but no *E. coli* AR are available for studied regions. Such classes are as follows: arsenicals, glycophospholipids, ionophores, lincosamides, orthosomycins, pleuromutilins, polypeptides, and streptogramins. It is important to note that most of the representatives of these antibiotic classes act against Gram-positive bacteria. However, the prevalence of resistance or resistance determinants in *E. coli* to some of these antibiotics was detected by Heir et al. (2004), Bonnet et al. (2009), Cervantes et al. (1994), and Hummel et al. (1979).

The above outlined evaluation and conclusions from review of AU and AR provide input for the monitoring of antibiotic use in poultry and underlie the need for harmonized global surveillance and detection of the AU and AR.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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2. RESEARCH PUBLICATION - **Effect of a feed additive based on organic acids and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in cecum of broilers**

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Effect of an organic acids based feed additive and enrofloxacin on the prevalence of antibiotic-resistant *E. coli* in cecum of broilers

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ABSTRACT Increasing antibiotic resistance is a major public health concern. Fluoroquinolones are used to treat and prevent poultry diseases worldwide. Fluoroquinolone resistance rates are high in their countries of use. The aim of this study was to evaluate the effect of an acids-based feed additive, as well as fluoroquinolone antibiotics, on the prevalence of antibiotic-resistant *E. coli*. A total of 480 broiler chickens (Ross 308) were randomly assigned to 3 treatments: a control group receiving a basal diet; a group receiving a feed additive (**FA**) based on formic acid, acetic acid and propionic acid; and an antibiotic enrofloxacin (**AB**) group given the same diet, but supplemented with enrofloxacin in water. A pooled fecal sample of one-day-old chicks was collected upon arrival at the experimental farm. On d 17 and d 38 of the trial, cecal samples from each of the 8 pens were taken, and the count of *E. coli* and antibiotic-resistant *E. coli* was determined.

The results of the present study show a high prevalence of antibiotic-resistant *E. coli* in one-day-old chicks. Supplementation of the diet with FA and treatment of broilers with AB did not have a significant influence on the total number of *E. coli* in the cecal content on d 17 and d 38 of the trial. Supplementation with FA contributed to better growth performance and to a significant decrease ($P \leq 0.05$) in *E. coli* resistant to ampicillin and tetracycline compared to the control and AB groups, as well as to a decrease ($P \leq 0.05$) in sulfamethoxazole and ciprofloxacin-resistant *E. coli* compared to the AB group. Treatment with AB increased ($P \leq 0.05$) the average daily weight compared to the control group and increased ($P \leq 0.05$) the number of *E. coli* resistant to ciprofloxacin, streptomycin, sulfamethoxazole and tetracycline; it also decreased ($P \leq 0.05$) the number of *E. coli* resistant to cefotaxime and extended spectrum beta-lactamase- (ESBL-) producing *E. coli* in the ceca of broilers.

Key words: poultry, antimicrobial, resistance, acidifier, intestinal

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INTRODUCTION

The efficacy of antibiotics has decreased due to the rapid emergence and dissemination of resistant bacteria (Sengupta et al., 2013). The application of antibiotics for the treatment of disease, disease prevention, and growth promotion in food-producing animals provides favorable conditions for the selection, persistence and spread of antibiotic-resistant bacteria and their resistance determinants at the farm level (Diarra et al., 2007; Diarrassouba et al., 2007; Miranda et al., 2008; Furtula et al., 2010; da Costa et al., 2011; Burow et al., 2014). Thus, resistance to antibiotics has become a global concern not only in human but also in animal health. Furthermore, antibiotic-resistant (**AR**) bacteria and determinants generated at the farm may spread to humans through direct contact, contamination of meat, or environmental pathways (Aarestrup et al., 2008; Dolejska et al., 2013).

To study the emergence of antibiotic resistance (**AR**) in gram-negative bacteria, *E. coli* are widely accepted as indicator bacteria (Kaesbohrer et al., 2012). They are commensal members of the normal gastrointestinal microbiota in humans and animals, can be rapidly altered by exposure to antibiotics, according to Francino (2016), and act as an important pool of resistance determinants (Schjorring and Krogfelt, 2011). Possible contamination of poultry meat with AR *E. coli* may also occur during slaughtering. Moreover, *E. coli* is also of widespread importance, as it is a major pathogen in commercially produced poultry that contributes to significant economic losses (Hammerum and Heuer, 2009).

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Antibiotic growth promoters were banned in the European Union in 2006 (European Commission, 2005). Accordingly, poultry producers are searching for alternatives that claim to enhance the performance of broiler chickens. One alternative to antibiotics is the use of organic acids as feed additives (Adil et al., 2010), as well as cinnamaldehyde-containing feed additives (Demir et al., 2005). Organic acids and cinnamaldehyde can improve chicken performance through their antimicrobial activity (Helander et al., 1998; Raftari et al., 2009; Wang et al., 2009; Adil et al., 2010), which improves protein and energy digestibility by reducing microbial competition with the host for nutrients. Additionally, organic acids lower the incidence of subclinical infections (Dibner and Buttin, 2002), and both compounds are recognized as safe. Their antimicrobial activities against various types of bacteria were found to be similar to those of antibiotics (Helander et al., 1998; Raftari et al., 2009; Wang et al., 2009; Adil et al., 2010). Thus, the supplementation of diets with acids results in lower numbers of pathogenic bacteria (Khan and Iqbal, 2015), such as *Salmonella*, *Campylobacter*, and *E. coli* (Van Immerseel et al., 2006; da Costa et al., 2008; Gharib Naseri et al., 2012; Khan and Iqbal, 2015; Upadhyaya et al., 2015).

The application of antibiotic classes in food-producing animals with therapeutically useful analogs in human medicine is a public concern (Turnidge, 2004; Collignon, 2009; da Costa et al., 2011). Fluoroquinolones are used to treat diseases in poultry in many countries worldwide (Randall et al., 2006). Enrofloxacin is a representative fluoroquinolone prescribed for the prevention of early chick mortality and the reduction of the spread of pathogens (da Costa et al., 2011). This antibiotic is licensed for use in poultry in many countries, including Brazil, China, and the European Union (German Federal Ministry of Health, 2016; Ministry of Agriculture of People's Republic of China, 2001; MAPA et al., 2008; Ministry of Agriculture of People's Republic of China, 2013; The office for registration of medicinal products, medical devices and biocidal products in Poland, 2016). Via de-ethylation of the ethyl group on the piperazine ring, enrofloxacin is metabolized to ciprofloxacin (Riviere and Papich (2009), which is a very potent antibiotic in human medicine (Ovando et al., 2004). Bacterial resistance to fluoroquinolone antibiotics has increased significantly since their introduction into medicine and agriculture in the late 1980s (Everett et al., 1996). The percentage of resistance to ciprofloxacin in *E. coli* isolates from broilers in the European Union is approximately 66% (EFSA/ECDC et al., 2016). Concerns about the development of fluoroquinolone-resistant *Campylobacter* species in poultry led to a withdrawal of enrofloxacin in poultry in the United States in 2005 (US Food and Drug Administration, 2005). Furthermore, use of enrofloxacin may increase the level of resistance to non-fluoroquinolones (da Costa et al., 2011). Due to the antimicrobial activity of organic acids, the level of AR bacteria in the gastrointestinal tract of broilers may decrease. The present study therefore evaluates the effect of enrofloxacin and feed additives based on organic acids on the prevalence of AR *E. coli* in the gastrointestinal tract of broilers.

Table 1. Composition of broiler diets (g/kg).

Ingredients	Starter (0–17 d)	Grower (18–38 d)
Corn	579.1	597.5
Soybean	312.5	296.0
Soybean oil	25.0	20.0
Plant oils and fats	12.5	25.0
Premix for broiler[1]	62.5	60.0
L-Lysine	3.8	1.5
DL-Methionine	0.8	-
Threonine	1.3	-
Limestone	2.5	-
Total	1000.0	1000.0
Formulated nutrients per kg diet		
ME, MJ	12.65	12.96
Crude protein, g	208.05	197.37
Crude fat, g	65.2	73.18
Crude fiber, g	25.43	25.23
Crude ash, g	75.57	68.68
Methionine, g	5.49	4.59
Met + Cys, g	9.02	8.02
Lysine, g	14.06	11.78
Threonine, g	9.27	7.77
Tryptophan, g	2.44	2.34
Ca, g	11.96	11.10
P, g	8.47	7.68
Na, g	2.02	1.94

[1]Contents (/kg premix): calcium 170 g; phosphorus 64 g; sodium 30 g; magnesium 6 g; methionine 30 g; vit. A 230,000 IU; vit. D3 80,000 IU; nicotinic acid 1420 mg; Ca-pantothenic acid 255 mg; vit. E 1600 mg; choline chloride 8400 mg; vit. K_3 56 mg; folic acid 36 mg; vit. C 1300 mg; biotin 4260 μg; vit. B1 53 mg; vit. B2 142 mg; vit. B6 106 mg; vit. B_{12} 710 μg; copper 400 mg; cobalt 20 mg; zinc 1200 mg; iodine 40 mg; iron 2000 mg; selenium 8 mg; manganese 1200 mg.

MATERIALS AND METHODS

Animals, Housing and Treatments

The animal experiment was conducted at the Center of Applied Animal Nutrition in Mank, Austria. All procedures involving animal handling and treatment were approved by the local state office "Amt der Niederösterreichischen Landesregierung Abteilung Agrarrecht," which is the authority for animal care in Lower Austria. The official number of the trial approval is LF1-TVG-39/030–2016. A total of 480 mixed-sex, one-day-old broiler chickens (Ross 308) were randomly assigned to 3 treatments, with 8 pens per treatment and 20 birds per pen. All groups received a common basal diet without coccidiostats from hatch until 38 d of age. The composition of the starter and grower diets mets or exceeded the requirements of the National Research Council (1994) and is presented in Table 1. Chicks had free access to feed and water supplied through nipple drinkers.

The first group of chickens was a negative control group fed a basal diet. The feed additive (**FA**) group also received the control group diet supplemented

"on top" with a feed additive based on 20% formic, 10% acetic, and 5% propionic acids, as well as 2.5% cinnamaldehyde (Biotronic® Top3; BIOMIN Holding GmbH, Getzersdorf, Austria) at a dosage of 2 kg/t of feed. The antibiotic (**AB**) treatment group received the same diet as the control group, but 10 mg enrofloxacin per kg body weight (Baytril, 10% oral solution, Bayer, Leverkusen, Germany) was provided via drinking water from d 14 to d 16 of the trial, before the change to the grower diet.

All groups were subjected to the same rearing, environmental and sanitary conditions. Ventilation and temperature control, light intensity and day-length were applied according to the management handbook's guidelines (Aviagen, 2014). Wood shavings were used for bedding. Clinical observations were done twice a day, and all incidents were recorded. The study was supervised by an independent, licensed local veterinarian.

Sampling

A pooled fecal sample of all 480 chicks was collected at arrival before the chicks were divided and placed into the pens. On d 17 of the trial, 3 chicks per pen were randomly selected and humanely euthanized by CO_2. The intestinal tract of each chick was dissected after slaughter, and cecal samples were collected. Cecal samples from 3 chicks per pen were pooled to one sample. Therefore, 8 samples per group were collected, resulting in a total of 24 cecal samples. The same sampling procedure was used on d 38 of the trial.

Performance Data

Body weight (**BW**) by pen was calculated as an average of the sum of the weight of 20 birds on d 1 and 17, and as the average of individual animal weight on d 38 of the trial. Average daily weight gain (**ADG**) was calculated for the periods d 1 to 17, d 18 to 38, and d 1 to 38 of the trial. Penwise feed intake was recorded at d 17 and at the end of the trial (d 38). Average daily feed intake (**ADFI**) was calculated accordingly. The feed conversion rate (**FCR**) was calculated penwise. The European Production Efficiency Factor (**EPEF**) was calculated using the following formula EPEF = (Livability [%] × BW[kg]/age[d]/FCR) × 100 (Smyth et al., 2010).

Microbiological Analysis

Intestinal samples were kept on ice during transport to the laboratory. All 49 samples were analyzed for *E. coli*, *E. coli* resistant to ampicillin, cefotaxime, ciprofloxacin, streptomycin, sulfamethoxazole, and tetracycline, and ESBL-producing *E. coli* within 24 h of arrival to the laboratory. A sample of approximately 3 g was filled to its 10-fold balance weight with buffered peptone water (Oxoid, Hampshire, United Kingdom) and homogenized (Stomacher® 400 Circulator, Seward Limited, UK). Using the first dilution, dilution series up to 10^{-6} were prepared. For each dilution, 0.1 mL was spread in duplicate onto plates containing MacConkey agar (Merck, Darmstadt, Germany) and ChromID ESBL medium (BioMérieux, Marcy-l'Étoile, France). Additionally, MacConkey agar respectively supplemented with one of the following antimicrobial agents at the corresponding breakpoint concentration (Clinical and Laboratory Standards Institute, 2012) was applied to obtain a maximum diversity of antibiotic-resistant *E. coli*: ampicillin (32 μg/mL), cefotaxim (4 μg/mL), ciprofloxacin (4 μg/mL), streptomycin (64 μg/mL), sulfamethoxazol (512 μg/mL) and tetracycline (16 μg/mL). All antibiotics originated from Sigma-Aldrich (St. Louis, USA). After incubating plates at 37°C for 24 h, colonies typical for *E. coli* were enumerated on countable plates: red colonies surrounded by a turbid zone on MacConkey agar and pink to burgundy colonies or translucent colonies with a pink to burgundy center on ChromID ESBL medium. The averages of duplicate plates were taken to calculate the number of CFU per gram sample.

Statistical Analysis

All normally distributed generated growth performance data (initial weight, BW d 17, ADG, d 1–17, ADFI, FCR, EPEF) were subjected to statistical analysis using analysis of variance (**ANOVA**) (IBM®SPSS® statistics 19.0). The non-normally distributed performance parameters (FI and BW d 38, ADG d 18–38, ADG d 1–38) were analyzed using a non-parametric Kruskal-Wallis test. The microbiological analyses were transformed into LOG prior to statistical evaluation. All normally distributed generated microbiological data were analyzed by means of ANOVA (IBM®SPSS® statistics 19.0). The non-normally distributed data were for *E. coli* resistant to cefotaxime and ESBL-producing *E. coli*, which were analyzed using a non-parametric Kruskal-Wallis test. The level of statistical significance for all measured parameters was expressed at $P \leq 0.05$, and means were separated using the LSD test.

RESULTS AND DISCUSSION

Performance Data

The influence of FA and AB on poultry performance is shown in Table 2. The results of the present study showed that dietary supplementation with FA enhanced ($P \leq 0.05$) broiler growth performance (BW and EPEF) compared to the control group. There was no significant influence of AB ($P \leq 0.05$) on the BW of birds compared to other feeding groups on d 17 and 38 of the trial. However, ADG was higher ($P \leq 0.05$) in the FA and AB group compared to the control group.

Table 2. Performance characteristics of broilers receiving feed additive based on organic acids (FA) and enrofloxacin (AB) compared to the control group.

	Control	FA	AB	*P*-value
Initial weight, g	46.00 ± 0.0002	46.00 ± 0.0002	46.00 ± 0.0002	1.00
BW d 17, g	548 ± 0.010	583 ± 0.007	554 ± 0.015	0.09
BW d 38, g	$2070^{b} \pm 0.03$	$2250^{a} \pm 0.03$	$2170^{a,b} \pm 0.03$	0.0001
ADG d 1- 17, g/d	29.55 ± 0.66	31.60 ± 0.40	29.86 ± 0.89	0.01
ADG d 18- 38, g/d	$72.33^{b} \pm 1.35$	$79.34^{a} \pm 0.74$	$77.19^{a,b} \pm 1.49$	0.002
ADG d 1- 38, g/d	$53.19^{b} \pm 0.67$	$57.98^{a} \pm 0.44$	$56.02^{a} \pm 1.17$	0.017
ADFI d 1 - 17, g/d	51.09 ± 1.51	49.74 ± 1.34	51.29 ± 1.92	0.77
ADFI d 18 - 38, g/d	158.24 ± 6.35	148.17 ± 3.69	162.00 ± 5.22	0.18
ADFI d 1 - 38, g/d	110.31 ± 4.05	104.13 ± 2.36	112.47 ± 3.69	0.22
FCR d 1- 17, g/g	1.73 ± 0.04	1.58 ± 0.05	1.73 ± 0.10	0.19
FCR d 18- 38, g/g	$2.19^{b} \pm 0.10$	$1.87^{a} \pm 0.05$	$2.11^{a,b} \pm 0.09$	0.023
FCR d1 – 38, g/g	$2.07^{b} \pm 0.07$	$1.80^{a} \pm 0.04$	$2.02^{a,b} \pm 0.09$	0.029
Mortality, %	4.38	2.50	4.38	0.57
EPEF	$228.5^{b} \pm 7.9$	$292.3^{a} \pm 9.9$	$249.1^{a,b} \pm 13.1$	0.001

AB, enrofloxacin; ADFI, average daily feed intake; ADG, average daily weight gain; BW, Body weight; EPEP—European Poultry Efficiency Factor; FA, feed additive based on organic acids; FCR, feed conversion ratio. EPEF = (Livability [%] × BW[kg]/age[d]/FCR) × 100; ^{a,b}means in the same row with no common superscripts are significantly different ($P \leq 0.05$); ± standard error.

Animals from the group fed with FA were heavier ($P \leq 0.05$) than the control group at the end of the trial. Nevertheless, feed intake was not affected by diet, but the FCR was lower ($P \leq 0.05$) in the FA group compared to the control group. Compared to birds fed with basal diet, the EPEF was improved ($P \leq 0.05$) by feeding FA.

A review of the role of organic acids in poultry nutrition confirms these results through comparison to other studies where organic acids were as efficient as, or even more efficient than, some antibiotics (Khan and Iqbal, 2015). ADG and FCR of broiler chicks was significantly increased by supplementation with a formic and propionic acid mixture (Senkoylu et al., 2007). The results of the study by Olarve et al. (2007) using 0.3 and 0.4% acidifier as a blend of formic, fumaric, lactic, propionic, and phosphoric acids in basal diets showed significant effects on the ADG and feed efficiency of broilers from d 28 to 42. Additionally, the use of fumaric, butyric, and lactic acids significantly improved BWG and FCR (Adil et al., 2010). The improvement in ADG and FCR was possibly achieved due to better nutrient digestibility; Ghazalah et al. (2011) reported that feed supplementation with formic or fumaric acid and acetic or citric acid improved nutrient digestibility. Dietary supplementation of formic acid in the broiler finisher diet improved the apparent ileal digestibility of dry matter and crude protein (Hernandez et al., 2006; Garcia et al., 2007). The positive effect of organic acids and cinnamaldehyde on performance may be due to a decrease in bacterial count, which will be discussed when considering the microbiological analysis. In the present study, continuous fed FA more efficiently improved growth performance than AB. Enrofloxacin is not used for growth promotion but is used for the treatment of disease, prevention of chick mortality, and reduction of the spread of pathogens (da Costa et al., 2011). AB was provided to animals for 3 d according to the recommended use, which differs from the continuous application of in-feed antibiotics. A positive effect of enrofloxacin on growth performance was observed in ADG, but not BW, compared to the control group. There was no significant effect of enrofloxacin on growth performance in studies by da Costa et al. (2011) and Dibner and Richards (2005).

Microbiological Analysis

Analysis of fecal samples on d 1 showed a total *E. coli* count of 8.18 $\log_{10}$ CFU/g of fecal content. The number $\log_{10}$ CFU/g of *E. coli* resistant to ampicillin was 7.83; cefotaxime, 2.40; ciprofloxacin, 8.00; streptomycin, 7.63; sulfamethoxazole, 7.56; tetracycline, 7.81; and ESBL-producing *E. coli* was at 2.82 $\log_{10}$ CFU/g of fecal content. These results indicate that AR *E. coli* were already present in newborn chicks after transport without any access to water or feed. This finding corresponds to other published studies. Rashid et al. (2013) reported that *E. coli* from the gut, liver, and lungs of one-day-old chicks was multiresistant to amoxicillin, enrofloxacin, erythromycin, kanamycin, nalidixic acid, and cloxacillin. *E. coli* resistant to ampicillin, cefotaxime, tetracycline, streptomycin, gentamicin, and enrofloxacin from one-day-old chick's meconium was reported by da Costa et al. (2011). Baron et al. (2014) also observed *E. coli* resistant to third-generation cephalosporins in one-day-old chicks. These authors suggest that the source of resistant *E. coli* may be eggshells or the immediate environment. Third-generation cephalosporins and fluoroquinolones are classified by the WHO as highest-priority critically important antibiotics, which means that this antibiotic is the sole therapy, or one of a limited set of available therapies, to treat serious human disease (World Health Organization, 2011). The source of antibiotic-resistant bacteria may be vertical transmission of resistance gene determinants along the poultry chain. Zurfluh et al. (2014) provided evidence that gene determinants of ESBL-producing *E. coli* are transmitted vertically in the broiler production

Table 3. *E. coli* count in cecum on d 17, log CFU/g.

	Control	FA	AB	*P*-value
E. coli	8.09 ± 0.19	7.90 ± 0.14	7.76 ± 0.34	0.35
Ampicillin-resistant *E. coli*	7.05 ± 0.28	6.87 ± 0.14	7.03 ± 0.23	0.81
Cefotaxime-resistant *E. coli*	2.13 ± 0.82^{a}	2.14 ± 0.59^{a}	0.00 ± 0.00^{b}	0.007
Ciprofloxacin-resistant *E. coli*	6.90 ± 0.66^{b}	7.04 ± 0.47^{b}	7.68 ± 0.36^{a}	0.014
Streptomycin-resistant *E. coli*	6.62 ± 0.18^{b}	6.97 ± 0.17^{b}	7.47 ± 0.12^{a}	0.004
Sulfomethoxazole-resistant *E. coli*	6.85 ± 0.21^{a}	7.10 ± 0.17^{a,b}	7.59 ± 0.13^{b}	0.020
Tetracycline-resistant *E. coli*	6.83 ± 21^{a}	6.97 ± 18^{a}	7.55 ± 15^{b}	0.024
ESBL-producing *E. coli*	2.82 ± 1.96^{a}	2.85 ± 1.23^{a}	0.00 ± 0.00^{b}	0.010

AB, enrofloxacin; amount of antibiotic in the media: ampicillin 32 μg/mL, cefotaxim 4 μg/mL, ciprofloxacin 4 μg/mL, streptomycin 64 μg/mL, sulfamethoxazol 512 μg/mL and tetracycline 16 μg/mL; FA, feed additive based on organic acids; ^{a,b}means in the same row with no common superscripts are significantly different ($P \leq 0.05$); ± standard error.

Table 4. *E. coli* count in cecum on d 38, log CFU/g.

	Control	FA	AB	*P*-value
E. coli	8.25 ± 0.20	8.24 ± 0.12	8.46 ± 0.16	0.59
Ampicillin-resistant *E. coli*	7.08 ± 0.31^{a}	5.28 ± 0.41^{b}	6.91 ± 0.31^{a}	0.002
Cefotaxime-resistant *E. coli*	3.09 ± 0.87^{a}	1.04 ± 0.52^{a,b}	0.24 ± 0.24^{b}	0.018
Ciprofloxacin-resistant *E. coli*	5.83 ± 0.28^{b}	5.68 ± 0.12^{b}	7.36 ± 0.33^{a}	0.001
Streptomycin-resistant *E. coli*	5.42 ± 0.23	5.05 ± 0.27	6.12 ± 40	0.07
Sulfomethoxazole-resistant *E. coli*	5.62 ± 0.36^{a,b}	5.16 ± 0.28^{b}	6.48 ± 0.34^{a}	0.034
Tetracycline-resistant *E. coli*	6.18 ± 0.27^{a}	5.28 ± 0.23^{b}	6.91 ± 0.35^{a}	0.003
ESBL-producing *E. coli*	3.09 ± 0.91^{a}	1.15 ± 0.58^{a,b}	0.30 ± 0.20^{b}	0.007

AB, enrofloxacin; amount of antibiotic in the media: ampicillin 32 μg/mL, cefotaxim 4 μg/mL, ciprofloxacin 4 μg/mL, streptomycin 64 μg/mL, sulfamethoxazol 512 μg/mL and tetracycline 16 μg/mL; FA, feed additive based on organic acids; ^{a,b}means in the same row with no common superscripts are significantly different ($P \leq 0.05$); ± standard error.

pyramid from the top (nucleus poultry flock level) to the bottom, with little evidence of any antibiotic selection pressure.

Because only one fecal samples was received on d 1 of the trial, findings from this day cannot be directly compared to the other days of the trial, where 24 cecal samples per sampling day were available. Additionally, on d 17 and 38 of the experiment, a high prevalence around 6 $\log_{10}$ CFU/g of *E. coli* resistant to ampicillin, ciprofloxacin, streptomycin, sulfamethoxazole, and tetracycline was detected in all groups (Tables 3 and 4). According to the European summary report on antibiotic resistance, the average percent of resistance to ampicillin is 59%, for ciprofloxacin is 66%, for sulfamethoxazole is 53% and for tetracycline is 50% in *E. coli* from poultry in 28 countries in the EU (EFSA/ECDC et al., 2016). Smith et al. (2007) found a high prevalence of resistance to tetracycline, sulfonamides, and streptomycin in commercial flocks, although these antibiotics were not used in most cases. The same study states that the ecology of bacterial communities present in animal environments plays an important role in the prevalence of antibiotic resistance. Supplementation of the diet with FA and treatment of broilers with AB did not have a significant influence on the total number of *E. coli* on d 17 and d 38 of the trial when enumerated without antibiotics present in the media. An influence of FA and AB on the microbial count of some AR *E. coli* in the cecum was shown in Tables 3 and 4. There was no effect ($P \leq 0.05$) of FA on resistant *E. coli* numbers on day 17, except for lower ($P \leq 0.05$) numbers of streptomycin-resistant *E. coli* compared to the AB group. In the AB group, the level of cefotaxime-resistant *E. coli* and ESBL-producing *E. coli* was lower compared to the other 2 feeding groups on d 17 of the trial. Treatment with AB reduced the level of ESBL-producing *E. coli* in the cecum of broilers. Similar results were found in other studies. In fact, da Costa et al. (2011) report that *E. coli* strains displaying resistance to cephalothin were selected after the use of enrofloxacin. Additionally, ESBL-producing *E. coli* may also be resistant to fluoroquinolones, as reported by Su et al. (2016).

The number of *E. coli* resistant to ciprofloxacin, sulfamethoxazole, and tetracycline was higher ($P \leq 0.05$) in the AB group than in the control group on d 17 of the trial. Da Costa et al. (2011) also reports increased prevalence of resistance to unrelated antibiotics in medicated broilers upon exposure to enrofloxacin. The level of *E. coli* resistant to streptomycin was higher ($P \leq 0.05$) in the enrofloxacin group compared to the FA group on d 17 of the trial. In general, 3 d of treatment with enrofloxacin resulted in increased ($P \leq 0.05$) numbers of *E. coli* resistant to ciprofloxacin, sulfamethoxazole and tetracycline; the treatment decreased ($P \leq 0.05$) the number of ESBL-producing *E. coli* in the AB group.

The microbiological analysis of control groups on d 17 and 38 of the trial show decreased numbers of *E. coli* resistant to ciprofloxacin, streptomycin, and sulfamethoxazole ($P \leq 0.05$) with time. The level of ampicillin- and tetracycline-resistant *E. coli* in the FA group was significantly lower ($P \leq 0.05$) than in the other 2 groups. Penicillins and tetracyclines are licensed for use in

poultry in many countries, including large poultry producers such as the United States, Brazil, China, and the EU (Food and Drug Administration, 2016; German Federal Ministry of Health, 2016; MAPA, Ministery of Agriculture, Livestock and Farming in Brazil, 2014; Ministry of Agriculture of People's Republic of China, 2013). The prevalence of tetracycline-resistant *E. coli* is approximately 90% in China, 70% in Brazil and 50% in the USA and EU (Pessanha and Filho, 2001; Lei et al., 2010; Ho et al., 2011; Jiang et al., 2011; Stella et al., 2013; Korb et al., 2015; Bezerra et al., 2016; EFSA/ECDC et al., 2016; Food and Drug Administration, 2016). In the USA, rates of *E. coli* resistant to ampicillin are lower (20%) compared to other large poultry producers (Food and Drug Administration (2016). The prevalence of ampicillin-resistant *E. coli* from broilers is 80% in China, 70% in Brazil and 60% in European Union (Pessanha and Filho, 2001; Yang et al., 2004; Dai et al., 2008; Lei et al., 2010; Lu et al., 2010; Ho et al., 2011; Jiang et al., 2011; Yu et al., 2012; Stella et al., 2013; Chen et al., 2014; Gai et al., 2015; Korb et al., 2015; Bezerra et al., 2016; EFSA/ECDC et al., 2016; Zhang et al., 2016). Therefore, reducing the numbers of ampicillin- and tetracycline-resistant *E. coli* is of special importance. Confirmation of the literature-reported ability of organic acids and cinnamaldehyde to decrease the prevalence of ampicillin resistant *E. coli* in poultry was not obtained. However, the ability of organic acids and cinnamaldehyde to reduce *E. coli* counts in poultry is known (Raftari et al., 2009; Adil et al., 2010; Yossa et al., 2014), and therefore, reduction of AR *E. coli* is possible. A significant reduction in total *E. coli* count was not observed in the present study. Therefore, a possible selective effect of FA on resistant *E. coli* should be investigated further. Additionally, the level of *E. coli* resistant to ciprofloxacin and sulfamethoxazole was lower ($P \leq 0.05$) in the FA group compared to the AB group, but there was no significant difference ($P > 0.05$) compared to the control group. Microbiological analysis at the end of the trial showed that the level of cefotaxime-resistant *E. coli* and ESBL-producing *E. coli* was lower in the AB group compared to other groups, similar to the results for d 17. As expected, the level of *E. coli* resistant to ciprofloxacin was higher ($P \leq 0.05$) in the group treated with enrofloxacin than in the 2 other groups. Thus, the use of enrofloxacin in broilers selects for ciprofloxacin resistant *E. coli* populations. This selection could also be detected in the present trial after 11 d of enrofloxacin use. Smith et al. (2007) reported that antibiotic use also creates resistant *E. coli* that can still compete with susceptible strains in the absence of antibiotic selective pressure. However, after increasing the prevalence of ciprofloxacin-resistant *E. coli* during enrofloxacin treatment for 3 d, as reported by da Costa et al. (2011), the ciprofloxacin resistance level progressively approached that of the control group within the subsequent 11 d.

The present trial shows that FA can improve growth performance. Furthermore, the number of *E. coli* resistant to ampicillin, ciprofloxacin, sulfamethoxazole, and tetracycline was higher in the AB group compared to the FA group. Whether AB can be replaced for disease prevention and reduction of mortality with FA should be clarified with further trials.

CONCLUSION

A high prevalence of AR *E. coli* in all experimental groups was observed throughout the study. Dietary supplementation with FA and treatment of broilers with AB did not have a significant influence on the total number of *E. coli* on d 17 and d 38 of the trial. Supplementation with FA contributed to better growth performance and a decrease in ampicillin- and tetracycline-resistant *E. coli* in the cecum of broilers compared to control and AB group. The decrease ($P \leq 0.05$) in sulfamethoxazole and ciprofloxacin-resistant *E. coli* compared to the AB group was observed in the FA group. Treatment of broilers with AB increased ($P \leq 0.05$) the number of *E. coli* resistant to ciprofloxacin, streptomycin, sulfamethoxazole, and tetracycline in the cecum. However, fewer ($P \leq 0.05$) *E. coli* were resistant to cefotaxime, and ESBL-producing *E. coli* was observed in the group treated with enrofloxacin.

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3. RESEARCH PUBLICATION - **Prevalence of antibiotic-resistant *E. coli* in broilers challenged with a multiresistant *E. coli* strain and received ampicillin, an organic acids based feed additive or a synbiotic preparation**

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Prevalence of antibiotic-resistant *E. coli* in broilers challenged with a multi-resistant *E. coli* strain and received ampicillin, an organic acid-based feed additive or a synbiotic preparation

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ABSTRACT The aim of this study was to evaluate the effect of ampicillin, an organic acid-based feed additive and a synbiotic preparation on the prevalence of antibiotic-resistant *E. coli* in the ceca of broilers. A total of 2000 broiler chickens (Ross 708) were randomly assigned to 5 groups with 8 replicates. The negative control group was the only group that was not subjected to avian pathogenic *E. coli* challenge, while all the other 4 groups received a multi-resistant *E. coli* strain that was resistant to ampicillin, cephalexin, and nalidixic acid as an oral challenge. The second group served as a challenge control, and the third group received the antibiotic ampicillin via water for 5 d. The fourth group received a feed additive based on organic acids and cinnamaldehyde, and the fifth group received a synbiotic preparation via feed and water. On day 17 and 38 of the trial, cecal samples from 3 birds from each of the 40 pens were obtained, and the *E. coli* counts and abundances of antibiotic-resistant *E. coli* were determined.

Oral challenge with an avian pathogenic *E. coli* strain did not influence the performance, and there was no significant difference in growth performance between groups. The total *E. coli* count was lower ($P < 0.05$) in the group supplemented with the synbiotic than in the challenge control group on day 38 of the trial. Administration of an antibiotic for 5 d led to a significant increase in the abundance of *E. coli* strains resistant to ampicillin, amoxicillin-clavulanic acid, cefoxitin, and ceftriaxone. There was no increase in the abundance of antibiotic-resistant *E. coli* observed in the groups that received feed supplemented with an organic acid/cinnamaldehyde-based feed additive or a synbiotic. Moreover, the effects of the tested feed additives on the prevalence of resistant *E. coli* are demonstrated by the lower ceftriaxone minimal inhibitory concentration values for this group than for the antibiotic group. Additionally, the synbiotic group exhibited lower ceftriaxone minimal inhibitory concentration values than the antibiotic group.

Key words: feed additive, poultry, antibiotic resistance, APEC, *E. coli* challenge

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INTRODUCTION

Antibiotics have been used for treatment and prevention of disease as well as growth promotion in livestock and poultry production (Allen et al., 2013). The use of antibiotics to treat food-producing animals provides favorable conditions for the spread of antibiotic-resistant (**AR**) bacteria and the corresponding resistance determinants at the farm level (Diarra et al., 2007; Diarrassouba et al., 2007; Miranda et al., 2008; Furtula et al., 2010; da Costa et al., 2011; Burow et al., 2014). The use of antibiotics has potentially increased the prevalence of resistance determinants in animal microbiomes (Pal et al., 2016). The development of resistant pathogens associated with animal diseases has increased, and the growing antibiotic resistance gene pool in commensal bacteria is a cause for concern, and intensive research is required for understanding the prevalence and dynamics of AR bacteria in poultry flocks. *Escherichia coli* is a commensal bacterium in broilers and has a higher prevalence in chicken excreta than some key pathogens (Chinivasagam et al., 2010). *E. coli* may frequently be exposed to selective pressures imposed by antibiotic treatments and may contribute considerably to the spread of antibiotic resistance (Simoneit et al., 2015). Moreover, avian pathogenic

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E. coli (**APEC**) causes various diseases, collectively termed colibacillosis, in chickens, and these diseases are responsible for significant economic loss in the chicken industry (Hammerum and Heuer, 2009; Mohamed et al., 2014). Moreover, poultry products contaminated with APEC are potential sources of foodborne extraintestinal pathogenic *E. coli* infections for humans, posing a threat to human health (Bergeron et al., 2012)

This study evaluated the effect of oral challenge of broilers with a multi-resistant APEC strain and the effects of the antibiotic ampicillin, a feed additive (**FA**) containing organic acids and cinnamaldehyde, and a synbiotic application (**SA**) on the prevalence of AR *E. coli* in the ceca of these broilers. The application in food-producing animals of antibiotics with therapeutically useful analogs has led to public health concerns (Turnidge, 2004; Collignon et al., 2009; da Costa et al., 2011). Ampicillin is an aminopenicillin that is characterized by broad-spectrum antimicrobial activity and is applied in poultry farming for the treatment of bacterial infections (Agunos et al., 2012; Wang et al., 2017). Bacterial resistance to ampicillin has increased significantly since the introduction of this antibiotic in medicine and agriculture in the late 1980s (Everett et al., 1996). The percentage of ampicillin-resistant *E. coli* isolates from broilers in the European Union is approximately 70% (EFSA/ECDC, European Food Safety Authority and European Centre for Desease Prevention and Control, 2016).

Organic acid-based FAs are frequently used in poultry production due to their bactericidal activities, in both feed and the gastrointestinal tract (Ricke, 2003). Organic acids and cinnamaldehyde are known to have antimicrobial activity as well as the ability to promote the growth of chickens (Helander et al., 1998a; Raftari et al., 2009; Wang et al., 2009a; Adil et al., 2010). The effects of non-antibiotic antimicrobial compounds such as organic acids and cinnamaldehyde on resistant *E. coli* are not clear. On the one hand, there is indication that exposure to non-antibiotic antimicrobial agents can induce or select bacterial adaptations that result in decreased susceptibility to one or more antibiotics (Wales and Davies, 2015). On the other hand, the reduction of extended-spectrum cephalosporin-producing *E. coli* has been associated with the use of acidified drinking water in a risk factor study performed in Belgian broiler farms (Persoons et al., 2010). In general, the extent to which antibiotic resistance is associated with the use of chemicals and biological agents—used expressly to control, deter, inhibit, or kill harmful microorganisms—is poorly understood (Food and Agriculture Organization of the United Nations, 2018). This study aims to clarify the effect of FAs based on organic acids as well as synbiotics on resistant *E. coli* in broilers.

Synbiotics may be defined as mixtures of probiotics and prebiotics that beneficially affect the host by improving survival and implantation of live microbial dietary supplements in the gastrointestinal tract via selective stimulation of growth and/or metabolic activation of one or a limited number of health-promoting bacteria, thus improving host welfare (Gibson and Roberfroid, 1995). Probiotics are defined as monocultures or mixed cultures of live microorganisms that beneficially affect the host animal by modulating the gut microbiota in livestock (Fuller, 1989). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). The application of a synbiotic preparation reduced the *E. coli* and total coliform populations in the intestines of broiler chickens (Dibaji et al., 2014). The antimicrobial activity of organic acid- and cinnamaldehyde-based FAs, as well as the application of a synbiotic preparation, may influence the AR *E. coli* levels in the gastrointestinal tracts of broilers. The present study therefore evaluates the effect of ampicillin and FAs (an organic acid/cinnamaldehyde-based product and a synbiotic preparation) on the prevalence of AR *E. coli* in the gastrointestinal tracts of broilers challenged with a multi-resistant APEC strain.

MATERIALS AND METHODS

Animals, Housing and Treatments

The animal experiment was conducted at the facility of the Southern Poultry Research Group (**SPRG**; Athens, GA, USA). A total of 2000 day-of-hatch Ross 708 male broiler chicks were assigned to 5 treatment groups, with 8 replicate blocks, and allocated into groups of 50 birds per pen (pen size was 1.5 meters x 3.0 meters). One empty pen or a 1.5 meter empty space was positioned between the trial pens to reduce cross-contamination. All the animal caretakers wore plastic boots dedicated to each pen and wore gloves when entering the pens.

Treatment groups were assigned to pens using a randomized complete block design. The SPRG completed the randomization and assignment of treatment groups to pens using random permutation tables (Cochran and Cox, 1992). The first group was the negative control (**NC**) and only group without APEC challenge was positioned at a distance of two empty pens from the next group. The second group served as a challenge control (**CC**), and the third group received 100 g of ampicillin trihydrate (**AB**) (= 86.6 g of ampicillin) per 1000 L of water (Ampiciph®; bela-pharm GmbH & Co. KG, Germany) from day 11 to day 15 of the trial. The fourth group received a top-dressed FA composed of 20% formic acid, 10% acetic acid, and 5% propionic acid, as well as 2.5% cinnamaldehyde (Biotronic® Top3; BIOMIN Holding GmbH, Austria) at a dose of 2 kg/t of feed during the entire trial period. The fifth SA group received the multistrain synbiotic product PoultryStar® (BIOMIN Holding GmbH, Austria), containing *Enterococcus*, *Pediococcus*, *Bifidobacterium*, and *Lactobacillus* isolated from healthy chicken guts

combined with inulin, via feed and water. PoultryStar® me was provided via feed at a concentration of 1 kg/t of feed (2×10^8 CFU/kg of feed), and PoultryStar® sol was provided via drinking water on days 1, 2, 3, 16, 17, and 18 of the trial at a dose of 20 g per 1000 birds per day.

All groups except NC received oral challenge with multi-resistant *E. coli* with resistance to ampicillin, cephalexin, and nalidixic acid. A total of 25 one-day-old chicks per pen were tagged; color-coded for identification; and orally administered (gavage, 0.1 ml into the crop) the APEC strain X-7122, isolated by Dr. Jonn Maurer (Georgia University, USA), at 4.0×10^6 CFU per chick on the first day of the trial. Seeder birds were placed only in treatment groups CC, AB, FA, and SA.

All birds received routine vaccinations and were sprayed with a commercial coccidia vaccine (Advent® Coccidiosis Control; Huvepharma, Bulgaria) at 1 d of age, as per the manufacturer's recommendations.

All birds received a common basal diet without coccidiostats, a starter diet from hatch until day 17 and a grower diet until day 38. Diets were fed as mash throughout the study. The nonmedicated commercial-type broiler starter and grower diets consisted of the feedstuffs commonly used in the United States, which were representative of local formulations and met or exceeded National Research Council (1994) standards. The chicks had free access to feed and water supplied through bell drinkers.

The birds were housed at 0.09 square meters/bird. All birds were subjected to the same rearing, environmental and sanitary conditions. Birds were reared under ambient humidity. Thermostatically controlled gas heaters were the primary heat source. One heat lamp per pen provided supplemental heat during brooding. Birds were provided controlled lighting and ventilation. At placement, each pen contained approximately 4 inches of fresh pine shavings. Litter was not replaced during the course of this study. Each pen contained one tube feeder and one bell drinker (50 birds/feeder and drinker).

Sampling

To determine total *E. coli* counts and ensure that no multi-resistant *E. coli* strains that were resistant to nalidixic acid, ampicillin, and cephalexin were present in day-of-hatch chicks, swabs of all 20 chick box papers were tested on arrival at the trial facility. Sterile chick box paper was placed on the bottom of the transport box. Chick box papers were aseptically collected at the farm, immediately placed into sterile Whirl Pack bags (Sigma-Aldrich, Germany), and transported on ice to the laboratory for analysis of the presence of AR *E. coli*. On the 17th and 38th days of the trial, 3 chicks per pen were randomly selected and humanely euthanized by using CO_2. The intestinal tract of each chick was dissected after slaughter, and a total of 240 cecal samples were collected and placed into sterile plastic bags (Fisher Scientific, USA). The samples were labeled, stored on ice, and delivered to the laboratory for *E. coli* analysis.

Performance Data

Body weight (BW) by pen was calculated as the average of the sum of the weights of 50 birds as determined on days 1, 17, and 38. Average daily weight gain was calculated for day 1 to day 17, day 18 to day 38, and day 1 to day 38 of the trial. Pen-wise feed intake was recorded at day 17 and at the end of the trial on day 38. The average daily feed intake (**ADFI**) was calculated accordingly. The feed conversion rate (**FCR**) was calculated per pen and corrected for mortality.

Microbiological Analysis

E. coli From Chick Papers. Intestinal samples were kept on ice during transport to the laboratory. Each chick paper was hand swabbed with a premoistened 4×4 gauze pad that was then placed into 50 ml of phosphate-buffered saline (**PBS**; Dulbecco's PBS, MP Biomedicals, Solon, Ohio, USA). One milliliter of PBS was spread plated onto MacConkey agar (Becton, Dickinson and Company, Sparks, Maryland, USA) containing 25 μg/ml nalidixic acid, 6.25 μg/ml ampicillin, and 25 μg/ml cephalexin.

E. coli Isolation, Identification, and Enumeration. For all samples, a 1-ml aliquot of PBS was transferred to three adjacent wells in the first row of a 96-well 2-ml-deep block. A 0.1-ml aliquot of the sample was transferred to 0.9 ml of PBS in the second row, and the process was repeated for the remaining rows (to produce five ten-fold dilutions). One microliter from each well was transferred onto standard MacConkey agar for total *E. coli* enumeration and onto MacConkey agar containing 25 μg/ml nalidixic acid, 6.25 μg/ml ampicillin, and 25 μg/ml cephalexin for challenge strain enumeration with a pin-tool replicator. The plates were incubated aerobically (37°C for 24 h). The final dilution of each sample was recorded and entered into the most probable number (**MPN**) calculator for determination of the MPN value of the sample (Berghaus et al., 2013).

Antibiotic Susceptibility Testing. The automated National Antibiotic Resistance Monitoring System (NARMS, Sensititre®, USA) was used to determine minimal inhibitory concentrations (**MICs**) for antibiotic resistance levels of 3 random isolates of *E. coli* from antibiotic-free media for each of 240 samples; a total of 720 *E. coli* isolates were used. The MICs of the following antibiotics were tested: ampicillin, amoxicillin-clavulanic acid, azithromycin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, meropenem, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole.

Statistical Analysis

Performance Data. Statistical evaluations were carried out using Statistical Package for Social Sciences (SPSS 22.0., IBM Corp., US) (SPSS, 2013), and the results were considered significant at $P < 0.05$. After checking the data for normal distribution (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test), ANOVA, followed by the Bonferroni test, was performed. If variances were not homogenous, the data were evaluated by the Welch test with Tamhane's T2 test as a post hoc test. Data that were not normally distributed were further analyzed by the Kruskal–Wallis test (nonparametric ANOVA), followed by pairwise comparison.

E. coli Enumeration. Analysis was performed using SAS Enterprise software (SAS 9.4 with SAS Enterprise Guide 7.1 © (64bit) 2014 by SAS Institute Inc., Cary, NC, USA).

Linear mixed models were used to compare *E. coli* counts on the basis of MPN results expressed as colony forming unit (**CFUs**) per g of sample between days and treatment groups (MIXED procedure, SAS). Days, treatments and their interactions served as fixed effects and pens and birds as nested random effects. Before starting the statistical analysis, CFUs were $\log_{10}$ transformed to obtain linearity (because of the decimal dilution schema used for MPN determination). Using the Tukey–Kramer method, least square means of days as well as treatments were compared at a significance level of 5%.

Additionally, the prevalence of resistant isolates was compared between days and treatment groups using generalized linear mixed models (GLIMMIX procedure, SAS). Logistic regression was applied with the logit function to account for the correlation of isolates obtained from the same pens. Days, treatments, and their interactions served again as fixed effects and pens and birds as nested random effects. Multiple comparisons were applied to test for significant differences of days as well as treatments. Differences between least square means were tested by the Tukey–Kramer method ($\alpha = 5\%$). Furthermore, an in-depth analysis of treatment effects on different days was performed to test for specific treatment group differences of interest.

Antibiotic Susceptibility Testing. MICs were $\log_2$ transformed prior to statistical analysis (based on the MICs, provided in concentration steps of two to the power of n). MICs reported as being greater than the upper limit of the assay or lower than the lower limit of the assay were set as being equal to the corresponding limit to be included in the statistical analysis. Linear mixed models were used again to compare the means of the MICs between days and treatment groups (MIXED procedure, SAS). Model effects were set and statistical analysis was performed in the same manner as described for the MPN results (see "*E. coli* enumeration").

Additionally, the prevalence of resistant isolates was compared between days and treatment groups using generalized linear mixed models (GLIMMIX procedure, SAS) as described above ("*E. coli* enumeration"). Logistic regression was applied with the logit function for binary response distributions. In the case of multinomial (ordered) response distributions, the cumulative logit function was used. Treatment differences were examined by means of the Tukey–Kramer test.

For multinomial responses, intermediate and resistant prevalence results were merged prior to analysis in order to obtain binary responses. For treatment and day combinations with no intermediate or resistant responses, analysis was conducted without considering the interaction terms in the model. The Tukey–Kramer test was applied again for in-depth analysis of treatment effects on different days to test for specific treatment group differences of interest. Furthermore, contingency analysis was used for interpretation of some of the results regarding resistance.

RESULTS AND DISCUSSION

Performance Data

The influence of oral challenge with multi-resistant *E. coli* as well as AB, FA, and SA on poultry performance is shown in Table 1. Oral challenge with APEC did not influence the performance of the birds. The

Table 1. Performance characteristics and standard deviations (± SD) of broilers (400/group) that received ampicillin, a feed additive based on organic acids (FA) or a synbiotic preparation (SA) compared to the control groups.

	NC	CC	AB	FA	SA	*P*-value
Initial weight, g	46 ± 0.62	46 ± 0.89	46 ± 0.24	46 ± 0.51	46 ± 0.37	0.15
BW d17, g	471 ± 18.1	453 ± 15.8	473 ± 50.8	468 ± 16.2	455 ± 29.5	0.32
BW d38, g	1967 ± 102,8	1928 ± 48.8	1972 ± 110.1	1995 ± 63.4	1960 ± 79.3	0.62
ADFI d1–17, g/d	31.0 ± 1.2	30.3 ± 1.0	30.5 ± 2.9	30.8 ± 1.0	30.3 ± 1.7	0.67
ADFI d -38, g/d	149.1 ± 5.1	148.9 ± 5.1	147.9 ± 7.0	146.8 ± 6.3	148.9 ± 7.8	0.95
FCR d1–17, g/g	1.34 ± 0.05	1.37 ± 0.05	1.30 ± 0.05	1.34 ± 0.05	1.36 ± 0.04	0.11
FCR d1–38, g/g	1.64 ± 0.14	1.68 ± 0.06	1.62 ± 0.03	1.62 ± 0.03	1.63 ± 0.04	0.13
Mortality, %	3.00	4.25	3.50	5.75	2.75	0.478

NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic; BW, body weight; ADG, average daily weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio; mean values ± standard errors.

Table 2. *E. coli* counts in cecal samples on days 17 and 38 of the trial on MacConkey medium without and with antibiotic supplementation, shown as $\log_{10}$ MPN/g values and standard deviations (± SD); 24 positive samples.

Antibiotic	Day	NC	CC	AB	FA	SA
None	17	6.83 ± 1.36	6.78 ± 1.14	7.83 ± 1.01	6.66 ± 1.70	6.27 ± 1.19
	38	6.04 ± 1.35	5.57 ± 1.47	5.23 ± 1.54	4.96 ± 1.2	4.46 ± 1.72
Ampicillin, cephalexin and nalidixic acid	17	0 (0/24)	0.97 ± 1.21 (16/24)	1.86 ± 0.31 (14/24)	1.66 ± 0.99 (16/24)	1.77 ± 1.18 (14/24)
	38	0.68 ± 0.53 (5/24)	1.38 ± 1.24 (11/24)	1.14 ± 1.28 (9/24)	1.46 ± 1.17 (16/24)	1.54 ± 1.38 (15/24)

NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic.

lack of change in performance in the challenged group compared to the non-challenged control shows that the APEC challenge strain did not have a significant impact on bird health and performance, perhaps due to the low competitiveness of APEC with other intestinal microorganisms. Non-significant differences in performance parameters between treatment groups may also be due to the determination of performance parameters per pen, without individual animal data or excessive variability between treatment pens.

The studies described below present the effects of antibiotics, organic acids, cinnamaldehyde, and synbiotics on growth performance. Penicillins are the most commonly used antibiotics in poultry (Hofacre et al., 2013). Ampicillin is registered for use in poultry in large poultry producing countries such as Brazil, China, Germany, and France (Roth et al., 2018) Stokstad and Jukes (1950), showed that small subtherapeutic doses of penicillin and tetracycline enhance weight gain in poultry. Antibiotics have been used in animals for the treatment of diseases, for the prevention and control of diseases, and as growth promoters (Economou and Gousia, 2015). The administration of antibiotics decreases or alters the bacterial populations present in the digestive tract, which protects animals from pathogenic organisms, increases animal weight and improves meat quality (Fairchild et al., 2001). Antibiotic resistance is the main undesirable side effect of antibiotic use (EFSA/ECDC, European Food Safety Authority and European Centre for Desease Prevention and Control, 2016). Replacement of antibiotics for disease prevention with non-antibiotic substances is essential for implementation of technological solutions that can reduce selection pressure and therefore reduce contamination with AR bacteria.

Organic acids and cinnamaldehyde improve chicken performance via antimicrobial activity (Helander et al., 1998; Raftari et al., 2009; Wang et al., 2009b; Adil et al., 2010). Olarve et al. (2007) showed significant effects on the weight gain and feed efficiency of broilers by using 0.3 and 0.4% (a blend of formic, fumaric, lactic, propionic, and phosphoric acids) in basal diets. The BWs and FCRs of broilers were significantly increased by supplementation with a mixture of formic and propionic acids (Senkoylu et al., 2007). Improvement in weight gain and FCR due to improved nutrient digestibility was detected in a study where formic or fumaric acid and acetic or citric acid were used (Ghazalah et al., 2011). Application of the same synbiotic product as that used in the present study has been previously shown to improve BW gain and FCR as well as the apparent ileal and total tract digestibility (Palamidi et al., 2016). Other studies with the same product showed improvement of zootechnical performance parameters and nutrient digestibility compared to the control (Ritzi et al., 2014; Mountzouris et al., 2015). The tested multistrain synbiotic showed significant modulation of the composition of the cecal microbiota, resulting in increased *Bifidobacterium* spp. and *Lactobacillus* spp. concentrations compared with the control (Mountzouris et al., 2010).

Microbiological Analysis

***E. coli* From Chick Papers.** Swabs of 20 chick box papers were collected on day 0 and cultured for detection of multi-resistant *E. coli* with resistance to ampicillin, cephalexin, and nalidixic acid. The objective was to confirm the absence of multi-resistant *E. coli* prior to challenge with the multi-resistant APEC strain with resistance to these antibiotics. For the cultures grown on MacConkey agar supplemented with ampicillin, cephalexin, and nalidixic acid, no *E. coli* was identified on any of the 20 swabs. This outcome can be seen as a prerequisite for the planned challenge with the multi-resistant APEC strain.

***E. coli* Enumeration.** *E. coli* counts in cecal samples on MacConkey medium without and with antibiotic supplementation are presented in Table 2, and the statistical evaluation of differences between treatments, days and the interactions between treatments and days is presented in Table 3.

The means of the *E. coli* counts in cecal samples grown on MacConkey medium that was not supplemented with any antibiotics were significantly high on day 17. Significant effects of treatment were observed between the NC-SA and AB-SA groups on both days. As the interaction term was also significant, in-depth analysis (multiple comparisons, Tukey–Kramer) showed significant differences between treatments AB-SA on day 17 and NC-SA on day 38, with low *E. coli* counts in SA observed in both cases. The influence of synbiotics on *E. coli* counts has also been shown in other studies. Gunal et al. (2006) demonstrated that probiotic supplementation decreased the abundances of gram-negative bacteria compared to the control group. The

Table 3. Statistical evaluation of differences in *E. coli* counts on MacConkey medium without and with antibiotic supplementation among treatments, days, and interactions between treatments and days.

Antibiotic	Effect of day	Effect of treatment	Effect of interaction
None	17>38 ($P < 0.0001$)	NC, AB>SA ($P = 0.0016$) *, **	$P = 0.02$
Ampicillin, cephalexin and nalidixic acid	17>38 ($P = 0.02$)	NC<FA, SA ($P < 0.0001$) *	$P = 0.30$
Ampicillin, cephalexin and nalidixic acid	$P = 0.56$)	NC<CC, AB, FA, SA ($P = 0.0036$) ***	n.a.

NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic; n.a., not available.

*MIXED procedure and multiple comparisons of *E. coli* count results adjusted according to the Tukey–Kramer test at a significance level of 5%.

**In-depth analysis of treatment effects by day (Tukey–Kramer test) showed significant differences between AB-SA on day 17 and NC-SA on day 38.

***GLIMMIX procedure with and without interaction terms and in-depth analysis of treatment effects by day showed significant differences between NC-CC, NC-AB, NC-FA, and NC-SA on day 17 only.

population of intestinal *E. coli* in broilers that were fed lactobacilli-supplemented feed was significantly lower ($P < 0.05$) than that of the control (Jin et al., 1996).

For the analysis of *E. coli* counts in cecal samples grown on MacConkey medium containing antibiotic supplements, all samples that showed a lack of growth were excluded from the statistical analysis. No *E. coli* growth was observed on day 17 in the NC group. Because a zero value would be undefined on the log scale after $\log_{10}$ transformation, this group could not be considered for comparison of means. Mixed model analysis indicated that the effects of the days and treatments were not significant. To include all the results, the detection limit (MPN code 0–0-0) was set to 0.3 CFU. This value was based on the fact that the MPN assay results, with MPN code 0–0-0, corresponded to a value of < 0.3 CFU per ml of medium. Under these conditions, we observed significant differences among treatments as well as days, whereas the interactions between the two had no significant effects, as per the linear mixed model analysis. Consequently, significant differences between the NC-FA and NC-SA groups could be identified using the Tukey–Kramer test for multiple comparisons.

Furthermore, based on the growth ability in the presence of antibiotic substances, the prevalence of multi-resistant isolates was compared between days and treatment groups using the *E. coli* count results in cecal samples on MacConkey medium supplemented with ampicillin, cephalexin, and nalidixic acid. When considering all culture-positive samples that exhibited growth on MacConkey agar as resistant and all other results as susceptible to the applied antibiotic mixture, categorical data analysis showed no day-related effects but significant treatment-related effects. No multi-resistant *E. coli* strain with resistance to ampicillin, cephalexin and nalidixic acid was detected in NC on day 17 of the trial, but a strain was detected on day 38 of the trial, indicating transition of resistance determinants between pens, despite separation with two empty pens between groups. Because there was no resistant strain in group NC on day 17, the interaction term had to be excluded for successful application of the GLIMMIX procedure. In-depth analysis showed significant differences between the NC-CC, NC-AB, NC-FA, and NC-SA groups on day 17 only. The *E. coli* count results and prevalence of *E. coli* are summarized by sampling day and treatment group in Table 2 and statistical evaluation in Table 3. *E. coli* were detected in all tested cecal samples except the NC group on day 17. No multi-resistant *E. coli* that were resistant to ampicillin, cephalexin, and nalidixic acid were detected in the negative control group on day 17 of the trial. However, resistant *E. coli* was detected in 21% (5/24) of samples in the negative control on day 38 of the trial, indicating the possible transmission of the multi-resistant APEC strain used for the oral challenge to the negative control.

Antimicrobial Susceptibility Testing. The mean MIC results and the corresponding standard deviations expressed as $\log_2$ values are shown in Table 4, and the statistical evaluation is presented in Table 5.

Generally, the mean MICs on day 38 were higher for all antibiotic substances compared to those on day 17, with the exception of the MICs of ceftriaxone. These results indicated higher antibiotic resistance levels on day 38 compared to day 17. Antibiotics were not used between days 16 and 38; therefore, the increase in MIC and resistance to antibiotics may not be due to selective pressure. However, resistant *E. coli* can compete with susceptible strains in the absence of selective antibiotic pressure (Smith et al., 2007). The incorporation and development of resistant *E. coli* strains in the intestinal tract depends on the composition of the intestinal microbiota, growth rates, transmission dynamics, persistence, and features affecting colonization, such as adherence and virulence (Karami et al., 2006; Marciano et al., 2007). All of these factors together affect the epidemiological fitness of resistant *E. coli* and the ability of these bacteria to competitively develop in the intestinal environment (Sundqvist, 2014).

Significant effects of treatment were evident with amoxicillin-clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftriaxone, and tetracycline, whereas the AB group often exhibited a different behavior. The MICs of amoxicillin-clavulanic acid and ampicillin exhibited significant treatment-related effects as well as effect of the interactions between days and treatments. Further analysis confirmed that the ampicillin-treated AB group exhibited a greater mean MIC

Table 4. Mean minimal inhibitory concentrations (MICs) of tested antibiotics and the corresponding standard deviations (± SD), shown as log2 values.

	Antibiotic	NC	CC	AB	FA	SA
Day 17	n	72	72	72	72	71
	Amoxicillin—clavulanic acid	1.38 ± 1.11	1.75 ± 1.2	3.24 ± 1.31	1.40 ± 0.80	1.08 ± 0.69
	Ampicillin	1.58 ± 1.55	1.71 ± 1.58	4.33 ± 1.39	1.44 ± 1.09	0.90 ± 0.74
	Azithromycin	2.60 ± 1.08	2.17 ± 0.67	1.79 ± 0.63	2.33 ± 0.92	2.15 ± 0.75
	Cefoxitin	2.36 ± 0.91	2.43 ± 0.90	3.21 ± 1.30	2.51 ± 0.69	2.06 ± 0.67
	Ceftriaxone	−1.76 ± 1.14	−1.79 ± 1.01	0.22 ± 2.71	−2.00 ± 0.00	2.00 ± 0.00
	Chloramphenicol	2.53 ± 0.67	2.49 ± 0.56	2.32 ± 0.53	2.64 ± 0.56	2.56 ± 0.67
	Ciprofloxacin	−5.91 ± 0.62	−5.89 ± 0.63	6.06 ± 0.00	−5.93 ± 0.33	6.06 ± 0.00
	Gentamycin	2.04 ± 2.34	1.79 ± 2.27	2.18 ± 2.34	2.33 ± 2.32	2.06 ± 2.15
	Meropenem	−4.06 ± 0.00	−4.06 ± 0.00	4.06 ± 0.00	−4.06 ± 0.00	4.06 ± 0.00
	Nalidixic acid	1.15 ± 1.02	1.15 ± 1.02	0.74 ± 0.56	1.22 ± 0.56	0.85 ± 0.40
	Streptomycin	4.57 ± 1.67	4.47 ± 1.54	4.60 ± 1.38	4.61 ± 1.67	4.96 ± 1.26
	Sulfisoxazole	6.51 ± 1.94	5.35 ± 1.80	5.49 ± 1.87	5.72 ± 1.92	6.04 ± 2.00
	Tetracycline	4.42 ± 1.20	4.04 ± 1.41	3.04 ± 1.44	4.25 ± 1.31	4.54 ± 1.09
	Trimethoprim-sulfamethoxazole	−2.02 ± 1.79	−2.59 ± 1.24	1.89 ± 2.10	−2.75 ± 1.17	2.63 ± 1.42
Day 38	n	72	72	72	72	72
	Amoxicillin—clavulanic acid	2.18 ± 09.94	2.49 ± 1.07	3.51 ± 1.06	2.14 ± 0.83	2.18 ± 0.84
	Ampicillin	2.24 ± 1.51	2.93 ± 1.76	4.96 ± 0.35	2.29 ± 1.42	2.51 ± 1.59
	Azithromycin	2.92 ± 0.98	2.36 ± 0.59	2.31 ± 0.66	2.68 ± 0.82	2.63 ± 0.57
	Cefoxitin	2.69 ± 0.74	2.79 ± 0.87	3.36 ± 1.18	2.78 ± 0.56	2.53 ± 0.63
	Ceftriaxone	−1.76 ± 1.14	−1.53 ± 1.58	0.29 ± 2.67	−1.92 ± 0.71	2.00 ± 0.00
	Chloramphenicol	2.81 ± 0.46	2.76 ± 0.46	2.67 ± 0.50	2.93 ± 0.42	2.90 ± 0.48
	Ciprofloxacin	−5.73 ± 0.90	−5.88 ± 0.66	6.02 ± 0.35	−5.81 ± 0.67	5.98 ± 0.50
	Gentamycin	3.61 ± 1.24	2.72 ± 1.99	3,08 ± 1.81	2.93 ± 1.95	2.67 ± 1.92
	Meropenem	−4.06 ± 0.00	−4.06 ± 0.00	4.06 ± 0.00	−4.06 ± 0.00	4.06 ± 0.00
	Nalidixic acid	1.61 ± 1.19	1.44 ± 0.98	1.08 ± 0.64	1.56 ± 0.89	1.18 ± 0.70
	Streptomycin	5.71 ± 0.72	5.18 ± 1.17	5.28 ± 1.05	5.21 ± 1.22	5.51 ± 0.93
	Sulfisoxazole	7.57 ± 1.23	6.39 ± 1.89	6.40 ± 1.96	6.56 ± 1.85	6.78 ± 1.86
	Tetracycline	4.83 ± 0.69	4.79 ± 0.77	4.13 ± 1.37	4.58 ± 1.04	4.71 ± 0.90
	Trimethoprim-sulfamethoxazole	−1.67 ± 1.97	−2.03 ± 1.76	0.60 ± 2.49	−2.42 ± 1.43	1.99 ± 1.97

Three isolates were evaluated from each of 3 birds per pen in each of 8 pens per treatment group (72 isolates per treatment group); mean ± standard error; NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic.

Table 5. Statistical evaluation of MICs and standard deviations (± SDs) of the antibiotics showing significant differences among treatments, days, and interactions between treatments and days.

Antibiotic	Effect of day	Effect of treatment*	Effect of interaction
Amoxicillin—clavulanic acid	38>17 ($P < 0.0001$)	AB>NC, CC, FA, SA ($P < 0.0001$)**	$P < 0.0001$
Ampicillin	38>17 ($P < 0.0001$)	AB>NC, CC, FA, SA ($P < 0.0001$)	$P < 0.0001$
Azithromycin	38>17 ($P < 0.0001$)	NC>AB ($P = 0.02$)	$P = 0.11$
Cefoxitin	38>17 ($P < 0.0001$)	AB>SA ($P = 0.02$)	$P = 0.24$
Ceftriaxone	$P = 0.38$	AB>FA, SA ($P = 0.03$)	$P = 0.44$
Chloramphenicol	38>17 ($P < 0.0001$)	$P = 0.14$	$P = 0.93$
Ciprofloxacin	38>17 ($P = 0.04$)	$P = 0.25$	$P = 0.44$
Gentamicin	38>17 ($P < 0.0001$)	$P = 0.81$	$P = 0.03$
Nalidixic acid	38>17 ($P < 0.0001$)	$P = 0.05$	$P = 0.86$
Streptomycin	38>17 ($P < 0.0001$)	$P = 0.72$	$P = 0.09$
Sulfisoxazole	38>17 ($P < 0.0001$)	$P = 0.14$	$P = 0.85$
Tetracycline	38>17 ($P < 0.0001$)	AB<NC, SA ($P = 0.02$)***	$P < 0.0001$
Trimethoprim-sulfamethoxazole	38>17 ($P < 0.0001$)	$P = 0.17$	$P < 0.002$

NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic.

*MIXED procedure and multiple comparisons of the MICs adjusted according to the Tukey–Kramer test at a significance level of 5%.

**In-depth analysis did not show significant differences between CC-AB on day 38.

***In-depth analysis did not show significant differences between NC-AB, AB-FA, and AB-SA on day 17 only.

than any other group. Additionally, the significance of the interaction term was evident with regard to tetracycline, trimethoprim-sulfamethoxazole, and gentamycin, whereas significant differences between treatments could be verified only in the case of tetracycline.

The effect of selective pressure of ampicillins on the increased MIC values of penicillins is clearly recognizable here. However, the selective pressure of ampicillin reduced the MIC values of tetracyclines with the *E. coli* isolates. In the present study, the MIC value of tetracycline was significantly lower in the AB group than in NC and SA. Antibiotic use may also lead to decreased abundances of some AR bacteria. The ability of enrofloxacin to decrease the prevalence of extended-spectrum beta-lactamase-producing *E. coli* was demonstrated by Roth et al. (2017). The effect of the applied FA and synbiotic preparation on the prevalence of resistant *E. coli* could be seen in the distribution of MIC values for cefoxitin-

Table 6. Statistical evaluation of the resistance to antibiotics, showing significant differences among treatments, days, and interactions between treatments and days.

Antibiotic	Effect of day	Effect of treatment	Effect of interactions
Amoxicillin—clavulanic acid***	38>17 ($P = 0.036$)	AB>NC, CC, FA, SA ($P < 0.0001$)	n.a.
Ampicillin**	38>17 ($P < 0.0001$)	AB>NC, CC, FA, SA ($P < 0.0001$)	n.a.
Cefoxitin***	$P = 0.36$	AB>NC, CC, FA, SA; SA<CC($P < 0.0001$)	n.a.
Ceftriaxone***	$P = 0.69$	AB>NC, CC, FA, SA ($P < 0.0001$)	n.a.
Chloramphenicol**	$P = 0.65$	$P = 0.49$	n.a.
Gentamicin*	38>17 ($P < 0.0001$)	$P = 0.70$	$P = 0.17$
Sulfisoxazole*	38>17 ($P < 0.0001$)	$P = 0.11$	$P = 0.75$
Tetracycline*	38>17 ($P < 0.0001$)	$P = 0.04$****	$P = 0.30$
Trimethoprim-sulfamethoxazole*	38>17 ($P = 0.0002$)	$P = 0.16$	$P = 0.48$

NC, negative control without *E. coli* challenge; CC, *E. coli* challenge control; AB, ampicillin; FA, feed additive based on organic acids; SA, multistrain synbiotic; n.a., not available.

*GLIMMIX procedure and multiple comparisons of the resistance results adjusted according to the Tukey–Kramer test at a significance level of 5%.

**GLIMMIX procedure without interaction terms and in-depth analysis of treatment effects by day according to the Tukey–Kramer test.

***Interpretation by contingency analysis (dependencies in contingency tables of treatments were tested by Pearson's Chi-squared test, and tests on subgroups were based on the Bonferroni correction to comply with the type I error rate).

****In-depth analysis showed significant differences between AB-SA on day 17 only.

and ceftriaxone-resistant *E. coli*. The MIC of cefoxitin was lower in the SA group, and the MIC of ceftriaxone was lower in the FA and SA groups, than in the AB group.

Given the antibiotic breakpoints defined by the Clinical and Laboratory Standards Institute (2012), MICs can be classified as resistant, susceptible or intermediate. Because there are no CLSI data for interpretation of azithromycin, nalidixic acid, and streptomycin MICs, the prevalence of resistant isolates could not be investigated. Based on the prevalence classification of the MIC results (see Table 6), the prevalence of some resistant isolates (sulfisoxazole, tetracycline, trimethoprim-sulfamethoxazole, and gentamycin) could be successfully analyzed with the GLIMMIX procedure of SAS. For these antibiotic substances, again, the day-related effects were significantly higher for resistant isolates on day 38, whereas, with the exception of the effects observed with tetracycline, no treatment-related or interaction-related effects could be identified. For tetracycline, some significance was observed between the prevalence results of AB-SA on day 17, in which the AB group exhibited the lowest abundance of resistant isolates.

Due to the unbalanced response matrix of some of the antibiotic substances (amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftriaxone, and chloramphenicol), the convergence criteria of the model (GLIMMIX procedure, SAS) could not be met. Therefore, the interaction term was excluded from the model in order to allow statistical analysis. Neither day- nor treatment-related effects were observed for chloramphenicol-resistant *E. coli*. Higher abundances ($P < 0.05$) of ampicillin-resistant were observed on day 38 than on day 17. There were significant differences between treatments, with the highest rate of ampicillin resistance observed in the AB group. The increased abundance of AR bacteria due to oral administration of antibiotics in the AB group corresponds with the outcome of the literature review for poultry conducted by Simoneit et al. (2015).

In the remaining 3 cases (amoxicillin-clavulanic acid, cefoxitin and ceftriaxone), analysis and interpretation of the resistance results was based on contingency analysis. Significant differences in the distribution of amoxicillin-clavulanic acid-resistant isolates were detected with respect to days and treatments, while for the cefoxitin- and ceftriaxone-resistant isolates, significant differences were observed with respect to only treatments. All 3 cases showed similarities in response patterns with the most resistant isolates in the AB group and, interestingly, with almost no resistant isolate on day 17 in groups FA and SA. Supplementation of the diet with FA in another trial contributed to a significant decrease ($P < 0.05$) in the abundance of *E. coli* that was resistant to ampicillin and tetracycline compared to the control and enrofloxacin-supplemented groups (Roth et al., 2017).

For azithromycin, ciprofloxacin, meropenem, nalidixic acid, and streptomycin, no statistical analysis could be performed because there was no variation in the resistance results (either the bacteria were susceptible or the results were not interpretable).

CONCLUSION

A high prevalence of AR *E. coli* in all experimental groups was observed throughout the study. It may be concluded that administration of ampicillin for 5 d led to a significant increase in the abundances of *E. coli* resistant to ampicillin, amoxicillin-clavulanic acid, cefoxitin, and ceftriaxone, all of which are ß-lactam antibiotics. The tested feed additives did not increase the prevalence of resistant determinants in the guts of the broilers. Moreover, the MIC of ceftriaxone was lower in the FA and SA groups than in the AB group. Additionally, administration of SA led to a decreased MIC value of cefoxitin in the SA group. Further studies are

needed to confirm these findings and to clarify the mode of action of FA and SA on *E. coli* strains resistant to cephalosporin and ß-lactams in the digestive tract.

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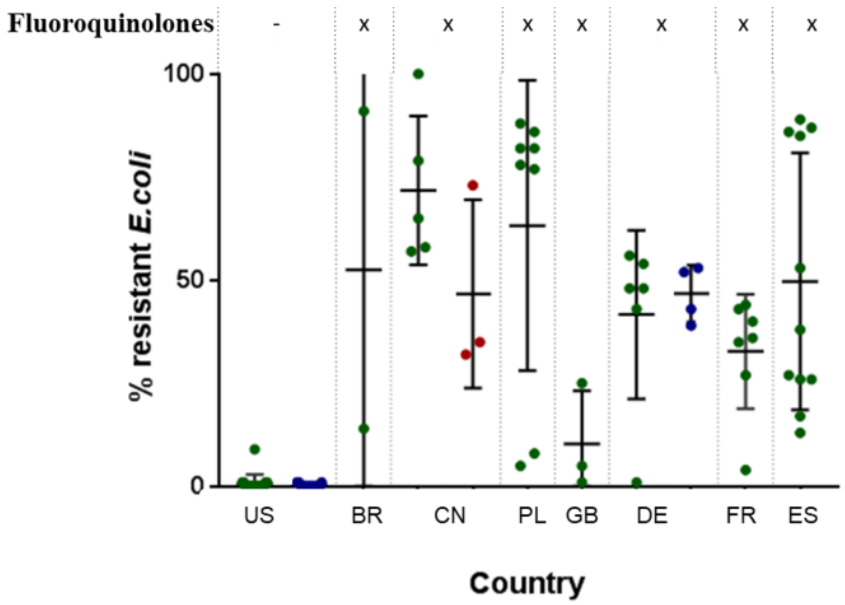
IV. CONCLUDING STATEMENTS AND FUTURE ASPECTS

Although, antibiotics were used in the human medicine since the introduction of sulphonamides in 1930, the phenomenon of antibiotic resistance is ancient as antibiotic resistance genes have been known to evolve billions of years ago, long before clinical use of antibiotics (D'Costa, et al., 2011). The use of high concentration of lethal dose of antibiotics as a consequence of human activity led to a major change in innate functional role to give rise to the emergence of antibiotic-resistant pathogens. Because of the rapid dissemination of antibiotic resistance in pathogens, many of the antibiotics, which were effective earlier, became obsolete during the past few decades (Sengupta, et al., 2013). In addition, only one new antibiotic was introduced in the recent years and all other entrants were just the variation of the existing one (Raghunath, 2008). As the results, antibiotic resistance became a threat to human and animal health worldwide.

Key measures are required to reduce the risks posed by antibiotic resistance. The literature review is the first comprehensive evaluation of data recording the authorized antibiotics for poultry production combined with AR data in *E. coli* isolates in large poultry producing regions that together produce more than 1/3 of poultry meat worldwide. The survey results clearly display the absence of a harmonized approach in the monitoring of antibiotics per animal species and the evaluation of resistances using the same methodology. The outcome of the review confirms finding demonstrated with other studies as summarized by Food and Agriculture Organization of the United Nations (2018), that the use of antibiotics in poultry production increases the selection pressure for antibiotic-resistant bacteria. The resistance rates to fluoroquinolones and quinolones in the US, where fluoroquinolones are not registered for use, are below 5%, while the average of resistant *E. coli* is above 40% in Brazil, China, and EU, where use of fluoroquinolones is legalized, as can be observed in Figure 5. These finding demonstrate the possibility to produce broilers without fluoroquinolones, which results in low resistance rates.

Figure 5. Registration of fluoroquinolones and resistance rates of ciprofloxacin in *E. coli* originating from broilers from the US, China, Brazil, Poland, United Kingdom, Germany, France and Spain.



E. coli isolates from healthy animals (green dots), chicken retail meat (blue dots) and diseased chickens (red dots) detected within scientific studies or national monitoring programmes. Each dot represents one study or data set in one year. On the top of the figure, status of approval for fluoroquinolones.

Data research also presents, that there are several classes of antibiotics that are approved for use in poultry, but no *E. coli* AR are available for studied regions. Such classes are: arsenicals, glycophospholipids, ionophores, lincosamides, orthosomycins, pleuromutilins, polypeptides and streptogramins. Although, most of the representatives of these antibiotic classes act against Gram-positive bacteria, the prevalence of resistance determinants in *E. coli* to some of these antibiotics was detected by (Bonnet, et al., 2009; Cervantes, et al., 1994; Heir, et al., 2004; Hummel, 1979). Additionally, the research gap was identified, concerning the detection of resistance to colistin and tylosin. Colistin, as a representative of the polymyxins, and tylosin, as representative of the macrolides, are both allowed for poultry use in all countries for oral treatment or injection solution, but there is only a limited amount of resistance data available.

Due to the detection of plasmid-located colistin resistance genes in some countries, the assessment of resistance rates to this antibiotic would be essential in the future.

The use of feed additives as alternatives to antibiotic for disease prevention may be possible. However, the gaps of knowledge were identified during the evaluation of alternatives to antibiotics by Murphy, et al. (2017). Therefore, further research for evaluation of efficacy of organic acid based feed additives as well as synbiotics in comparison to antibiotics was needed. Additionally, the effect of feed additives with biocidal activity needed to be clarified as stated by Food and Agriculture Organization of the United Nations (2018). Two trials in different countries (Austria and US) and using different conditions (non-challenge and challenge with resistant pathogenic *E. coli* were conducted in order to evaluate the efficacy of tested feed additives and antibiotics.

A high prevalence of antibiotic resistant *E. coli* in both trials was observed throughout the study period. Which also corresponds with prevalence of resistant *E. coli* evaluated in the process of literature review conducted in this thesis. The source of antibiotic-resistant bacteria is the vertical transmission of resistance gene determinants along the poultry chain. (Zurfluh, et al., 2014) showed that gene determinants of ESBL-producing *E. coli* are transmitted vertically in the broiler production pyramid from the top (nucleus poultry flock level) to the bottom, with little evidence of any antimicrobial selection pressure.

The presence of the multiresistant *E. coli* in the trial conducted in the US did not increase the prevalence of resistant *E. coli*. The use of antibiotics was recognized as a reason for significant increase of resistant *E. coli*. The administration of enrofloxacin as well as ampicillin for few days led to significant increase of *E. coli* resistant to different antibiotics. Use of ampicillin led to an increase of ampicillin, amoxicillin-clavulanic acid, cefoxitin and ceftriaxone that all belong to *ß*-lactam antibiotics. The use of enrofloxacin led to an increase not only fluoroquinolones, but also antibiotics streptomycin, sulfamethoxazole and tetracycline belonging to other antimicrobial classes. On another hand, use of enrofloxacin led to a decrease of cefotaxime resistant antibiotics as well as ESBL producing *E. coli*.

Tested feed additives did not increase the prevalence of resistant determinants in the gut of broilers during the two experiments. Moreover, supplementation with feed additives based on organic acids contributed to a decrease in ampicillin- and tetracycline-resistant *E. coli* in

the cecum of broilers compared to the negative control group and group received enrofloxacin. Decrease of resistant *E. coli* in the groups supplemented tested feed additives was indicated also during the challenge trial in the US. The reason for such a decrease is not clear and needs further experiments.

Additionally, it would be important to evaluate the prevalence of resistant bacteria as well as the effect of feed additives on resistant bacteria using the technology of gene sequencing, which is able to detect the whole resistome. The studies of antibiotic resistance have grown from focusing on single organisms in culture to studying antibiotic resistance in pathogenic, commensal and environmental bacteria at the level of microbial communities (Crofts, et al., 2017). As the study of antibiotic resistance advances, it is important to incorporate this comprehensive approach to better inform global antibiotic resistance surveillance. It is increasingly becoming apparent that although not all resistance genes are likely to geographically and phylogenetically disseminate, the threat presented by those that are is serious and warrants an interdisciplinary research focus.

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LIST OF ABBREVIATIONS

APEC ……avian pathogenic *E. coli*

BR ……Brazil

CN ……China

DE ……Germany

E. coli ……*Escherichia coli*

EFSA ……European Food Safety Authority

EMA ……European Medicines Agency

ES ……Spain

ESBL …… extended spectrum beta-lactamase

ESVAC ……European Surveillance of Veterinary Antimicrobial Consumption

EU ……European Union

FA ……acid-based feed additives

FAO …… Food and Agriculture Orgainzation

FR ……France

GB ……United Kingdom

MIC ……minimal inhibitory concentration

NIR ……not Independently Reported

PL ……Poland

US …… United States of America

WHO……World Health Organization