Preventive Veterinary Medicine 186 (2021) 105222

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Resistance patterns to C and D antibiotic categories for veterinary use of *Campylobacter* spp., *Escherichia coli and Enterococcus* spp. commensal isolates from laying hen farms in Spain during 2018

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ARTICLE INFO

Keywords:
Antimicrobial resistance
Campylobacter spp.
E. coli
Enterococcus spp.
Laying hen
Minimum inhibitory concentration

ABSTRACT

Antimicrobial Resistance (AMR) is a global threat for human and animal health. Few studies have been carried out on laying hens. The aim of this work was to evaluate the antimicrobial susceptibility of commensal *Campylobacter* spp., *E. coli, and Enterococcus* spp. isolates in Spanish laying hens in 2018. Samples were collected from 39 laying hen farms. The microorganisms of interest were isolated and confirmed by PCR. The Minimum Inhibitory Concentration (MIC) to antimicrobials of C and D categories were determined. 195 *E. coli*, 195 *Enterococcus* spp. and 25 *Campylobacter* spp. isolates were obtained. *E. coli* isolates showed high resistance to D category antimicrobials (sulfamethoxazole 76.41 %, tetracycline 62.05 %, trimethoprim 50.77 %, ampicillin 30.77 %) and lower resistance to C category (azithromycin 30.26 %, gentamicin 12.31 %, chloramphenicol 4.62 %). A 10.26 % of *E. coli* isolates were susceptible to all antimicrobials tested, Multi Drug Resistance (MDR) to 3 antimicrobial families was found in 23.08 % of the isolates and 13.85 % were MDR to 4 families, being Erythromycin-Sulfamethoxazole-Tetracycline the most common resistance profile (10.77 %). *Enterococcus* spp. showed very high resistance to D category tetracycline (78.47 %) and C category erythromycin (76.42 %). The 11.79 % of *Enterococcus* spp. isolates were susceptible to all antimicrobials and 53.33 % were resistant to 2 families, being Erythromycin-Tetracycline the most common AMR profile (51.79 %). Regarding *Campylobacter* spp., resistance to tetracycline (48 %) was higher than resistance to C category antimicrobials (erythromycin 28 %, streptomycin 24 %, gentamicin 16 %). There was a 52 % sensitivity to all tested antimicrobials and 24 % showed MDR to aminoglycosides, macrolides and tetracyclines (Gentamicin-Streptomycin-Erythromycin-Tetracycline MDR profile). Novel data on AMR in laying hen commensal isolates in Spain was provided. High resistance to several antimicrobials was found, especially to key drugs for the treatment of zoonosis, which represents a public health risk. Better surveillance and careful regulation of antimicrobial use is required in laying hen production.

1. Introduction

Antimicrobial resistance (AMR) is a global health threat (WHO, 2014). This situation is the consequence of the excessive prescription of antibiotics, their inappropriate use by patients, and the overuse of these substances in livestock (Capita and Alonso-Calleja, 2013; Van Boeckel et al., 2015). The cause of excessive use of antibiotics in farm animals is the need of controlling disease associated with intensive farming and the use of antibiotics as growth promoters (Dibner and Richards, 2005; Marshall and Levy, 2011). This situation accelerates the selection and spread of AMR genes in pathogenic and commensal microorganisms, which represent a risk to human health (Wegener, 2003). The World

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https://doi.org/10.1016/j.prevetmed.2020.105222
Received 6 July 2020; Received in revised form 17 November 2020; Accepted 23 November 2020
Available online 25 November 2020

Health Organization (WHO) established the concept of "One Health", by which AMR is an ecological problem characterized by complex interactions involving diverse microbial populations that affect the health of humans, animals, and the environment. Therefore, it is necessary to tackle AMR using a coordinated and multi-sectoral approach in order to take into account its ecological nature and complexity (McEwen and Collignon, 2018; WHO, 2015).

Poultry is one of the most important food producing industries worldwide. The main reasons for this are the relatively low production costs and the absence of cultural and religious restrictions for poultry products consumption (Nhung et al., 2017). The European Union (EU) produces around 15 million tons of poultry meat and 7.5 million tons of eggs (400 million laying hens) a year (EU Parliament, 2019). A variety of antimicrobials are used on poultry in most countries (Agunos et al., 2012; Landoni and Albarellos, 2015), mostly through oral route, with the aim to prevent and treat disease, but also to enhance growth and productivity (Page and Gautier, 2012). Many of such antimicrobials are considered to be of critical importance for human medicine (WHO, 2017). Additionally, AMR in poultry pathogens is likely to cause economic losses due to the expenses on ineffective treatments, as well as due to the burden of uncured poultry disease. Because of this, the EU banned antibiotics as growth promoters in animal feed (EU Commission, 2003a).

Moreover, the Antimicrobial Advice Ad Hoc Expert Group (AMEG) of the European Medicines Agency has categorized antibiotics based on the potential consequences to public health of increased antimicrobial resistance when used in animals and on the need for their use in veterinary medicine. The categorization of antibiotics for a prudent use is intended as a tool to support decision-making by veterinarians on which antibiotic to use but does not replace treatment guidelines. There are four categories in the AMEG categorization: A (Avoid), B (Restrict), C (Caution) and D (Prudence), where the risk decreases from A to D categories (EMA, 2019). D category should be used as first option treatment if possible and only when medically needed. Antibiotics in the C category should be used only when there are no antibiotics in the D category that could be clinically effective.

Standardized and continuous surveillance programmes are necessary to monitor the occurrence of AMR in food animals (WHO, 2015; Wallmann, 2006). Indicator bacteria that can be commonly found in healthy animals are generally used to monitor AMR since. Additionally, these bacteria acquire AMR faster than other commonly found bacteria (Miranda et al., 2008; Wallmann, 2006). Commensal *Escherichia coli*, *Enterococcus* spp., and *Campylobacter* spp. are internationally used as indicator bacteria for monitoring AMR in poultry because of their common presence in the avian intestinal tract (EU Commission, 2013).

Some *Campylobacter* spp. bacteria, such as *Campylobacter jejuni*, colonize the gut of birds as commensals. The common presence of *Campylobacter* spp. that contaminate slaughterhouses and poultry products has been reported worldwide, representing a major source of human infections. In addition, the increasing number of reports on *Campylobacter* spp. antibiotic resistance (to fluoroquinolones, tetracycline, erythromycin, gentamicin) and virulence are motivating the implementation of measures against *Campylobacter* spp. (Marotta et al., 2015; Nowaczek et al., 2019).

Escherichia coli is a Gram-negative bacillus, a usual inhabitant of the digestive tract of birds, and is widely disseminated with feces. Strains from the bird's gut can contaminate poultry carcasses during slaughter (Turtura et al., 1990) and eggs become contaminated during laying (Lakhotia and Stephens, 1973). Some strains are food-borne pathogens responsible for serious human diseases worldwide. Numerous studies provide evidence for significant AMR of *E. coli* isolates from broiler chickens raised in farms, even without recorded antimicrobial use (Miles et al., 2006).

Enterococcus spp. are ubiquitous among the commensal microbiota of terrestrial vertebrates. *Enterococcus* spp. have been associated with septicemia, endocarditis, and other diseases in poultry, but certain species, such as *Enterococcus faecalis* and *Enterococcus faecium*, are opportunistic pathogens in humans. The treatment of these infections in humans can be compromised due to the transmission of AMR enterococci to humans (O'Dea et al., 2019; Rehman et al., 2018).

Table 1
Origin and number of commensal bacterial isolates.

Spanish Region	Province	*E. coli*	*Enterococcus* spp.	*Campylobacter* spp.
Castilla-La Mancha	Cuenca	15	15	
	Toledo	15	15	10
Castilla y León	León	10	10	5
Comunidad de Valencia	Alicante	10	10	
	Castellón	5	5	
	Valencia	50	50	5
Extremadura	Badajoz	25	25	
Andalucía	Cadiz	5	5	
	Huelva	10	10	
	Granada	5	5	
	Sevilla	5	5	
Región de Murcia	Murcia	40	40	5
TOTAL		195	195	25

Consequently, the poultry industry has been recognized as a source of AMR bacteria that causes disease in humans, due to the widespread presence of chicken meat and eggs in the diet of humans, and the documented wide use of antimicrobials within the poultry productive cycle. AMR poses a serious threat to animal and public health. Food from animal origin has a pivotal role in the transmission of genes and bacterial strains resistant to antimicrobials. No data on AMR profiles in commensal bacteria circulating in laying hens has been presented regarding antibiotic classes.

Because of this, the monitoring of AMR is imperative. However, much of our knowledge on the prevalence and evolution of AMR in poultry is based on the study of commensal bacteria isolated from broiler chickens. There are few data on AMR in laying hens. Therefore, the aim of this study was to investigate the prevalence of AMR and quantify multi-resistance to several antimicrobials in commensal *E. coli, Enterococcus* spp. and *Campylobacter* spp. isolates from healthy laying hens in Spain in 2018.

2. Materials and methods

2.1. Sampling

Samples were collected from 39 laying hen farms located in 12 provinces of 6 Spanish regions from April to November 2018 (Table 1). Those areas ensure 64 % of the national production of eggs. All farms had similar breeding and biosecurity/biosafety protocols. The samples were taken at one point in time during the 40–50 laying weeks in each farm according to EU Commission (2003b).

From each farm, a fecal sample of 250 g was taken from 10 randomly selected sampling points. The samples (250 g) were collected in sterile plastic containers and transported refrigerated by courier services to the laboratory. After arrival, the samples were refrigerated and processed in less than 24 h.

2.2. Isolation and molecular identification of Escherichia coli, Enterococcus spp. and Campylobacter spp. commensal isolates

Fecal samples were diluted in peptone water (1:10). Commensal *E. coli* were isolated using the chromogenic selective and differential medium Rapid'E. Coli2 (Bio-Rad) (ISO 16649-2). Three isolates per sample were analyzed by a simplex PCR assay for genus confirmation (ISO 22174: 2005).

Campylobacter spp. commensal isolates were obtained according to ISO 10272-1:2006. Fecal samples were diluted in peptone water (1:10), inoculated directly in the modified Charcoal Cefoperazone

Table 2
Antimicrobials tested for each commensal microorganism studied.

Microorganism	C category antimicrobials	D category antimicrobials
E. coli	Aminoglycosides (Gentamicin, GEN) Macrolides (Azithromycin, AZI) Amphenicols (Chloramphenicol, CHL)	Tetracyclines (Tetracycline, TET), Aminopenicillins (Ampicillin, AMP) Sulfonamides (Trimethoprim, TRI; Sulfamethoxazole, SME)
Enterococcus spp.	Aminoglycosides (Gentamicin, GEN) Macrolides (Erythromycin, ERY) Amphenicols (Chloramphenicol, CHL)	Tetracyclines (Tetracycline, TET) Aminopenicillins (Ampicillin, AMP),
Campylobacter spp.	Aminoglycosides (Gentamicin, GEN, Streptomycin, STR), Macrolides (Erythromycin, ERY)	Tetracyclines (Tetracycline, TET)

Deoxycholate Agar (mCCDA, Oxoid, UK) plates and incubated at 42 °C for 48 h in a micro aerobic environment (5 % 0_2, 10 % CO_2, 85 % N_2) (OIE, 2008) created by Campy Gen generators (Oxoid, UK). Three microscopically confirmed *Campylobacter* isolates per sample were subjected to a simplex PCR assay for genus confirmation. The primer sequence and the cyclic conditions used were according to Linton et al. (1997) for *Campylobacter* genus.

Enterococcus spp. were isolated using Billis Esculine Agar (Thermo Scientific™ CM0888B) medium and identified by PCR (Ke et al., 1999).

From each sample, five commensal PCR confirmed isolates were stored in Brain Heart Infusion broth (Bio-Rad) with 20 % glycerol at -80 °C for further analysis.

2.3. Antimicrobial susceptibility testing

The growth suspension, prepared with Tryptic Soy broth from a 24 h culture and compared with 0.5 McFarland standard, was inoculated on Mueller-Hinton broth and incubated at 37 °C for 24 h. For *Campylobacter* spp., cation-adjusted Mueller–Hinton broth supplemented with 2.5–5% lysed horse blood incubated at 37 °C for 24 h in microaerobic atmosphere was used (Ge et al., 2013).

Isolates were tested with C category (aminoglycosides, amphenicols and macrolides) and D category (aminopenicillins, sulfonamides and tetracyclines) antimicrobials (Table 2). *C jejuni* (ATCC 33560), *E coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were used as quality control organism.

Detection of antibiotic resistance of commensal isolates was carried out by determinations of the Minimum Inhibitory Concentration (MIC). The SensitreTM system (Thermo Fisher) was used to determine the MIC of the different antimicrobial compounds by broth microdilution. The reading was manual. The broth microdilution plates used were Sensititre™ EU Surveillance Salmonella/E. coli EUVSEC AST Plate for *E. coli*, Sensititre™ EU Surveillance Enterococcus EUVENC AST Plate for *Enterococcus* spp. and Sensititre™ EU Surveillance Campylobacter EUCAMP2 AST Plate for *Campylobacter* spp. (Thermo Fisher). Results were interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EFSA, 2020; EU Commission, 2013; Eucast, 2000).

The MICs at which 50 % and 90 % of the isolates are inhibited by antimicrobials are defined as MIC_{50} and MIC_{90}, respectively. Isolates were considered multidrug-resistant (MDR) when phenotypic resistance was shown to three or more antimicrobial classes (Magiorakos et al., 2012).

2.4. Data analysis

All the data collected within the present study were analyzed using SPSS software (version 16) to generate frequency and proportion values of AMR profiles.

Table 3
Minimum Inhibitory Concentration (mg/L) distributions of antimicrobials against 195 *E. coli* isolates.

EMA Category	Antimicrobial agent	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	MIC_{50}	MIC_{90}	ECOFF
C Category	**Gentamicin**	0	0	**7.69**	**15.38**	**56.41**	**8.20**	**5.13**	**6.67**	**0.51**	0	0	0	0	0	0	1	4	>2
	Nº Isolates	0	0	15	30	110	16	10	13	1	0	0	0	0	0	0			
	Azithromycin	0	0	0	0	**2.05**	**5.64**	**24.62**	**34.36**	**3.08**	**1.03**	**29.23**	0	0	0	0	8	≥64	>16
	Nº Isolates	0	0	0	0	4	11	48	67	6	2	57	0	0	0	0			
	Chloramphenicol	0	0	0	0	0	0	**22.05**	**63.08**	**10.26**	**3.08**	**1.54**	0	0	0	0	8	16	>16
	Nº Isolates	0	0	0	0	0	0	43	123	20	6	3	0	0	0	0			
	Sulfamethoxazole	0	0	0	0	0	**0.51**	**3.59**	**1.54**	**8.21**	**4.62**	**5.13**	**1.03**	**1.54**	**4.10**	**69.74**	≥1024	≥1024	>64
	Nº Isolates	0	0	0	0	0	1	7	3	16	9	10	2	3	8	136			
	Tetracycline	0	0	0	0	**12.31**	**18.46**	**5.13**	**2.05**	**2.56**	**11.28**	**48.21**	0	0	0	0	32	≥64	>8
	Nº Isolates	0	0	0	0	24	36	10	4	5	22	94	0	0	0	0			
D Category	**Trimethoprim**	0	**7.18**	**10.26**	**13.33**	**7.18**	**11.28**	**9.74**	**5.13**	**5.13**	**30.26**	**0.51**	0	0	0	0	4	≥32	>2
	Nº Isolates	0	14	20	26	14	22	19	10	10	59	1	0	0	0	0			
	Ampicillin	0	0	0	**2.05**	**7.18**	**14.87**	**36.41**	**8.72**	**1.54**	**3.59**	**25.64**	0	0	0	0	4	≥64	>8
	Nº Isolates	0	0	0	4	14	29	71	17	3	7	50	0	0	0	0			

MIC_{50}: the minimum inhibitory concentration at which 50 % of the isolates were inhibited.
MIC_{90}: the minimum inhibitory concentration at which 90 % of the isolates were inhibited.
For each drug, vertical bars show the positions of the ECOFF values (EU Commission, 2013/652/EU).

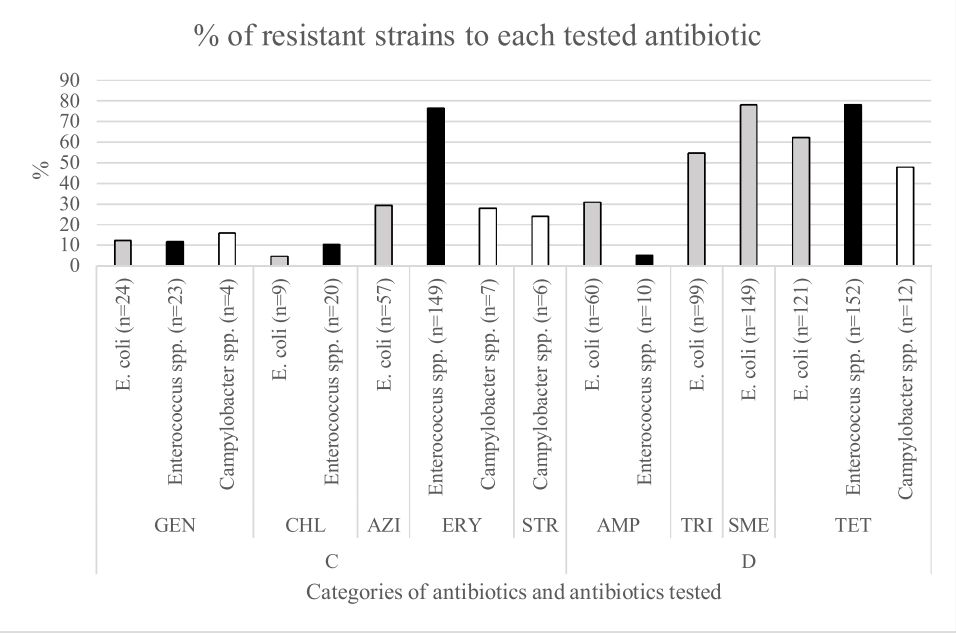

Fig. 1. Proportion of resistant isolates to each tested antibiotic for all three studied microorganisms[1].
[1]Abbreviations: Gentamicin, GEN; Azithromycin, AZI; Chloramphenicol, CHL; Erythromycin, ERY; Streptomycin, STR; Tetracycline, TET; Ampicillin, AMP; Trimethoprim, TRI; Sulfamethoxazole, SME.

3. Results

3.1. MIC distributions

The MIC distributions of the antimicrobials tested against *E. coli* isolates identified by PCR are summarized in Table 3. A very high percentage of the tested isolates showed resistance to antibiotics from the D category (sulfamethoxazole 76.41 %, tetracycline 62.05 %, trimethoprim 50.77 %, ampicillin 30.77 %). Much lower percentages of resistant isolates were found for antibiotics from the C category (azithromycin 30.26 %, gentamicin 12.31 %, chloramphenicol 4.62 %) (Fig. 1).

The MIC distributions of the antimicrobials tested against *Enterococcus* spp. isolates identified by PCR are summarized in Table 4. A very high percentage of the tested isolates showed resistance to tetracycline (78.47 %, D category) and erythromycin (76.42 %, C category). Very low percentages of resistant isolates were found for ampicillin (5.13 %, D category) and for the other C category antibiotics (gentamicin 11.80 % and chloramphenicol 10.25 %) (Fig. 1).

The MIC distributions of the antimicrobials tested against *Campylobacter* spp. isolates identified by PCR are summarized in Table 5. The antibiotic with the highest percentage of resistant isolates was tetracycline (48 %) from the D category. Regarding the antibiotics from C category, there were medium to low proportions of resistant isolates (erythromycin 28 %, streptomycin 24 %, gentamicin 16 %) (Fig. 1).

3.2. AMR pattern to antibiotics from classes C and D

Among the 195 *E. coli* isolates, 10.26 % were susceptible to all antimicrobial tested and 1.03 % were MDR to all the families of antimicrobials tested (Table 6). MDR to 3 families of antimicrobials was observed in 23.08 % of the isolates. MDR to 4 families of antimicrobials was observed in 13.85 % of the isolates.

The most common *E. coli* MDR profiles observed were ERY-SME-TET, macrolides (C category), sulfonamides (D category) and tetracyclines (D category) (10.77 %) and SME-TET-AMP, sulfonamides, tetracyclines and aminopenicillins (D category) (10.26 %).

Among the 195 *Enterococcus* spp. isolates, 11.79 % were susceptible to all antimicrobials and 5.64 % were MDR to all the antimicrobial classes tested from both C and D categories (Table 6). A high proportion of isolates (53.33 %) were resistant to 2 families of antimicrobials, and MDR to 3 families was observed in 9.23 % of the isolates (Table 6).

The most common *Enterococcus* spp. resistance profile was to macrolides (C category) and tetracyclines (D category) (ERY-TET), found in more than half of the isolates (51.79 %). 6.67 % of the isolates were MDR to macrolides, amphenicols, aminoglycosides and tetracyclines (ERY-CHL-GEN-TET).

Regarding the 25 *Campylobacter* spp. isolates, 52 % were sensitive to all and 24 % showed MDR to all three tested families of antimicrobials (aminoglycosides, C category; macrolides, C category and tetracyclines, D category) (Table 6). The most common MDR profile was GEN-STR-ERY-TET (24 %).

4. Discussion

This work investigated the AMR profile of bacteria present in healthy laying hens in Spain in 2018 regarding C category and D category antimicrobials. Presence of AMR bacteria, specific levels and profiles of resistance were identified in commensal *E. coli*, *Enterococcus* spp. and *Campylobacter* spp. isolates. The antimicrobial susceptibility data generated from commensal bacteria in this study can be a valuable indicator of AMR and antimicrobial use in Spanish laying hens.

Regarding AMR values for *Campylobacter* spp., we found a 48 % of resistant isolates to tetracycline (D category) in laying hens. This value contrasts with the 88.59 % of resistance to tetracycline of *Campylobacter* spp. isolates from broilers in Spain in 2016 (ECDC et al., 2017) but is similar to the values found in poultry at a European level, which ranged from high to very high (61.36 % in broilers) (EFSA and ECDC, 2020). This high level of antibiotic resistance is probably related to the inappropriate use of antimicrobial drugs.

The 28 % of *Campylobacter* spp. resistance to erythromycin (C category) is specially worrying, as this compound is used for the treatment of campylobacteriosis in humans (WHO, 2019). Furthermore, 16 % of the isolates showed an MIC value of 128 mg/L. Further research on this subject should be carried out to clarify the cause of this level of resistance to erythromycin in Spanish laying hens and the mechanisms involved. This high percentage of resistance adds more evidence for the

Table 4
Minimum Inhibitory Concentration (mg/L) distributions of antimicrobials against 195 *Enterococcus* spp. isolates.

EMA Category	Antimicrobial agent	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	MIC50	MIC90	ECOFF
C Category	**Gentamicin**	0	0	0	0	0	0	**32.82**	**7.69**	**37.44**	**10.26**	**1.03**	**0.51**	**1.03**	**0.51**	**8.72**	16	256	>32
	Nº Isolates	0	0	0	0	0	0	64	15	73	20	2	1	2	1	17			
	Erythromycin	0	0	0	**13.85**	**0.51**	**4.10**	**5.13**	**1.54**	**5.13**	**1.03**	**3.08**	**65.64**	0	0	0	≥128	≥128	>4
	Nº Isolates	0	0	0	27	1	8	10	3	10	2	6	128	0	0	0			
	Chloramphenicol	0	0	0	0	0	**8.21**	**2.56**	**48.72**	**26.67**	**3.59**	**2.56**	**7.69**	0	0	0	8	64	>32
	Nº Isolates	0	0	0	0	0	16	5	95	52	7	5	15	0	0	0			
	Tetracycline	0	0	0	**15.90**	**2.56**	**2.56**	**0.51**	**3.08**	**1.03**	**6.67**	**15.38**	**52.31**	0	0	0	≥128	≥128	>4
	Nº Isolates	0	0	0	31	5	5	1	6	2	13	30	102	0	0	0			
D Category	**Ampicillin**	0	0	**9.74**	**2.56**	**32.31**	**28.21**	**22.05**	**4.10**	**1.03**	0	0	0	0	0	0	2	4	>4
	Nº Isolates	0	0	19	5	63	55	43	8	2	0	0	0	0	0	0			

MIC_{50}: the minimum inhibitory concentration at which 50 % of the isolates were inhibited.
MIC_{90}: the minimum inhibitory concentration at which 90 % of the isolates were inhibited.
For each drug, vertical bars show the positions of the ECOFF values (EU Commission, 2013/652/EU).

urgency of increasing AMR monitoring in laying hens.

Regarding *E. coli* findings for D category antimicrobials, 76.41 % of the isolates showed resistance to the D category antibiotic sulfamethoxazole. According to EFSA and ECDC (2020), *E. coli* resistance to sulfamethoxazole in Spain was 33.53 % in poultry in 2018, and 43.72 % at the EU level. Trimethoprim resistance values were much higher than those found in broilers. We detected 50.77 % compared to 24.71 % reported in Spain and 34.57 % in the EU. Since 2015, the consumption of sulfonamides and trimethoprim in Spain is among the highest in the EU, which justifies the development of resistance to these antibiotics in the microbial populations living under this selection pressure (ECDC, 2017).

The proportion of resistant isolates to tetracycline (62.05 %) was very similar to what found in Canadian avian isolates by Agunos et al. (2012) and was relatively close to the 50.59 % identified in Spain by EFSA and ECDC (2020), although it differed from the EU mean value (40.34 %). At the European level, other studies identified lower proportions of resistant isolates in Norway (27.7 %) (Kaspersen et al., 2018) and between 40–60 % in France (Roth et al., 2019) and Poland (Wasyl et al., 2013). Ampicillin (D category) had the lowest resistance value, being 30.77 % in this study, compared to the 50 % identified in Spanish broilers and 52.08 % at the EU level.

Regarding *E. coli* resistance to C category antimicrobials, the most significant finding was a value of 30.26 % of resistant isolates to azithromycin. This antimicrobial is recognized by the WHO as important for the treatment of human infections (WHO, 2019). The values of resistance found to this antimicrobial in 2018 by EFSA and ECDC (2020) in broilers (4.12 % for Spain and 2.59 % for the EU) are much lower than what found in this study in laying hens. Resistance to azithromycin in food-producing animals is attributed to resistance to other macrolides of veterinary use, as azithromycin is not used in animals (EFSA and ECDC, 2020).

Gentamicin and chloramphenicol (C category) showed lower values in laying hens (12.31 % and 4.62 % respectively) than what reported by Spain in 2018 for broilers (23.53 % and 11.76 % respectively). The AMR values found for these antimicrobials in laying hens were similar to values in European broilers (7.06 % for gentamicin and 13.90 % for chloramphenicol) (EFSA and ECDC, 2020).

MDR levels in *E. coli* isolated from laying hens are similar to what found in Spanish broilers. In layers, 23.08 % of isolates were MDR to 3 families of antimicrobials and 13.85 % were resistant to 4 families. The total MDR value was close to the 49.41 % of MDR found in Spanish broilers in 2018 (EFSA and ECDC, 2020). The resistance patterns found were also similar, including macrolides (C category), sulfonamides, tetracyclines and aminopenicillins (D category). The proportion of *E. coli* isolates susceptible to all antimicrobials tested was 10.26 % in laying hens, which was higher than the value for Spanish broilers (7.06 %) but lower than the average EU value (22.88 %).

Much less data is available at the EU level for *Enterococcus* spp. in poultry, despite it being included in the EU Commission Decision 2013/652/EU (2013). Regarding our findings for *Enterococcus* spp. in laying hens, our study showed a high percentage of resistance to tetracycline (78.47 %) and erythromycin (76.42 %), and low resistance to gentamicin (11.80 %), chloramphenicol (10.25 %) and ampicillin (5.13 %). More than half of the *Enterococcus* spp. isolates (51.79 %) were resistant to both tetracyclines and macrolides. These levels of AMR were lower than those reported in poultry by Van den Bogaard et al. (2002) in the Netherlands, where higher percentages of resistant isolates were found (oxytetracycline 98 %, erythromycin 98 %, gentamicin 40 %). Similar results were obtained by de Jong et al. (2019), where very high resistance to tetracycline and erythromycin was reported for *E. faecium* and *E. faecalis* in chickens.

The dissemination of resistant enterococci from animals to humans and the exchange of resistance genes between poultry and human enterococci has been demonstrated (Van den Bogaard et al., 2002). During the last decade, enterococci have emerged as an important cause of nosocomial infections in hospitals. Increasing attention is paid to the

Table 5
Minimum Inhibitory Concentration (mg/L) distributions of antimicrobials against 25 *Campylobacter* spp. isolates.

EMA Category	Antimicrobial agent	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	MIC_{50}	MIC_{90}	ECOFF
C Category	**Gentamicin**	**56**	**0**	**12**	**8**	**0**	**8**	**4**	**0**	**12**	0	0	0	0	0	0	≤0.125	≥16	>2
	Nº Isolates	14	0	3	2	0	2	1	0	3	0	0	0	0	0	0			
	Streptomycin	0	**52**	**0**	**0**	**12**	**4**	**8**	**4**	**20**	0	0	0	0	0	0	≤0.25	≥16	>4
	Nº Isolates	0	13	0	0	3	1	2	1	5	0	0	0	0	0	0			
	Erythromycin	0	0	0	**72**	**0**	**0**	**0**	**4**	**8**	**0**	**0**	**16**	0	0	0	≤1	≥128	>4
	Nº Isolates	0	0	0	18	0	0	0	1	2	0	0	4	0	0	0			
D Category	**Tetracycline**	0	0	**52**	**0**	**0**	**0**	**8**	**0**	**0**	**8**	**32**	0	0	0	0	≤0.5	≥64	>1
	Nº Isolates	0	0	13	0	0	0	2	0	0	2	8	0	0	0	0			

MIC_{50}: the minimum inhibitory concentration at which 50 % of the isolates were inhibited.
MIC_{90}: the minimum inhibitory concentration at which 90 % of the isolates were inhibited.
For each drug, vertical bars show the positions of the ECOFF values (EU Commission, 2013/652/EU). When different ECOFF values for different *Campylobacter* spp. species were established in the EU Commission, 2013/652/EU (2013), the lower value among them was used.

Table 6
Resistance profiles observed.

Resistance	Microorganism	Nº of isolates	%	Resistance profile	Nº of isolates	%
Pan-sensitive	*E. coli*	20	10.26	–	20	10.26
	Enterococcus spp.	23	11.79	–	23	11.79
	Campylobacter spp.	13	52	–	13	52
Resistant to 2	*E. coli*	45	23.08	Sulfonamides Tetracyclines	29	14.87
				Tetracyclines Aminopenicillins	5	2.56
				Sulfonamides Aminopenicillins	7	3.59
	Enterococcus spp.	104	53.33	Aminoglycosides Sulfonamides	4	2.05
				Aminopenicillins Tetracyclines	1	0.51
				Aminoglycosides Tetracyclines	1	0.51
				Macrolides Tetracycline	101	51.79
	Campylobacter spp.	2	8	Macrolides Aminopenicillins	1	0.51
				Macrolides Tetracyclines	1	4
				Aminoglycosides Tetracyclines	1	4
Resistant to 3	*E. coli*	45	23.08	Sulfonamides Tetracyclines Aminopenicillins	20	10.26
				Amphenicols Sulfonamides Aminopenicillins	1	0.51
				Macrolides Tetracyclines Aminopenicillins	3	1.54
	Enterococcus spp.	18	9.23	Macrolides Sulfonamides Tetracyclines	21	10.77
				Amphenicols Aminoglycosides Tetracyclines	1	0.51
				Macrolides Aminopenicillins Tetracyclines	6	3.08
				Macrolides Aminoglycosides Tetracyclines	6	3.08
	Campylobacter spp.	6	24	Macrolides Amphenicols Tetracyclines	4	2.05
				Macrolides Amphenicols Aminoglycosides	1	0.51
				Macrolides Aminoglycosides Tetracyclines	6	24
Resistant to 4	*E. coli*	27	13.85	Amphenicols Sulfonamides Tetracyclines Aminopenicillins	2	1.03
				Macrolides Sulfonamides Tetracyclines Aminopenicillins	10	5.13
				Macrolides Amphenicols Sulfonamides Tetracyclines	2	1.03
	Enterococcus spp.	13	6.67	Aminoglycosides Macrolides Sulfonamides Tetracyclines	13	6.67
				Macrolides Amphenicols Aminoglycosides Tetracyclines	13	6.67
Resistant to 5	*E. coli*	7	3.59	Macrolides Amphenicols Sulfonamides Tetracyclines Aminopenicillins	2	1.03
				Aminoglycosides Macrolides Sulfonamides Tetracyclines Aminopenicillins	5	2.56
	Enterococcus spp.	1	0.51	Macrolides Amphenicols Gentamicin Aminopenicillins Tetracyclines	1	0.51
Resistant to 6	*E. coli*	2	1.03	Aminoglycosides Macrolides Amphenicols Sulfonamides Tetraciclines Aminopenicillins	2	1.03

rising numbers of vancomycin-resistant or multi-resistant enterococci, as vancomycin is used to treat human infections caused by multi-resistant *Enterococcus* spp. (Arias et al., 2010).

5. Conclusions

Our data show that worrisome levels of AMR to the C and D EMA categories of antimicrobials (EMA, 2019) can be found in Spanish laying hens farms. These high AMR levels are significant to both farmers and veterinarians and should be taken into account when choosing the antimicrobial and dose needed to treat pathologies in laying hens. Therefore, treatment options at the farm level could be severely affected by AMR. Our results are also relevant in a Public Health context, as bacteria found in laying hens represent a source of AMR that could reach pathogenic microorganisms that affect humans, such as pathogenic *E. coli* and *Campylobacter* spp.

A more detailed monitoring of the laying hens sector is needed, as significant values of AMR were found. The information available for the poultry sector was not representative for laying hens regarding the levels of AMR and resistance to antibiotics of special importance, such as erythromycin resistance in *Campylobacter* spp. or azithromycin resistance in *E. coli*. Further research is needed on AMR levels in laying hens at the EU level and on the risk it poses to humans through egg consumption, direct contact and environmental spread of AMR genetic material and bacteria from laying hens farms. A method of sampling more representative of the AMR situation in the laying hens Spanish productive sector, such as those carried out for other productive sectors (EFSA and ECDC, 2020), should be used to increase the representativeness of the samples in order to implement measures according to the results. Finally, laying hens should be included in programmes for the reduction of antibiotic use in animal production, such as the Spanish PRAN programme (2020), as AMR is widely present in this sector, as shown by the results of this study.

Author's contributions

MJC and CM designed the study and carried out the laboratory analysis. JR, PM, JSG and MJC edited the manuscript. JO and JR performed the data analysis. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements

We would like to thank the farmers for providing samples and participating in this study as well as the veterinarians that facilitated sample collection and transport to the laboratory for processing. This research was carried out within the Project RTA2015-00075-C04-04 "Reduction of the use of antibiotics in intensive livestock production: comprehensive program for management and application of alternative products" granted by the Spanish Ministry of Economy, Industry and Competitiveness (2015-2018), "Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016, Programa Estatal de I + D+I Orientada a los Retos de la Sociedad".

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.105222.

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