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Prevalence, quantification and antibiotic resistance of *Listeria monocytogenes* in poultry preparations

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ABSTRACT

A total of 100 samples of fresh poultry preparations were obtained from 10 retail outlets in North-Western Spain. *Listeria* spp. were found in 73 samples. Isolates were identified through polymerase chain reaction (PCR) as *Listeria monocytogenes* (56 samples), *Listeria innocua* (32), *Listeria grayi* (3), *Listeria seeligeri* (1) and *Listeria* spp. (6). In 24 samples, several different *Listeria* species were found. The loads of *L. monocytogenes* detected by quantitative polymerase chain reaction (q-PCR) in the 56 positive samples ranged from <2.15 log units (limit of detection) up to 5.96 log units. Neither the type of sample nor the retail outlet involved had any significant influence ($P > 0.05$) on concentrations of *L. monocytogenes*. A total of 163 *L. monocytogenes* isolates were tested (disc diffusion) against 15 antimicrobials of clinical significance. The average number of resistances per isolate was 5.83 ± 1.64. All strains showed resistance to multiple antimicrobials (between 4 and 11). In all, 80 isolates (49.1%) showed a multi-drug resistant (MDR) phenotype, and two isolates (1.2%) showed an extensively drug-resistant (XDR) phenotype. More than 50.0% of isolates showed resistance or reduced susceptibility to oxacillin, cefoxitin, cefotaxime, cefepime, rifampicin, ciprofloxacin, enrofloxacin or nitrofurantoin. This is a cause for concern because these substances are among the antibiotics used to treat human listeriosis, with rifampicin and fluoroquinolones frequently being used. The results from this research work show that poultry preparations are a potential major source of resistant *L. monocytogenes* strains, since these are present in some samples at high concentrations. This highlights the pressing need to handle poultry preparations correctly, so as to ensure they are sufficiently cooked and to avoid cross-contamination events.

1. Introduction

The worldwide production of poultry is second only to that of pork. In 2017, output was 109.1 million tonnes of poultry and 119.9 million tonnes of pork. The consumption of these two types of meat in the European Union in 2013 was 39.0 kg of pig products and 22.5 kg of poultry, principally chicken, per person per year (FAOSTAT, 2020). In Spain, poultry is the most widely eaten meat, accounting for 37.7% of the overall volume of fresh meat (MAPA, 2020).

Over recent years, saving time in the preparation of food has become a priority for most households (Buzón-Durán, Capita, & Alonso-Calleja, 2017; Selvan, Narendra Babu, Sureshkumar, & Venkataramanujam, 2007), which has triggered growth in the consumption of meat preparations. In Spain, this type of foodstuff constitutes 24.8% of the total consumption of meat (MAPA, 2020). Meat preparations are defined as fresh meat, including meat that has been reduced to fragments, which has had foodstuffs, seasonings, or additives added to it or which has undergone processes insufficient to modify the internal muscle fibre structure of the meat and thus to eliminate the characteristics of fresh meat (Capita et al., 2020).

The considerable consumption of poultry and poultry preparations makes it crucial for these foodstuffs to be safe for consumers (Del Río, Panizo-Morán, Prieto, Alonso-Calleja, & Capita, 2007). Contamination of meat with pathogenic microorganisms can be a cause of food-borne illness, whether as an outcome of insufficient cooking or through cross-contamination from other foodstuffs. Keeping raw poultry preparations under refrigeration does not hinder the multiplication of psychrotrophic microorganisms, of which *Listeria monocytogenes* is an example. This bacterium is able to multiply at temperatures close to 0 °C, and can increase its concentration over the course of storage

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(Rodríguez-Campos, Rodríguez-Melcón, Alonso-Calleja, & Capita, 2019; Zhang, Wu, & Guo, 2016).

Listeria monocytogenes is the etiological agent of listeriosis, a foodborne zoonosis that causes every year 23,000 cases of invasive human infections worldwide (Rodríguez-Campos et al., 2019). Moreover, it is the illness transmitted by foodstuffs with the highest level of lethality, reaching 17.6% in the European Union in 2019 (EFSA-ECDC, 2021). The classic methods for enumerating or detecting *L. monocytogenes* in food do not allow results to be obtained until some 24–48 or 72–96 h have elapsed, respectively. An alternative for rapid detection of this microorganism, also permitting its quantification, is quantitative (real-time) polymerase chain reaction or q-PCR. However, whilst there are numerous publications on the prevalence of *L. monocytogenes* in food, there has been little research aimed at its quantification, especially in raw poultry, and this sort of studies are lacking in North-Western Spain (Jandaghi, Seno, Farzin, & Mohsenzadeh, 2020; Rørvik & Yndestad, 1991; Sugiri et al., 2014).

There is at present considerable concern about the increase in the prevalence of bacteria resistant to antibiotics, this constituting a critical problem worldwide (Capita & Alonso-Calleja, 2013). In the past, *L. monocytogenes* has usually been sensitive to the majority of antibiotics of clinical interest used to treat infections by Gram-positive bacteria. However, in recent years a growth has been observed in the prevalence of resistance in this bacterial species, principally in strains isolated from foodstuffs (Komora, Bruschi, Magalhães, Ferreira, & Teixeira, 2017; Olaimat et al., 2018). Monitoring of antibiotic resistance, both in the food system and in clinical contexts, allows the identification of trends in the prevalence of resistance, as well as permitting the planning and evaluation of strategies to prevent its spread (Capita, Felices-Mercado, García-Fernández, & Alonso-Calleja, 2019).

The aims of this research were 1) to know the prevalence of *Listeria* spp. and *L. monocytogenes* in poultry preparations from North-Western Spain, 2) to determine the levels of *L. monocytogenes* in such foodstuffs, and 3) to establish the patterns of resistance to antibiotics in the strains of *L. monocytogenes* isolated.

2. Material and methods

2.1. Samples

A total of 100 samples, each weighing approximately 200 g, of raw poultry preparations (chicken and turkey) were obtained from 10 retail outlets in the city of León, with between 4 and 23 samples being taken from each shop. The samples analysed included breaded breast fillets stuffed with ham and cheese, a form of chicken cordon bleu, known in Spain as a "*San Jacobo*" (13 samples), other breaded poultry products, known in Spain as "*flamenquines*" (5), strips of meat, or "*fajitas*" (5), ground patties (4), hamburgers (16), marinated wings (8), meatballs (9), minced meat (7), nuggets (12), "creole" (spiced) sausages (3), red sausages (5), white sausages (8) and skewers (5). All products were prepared at the retail outlets and they sold unpacked. The samples were taken individually to the laboratory, where they were processed within a maximum of 1 h after arrival.

2.2. Isolation and identification of Listeria spp.

In detecting *Listeria* spp. and *L. monocytogenes* the UNE-EN ISO 11290-1 method was used. From each sample, 25 g were taken and homogenized using a Stomacher (IUL Instruments, Barcelona, Spain) in 225 ml of half-Fraser broth for 120 s. The homogenate was incubated for 24 h at 30 °C, and thereafter amounts of 100 μl were transferred to test-tubes with 10 ml of Fraser broth, these being incubated at 37 °C for 24 h. After this time had elapsed, they were streaked onto plates with Oxoid Chromogenic *Listeria* Agar (OCLA) medium, these being incubated for 48 h at 37 °C. From each positive sample three colonies with typical characteristics of *Listeria* spp. (green colonies), three colonies of *L. monocytogenes* (green colonies with a halo), or both, were taken and inoculated in tryptone soya broth (TSB) for later identification. Cultures were stored at −50 °C in 20% glycerol. All the media utilized in this research were obtained from Oxoid Ltd. (Hampshire, United Kingdom).

Identification of isolates was carried out using conventional polymerase chain reaction (PCR) to detect the genes *lmo1030* (*L. monocytogenes*), *lin0464* (*L. innocua*), *oxidoreductase* (*L. grayi*), *lmo0333* (*L. seeligeri*) and *prs* (*Listeria* spp.), as indicated in Table 1 (Ryu et al., 2013). To extract deoxyribonucleic acid (DNA), 20 μl of each strain, kept frozen, were inoculated into test-tubes holding 9 ml of TSB, which were then incubated for 24 h at 37 °C. After this period had elapsed, DNA was extracted from 1.5 ml of the culture by carrying out two cycles of centrifugation at 13,000 rpm for 60 s, and leaving it in a water bath at 100 °C for 30 min. The purity and concentration of the DNA were determined with a Nano-Drop One spectrophotometer (Thermo Scientific, Wilmington, Delaware, United States), a wavelength of 260 nm being used. Those samples whose DNA concentration fell in the range of 80 ng/μl to 180 ng/μl were deemed useable.

In amplifying samples, use was made of 5 μl of DNA, reaction buffer at a 1 × concentration (EUR_X Sp. z o.o., Gdansk, Poland), $MgCl_2$ at a concentration of 3 mM (EURx), a mix of deoxynucleotide triphosphates (dNTPs) each with a concentration of 0.2 mM (EURx), primers at 0.5 μM each (Isogen Life Science, Barcelona, Spain), 1.25 U of Taq DNA polymerase (BIORON GmbH, Ludwigshafen, Germany) and sterile Milli-Q water to make up a final volume of 25 μl. Trials included negative (samples lacking DNA) and positive (previously identified strains of *Listeria* spp.) controls.

All amplification reactions took place in a thermocycler manufactured by Bio-Rad (Hercules, California, United States). This was programmed as follows: denaturation at 94 °C for 5 min, followed by 35 amplification cycles (denaturation for 30 s at 94 °C, annealing for 30 s and elongation at 72 °C for 45 s), followed by a final elongation period of 5 min at 72 °C.

The amplification products were separated by horizontal electrophoresis on agarose gel (BIORON) at 1.0% in 1 × tris-acetate-EDTA buffer stained with SimplySafe (EURx) diluted to 1:10.000. For visualization an ultra-violet transilluminator (Gel Doc EZ System, Bio-Rad) was used. The amount of each PCR product was estimated using markers with a standard molecular weight (Perfect Plus 1 kb DNA Ladder, EURx).

2.3. Quantification of L. monocytogenes through q-PCR

In order to quantify the *L. monocytogenes* cells, 25 g from each sample were homogenized with 225 ml of 0.1% peptone water. DNA was extracted from this homogenate using the commercial protocol PrepSEQ™ Rapid Spin Sample Preparation Kit with Proteinase K (Thermo Fisher Scientific, Waltham, Massachusetts, United States). For this purpose, 750 μl of homogenate were loaded into an extraction column, which was centrifuged at 13,000 rpm for 3 min. The column and supernatant were discarded, while the pellet was re-suspended in 50 μl of lysis buffer with proteinase K, incubated for 30 min at 56 °C in a thermal block, and thereafter at 97 °C for 12 min, so as to deactivate the proteinase K. After centrifuging at 13,000 rpm for 60 s and the addition of 250 μl Milli-Q water (to make up a total volume of 300 μl), there was a further centrifuging at 13,000 rpm for 120 s, with the DNA ending up suspended in the aqueous phase.

Amplification by means of q-PCR was performed using the commercial product MicroSEQ™ *Listeria monocytogenes* Detection Kit (Thermo Fisher Scientific). In doing this, 30 μl of the sample with DNA were placed in each reaction tube, which was then shaken to achieve blending with the freeze-dried master mix in the bottom of the tube. The amplification reaction took place in a StepOne™ thermal cycler (Applied Biosystems, Foster City, California, United States), a fluorescence threshold of 0.3 being set. To transform the results of the amplification into a quantity of DNA a standard straight line was used ($y =$

Table 1
Genes and primers utilized in identifying *Listeria* spp. through PCR.

Species	Gene	Primer	Secuence (5′ → 3′)	Annealing T[a] (°C)	Product size (bp)
L. monocytogenes	*lmo1030*	Lmo1030-F	GCTTGTATTCACTTGGATTTGTCTGG	62	509
		Lmo1030-R	ACCATCCGCATATCTCAGCCAACT		
L. innocua	*lin0464*	Lin0464-F	CGCATTTATCGCCAAAACTC	60	749
		Lin0464-R	TCGTGACATAGACGCGATTG		
L. grayi	*oxidoreductase*	JOgrayi-F	GCGGATAAAGGTGTTCGGGTCAA	62	201
		JOgrayi-R	ATTTGCTATCGTCCGAGGCTAGG		
L. seeligeri	*lmo0333*	Lseelin-F	GTACCTGCTGGGAGTACATA	58	673
		Lseelin-R	CTGTCTCCATATCCGTACAG		
Listeria spp.	*prs*	Prs-F	GCTGAAGAGATTGCGAAAGAAG	58	370
		Prs-R	CAAAGAAACCTTGGATTTGCGG		

-3.0525 x + 23.206; $R^2 = 0.966$), this being obtained on the basis of pattern samples having known quantities of *L. monocytogenes* DNA (Fig. 1).

The quantity of DNA was extrapolated to $\log_{10}$ cfu/g in the sample of food, taking into account the size of the genome of *L. monocytogenes* (Glaser et al., 2001). It was established that 1 ng of DNA equated to approximately 340,000 cfu. Calculations were performed on the basis of the following equation:

$$L.\ monocytogenes\ concentration\left(Log_{10}\frac{cfu}{g}\right) = Log_{10}\left(\frac{10^{\frac{Ct-23.206}{-3.0525}} \times 340,000 \times 10^5}{750}\right) cfu \Big/ g$$

In establishing this equation, various items were taken into account. These were: 1) the total volume of the homogenization bag (250 ml, or 250,000 μl), 2) the decimal dilution performed to produce the homogenate (25 g of sample in 225 ml of diluent), 3) the fact that the reaction tube receives one-tenth of the total amount of DNA extracted (30 μl out of 300 μl) and 4) DNA was extracted solely from 750 μl.

2.4. Resistance to antibiotics

Three isolates of *L. monocytogenes* from each positive sample were taken from the OCLA medium and identified using PCR, and their susceptibility to a panel of 15 antibiotics of clinical importance was determined. A disc diffusion method, as described by the Clinical and Laboratory Standards Institute (CLSI, 2013), was used. The isolates kept frozen were inoculated into tubes with Mueller Hinton broth (MHB), these being incubated for 6 h at 37 °C. Thereafter, they were inoculated onto Mueller Hinton agar (MHA) plates using a spread plate technique, and then the antibiotic discs were placed (5 antibiotics per plate).

The following antibiotic discs (Oxoid) were used: ampicillin (AMP, 10 μg), oxacillin (OX, 1 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), gentamycin (CN, 10 μg), erythromycin (E, 15 μg), vancomycin (VA, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), rifampicin (RD, 5 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 5 μg) and nitrofurantoin (F, 300 μg). After incubation at 37 °C for 18–24 h, the inhibition halos were measured and the strains were classified as

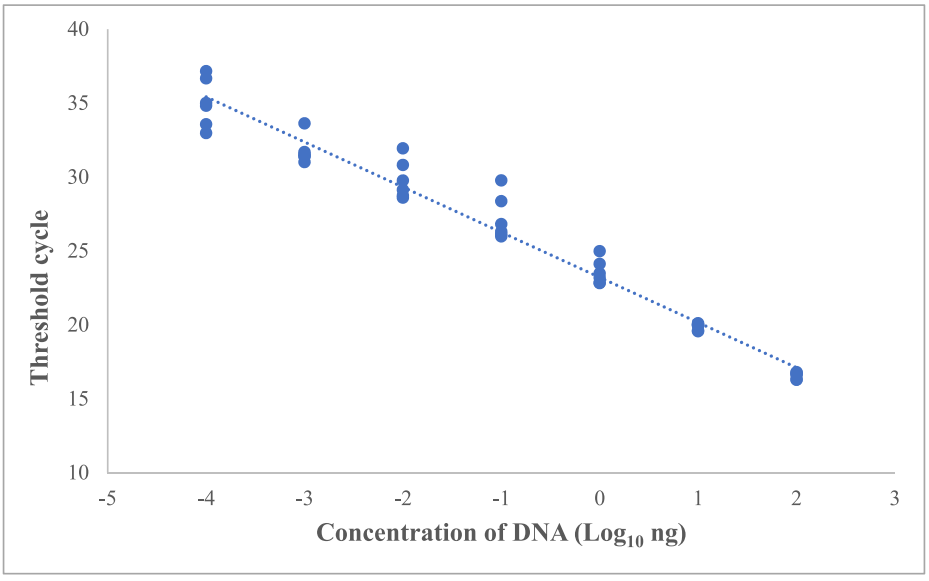

Fig. 1. Standard straight line. Known quantities of *Listeria monocytogenes* DNA act as abscissas: 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng and 0.0001 ng. On the ordinate axis the number of cycles corresponding to each of these quantities of DNA are shown.

susceptible, with reduced susceptibility, or resistant, in accordance with the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) for E, SXT (*L. monocytogenes*), CN, RD, TE, C, CIP (*Staphylococcus* spp.), CTX, FEP (*Streptococcus* spp.) and VA (*Enterococcus* spp.), of the CLSI (2018a) in the case of OX, FOX, F (*Staphylococcus* spp.), AMP (*Enterococcus* spp.) and the VET08 norms from the CLSI (2018b) for ENR (*Staphylococcus* spp.).

A group of international experts set up under a joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) in the United States established a standard definition for phenotypes seen as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR) in bacteria of interest for Public Health. The MDR phenotype is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, with one or more antibiotics from each category being applied. The XDR phenotype is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories, so that the bacterial isolates remain susceptible to only one or two categories. Finally, the PDR phenotype refers to a lack of susceptibility affecting all agents in all antimicrobial categories (Magiorakos et al., 2012). These criteria were utilized to characterize the profile of resistance to antibiotics of the strains tested in the present research.

2.5. Statistical analysis

The prevalence data for *Listeria* spp. and *L. monocytogenes*, as also their resistance to antibiotics, were analysed using exact Chi-squared tests. The quantification results were compared by means of an analysis of variance (ANOVA), after their normality had been checked with a Shapiro-Wilk test. In making multiple comparisons, a Tukey Kramer test was utilized. When a sample proved to be positive for *L. monocytogenes* with OCLA-PCR and do not surpassed the fluorescence threshold in q-PCR before 40 cycles of amplification, this number of cycles (detection limit, corresponding to -5.50 $\log_{10}$ ng DNA in the reaction tube or 2.15 $\log_{10}$ cfu/g of sample) was used in carrying out statistical analyses. In all instances, significant differences were set at a probability level of 95% ($P < 0.05$). All statistical analyses were performed with RStudio software (RStudio RTeam, 2019).

3. Results

3.1. Prevalence of Listeria spp. and Listeria monocytogenes

In 73 of the 100 samples of poultry preparations studied (73.0%) *Listeria* spp. were detected. The isolates were identified by PCR (Fig. 2), and it was noted that 56 samples were contaminated with *L. monocytogenes*, 32 with *L. innocua*, 3 with *L. grayi* and one with *L. seeligeri*. The strains isolated from 6 of the samples could not be identified at species level. A total of 24 samples were contaminated with more than one species of *Listeria*: *L. monocytogenes* and *L. innocua* (21 samples), *L. monocytogenes* and *L. grayi* (2), and *L. monocytogenes*, *L. grayi and L. seeligeri* (1).

Figs. 3 and 4 show the prevalence of *Listeria* spp. and *L. monocytogenes* as a function of the type of product and the retail outlet from which the sample was acquired. With regard to the type of meat preparation, significant differences were noted both for *Listeria* spp. ($X^2 = 89.00$; $P = 7.70 \times 10^{-14}$) and for *L. monocytogenes* ($X^2 = 134.43$; $P = 2.20 \times 10^{-16}$). In the case of *Listeria* spp., the samples with the lowest prevalence were the "*San Jacobo*" stuffed breast fillets (30.8%), and the skewers (50.0%), whilst those with the highest percentage of positives (100%) were the marinated wings, the "creole" saussages and the red sausages. In respect of *L. monocytogenes*, the samples with the lowest prevalence were the "*San Jacobo*" stuffed breaded fillets (30.8%) and those with the highest, the "creole" sausages (100%). No *L. monocytogenes* was found in any sample of skewers.

As for the outlets from which samples were procured, there were also differences in prevalence for *Listeria* spp. ($X^2 = 93.01$; $P = 4.04 \times 10^{-16}$) and for *L. monocytogenes* ($X^2 = 134.32$; $P = 2.20 \times 10^{-16}$). The establishments with the lowest prevalence of *Listeria* spp. were E4 (25.0%) and E5 (30.0%), whilst those with the highest percentages of positive samples were E3 (100%) and E6 (100%). For *L. monocytogenes*, the outlet with the lowest prevalence was establishment E4 (12.5%), whilst E8 (91.7%) and E10 (87.0%) were the outlets with the greatest percentages of the microorganism isolated from their samples.

3.2. Quantification of Listeria monocytogenes

Table 2 shows the results of quantification of *L. monocytogenes* in the 56 samples of poultry preparations that were positive in the OCLA medium. The concentration of bacteria was calculated keeping in mind the standard straight line established and the equivalence between ng of DNA in the reaction tube and cfu/g of sample. Samples with *L. monocytogenes* detected in OCLA medium but which did not amplify in q-PCR (6 in total), were considered positive, but with a concentration of the microorganism lower than the detection limit of q-PCR (40 cycles, equivalent to 2.15 $\log_{10}$ cfu/g). The maximum estimated level of contamination was 5.96 $\log_{10}$ cfu/g, in a sample of ground patties.

Fig. 5 shows an example of the results obtained. A comparison of this image with the data in Table 2 makes it possible to observe how the samples with a higher concentration of *L. monocytogenes* have a lower figure for Ct, since they needed fewer cycles to exceed the threshold value stipulated (0.3).

Neither the type of sample ($F = 1.46$; $P = 0.18$) nor the establishment involved ($F = 2.87$; $P = 0.10$) influenced the results obtained. The concentration of *L. monocytogenes* ranged between 3.19 ± 1.14 $\log_{10}$ cfu/g for the meatballs and 5.30 ± 0.92 $\log_{10}$ cfu/g for the ground patties (Fig. 6). In respect of the establishment where procured, concentrations of *L. monocytogenes* varied from 3.06 ± 0.68 $\log_{10}$ cfu/g (E7)

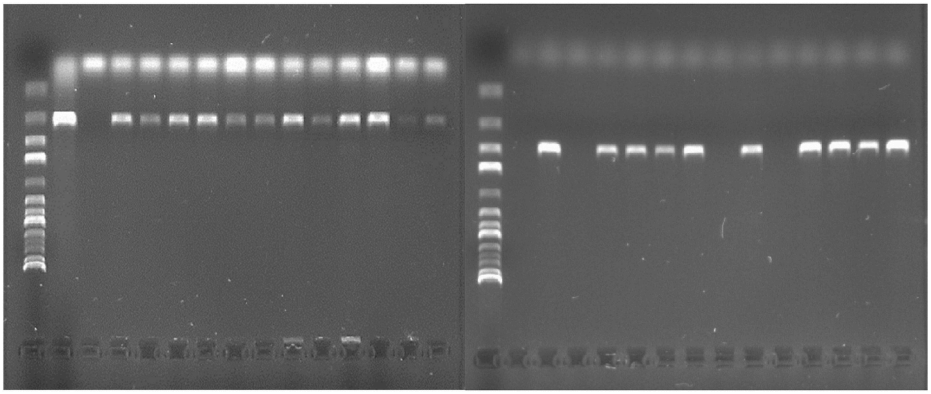

Fig. 2. Agarose gel electrophoresis of PCR products. Left: agarose gel (1.0%) on which may be observed, from left to right, the size marker (from 0.25 kb to 10 kb), a positive control, a negative control, and the results of amplifying 12 samples positive for *Listeria monocytogenes* (*lmo1030* gene). Right: agarose gel (1.0%) on which may be seen, from left to right, the size marker (from 0.25 kb to 10 kb), a negative control, a positive control, and the results of amplifying twelve samples, nine positive and three negative, for *Listeria innocua* (*lin0464* gene).

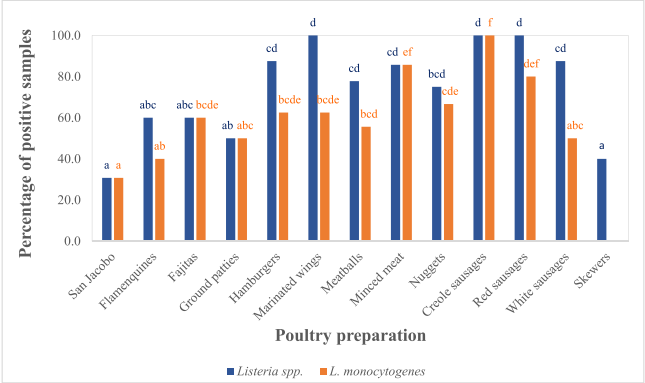

Fig. 3. Percentage of samples positive for *Listeria* spp. and *Listeria monocytogenes* as a function of the type of product under consideration. Columns that do not share any letter for the same microbial group show significant differences one from another ($P < 0.05$).

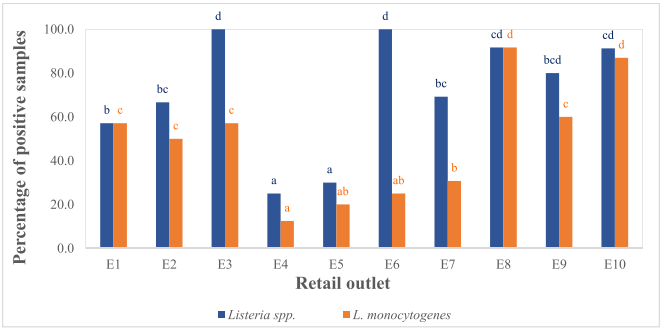

Fig. 4. Percentage of samples positive for *Listeria* spp. and *Listeria monocytogenes* as a function of the outlet involved. Columns that do not share any letter for the same microbial group show significant differences one from another ($P < 0.05$).

to 3.99 ± 1.12 $\log_{10}$ cfu/g (E10). Outlets E4 (<2.15 $\log_{10}$ cfu/g) and E6 (4.28 $\log_{10}$ cfu/g) were excluded from analysis, as they each had just one positive sample (Fig. 7).

3.3. Resistance to antibiotics of Listeria monocytogenes

A total of 163 colonies of *L. monocytogenes* were isolated from 56 samples of poultry preparations. These were tested to determine their susceptibility to a panel of 15 antibiotics of clinical importance. All the isolates presented multiple resistances, to between 4 and 11 antibiotics (Fig. 8). No significant differences were found in the number of antibiotics to which strains were resistant either between type of sample ($X^2 = 83.99$; $P = 0.27$), or as a function of the outlet where a given sample had been acquired ($X^2 = 73.32$; $P = 0.17$).

Taking all the isolates and antibiotics tested together, resistance was found in 38.9% of cases, reduced susceptibility in 12.2% and susceptibility in 48.9%. An average of 5.83 ± 1.64 resistances per isolate was observed. This value rose to 7.66 ± 1.52 when resistant isolates were bundled together with those having reduced susceptibility.

Table 3 indicates the patterns of resistance encountered. There were three main phenotypes present: OX-FOX-CTX-FEP was shown by 35 isolates, OX-FOX-CTX-FEP-RD by 14, and OX-FOX- CTX-FEP-RD-CIP by 12. The remaining patterns of resistance were each exhibited by between one and nine isolates.

This work trialled ten categories of antibiotics: beta-lactams (AMP, OX, FOX, CTX and FEP), aminoglycosides (CN), macrolides (E), glycopeptides (VA), sulphonamides (SXT), rifamycins (RD), tetracyclines (TE), phenicols (C), fluoroquinolones (CIP, ENR) and nitrofurans (F). No PDR strains were found, nor any resistant to just one antibiotic. Thirty-five isolates (21.5% of the total) demonstrated resistance to four antibiotics, 43 isolates (26.4%) to five antibiotics, three isolates (1.8%) to six antibiotics, 80 isolates (49.1%) presented a MDR phenotype, being resistant to between 3 and 7 categories of antibiotics, and two isolates (1.2%) were of a XDR phenotype, having resistance to eight categories of antibiotics. The strains of MDR phenotype evinced resistance to five (1 isolate; 0.6% of the total), six (41 isolates; 25.2%), seven (18 isolates; 11.0%), eight (8 isolates; 4.9%), nine (8 isolates; 4.9%), ten (2 isolates; 1.2%) and eleven (2 isolates; 1.2%) different antibiotics. Finally, the strains (2 isolates; 1.2%) assigned to the XDR phenotype presented resistance to 11 antimicrobial substances. These data may be observed from Table 3.

More than 90.0% of isolates presented resistance to OX, FOX, CTX and FEP, whilst the lowest percentages of resistance related to VA (0.0% of isolates) and C and AMP (2.5%). For the other antibiotics, the prevalence of resistance ranged from 3.1% for ENR to 44.8% for RD. When resistant strains are bundled together with those having reduced susceptibility, prevalence was above 70% for OX, FOX, CTX, FEP, CIP, ENR and RD. Among the remaining antibiotics, these values varied from 2.5% for C and AMP to 55.8% for F (Fig. 9).

Comparisons by type of sample showed significant differences ($P < 0.05$) for two of the 15 antibiotics tested. The greatest prevalence of isolates resistant to CN was found in creole sausages, while "*flamenquines*" showed the greatest prevalence of isolates resistant to SXT. By outlets, the highest levels ($P < 0.05$) of resistant isolates were observed in E8 and E10 for 3 antibiotics (E, RD and CIP).

Table 2
Results of quantification with q-PCR of *Listeria monocytogenes* in raw poultry preparations. The data are given in the form of values of Ct (threshold cycle), ng of DNA in the reaction tube and $\log_{10}$ cfu/g of sample.

Sample	Threshold cycle (Ct)	ng de DNA in the reaction tube	$\log_{10}$ cfu/g of sample
MM1	>40	>0.000003	<2.15
MB1	>40	>0.000003	<2.15
HAM1	>40	>0.000003	<2.15
WS1	>40	>0.000003	<2.15
MB2	>40	>0.000003	<2.15
FAJ1	>40	>0.000003	<2.15
HAM2	39.43	0.000005	2.34
SJ1	38.68	0.000009	2.59
FL1	38.58	0.000009	2.62
RS1	37.85	0.000016	2.86
MM2	37.80	0.000017	2.88
NUG1	37.78	0.000017	2.88
NUG2	37.74	0.000017	2.90
MM3	37.55	0.000020	2.96
MB3	37.29	0.000024	3.04
HAM3	37.27	0.000025	3.05
MM4	37.01	0.000030	3.13
MW1	36.74	0.000037	3.22
NUG3	36.51	0.000044	3.30
HAM4	36.46	0.000045	3.31
HAM5	36.32	0.000050	3.36
SJ2	36.26	0.000053	3.38
FAJ2	36.26	0.000053	3.38
NUG4	36.15	0.000058	3.42
RS2	36.10	0.000060	3.43
MW2	36.09	0.000060	3.44
MW3	36.03	0.000063	3.46
NUG5	35.78	0.000076	3.54
SJ3	35.78	0.000076	3.54
NUG6	35.62	0.000086	3.59
CS1	35.29	0.000110	3.70
MM5	35.14	0.000123	3.75
MW4	35.10	0.000127	3.76
HAM6	34.96	0.000141	3.81
MB4	34.90	0.000147	3.83
FL2	34.89	0.000149	3.83
NUG7	34.44	0.000208	3.98
WS2	34.34	0.000226	4.01
HAM7	33.84	0.000329	4.17
WS3	33.82	0.000335	4.18
MW5	33.66	0.000376	4.23
NUG8	33.52	0.000418	4.28
WS4	33.36	0.000472	4.33
HAM8	33.25	0.000512	4.37
SJ4	32.68	0.000786	4.55
RS3	32.56	0.000862	4.59
GP1	32.36	0.001002	4.66
FAJ3	32.19	0.001140	4.71
CS2	32.13	0.001193	4.73
HAM9	32.05	0.001265	4.76
MM6	32.01	0.001306	4.77
MB5	31.87	0.001451	4.82
CS3	31.79	0.001545	4.85
RS4	31.61	0.001763	4.90
WS5	31.57	0.001819	4.92
GP2	28.40	0.019896	5.96

SJ, *San Jacobo*; FL, *flamenquines*; FAJ, *fajitas*; GP, ground patties; HAM, hamburgers; MW, marinated wings; MB, meat balls; MM, minced meat; NUG, nuggets; CS, creole sausages; RS, red sausages; WS, white sausages; SK, skewers.

4. Discussion

4.1. Prevalence of Listeria spp. and L. monocytogenes

The large percentage of samples contaminated with *Listeria* spp. (73.0%) that was recorded is similar to the figures from other studies: 40.0% in chicken carcasses (Zeinali, Jamshidi, Bassami, & Rad, 2017), 48.0% in fresh chicken (Soultos, Koidis, & Madden, 2003), 50.3% in processed chicken products (Osaili, Alaboudi, & Nesiar, 2011), 76.3% in free-range poultry (Vitas, Aguado, & Garcia-Jalon, 2004), 92.1% in raw chicken (Alonso-Hernando, Prieto, García-Fernández, Alonso-Calleja, & Capita, 2012) and 95.0% in chicken carcasses (Capita, Alonso-Calleja, Moreno, & García-Fernández, 2001). The presence of *Listeria* spp. in poultry may be due to faecal contamination during evisceration, since birds are quite often asymptomatic carriers of this bacterium. Moreover, because of their widespread incidence, *Listeria* spp. may be present on the surfaces of equipment and installations in processing plants, from where they can contaminate the exterior of meat (Alonso-Hernando et al., 2012; Gonçalves-Tenório, Nunes Silva, Rodrigues, Cadavez, & Gonzales-Barron, 2018).

In respect specifically of *L. monocytogenes*, the prevalence observed in the current research (56.0% of the samples) does lie within the broad range of figures recorded by other authors, who found incidences between 0.0% and 58.0% (Jamshidi & Zeinali, 2019; Ristori et al., 2014). Most of the researchers whose works were consulted, though, recorded incidences lower than those in the present study. In previous research works carried out in North-Western Spain, the prevalence of *L. monocytogenes* observed in chicken was 24.5% (Alonso-Hernando et al., 2012) and 32.0% (Capita et al., 2001). Other contamination prevalence values observed are 0.2%–2.5% (Kanarat, Jitnupong, & Sukhapesna, 2011), 4.3%–7.1% (Kosek-Paszkowska, Bania, Bystroń, Molenda, & Czerw, 2005), 8.6%–44.2% (Schäfer et al., 2017), 9.4% (Osaili et al., 2011), 11.4%–14.1% (Fallah, Saei-Dehkordi, Rahnama, Tahmasby, & Mahzounieh, 2012), 12.7% (Bilir Ormanci, Erol, Ayaz, Iseri, & Sariguzel, 2008), 15.8% (Sugiri et al., 2014), 17.9% (Santos Oliveira et al., 2018), 18.0% (Soultos et al., 2003), 18.2% (Osaili et al., 2011), 19.2% (Van Nierop et al., 2005), 19.3% (Gonçalves-Tenório et al., 2018), 20.0% (Saludes, Troncoso, & Figueroa, 2015), 22.2% (Gudbjörnsdóttir et al., 2004), 26.4% (Kuan et al., 2013), 34.0% (Gunasena, Kodikara, Ganepola, & Widanapathirana, 1995), 36.1% (Vitas et al., 2004), 38.0% (Sakaridis et al., 2011), 38.2% (Uyttendaele, De Troy, & Debevere, 1999), 40.0% (Zeinali et al., 2017), 41.0% (Antunes, Réu, Sousa, Pestana, & Peixe, 2002), or 45.0% (Elmali, Can, & Yaman, 2015). For their part, Ristori et al. (2014) noted a prevalence of 58.0%, very similar to the figure being reported here. The differences between studies may be due, at least in part, to the type of samples analysed, which were whole carcasses or chicken parts in the majority of the research checked. During the processing and manipulation needed to manufacture meat preparations, there is an increased risk of contamination with *L. monocytogenes*, which may explain the higher prevalence of *L. monocytogenes* observed during the current work (Santos Oliveira et al., 2018).

With regard to other species of *Listeria*, the second most abundant after *L. monocytogenes* was *L. innocua*, present in 32.0% of the samples. This figure is lower than those recorded in previous research conducted in Spain relating to fresh chicken (57.8% in Capita et al., 2001, and 59.5% in Alonso-Hernando et al., 2012) and to free-range poultry

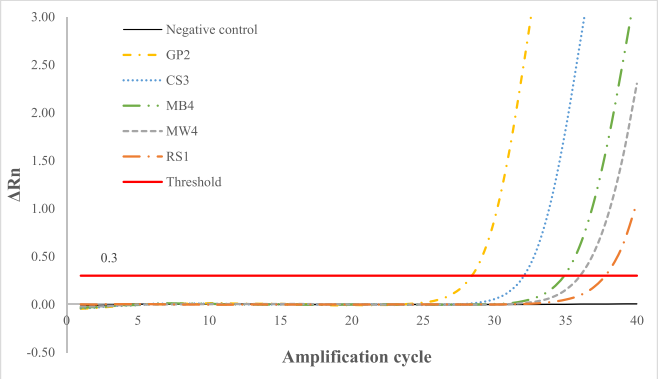

Fig. 5. Example of results of amplification obtained with q-PCR. The image shows five samples with varying quantities of *Listeria monocytogenes*. It is also possible to see a negative control (in black) and the threshold fluorescence value (in red; 0.3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

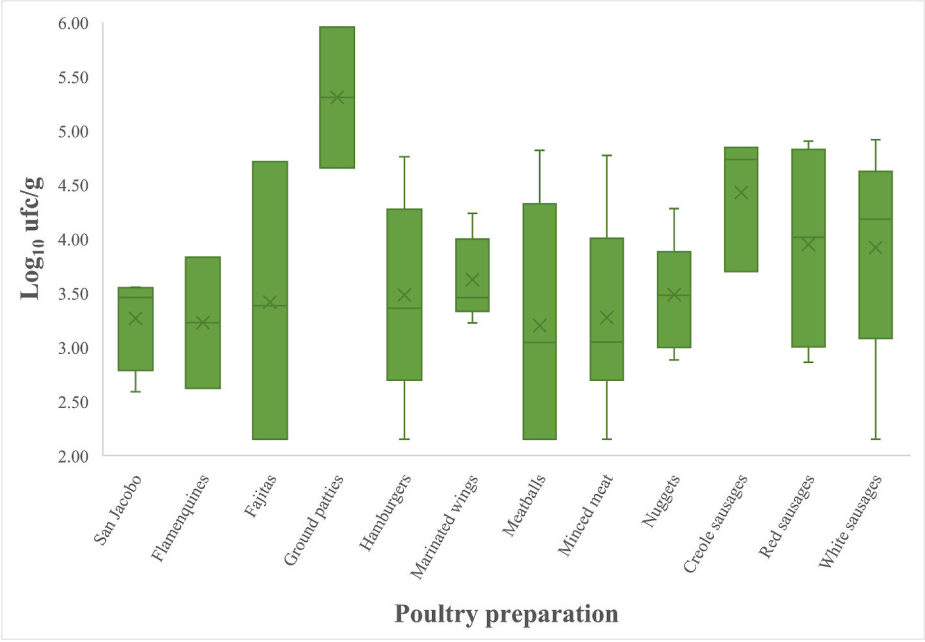

Fig. 6. Box-and-whisker diagrams showing the concentrations of *Listeria monocytogenes* ($\log_{10}$ cfu/g) as a function of the type of sample analysed. The boxes run from the 25th to the 75th percentile and are intersected by the median line. The mean of each type of sample is indicated inside the box as an individual data point (×). Whiskers extend below the box range, from the lowest to the highest values, respectively.

(67.4% in Vitas et al., 2004). Other work, however, did not detect this species in processed chicken products (Osaili et al., 2011). The prevalence of *L. grayi* recorded (2.0%) is similar to what was observed in earlier studies investigating fresh meat (2.2%; Alonso-Hernando et al., 2012) and in processed chicken products (3.5%; Osaili et al., 2011). Likewise, the incidence of *L. seeligeri* (1.0%) coincides with other research investigating free-range poultry (1.4%; Vitas et al., 2004) and processed chicken products (1.8%; Osaili et al., 2011).

No strain of *L. ivanovii* was identified, a finding similar to results previously obtained in work on chicken meat (1.1%; Alonso-Hernando et al., 2012) and free-range poultry (0.0%; Vitas et al., 2004). Nevertheless, other authors (Osaili et al., 2011) did detect strains of *L. ivanovii* in 26.1% of the samples of chicken meat they analysed. Finally, as was pointed out in the preceding paragraphs, strains found in 5.0% of the samples could not be identified at species level, as had been noted in previous studies (6.7%–12.8% of strains unidentified as particular species; Alonso-Hernando et al., 2012). It is also commonplace for different species of *Listeria* to be isolated from one and the same sample of poultry (Capita et al., 2001).

4.2. Quantification of Listeria monocytogenes

The levels of *L. monocytogenes* in the 56 positive samples were determined by q-PCR. In 50 of these samples the values fell between 2.34 and 5.96 $\log_{10}$ cfu/g. In 6 of the samples strains of *L. monocytogenes* were isolated on plates of OCLA medium, but no amplification was observed with q-PCR, so that the concentration was deemed lower than the detection limit for this technique, which had been fixed at 40 cycles of amplification, corresponding to 2.15 $\log_{10}$ cfu/g of sample. This detection limit is lower to that established by other authors (3–4 $\log_{10}$ cfu/g; Rantsiou, Alessandria, Urso, Dolci, & Cocolin, 2008).

In recent years, numerous publications have been published

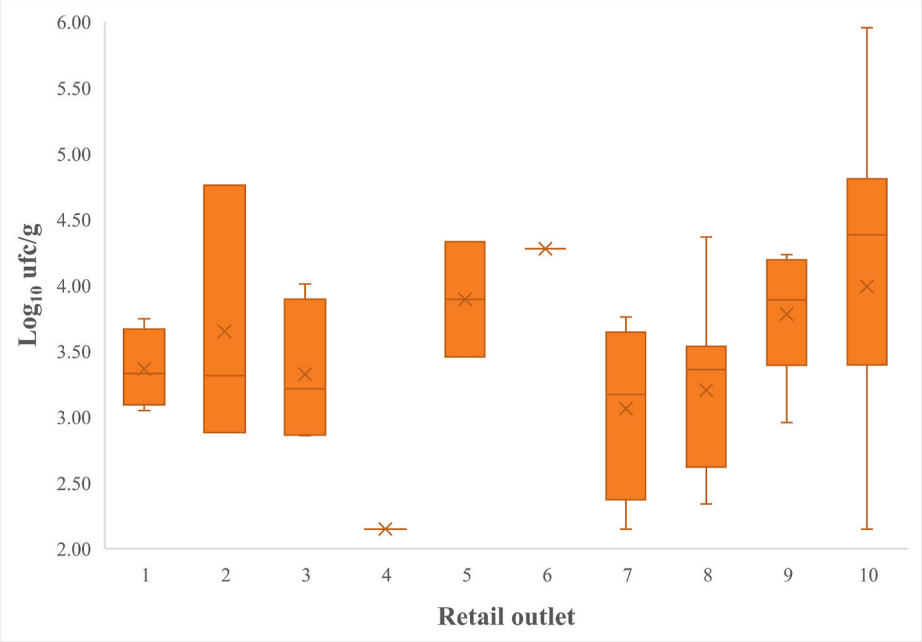

Fig. 7. Box-and-whisker diagrams showing the concentrations of *Listeria monocytogenes* (log_{10} cfu/g) as a function of the retail outlet. The boxes run from the 25th to the 75th percentile and are intersected by the median line. The mean of each outlet is indicated inside the box as an individual data point (×). Whiskers extend below the box range, from the lowest to the highest values, respectively. Outlets E4 and E6 are represented with a line, as they each had just one positive sample.

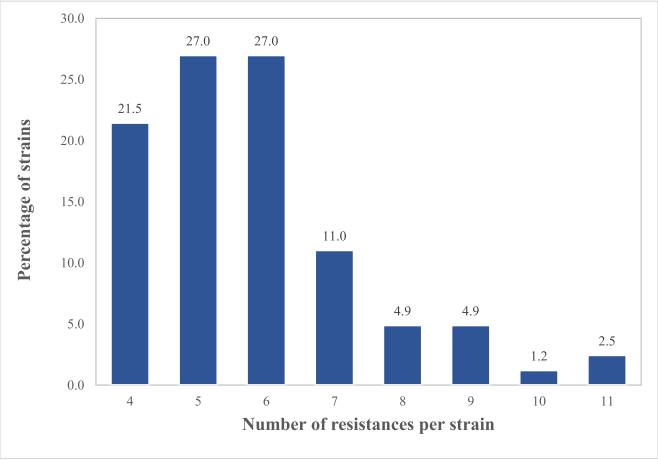

Fig. 8. Distribution of *Listeria monocytogenes* strains isolated by number of resistances to antibiotics found. Above each column is shown the exact value of the percentage of resistant strains.

proposing different protocols for detecting and quantifying pathogenic microorganisms. However, very few have focused on quantification of *L. monocytogenes* directly from samples of food. The concentration of *L. monocytogenes* recorded in the present work was similar to that observed by other authors in raw chicken, as the levels they noted did not exceed a figure of 3 log_{10} cfu/g of sample (Sugiri et al., 2014). Other researchers did find levels above 3 log units, but in only a small percentage of samples (Rørvik & Yndestad, 1991). The results obtained in the present study for poultry preparations are also similar to those recorded by other authors in various foodstuffs, such as fermented sausages (2.85–3.38 log_{10} cfu/g; Martín, Jofré, Garriga, Hugas, & Aymerich, 2004) or fresh cheeses (3.60 log_{10} cfu/g; Rantsiou et al., 2008).

The high levels of contamination found in several samples of poultry preparations from North-West Spain are worrying. This is because the model utilized by the European Food Safety Authority indicates that 92% of the cases of invasive listeriosis are to be attributed to doses higher than 5 log_{10} cfu per 50-g portion (AESAN, 2019), and this equates to 3.30 log_{10} cfu/g. In the work being presented here, 38 samples (67.9% of the samples of poultry that were positive for *L. monocytogenes*, and 38.0% of all samples tested) showed values greater than 3.30 log_{10} cfu/g. On the other hand, 33.9% of the positive samples showed values greater than 4 log_{10} cfu/g, and 1.8% had levels above 5 log_{10} cfu/g.

It is true that poultry preparations are supposed to be cooked before consumption. However, the risk associated with the presence of *L. monocytogenes* in such foodstuffs comes from the possibility of inadequate cooking or cross-contamination affecting other foods. Moreover, *L. monocytogenes* is a psychrotrophic microorganism whose concentration in meat preparations can increase during storage under refrigeration (Capita, Felices-Mercado, et al., 2019).

Table 3
Antibiotic resistance patterns shown by 163 isolates of *Listeria monocytogenes* from poultry preparations.

Number of resistances	Pattern of antibiotic resistance	Number of isolates
4	OX-FOX-CTX-FEP	35
5	OX-FOX-CTX-FEP-F	8
5	OX-FOX-CTX-FEP-CIP	9
5	OX-FOX-CTX-FEP-RD	14
5	OX-CTX-FEP-CN-RD	1
5	OX-FOX-CTX-FEP-SXT	7
5	OX-FOX-CTX-FEP-E	1
5	OX-FOX-CTX-FEP-CN	3
5	OX-FOX-CTX-FEP-TE	1
6	OX-FOX-CTX-FEP-RD-F	5
6	OX-FOX-CTX-FEP-E-F	1
6	OX-FOX-CTX-FEP-SXT-CIP	3
6	OX-FOX-CTX-FEP-CIP-F	8
6	AMP-OX-FOX-CTX-FEP-RD	2
6	OX-FOX-CTX-FEP-CN-CIP	1
6	OX-FOX-CTX-FEP-SXT-RD	5
6	OX-FOX-CTX-FEP-SXT-F	2
6	OX-FOX-CTX-FEP-RD-CIP	12
6	OX-CTX-FEP-RD-CIP-F	1
6	OX-FOX-CTX-FEP-CIP-ENR	1
6	OX-FOX-CTX-CN-E-CIP	1
6	OX-FOX-CTX-FEP-E-CIP	1
6	OX-FOX-CTX-FEP-TE-C	1
7	OX-FOX-CTX-FEP-E-RD-CIP	4
7	OX-FOX-CTX-FEP-SXT-CIP-F	2
7	OX-FOX-CTX-FEP-E-CIP-F	3
7	OX-FOX-CTX-FEP-RD-CIP-F	2
7	AMP-OX-FOX-CTX-FEP-RD-CIP	1
7	OX-FOX-CTX-FEP-SXT-RD-CIP	2
7	OX-FOX-CTX-FEP-E-SXT-RD	2
7	OX-FOX-CTX-FEP-RD-TE-CIP	1
7	OX-FOX-CTX-FEP-SXT-RD-TE	1
8	OX-FOX-CTX-FEP-E-RD-CIP-F	2
8	OX-FOX-CTX-FEP-SXT-RD-CIP-F	2
8	AMP-OX-FOX-CTX-FEP-E-RD-F	1
8	OX-FOX-CTX-FEP-RD-TE-CIP-F	1
8	OX-FOX-CTX-FEP-CN-E-SXT-RD	1
8	OX-FOX-CTX-FEP-E-SXT-RD-TE	1
9	OX-FOX-CTX-FEP-CN-E-SXT-RD-TE	2
9	OX-CTX-FEP-CN-E-SXT-CIP-ENR-F	2
9	OX-FOX-CTX-FEP-CN-E-RD-TE-C	1
9	OX-FOX-CTX-FEP-E-SXT-RD-CIP-F	1
9	OX-FOX-CTX-FEP-CN-E-SXT-RD-CIP	1
9	OX-FOX-CTX-FEP-E-SXT-RD-TE-F	1
10	OX-FOX-CTX-FEP-CN-E-SXT-RD-TE-CIP	2
11	OX-FOX-CTX-FEP-CN-E-SXT-RD-CIP-ENR-F	1
11	OX-FOX-CTX-FEP-CN-E-SXT-RD-TE-CIP-ENR	1
11	OX-FOX-CTX-FEP-CN-E-SXT-RD-TE-C-CIP	2

Ampicillin (AMP, 10 μg), oxacillin (OX, 1 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), gentamycin (CN, 10 μg), erythromycin (E, 15 μg), vancomycin (VA, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), rifampicin (RD, 5 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 5 μg) and nitrofurantoin (F, 300 μg).

4.3. Resistance to antibiotics in Listeria monocytogenes

The susceptibility of 163 isolates of *L. monocytogenes* from poultry preparations was tested against 15 antibiotics of clinical interest. The average number of resistances per strain was 5.83 ± 1.64 when resistance was understood in the strict sense, and 7.66 ± 1.52 if resistance and reduced susceptibility were taken together. This is much higher than the values recorded in previous work with strains of *L. monocytogenes* obtained from poultry in North-West Spain, in which the number of resistances per strain observed was 1.60 in 1993 and 4.24 in 2006 (Alonso-Hernando et al., 2012). The larger figures noted in the present study are a matter for concern, since resistance to antibiotics compromises the usefulness of these substances as treatment options, and this implies a major challenge for Public Health (Capita & Alonso-Calleja, 2013). On this point it should be indicated that infections by multi-resistant bacteria not only increase morbidity and mortality rates, but also cause a considerable growth in the costs arising from the medical treatments required (Capita, Cordero, et al., 2019; Cosgrove, 2006).

Although *L. monocytogenes* is a bacterium that in the past has been sensitive to the majority of antimicrobials used to treat infections by Gram-positive organisms, in recent years a marked increase has occurred in the prevalence of resistance in this microorganism (Fallah et al., 2012), a situation that is also made plain by the outcome of the present study. Among other causes, this growth in resistance in *L. monocytogenes* is due to the progressive acquisition from the cells of various different genera of bacteria of mobile genetic elements, such as plasmids or transposons (Olaimat et al., 2018). It must be pointed out that resistance to antibiotics has been commonplace for some years in other Gram-positive bacteria. This has been noted previously in strains obtained from poultry in the North-West of Spain, where an average of 4.48 (Castaño-Arriba et al., 2020) or 5.58 (Cordero, Alonso-Calleja, García-Fernández, & Capita, 2019) resistances per strain has been observed in *Enterococcus* spp., and 6.35 resistances per strain in *Staphylococcus aureus* (Buzón-Durán et al., 2017).

More than 90.0% of the isolates of *L. monocytogenes* showed resistance or reduced susceptibility to OX, FOX, CTX, FEP and CIP. These figures were higher than 50.0% in the case of ENR, F and RD. Previous studies have also observed strains of *L. monocytogenes* derived from poultry resistant to the antibiotics indicated (Alonso-Hernando et al., 2012; Capita, Felices-Mercado, et al., 2019). It must be pointed out that these antibiotics are classified as "critically important" (CTX, FEP, RD, CIP), "highly important" (OX, FOX) or "important" (F) antimicrobial agents in human medicine (WHO, 2019). In the list published by the World Organization for Animal Health (OIE), OX, and CIP are considered "critically important" antibiotics, and RD "highly important" in veterinary medicine (OIE, 2018). Moreover, some of these compounds are employed in treating human listeriosis, for which beta-lactam antibiotics, generally ampicillin, administered alone or in combination with gentamycin, are the first choice of medicine. In cases of allergy to beta-lactams, possible alternatives include erythromycin, vancomycin, trimethoprim-sulfamethoxazole and fluoroquinolones. On occasion treatment for listeriosis is undertaken with rifampicin, tetracycline and chloramphenicol (Capita, Felices-Mercado, et al., 2019).

The high percentage of strains resistant to antibiotics observed in this work is very likely to be related to the extensive use of these substances in veterinary medicine. Indeed, the selective pressure brought to bear by the use of antibiotics, especially when this is done under inappropriate conditions, whether in humans or in animals, must be seen at the principal risk factor in the emergence of resistance to antibiotics (Capita & Alonso-Calleja, 2013). The present study highlighted a striking prevalence of resistance to antibiotics that are widely used in poultry rearing (Roth et al., 2019).

One especially reassuring feature is the great prevalence of susceptibility to ampicillin (97.6% of strains were susceptible). This is because of the major role of this antibiotic in treating listeriosis and because of the increase in the percentage of resistant strains that has taken place over the last two decades. On this point, it should be noted that actual results agree with previous work done on chicken meat from North-Western Spain which indicated that all isolates were susceptible to this antibiotic (Alonso-Hernando et al., 2012).

It should be noted that the majority of strains of *L. monocytogenes* show natural resistance to cephalosporins, especially third- and fourth-generation drugs of this group (Capita, Felices-Mercado, et al., 2019). This situation is corroborated by the results of the work being reported here, in which a considerable prevalence of resistance to cephalosporins

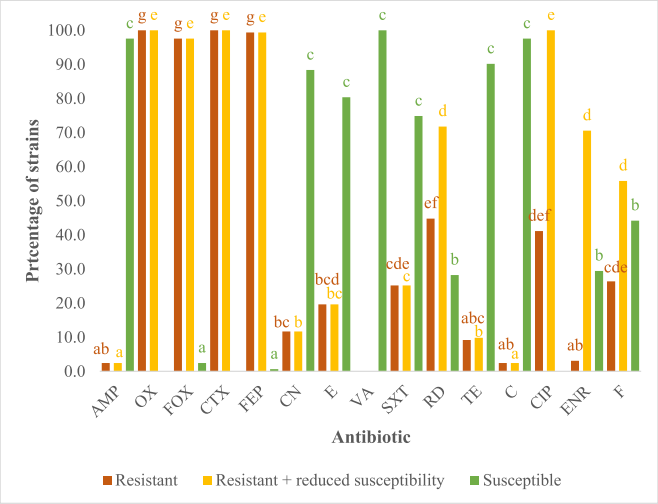

Fig. 9. Percentage of strains of *Listeria monocytogenes* resistant, resistant + with reduced susceptibility or susceptible for each antibiotic tested. Ampicillin (AMP, 10 μg), oxacillin (OX, 1 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), gentamycin (CN, 10 μg), erythromycin (E, 15 μg), vancomycin (VA, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), rifampicin (RD, 5 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, five μg) and nitrofurantoin (F, 300 μg). Columns that do not share any letter for the same group (resistant, resistant + reduced susceptibility or susceptible) show significant differences one from another ($P < 0.05$).

of the second (cefoxitin), third (cefotaxime) and fourth (cefepime) generations was detected.

Strains resistant to nitrofurantoin (55.8% of isolates showed resistance or intermediate susceptibility to this antibiotic) were recorded. This compound was routinely employed in veterinary medicine in the past, although their use was banned some three decades ago owing to the toxicological dangers for consumers. The strong prevalence of resistance to this antibiotic detected in the present study may be due to mechanisms of cross-resistance or co-resistance, as has previously been suggested (Capita & Alonso-Calleja, 2013; Cordero et al., 2019; Álvarez-Fernández, Cancelo, Díaz-Vega, Capita, & Alonso-Calleja, 2013).

5. Conclusions

The samples of fresh poultry preparations analysed showed a considerable prevalence of *L. monocytogenes* and, in some cases, presented high concentrations of this bacterium, which implies potential dangers to consumers. Testing with q-PCR proved itself to be a useful technique for detecting and quantifying *L. monocytogenes* in food, besides being rapid, as no prior enrichment stages are necessary. Furthermore, all the strains of *L. monocytogenes* presented multiple resistances, which is a worrying fact in the context of Food Safety and Public Health, because some of the antibiotics habitually used in treating listeriosis are ruled out as a therapeutic option. The results of this research stress how vital it is for those handling poultry preparations to apply correct hygiene practices, avoiding the under-cooking of such meats and cross-contamination to other foodstuffs, with the aim of reducing risks to consumers.

CRediT authorship contribution statement

Sarah Panera-Martínez: Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Cristina Rodríguez-Melcón:** Methodology, Validation, Investigation. **Víctor Serrano-Galán:** Methodology, Investigation, Resources. **Carlos Alonso-Calleja:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Rosa Capita:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing interest

None.

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