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Research Paper

Occurrence and Antimicrobial Profile of Bacterial Pathogens in Former Foodstuff Meat Products Used for Pet Diets

CRISTINA BACCI,* ALICE VISMARRA, SILVIA DANDER, ELENA BARILLI, AND PAOLA SUPERCHI

Department of Veterinary Science, University of Parma, Via del Taglio 10, 43126 Parma, Italy (ORCID: http://orcid.org/0000-0002-1332-6080 [C.B.])

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ABSTRACT

European legislation stipulates that food no longer intended for human consumption, due to commercial reasons, manufacturing problems, or some defect, can be used in pet feed. However, the presence of former foodstuffs in pet diets could constitute a public health issue because pets can act as reservoirs of antimicrobial resistance genes. In this study, for the first time, biological hazards due to the presence of *Escherichia coli* and *Salmonella* in former foodstuff meat products were evaluated. Among the 112 samples of packaged fresh meat (poultry, pork, and beef) collected from cold storage warehouses of a mass market retailer, the overall prevalence of *E. coli* and *Salmonella,* the prevalence of strains with multidrug resistance, the phenotypic and genotypic characteristics of strains that produce extended-spectrum β-lactamase, and the presence of biofilm producers were assessed. A high prevalence of *E. coli* was observed in former foodstuffs of poultry (100%), pork (100%), and beef (93.3%). *Salmonella* Derby and *Salmonella* Typhimurium were found in 11.5% of the poultry samples, and *Salmonella* Typhimurium was found in 13.3% of the pork samples. *Salmonella* was not recovered from the beef samples. Multidrug resistance was found in *E. coli* and *Salmonella* isolates from poultry, pork, and beef. Overall, 18.2% of *E. coli* isolates and 20% of *Salmonella* isolates were resistant to various types of antibiotics with various mechanisms of action. *Salmonella* isolates from pork are an important source of extended-spectrum β-lactamase production. Both *E. coli and Salmonella* were carriers of antibiotic resistance marker genes (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$) associated with β-lactamase production in poultry and pork. Approximately 30% of the *E. coli* isolates from the various types of meat were phenotypically biofilm producers, and all carried the *fimH* gene. These findings indicate that the use of former foodstuff meat products in pet diets can represent a risk for public health.

Key words: Antimicrobial resistance; Biofilm; *Escherichia coli*; Extended-spectrum β-lactamase; Former foodstuffs; *Salmonella*

The European Commission *(12)* has issued guidelines regarding former foodstuffs as feed materials that can be used raw in pet diets. Former foodstuffs are identified as food products that were manufactured for human consumption but that are no longer intended for human consumption for practical or logistical reasons or due to manufacturing or packaging defects and that do not present any health risks when used as feed *(11)*. Allowing the feed industry to use meat products from former human foodstuffs as an alternative to animal by-products in raw meat pet diets can improve the nutritional value and safety of these diets. Raw meat diets for pets have become increasingly popular, and this trend has been partially driven by a parallel movement in the human food market for organic products. Many pet owners have abandoned conventional pet diets in search of more "natural" choices, which has led to preferences for raw meat diets. The belief is that a raw diet reflects the unprocessed food that dogs and cats have evolved eating, and these pet diets should mimic the diets of wild canids and felids.

In ordinary commercial raw meat diets, the topic most discussed is the risk to human or animal health because of possible contamination with zoonotic bacteria *(49)*. Humans can be exposed to pet-associated zoonotic pathogens directly by touching animals or indirectly such as through pet food *(9, 35)*. A high prevalence of zoonotic microorganisms has been reported in pet food that include raw meat from various sources *(32)*. Concern has increased following reports of several outbreaks in humans and large-scale recalls linked to commercial raw meat pet diets. Common contaminants in raw meat are *Salmonella* and *Escherichia coli,* both of which can cause gastrointestinal illness in humans.

Salmonella is one of the most well-known foodborne pathogens because of its worldwide distribution and its ability to cause human infection, with 94,530 confirmed salmonellosis cases in the European Union (EU) *(15)*. A high proportion of human *Salmonella* isolates are resistant to various antibiotics, such as tetracyclines, sulfonamides, sulfamethoxazole, and ampicillin *(16)*. Some isolates of

* Author for correspondence. Tel: (+ 39) 0521902740; Fax: (+ 39) 0521902741; E-mail: cristina.bacci@unipr.it.

Salmonella are resistant against third-generation cephalosporins (β-lactams), which are fundamental for the treatment of systemic gram-negative infections in humans *(8)*.

Genes for antimicrobial resistance, encoded by plasmids, have been identified especially in *E. coli* in the intestines of humans and animals and in the environment. *E. coli* is usually a commensal component of the gut flora, but it can cause various complications when it becomes resistant to antibiotics, in particular infections of the urinary tract and skin *(5)*. The importance of *E. coli* in the spread of antibiotic resistance among bacteria is related to its widespread presence in the environment and to its ability to exchange mobile genetic elements (plasmids and transposons) and genes with other bacteria.

The interplay between different ecologies (i.e., animal, environment, and human) is also important in the context of antimicrobial resistance, which is an important global problem *(54)*. Antimicrobial resistance is responsible for an estimated 25,000 deaths per year in the EU with healthcare costs of €1.5 billion per year and high productivity losses *(41)*. Microbial strategies to resist the effects of antimicrobial agents include the formation of biofilms, which is one of the main reasons for the failure of treatment with antibiotics *(24)*. Thus, the role that raw meat pet diets play in transmission of antimicrobial resistant bacteria has received specific attention *(3, 23)*. However, attention has not been paid to former human foodstuffs, i.e., products derived from foods through compliance with the EU food regulations.

The aim of the present study was to evaluate the occurrence and antimicrobial profiles of *E. coli* and *Salmonella* in former foodstuff meat products. Raw meat samples were evaluated for the prevalence of *E. coli* and *Salmonella,* the frequency of occurrence of isolates with multidrug resistance (MDR), the phenotypic and genotypic expressions of isolates that produced extended-spectrum β-lactamase (ESBL), and the ability of these isolates to form biofilms.

MATERIALS AND METHODS

Sampling, isolation, and serotyping. From March 2017 to December 2017, samples of former foodstuffs in the form of packaged raw and expired poultry ($n = 52$), pork ($n = 30$), and beef ($n = 30$) were collected from cold storage warehouses of a mass market retailer in Emilia Romagna, Italy. Samples were immediately transported to the laboratory under refrigeration (4°C) and analyzed.

E. coli and *Salmonella* isolates were recovered according to ISO 16649-2:2001 *(26)* and the ISO 6579:2008 *(27),* respectively. For both bacteria, a 10-fold dilution of each sample was prepared in buffered peptone water (BPW; Biolife Italiana, Milan, Italy).

To obtain *E. coli* isolates, the broth culture was seeded onto tryptone bile X-gluc agar (Biolife Italiana) plates. Typical blue-green colonies were selected and subjected to the indole test. To obtain *Salmonella* isolates, the samples in preenrichment media were selectively enriched in Mueller Kauffmann tetrathionate broth (Biolife Italiana) and Rappaport-Vassiliadis soya broth (Biolife Italiana). An aliquot of the each enrichment culture was streaked onto xylose lysine desoxycholate agar (Biolife Italiana) and chromogenic *Salmonella* agar (Oxoid, Basingstoke, UK) plates. Suspect colonies were seeded into triple sugar iron agar (Biolife Italiana), lysine iron agar (Biolife Italiana), and Christensen's urea agar (Biolife Italiana). Cultures with typical *Salmonella* reactions were tested by slide agglutination with an O-omnivalent *Salmonella* serum (Denka Seiken Co., Tokyo, Japan). Agglutinating cultures were streaked onto tryptone soy agar (TSA; Biolife Italiana) plates. *E. coli* ATCC 25922, S*almonella* Enteritidis ATCC 13076, and *Salmonella* Typhimurium ATCC 14028 were used as quality controls. Biochemical identification of the microorganisms was performed using the API 20E microsubstrate system (bioMérieux, Marcy l'Étoile, France). All *Salmonella* isolates were serotyped according to the White Kauffmann–Le Minor scheme by slide agglutination with O and H antigen-specific sera at the Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna (Brescia, Italy).

Antimicrobial susceptibility test. Confirmed colonies of *E. coli* and *Salmonella* were tested for antimicrobial susceptibility. The disk diffusion test on agar plates was used following the protocol defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *(13)*. Strains were seeded onto TSA and incubated at 37°C for 24 h. One colony was put in tryptic soy broth (Biolife Italiana) and then incubated at 37°C for 4 to 6 h. The broth culture was seeded uniformly onto Mueller-Hinton agar (Biolife Italiana) plates, and antimicrobial disks were added. The susceptibility profile was evaluated with the following antimicrobial agents (Biolife Italiana): cefotaxime (CTX; 5 μg), ceftazidime (CAZ; 10 μg), ampicillin (AMP; 10 μg), chloramphenicol (CHL; 30 μg), ciprofloxacin (CIP; 5 μg), gentamicin (GEN; 10 μg), nalidixic acid (NAL; 30 μg), trimethoprim-sulfamethoxazole (SXT; 1.25/23.75 μg), tetracycline (TET; 30 μg), imipenem (IMI; 10 μg), and streptomycin (STRE; 10 μg). The plates were incubated at 37°C for 18 ± 2 h. The breakpoint of each drug was interpreted (based on the diameter of the growth inhibition zone) as sensitive, intermediate, or resistant using the EUCAST criteria *(14)*. The selected antibiotics have different mechanisms of action: A, interference with cell wall synthesis (AMP, CAZ, CTX, and IMI); B, interference with DNA synthesis (CIP and NAL); C, enzymatic inhibition (SXT); and D, interference with protein synthesis (CHL, GEN, STRE, and TET) *(28)*. The MDR classification of *Enterobacteriaceae* was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories *(34)*.

Phenotypic assessment of ESBL production and biofilm synthesis. The isolates that were resistant or had intermediate susceptibility to CTX and CAZ were submitted to a combined-disk test for the phenotypic evaluation of ESBL production, according to the methods of the Clinical and Laboratory Standards Institute *(7)*. ESBL production was assessed by the growth inhibition zones around both CTX (30 μg) and CAZ (30 μg) disks with or without clavulanate (10 μg). The combined-disk test was considered positive when the growth inhibition zone around the CTX or the CAZ disks with clavulanate was ≥5 mm wider than the diameter around the disks containing CTX or CAZ without clavulanate.

Bacterial biofilm formation was evaluated according to the protocol described by Christensen et al. *(6)*. Single *E. coli* and *Salmonella* colonies in BPW plus 1% glucose were incubated at 37°C for 2 h, 200 μL of this broth culture was seeded into five wells for three replications, and the 96-well plates were incubated at 37°C for 24 h. The plates were then washed with 300 μL of phosphate-buffered saline (Biolife Italiana) and dried at 42°C for 30 min, 200 μL of crystal violet (VWR International, Milan, Italy) was added to each well, and plates were incubated in the dark at

TABLE 1. *Oligonucleotide sequences for ESBL genes*

Gene	Primer sequence (5′–3′)[a]	Reference
bla_{TEM}	f: GCGGAACCCCTATTTG	*40*
	r: ACCAATGCTTAATCAGTGAG	
bla_{SHV}	f: TTATCTCCCTGTTAGCCACC	*53*
	r: GATTTGCTGATTTCGCTCGG	
bla_{CTX-M}	f: CGATGTGCAGTACCAGTAA	*4*
	r: TTAGTGACCAGAATCAGCGG	

[a] f, forward; r, reverse.

room temperature for 15 min. After a second washing cycle, the plates were incubated overnight under the same conditions. To fix the formed biofilm, 200 µL of 95% ethanol was added. Plates were evaluated at 620 nm with a spectrophotometer (Victor3, PerkinElmer, Waltham, MA) to obtain the optical density (OD). The cutoff OD value (ODc) for the test was five standard deviations above the mean OD of the negative controls (a well containing only medium). A positive control (*Pseudomonas aeruginosa* PAO1) also was used in the test. The isolates were classified according to the method of Stepanović et al. *(46)* into four categories: OD $\leq$ ODc, nonadherent; ODc $<$ OD $\leq 2 \times$ ODc, weak biofilm formation; $2 \times$ ODc $<$ OD $\leq 4 \times$ ODc, moderate biofilm formation; and $4 \times$ ODc $<$ OD, strong biofilm formation.

Genetic assessment of ESBL and biofilm producers. A real-time PCR assay was used for each *E. coli* and *Salmonella* isolate with an ESBL profile. Three of the more common genes *(33, 37)* implicated in coding for ESBLs were chosen: bla_{TEM}, bla_{SHV}, and bla_{CTX-M} (Bio-Rad, Milan, Italy).

DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fischer Scientific, Waltham, MA) from the broth culture obtained from a single colony diluted in BPW and grown at 37°C overnight. The real-time PCR protocol was adapted to match that described by Roschanski et al. *(43)*. Real-time amplifications were performed in 25-µL volumes containing 10 pmol of each primer, 1× SYBR Green buffer (Bio-Rad), water, and 2 µL of DNA. All samples and positive and negative controls were tested in triplicate. The thermal protocol was initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 50°C for 15 s, and 70°C for 20 s (CFX 96 Real-time PCR, Bio-Rad). A melting curve was added at the end to avoid the amplification of nonspecific products. Isolates with a Cq (quantification cycle) value above 31 or 32 were considered negative for ESBL genes. The sequences of the oligonucleotides used are listed in Table 1.

Three genes involved in biofilm production by *E. coli* were selected: *agn43* and *flu, fimH,* and *papC* (Bio-Rad). A classic end-point PCR protocol was used with the following thermal conditions: 95°C for 5 min, 94°C for 30 s, 55°C for 30 s (annealing at 60°C for *agn43* F and *flu* R), 72°C for 1 min, and a final elongation at 72°C for 7 min. The amplified products were separated on a 2% agarose gel, stained with SYBR safe (Invitrogen, Thermo Fischer Scientific), and visualized with an UV transilluminator (Elettrofor, Rovigo, Italy). The sequences of the oligonucleotides used are listed in Table 2.

Statistical analysis. Differences among the proportions of isolates resistant to each of the antibiotics were compared for each type of former foodstuff (poultry, pork, and beef) using Fisher's exact test with SPSS version 25.0 (IBM, Armonk, NY). Differences were considered significant at $P < 0.05$ and trending at $0.05 < P \leq 0.10$.

RESULTS

Prevalence of *E. coli* and *Salmonella*. Fifty-two *E. coli* isolates were recovered from poultry (100% of poultry samples), 30 from pork (100%), and 28 from beef (93.3%). *Salmonella* isolates were recovered from only the poultry and pork samples. *Salmonella* Derby ($n = 2$) and *Salmonella* Typhimurium ($n = 4$) were found in 11.5% of the poultry meat, and *Salmonella* Typhimurium ($n = 4$) was found in 13.3% of the pork meat.

Antibiotic resistant isolates. Among the *E. coli* isolates, the antibiotic resistance rate was >50% for AMP, TET, and STRE but was lower for CHL, SXT, CIP, NAL, and GEN (Table 3). A tendency was noted for a higher prevalence of isolates resistant to AMP ($P = 0.071$) in pork than in poultry and beef and a higher prevalence of isolates resistant to CHL ($P = 0.069$) in beef than in poultry and pork. All *E. coli* isolates were susceptible to IMI. Significant differences ($P < 0.05$) were detected among the percentage of isolates resistant to TET and NAL in the three meat types. Five isolates from poultry were resistant to cephalosporins (CTX, 5.8%; CAZ, 3.8%). GEN resistance was not found in any isolates from beef.

Salmonella isolates from poultry ($n = 6$) and pork ($n = 4$) had high resistance to AMP (66.6 and 75%, respectively), TET (66.6 and 100%, respectively), and STRE (66.6 and 100%, respectively). All *Salmonella* isolates were susceptible to IMI, GEN, and CTX. A significant difference was not detected between the percentage of resistant isolates recovered from poultry and pork meat ($P > 0.05$).

Twenty-six isolates of *E. coli* (15 of 52 from poultry, 4 of 30 from pork, and 7 of 28 from beef) were sensitive to all antibiotics tested (Tables 4 through 6). The antimicrobial

TABLE 2. *Oligonucleotide sequences and molecular weights of biofilm-associated genes*

Gene	Primer sequence (5′–3′)[a]	Weight (bp)	Reference
agn43, flu	f: GGGTAAAGCTGATAATGTCG	508	*44*
	r: GTTGCTGACAGTGAGTGTGC		
fimH	f: TGCAGAACGGATAAGCCGTGG	506	*44*
	r: GCAGTCACCTGCCCTCCGGTA		
papC	f: TGATATCACGCAGTCAGTAGC	482	*51*
	r: CCGGCCATATTCACATAAC		

[a] f, forward; r, reverse.

TABLE 3. *Antibiotic resistance of* E. coli *isolates from poultry, pork, and beef*

Antibiotic	No. (%) of resistant isolates				*P* value[a]
	Total	Poultry ($n = 52$)	Pork ($n = 30$)	Beef ($n = 28$)	
Ampicillin	61 (55.5)	29 (55.8)	21 (70.0)	11 (39.3)	0.071
Tetracycline	61 (55.5)	22 (42.3)	24 (80.0)	15 (53.6)	0.004
Streptomycin	56 (50.9)	22 (42.3)	19 (63.3)	15 (53.6)	0.176
Chloramphenicol	40 (36.4)	14 (26.9)	11 (36.7)	15 (53.6)	0.069
Trimethoprim-sulfamethoxazole	33 (30.0)	14 (26.9)	12 (40.0)	7 (25.0)	0.393
Ciprofloxacin	32 (29.1)	17 (32.7)	10 (33.3)	5 (16.7)	0.331
Nalidixic acid	31 (28.2)	21 (40.4)	7 (23.3)	3 (10.7)	0.015
Gentamicin	9 (8.2)	5 (9.6)	4 (13.3)	0	0.152
Cefotaxime	3 (2.7)	3 (5.8)	0	0	0.336
Ceftazidime	2 (1.8)	2 (3.8)	0	0	0.497

[a] Differences were considered significant at $P < 0.05$.

resistance pattern of the *E. coli* isolates differed depending on the meat type of the former foodstuff. In poultry, 21 isolates showed MDR, and 4 of these were resistant to six or more antibiotic categories; 19.2% of the isolates were resistant to antibiotics with different mechanisms of action (Table 4).

In pork, 20 *E. coli* isolates showed MDR, and 5 of these were resistant to six antibiotic categories. Three isolates were resistant to AMP, CIP, CHL, STRE, SXT, and TET, and two were resistant to AMP, CIP, CHL, NAL, STRE, SXT, and TET; 26.7% of the isolates were resistant to antibiotics with different mechanisms of action (Table 5).

In beef, 12 *E. coli* isolates showed MDR, and 4 of these were resistant to five antibiotic categories. Two isolates with the resistance pattern AMP, CIP, NAL, STRE, SXT, and TET were resistant to antibiotics with four different mechanisms of action (Table 6).

Seven *Salmonella* isolates from poultry and pork showed MDR; no isolates were recovered from beef. Two isolates from poultry with resistance pattern AMP, CHL,

TABLE 4. *Antimicrobial resistance patterns for 52* E. coli *isolates from poultry*

Antimicrobial resistance pattern[a]	No. of isolates	Mechanism(s)[b]	Antibiotic category(s)[c]
No resistance	15		
AMP	2	A	6
NAL	1	B	4
AMP, TET	3	A, D	6, 8
GEN, STRE	1	D	1
NAL, CIP	3	B	4
STRE, TET	2	D	1, 8
AMP, CIP, NAL	3	A, B	4, 6
CIP, NAL, STRE	1	B, D	1, 4
AMP, CIP, NAL, TET	2	A, B, D	4, 6, 8
AMP, CHL, CTX, STRE	2	A, D	1, 3, 6, 7
AMP, CHL, STRE, TET	1	A, D	1, 6, 7, 8
AMP, GEN, STRE, TET	1	A, D	1, 6, 8
AMP, CIP, CHL, NAL, STRE	1	A, B, D	1, 4, 6, 7
AMP, CHL, NAL, SXT, TET	1	A, B, C, D	4, 5, 6, 7, 8
AMP, CHL, STRE, SXT, TET	4	A, C, D	1, 5, 6, 7, 8
AMP, CHL, NAL, STRE, SXT	1	A, B, C, D	1, 4, 5, 6, 7
AMP, CIP, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 8
AMP, CHL, NAL, STRE, SXT, TET	1	A, B, C, D	1, 4, 5, 6, 7, 8
AMP, CIP, GEN, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 8
AMP, CAZ, CIP, CHL, NAL, STRE, SXT, TET	1	A, B, C, D	1, 3, 4, 5, 6, 7, 8
AMP, CIP, CHL, GEN, NAL, STRE, SXT, TET	1	A, B, C, D	1, 4, 5, 6, 7, 8
AMP, CAZ, CIP, CHL, CTX, NAL, STRE, SXT, TET	1	A, B, C, D	1, 3, 4, 5, 6, 7, 8

[a] AMP, ampicillin; NAL, nalidixic acid; TET, tetracycline; GEN, gentamicin; STRE, streptomycin; CIP, ciprofloxacin; CHL, chloramphenicol; CTX, cefotaxime; SXT, trimethoprim-sulfamethoxazole; CAZ, ceftazidime.

[b] A, interference with cell wall synthesis; B, interference with DNA synthesis; D, interference with protein synthesis; C, enzymatic inhibition.

[c] 6, penicillins; 4, fluoroquinolones; 8, tetracyclines; 1, aminoglycosides; 3, extended-spectrum cephalosporins; 7, phenicols; 5, folate pathway inhibitors.

TABLE 5. *Antimicrobial resistance patterns of 30* E. coli *isolates from pork*

Antimicrobial resistance pattern[a]	No. of isolates	Mechanism(s)[b]	Antibiotic category(s)[c]
No resistance	4		
STRE	1	D	1
TET	2	D	8
AMP, NAL	1	A, B	4, 6
AMP, TET	1	A, D	6, 8
AMP, STRE, TET	2	A, D	1, 6, 8
CIP, STRE, TET	1	B, D	1, 4, 8
GEN, STRE, TET	1	D	1, 8
AMP, CIP, NAL, TET	1	A, B, D	4, 6, 8
AMP, CHL, STRE, TET	3	A, D	1, 6, 7, 8
AMP, CHL, SXT, TET	1	A, C, D	5, 6, 7, 8
AMP, GEN, STRE, TET	1	A, D	1, 6, 8
AMP, STRE, SXT, TET	1	A, C, D	1, 5, 6, 8
AMP, CIP, NAL, SXT, TET	1	A, B, C, D	4, 5, 6, 8
AMP, CHL, STRE, SXT, TET	2	A, C, D	1, 5, 6, 7, 8
AMP, CIP, CHL, STRE, SXT, TET	3	A, B, C, D	1, 4, 5, 6, 7, 8
AMP, CIP, CHL, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 7, 8
AMP, CIP, GEN, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 8

[a] STRE, streptomycin; TET, tetracycline; AMP, ampicillin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole.
[b] D, interference with protein synthesis A, interference with cell wall synthesis; B, interference with DNA synthesis; C, enzymatic inhibition.
[c] 1, aminoglycosides; 8, tetracyclines; 4, fluoroquinolones; 6, penicillins; 7, phenicols; 5, folate pathway inhibitors.

NAL, STRE, SXT, and TET were resistant to six antibiotic categories with different mechanisms of action (Table 7).

Presence of ESBL and biofilm producers. The combined-disk test was used with 19 isolates (13 *E. coli* and 6 *Salmonella*) with resistance or intermediate susceptibility profiles. Three *E. coli* isolates from poultry and four *Salmonella* isolates from pork were ESBL producers (Table 8). No *E. coli* isolates from beef were confirmed as ESBL producers.

Seventy-eight of the 110 *E. coli* isolates showed no adherence and thus were not able to produce biofilms. Isolates from poultry (30.7%) and pork (10%) showed weak adherence. Isolates from beef showed weak (28.6%), moderate (7.1%), and strong (10.7%) adherence (Table 9). None of the *Salmonella* isolates produced biofilms.

TABLE 6. *Antimicrobial resistance patterns of 28* E. coli *isolates from beef*

Antimicrobial resistance pattern[a]	No. of isolates	Mechanism(s)[b]	Antibiotic category(s)[c]
No resistance	7		
CHL	1	D	7
STRE	1	D	1
TET	3	D	8
AMP, TET	1	A, D	6, 8
STRE, TET	1	D	1, 8
CIP, STRE	1	B, D	1, 4
CIP, TET	1	B, D	4, 8
AMP, STRE, TET	2	A, D	1, 6, 8
CIP, STRE, TET	1	B, D	1, 4, 8
AMP, CIP, STRE, TET	1	A, B, D	1, 4, 6, 8
AMP, CHL, STRE, TET	1	A, D	1, 6, 7, 8
AMP, STRE, SXT, TET	3	A, C, D	1, 5, 6, 8
AMP, CHL, STRE, SXT, TET	1	A, C, D	1, 5, 6, 7, 8
CHL, NAL, STRE, SXT, TET	1	B, C, D	1, 4, 5, 7, 8
AMP, CIP, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 8

[a] CHL, chloramphenicol; TET, tetracycline; STRE, streptomycin; AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid.
[b] D, interference with protein synthesis; A, interference with cell wall synthesis; B, interference with DNA synthesis; C, enzymatic inhibition.
[c] 7, phenicols; 1, aminoglycosides; 8, tetracyclines; 6, penicillins; 4, fluoroquinolones; 5, folate pathway inhibitors.

TABLE 7. *Antimicrobial resistance patterns of* Salmonella *isolates from poultry and pork*

Meat type	Antimicrobial resistance pattern[a]	No. of isolates	Mechanism(s)[b]	Antibiotic category(s)[c]
Poultry ($n = 6$)	CAZ, CIP, NAL	2	A, B	3, 4
	AMP, CHL, STRE, TET	2	A, B, D	1, 6, 7, 8
	AMP, CHL, NAL, STRE, SXT, TET	2	A, B, C, D	1, 4, 5, 6, 7, 8
Pork ($n = 4$)	STRE, TET	1	D	1, 8
	AMP, STRE, TET	3	A, D	1, 6, 8

[a] CAZ, ceftazidime; CIP, ciprofloxacin; NAL, nalidixic acid; AMP, ampicillin; CHL, chloramphenicol; STRE, streptomycin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole.
[b] A, interference with cell wall synthesis; B, interference with DNA synthesis; D, interference with protein synthesis; C, enzymatic inhibition.
[c] 3, extended-spectrum cephalosporins; 4, fluoroquinolones; 1, aminoglycosides; 6, penicillins; 7, phenicols; 8, tetracyclines; 5, folate pathway inhibitors.

Genes in ESBL and biofilm producers. All isolates confirmed to be ESBL producers carried the bla_{TEM} gene, and four isolates (one *E. coli* and three *Salmonella* isolates from poultry and pork) were positive for the presence of all three genes tested (Table 8). *E. coli* isolates positive for biofilm synthesis (32 of 110 isolates) carried the *fimH* gene, and only three isolates from poultry were positive for all three biofilm-associated genes tested (Table 9).

DISCUSSION

Promoting the use of former human foodstuffs as part of animal feeds without compromising animal and public health is one of the key objectives of the European Commission circular economy action plan on food *(12)*. However, the degree to which use of these foodstuffs could have consequences for both veterinary and human medicine must be evaluated. Among former foodstuffs, those of animal origin can be used raw in the formulation of pet diets. A review of the role of pet food in the transmission of zoonotic agents revealed the risk associated with raw meat pet diets *(32)*. In several studies, the presence of bacteria in raw meat and the potential for contaminating or shedding these bacteria in the pet's environment have been documented *(18, 47, 49)*.

In our study, 112 samples of former human foodstuff meat products were evaluated. The prevalence of *E. coli* in these former foodstuffs was similar to that in previous studies. Schlesinger and Joffe *(45)* reported that *E. coli* was isolated from all samples collected from 20 commercially prepared raw meat dog diets. In another study, the prevalence of *E. coli* in commercial raw diets for dogs and cats (64%) *(52)* was lower than that found in our study (98%). A lower prevalence of *Salmonella* was found by some researchers in the former foodstuffs than in commercial raw meat pet diets *(18, 49),* whereas our data were comparable to those obtained from frozen raw meat pet diets *(19)*. By-products in commercial raw meat diets for dogs and cats can comprise carcasses or parts of animals, which are rejected as unfit for human consumption in accordance with EU legislation *(10)* and therefore are more easily contaminated by bacterial pathogens than are food products. The freezing process may have contributed to the reduced growth of any *Salmonella* in these products. When microorganisms are subjected to an environmental stress such as freezing, some cells are killed and some may be only injured *(20)*.

TABLE 8. *ESBL* E. coli *and* Salmonella *isolates from poultry and pork and their genotypes*

		Genotype		
Meat type	Isolate	bla_{TEM}	bla_{SHV}	$bla_{\text{CTX-M}}$
Poultry	*E. coli* 28	+	−	−
	E. coli 30	+	+	−
	E. coli 31	+	+	+
Pork	*Salmonella* 1	+	+	+
	Salmonella 2	+	+	+
	Salmonella 3	+	−	−
	Salmonella 4	+	+	+

Although *E. coli* and *Salmonella* usually cause mild to moderate self-limiting gastroenteritis, complications may occur, resulting in pathological conditions such as urinary tract infections and inflammatory bowel diseases in humans and animals *(25)*. In the present study, *E. coli* isolates differed in antibiotic resistance patterns depending on the type of meat from which they were recovered. Isolates from poultry were characterized by high resistance to NAL, and those from pork had high resistance to TET. Use of fluoroquinolones and tetracyclines is widespread in poultry and pork breeding facilities *(21, 22)*. Although CHL is not authorized for use in food-producing animals in the EU, resistance to this agent was found in all types of meat, which is problematic because this antibiotic has been associated with aplastic anemia in humans and has reproductive and hepatotoxic effects in animals *(17)*. In accordance with the findings of Miranda et al. *(39),* we observed high resistance to older antimicrobial agents except for GEN, which is used less often in veterinary medicine. The lack of resistance to IMI is encouraging. Carbapenems are broad-spectrum β-lactam antimicrobial agents used mostly for the treatment of serious infections in humans and are considered the last line of therapy for infections caused by multidrug-resistant gram-negative bacteria.

The main concern is not related to resistance of bacteria to single antibiotic agent but rather to the spread of MDR among microorganisms. The prevalence of MDR is closely linked to the use of broad-spectrum antibiotics in various categories. The continuous use of these kinds of antibiotics leads to increased MDR in bacteria, thus creating a vicious

TABLE 9. E. coli *biofilm producers isolated from poultry, pork, and beef and their genotypes*

	No. of isolates with adherence type of:				Genotype		
Meat type	None	Weak	Moderate	Strong	*agn43* F, *flu* R	*fimH* F, *fimH* R	*papC* P1, *papC* P2
Poultry	36	3			+	+	+
		5			−	+	−
		8			+	+	−
Pork	27	3			−	+	−
Beef	15	3			−	+	−
		5			+	+	−
			1		+	+	−
			1		−	+	−
				3	+	+	−

cycle. In our study, the prevalence of MDR patterns in *E. coli* isolates from former foodstuffs of poultry, pork, and beef were 40.4, 66.7, and 42.9%, respectively. On average, MDR in bacteria from former foodstuffs is higher in pork and lower in poultry and beef compared with findings for raw meats *(1, 29, 39, 42)*. Kim et al. *(30)* reported that 87.2% of the *Salmonella* isolates from raw poultry were MDR, whereas in a study conducted on pork meat the MDR rate was 64.6% *(38)*. In the present study, the prevalence of MDR among *Salmonella* isolates from former foodstuffs was 66.7 and 75% for poultry and pork, respectively. Overall, 18.2% of the *E. coli* isolates and the 20% of the *Salmonella* isolates were resistant to antibiotics from different categories and with various mechanisms of action. Simultaneous resistance of isolates to compounds that act on key bacterial functions decreases the effectiveness of treatments for infections in both human and veterinary medicine.

Under the selective pressure over time of earlier generation β-lactams, *Enterobacteriaceae* have developed novel types of ESBL that can hydrolyze third- and fourth-generation cephalosporins *(42, 48, 50, 55)*. Our data clearly indicate that former foodstuffs derived from poultry and pork are a source of ESBL-producing *E. coli* and *Salmonella*. In particular, an alarmingly high prevalence of ESBL-producing *Salmonella* (100%) was detected in pork meat. Among the risk factors is the direct transmission of genes through mobile elements between bacteria of the same species or different families. The acquisition of resistance genes leads to an increase in phenotypic resistance regardless of the growth conditions or genetic characteristics of the microorganism *(2, 56)*. In the present study, most of the ESBL-producing *E. coli* and *Salmonella* isolates carried the correlated antibiotic resistance genes (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$).

In bacteria, one of the ways to counter the action of antimicrobial agents is biofilm production, which reduces the susceptibility of bacterial cells to these agents by slowing penetration into the biofilm and modifying the chemical microenvironment *(31, 36)*. Biofilm synthesis requires a set of genes facilitating the initial adhesion, maturation, and production of the extracellular polymeric matrix. Approximately 30% of the *E. coli* isolates from former foodstuffs were biofilm producers, and all of them harbored the *fimH* gene. The expression of this gene is involved in the colonization, invasion, and formation of biofilms *(44)*.

The results of this study suggest that former foodstuffs of animal origin may contain pathogens and can be a biological hazard when eaten raw by pets. The idea that a raw meat diet is natural and thus healthier for pets than canned, dried, and/or cooked food poses a risk for human health considering the close connection between humans and animals.

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