Microbial contamination, antimicrobial resistance and biofilm formation of bacteria isolated from a high-throughput pig abattoir

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Abstract

The aim of this work was to assess the level of microbial contamination and resistance of bacteria isolated from a high-throughput heavy pig slaughterhouse (approx. 4600 pigs/day) towards antimicrobials considered as critical for human, veterinary or both chemotherapies. Samples, pre-operative and operative, were obtained in 4 different surveys. These comprised environmental sampling, *i.e.* air (n_{total} = 192) and surfaces (n_{total} = 32), in four different locations. Moreover, a total of 40 carcasses were sampled in two different moments of slaughtering following Reg. (CE) 2073/2005. Overall, 60 different colonies were randomly selected from VRBGA plates belonging to 20 species, 15 genera and 10 families being *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* the most represented ones. Thirty-seven isolates presented resistance to at least one molecule and seventeen were classified as multi-drug resistant. *Enterobacteriaceae*, particularly *E. coli*, displayed high MIC values towards trimethoprim, ampicillin, tetracycline and sulphametoxazole with MIC_{max} of 16, 32, 32 and 512 mg/L, respectively. Moreover, isolated *Pseudomonas* spp. showed high MIC values in critical antibiotics such as ampicillin and azithromycin with MIC_{max} of 32 and 64 mg/L, respectively. Additionally, *in vitro* biofilm formation assays demonstrated that fifteen of these isolates can be classified as strong biofilm formers. Results demonstrated that a high diversity of bacteria containing antibiotic resistant and multiresistant species is present in the sampled abattoir. Considering these findings, it could be hypothesised that the processing environment could be a potential diffusion determinant of antibiotic resistant bacteria through the food chain and operators.

Introduction

In northern Italy, the breeding of heavy pigs (slaughtered at an approximate live weight of 170 kg) represents nearly the 90% of the national production with nearly 1 million slaughtered animals per month (I.Stat, 2021). At abattoir level, carcasses can be contaminated by bacteria in any of the different stages of processing (Pearce *et al.*, 2006). In order to control such hazards, it is compulsory that Food Business Operators (FBOs) implement Good Manufacturing and Hygienic Practices (GMP/GHP), proper Sanitation Standard Operating Procedures (SSOP) and Hazard Analysis and Critical Control Point (HACCP) based procedures along the entire slaughtering chain (Blagojevic *et al*., 2021) so as to meet the hygienic criteria established by the Reg. (CE) 2073/2005 (European Commission, 2005).

The microbiota present in the abattoir environment is considered a major risk for carcass contamination mainly due to transfer of bacteria from food and non-food contact surfaces (Di Ciccio *et al.,* 2016) and from bioaerosols (Cosenza-Sutton, 2004). The first one is referred to the inherent ability of bacteria to form multispecies biofilms that can persist through time (Rodríguez-López *et al*., 2020). Furthermore, these structures can facilitate horizontal transfer of antibiotic resistance genes (ARGs) raising concerns regarding the spreading of antimicrobial-resistant bacteria (ARB) through the food chain (EFSA, 2021).

On the other hand, it is nowadays accepted that air plays a critical role as a reservoir for ARB in pig abattoirs (EFSA, 2021; Masotti *et al.*, 2019). For instance, high levels of resistance to tetracycline, trimethoprim-sulfamethoxazole and ampicillin in two hundred forty-three aerobic bacteria isolated from a pig slaughterhouse were found (Li *et al.*, 2016). Similarly, a high occurrence of colistin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from process water and wastewater taken in pig slaughterhouses was reported (Savin et al., 2020). Additionally, recently published data report that Gram-negative bacteria play a central role in the transmission of the genes responsible for the onset of antimicrobial resistance (EFSA, 2021). Regarding this, the study carried out by Cosenza-Sutton, (2004) has shown that bacteria such as *Pseudomonas* spp. or *Enterobacteriaceae* are part of the normal air bioburden in both wet and clean zones of pig abattoirs (Cosenza-Sutton, 2004).

Therefore, the main aim of the present study was to investigate the level of contamination and the antimicrobial resistance and biofilm formation capability of Gram-negative bacteria present in a high-throughput heavy pig abattoir located in Emilia-Romagna (northern Italy).

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Materials and Methods

Rationale of the study

This study was performed in a pig slaughterhouse located in Emilia-Romagna, one of the regions included in the so-called

Italian *Food Valley* together with Lombardy, Piedmont and Veneto. This abattoir was characterised by a daily slaughtering capacity of about 480 fatteners per hour destined for Protected Designation of Origin (PDO) production. The premise had a linear design and was divided into two zones for sampling purposes: a 'wet' room (slaughter) and a 'clean' room (dressing). Sampling was performed weekly over a month.

Sampling

Air

Air samples were collected from four different locations: two in the wet room (WR), next to the gambrelling table (A1) and the polisher (A2), and two in the clean room (CR), near evisceration (A3) and precutting (A4) areas. At each survey, samples were collected one hour before (pre-operative) and five hours after the commencement of the slaughtering process (operative) using a portable SAS Super ISO-100 sampler (PBI International, Milan, Italy) placed at 1.5 m distance from the floor and 1.5 m distance from the processing line. Sampler's head was autoclaved at 121°C for 15 min before and after each sampling day and decontaminated with 70% ethanol between samples. Sampled volume in each location was determined in a preliminary survey (data not shown).

Samples were collected in triplicate on Plate Count Agar (PCA, Oxoid, Hampshire, UK) for enumeration of the total aerobic count (TVC; n_{total} = 96 plates) and on Violet Red Bile Glucose Agar (VRBGA, Oxoid) for the enumeration of Gram-negative bacteria (GNB; n_{total} = 96 plates).

Surfaces

Four different surfaces before (pre-operative) and after the commencement (operative) of the plant activity were sampled. Specifically, from the WR the gambrelling table (S1) and the polisher (S2), and from the CR the saw (S3) and the Fat-O-Meter (FOM, S4) were chosen (n_{total} = 32). In each location, an area of 100 cm^2 was sampled by thoroughly rubbing the surface using a sterile sponge (Whirl-Pak®, Madison, WI) moistened in 10 mL of Buffered Peptone Water (BPW, Oxoid) and kept refrigerated until analysis.

Carcasses

en carcasses were sampled in each sampling day as described in Reg. (CE) 2073/2005 (European Commission, 2005). In each sampling day, carcasses were sampled in two different rounds, at the beginning of the slaughtering activities, first batch of the day, and five hours after the initiation of the plant activity (n_{total} = 40).

Bacterial enumeration

Air samples were directly incubated. Sponges were mixed with 90 mL of BPW and stomached for 2 min. Resulting suspensions were serially diluted in phosphate buffered saline (PBS, Oxoid) and a 1 mL aliquot was spread onto PCA and VRBGA plates. In all cases, PCA plates were incubated at 30°C for 48 h and VRBGA plates at 37°C for 24 h for enumeration of TVC and GNB, respectively.

For air sampling, results were expressed in log CFU/m^3 representing the mean value of three plates whereas for surfaces and carcasses outcomes were expressed in log CFU/cm^2.

Isolation and identification of GNB

Bacterial colonies were randomly selected from VRBGA plates, stroked on Trypticase Soy Agar (TSA, Oxoid) and incubated at 37°C for 24 h. To ensure purity, the latter was repeated twice.

Next, colonies were subjected to oxidase test using Oxidase strips (Oxoid). Oxidase negatives were identified using the Microgen® GN-ID A/B system (Microgen bioproducts, Camberley, UK) whereas for oxidase positive isolates the API 20 NE system (bioMérieux, Marcy-l'étoile, France) was preferred.

Stock cultures of each isolate were kept at −80 °C in Brain-Heart Infusion (BHI, Oxoid) containing 50% glycerol 1:1 (v/v) mixed. Working cultures were maintained at −20 °C in Trypticase Soy Broth (TSB, Oxoid) containing 50% glycerol 1:1 (v/v) mixed.

Minimum inhibitory concentration (MIC) assays

The antimicrobial susceptibility of the GNB isolates was assessed via determination of MIC using the Sensititre™ – VIZION™ system (ThermoFisher Scientific, Waltham, MA, USA) using the EUVSEC3 panel, containing antibiotics considered as critical for antimicrobial chemotherapy in veterinary (Cloramphenicol, C; Sulphametoxazole, SMX; Tetracycline, TET; Trimethoprim, TRI), human (Nalidixic Acid, NA; Colistin, COL; Meropenem, MER) or both (Amikazin, AMI; Ampicillin, AMP; Azitromycin, AZI; Ceftazidime, CAZ; Ciprofloxacin, CIP, Cefoxitin, CTX; Gentamicin, GEN; Tigecycline, TIG) following manufacturer's instructions.

Biofilm assays

The production of biofilm was assessed by the method described by Stepanovic et al. (2007). Strains were classified as non-producers or weak, moderate, strong formers.

Statistical analyses

Statistical tests were performed using OriginPro 2021 software for Windows, v. 9.8.0.200 (OriginLab, Northampton, MA, USA) expressing significance at the 95% confidence level (α=0.05) or greater.

Specifically, to statistically compare TVC and GNB values between locations a one-way ANOVA with Tukey's post hoc test was chosen. Contrarily, a two-tailed Student's t-test was preferred to determine significance between pre-operative and operative samples.

Lastly, a two-sided chi-square (X^2) test was used to determine the correlation between the biofilm formation and antimicrobial multiresistance of GNB isolated.

Results

Microbial counts

Air

In pre-operative air samples, total viable count (TVC) presented mean values ranging from 2.19±0.11 and 3.67±0.27 log CFU/m^3 in locations A4 and A1, respectively (Table 1). One-way ANOVA analysis showed significance ($P<0.05$) in all sampling sites but those belonging to the CR, *i.e.* locations A3 and A4. Conversely, operative samples TVC values ranged from 2.91±0.13 and 4.33±0.08 log CFU/m^3 belonging to locations A4 and A1, respectively. No significance among operative samples ($P>0.05$) in TVC mean values was observed between the two locations of the WR (*i.e.* A1 and A2) whereas TVC values of the rest of sampling locations were significantly different ($P<0.05$; Table 1).

Of note, initiation of the normal activity of the slaughterhouse increased all TVC mean values of 0.65, 1.11, 0.79 and 0.71 log CFU/m^3 in locations A1, A2, A3 and A4, respectively, which represented a significant increase ($P<0.05$; two-tailed *t* Student) if TVC values were compared individually in terms pre-operative and operative sampling momenta (Table 1).

Overall, Gram-negative bacteria (GNB) counts in air pre-operative samples were lower compared to TVC values (Table 1). Additionally, GNB outcomes in some of the preoperative samples, from both wet and clean areas, demonstrated that the amount of GNB were below the limit of detection (10 CFU/m^3) considering the sampled volume (results not shown). Moreover, no significance ($P>0.05$) in GNB values were observed between any location among pre-operative samples (Table 1).

Slaughtering activities increased GNB

Table 1. Mean values (n = 3 agar plates, each) expressed in log CFU/m³ for total viable count (TVC) and Gram-negative bacteria (GNB) obtained in air active sampling at the selected locations at a pig abattoir. In each column, values with a different superscript indicate statistically significant differences between mean values (One-way ANOVA; α = 0.05). Additionally, a two-tailed Student's *t* test (α = 0.05) was performed to assess the significance between PreOperative and Operative samples.

Zone	Location	Operation	TVC (log CFU/m³)			GNB (log CFU/m³)		
			PreOperative	Operative	Dif Pre/OP	PreOperative	Operative	Dif Pre/OP
Wet room	A1	Gambrelling	3.67 ± 0.27^{A}	4.33 ± 0.08^{A}	Yes	1.42 ± 0.55^{A}	1.93 ± 0.18^{A}	No
	A2	Polishing	3.15 ± 0.19^{B}	4.26 ± 0.22^{A}	Yes	1.06 ± 0.13^{A}	$1.36 \pm 0.37^{A,B}$	No
Clean room	A3	Evisceration	2.38 ± 0.45^{C}	3.17 ± 0.14^{B}	Yes	1.10 ± 0.17^{A}	1.16 ± 0.22^{B}	No
	A4	Pre-cut	2.19 ± 0.11^{C}	2.91 ± 0.13^{C}	Yes	0.92 ± 0.37^{A}	$1.39 \pm 0.25B$	No

Dif Pre/OP: Significance between pre-operative and operative samples.

Table 2. Mean values in log CFU/cm² for total viable count (TVC) and Gram-negative bacteria (GNB) obtained in surface sampling at the selected locations at a pig abattoir. In each column, values with a different superscript indicate statistically significant differences between mean values (One-way ANOVA; α = 0.05). Additionally, a two-tailed Student's *t* test (α = 0.05) was performed to assess the significance between PreOperative and Operative samples.

Zone	Location	Operation	TVC (log CFU/cm²)			GNB (log CFU/cm²)		
			PreOperative	Operative	Dif Pre/OP	PreOperative	Operative	Dif Pre/OP
Wet room	S1	Gambrelling	5.05 ± 0.62^{A}	6.11 ± 0.23^{A}	Yes	3.02 ± 0.78	4.01 ± 0.33^{A}	Yes
	S2	Polishing	3.95 ± 0.24^{A}	5.65 ± 0.59^{A}	Yes	N.D.	$3.45 \pm 0.85^{A,B}$	N.A.
Clean room	S3	Saw	1.42 ± 0.60^{A}	3.66 ± 0.66^{B}	Yes	N.D.	$2.02 \pm 0.63^{B,C}$	N.A.
	S4	FOM	3.76 ± 0.68^{B}	4.37 ± 0.49^{B}	No	N.D.	2.74 ± 0.13^{C}	N.A.

Dif Pre/OP: Significance between pre-operative and operative samples; N.D.: Not detected; N.A.: Not applicable.

counts in 0.51, 0.3, 0.06 and 0.47 log CFU/m³ in locations A1 to A4, respectively. Taking a closer look, among operative samples GNB in location A1 (WR) was of 1.93±0.48 log CFU/m³ which was significantly different (P<0.05) to locations A3 and A4 (CR). Of note, location A2 (WR) presented no significance compared to the rest of the locations (Table 1).

Surfaces

Among pre-operative samples, mean TVC ranged from 1.42±0.60 to 5.05±0.62 log CFU/cm² corresponding to locations S3 and S1, respectively (Table 2). Of note, in locations A2, A3 and A4, collected samples had TVC that were below the limit of detection (results not shown). In all cases, normal slaughter activity of the plant increased the TVC mean values, being especially relevant in samples coming from the cutting saw presenting a significant increment of 2.23 log CFU/cm². For the rest of the locations the observed increase was of 1.06, 1.7 and 0.6 log CFU/cm² corresponding to locations S1, S2 and S4, respectively (Table 2). Moreover, a sharp decrease in TVC between pre and operative samples of 2.53 and 1.99 log CFU/cm² was observed in locations S2 and S3, respectively (Table 2).

GNB in preoperative samples were detected only among those coming from location S1. Mean value was significantly lower (P>0.05) compared to the GNB mean obtained in operative samples (4.01±0.33 log CFU/cm²). In all the other locations, samples collected during the slaughtering activities, presented detectable GNB counts ranging from 2.02 to 3.45 log CFU/cm² (Table 2).

Table 3. Mean values in log CFU/cm² for total viable count (TVC) and Gram-negative bacteria (GNB) obtained from pig carcasses in both sampling times sampling using the method described in Reg. (CE) 2073/2005. Of note, there was no significance between the two different moments of sampling (two-tailed Student's *t* test, α = 0.05).

TVC		GNB	
Sampling 1	Sampling 2	Sampling 1	Sampling 2
3.81 ± 0.38	3.95 ± 0.29	2.45 ± 0.49	2.09 ± 0.42

Carcasses

Mean TVC coming from pig carcasses did not present variations in mean values comparing both sampling times. Similarly, GNB outcomes neither presented statistically significant differences (two-tailed Student's *t* test; P>0.05) comparing the GNB values obtained in the first and the second moment of sampling, respectively. Of note, considering both counts, it can be inferred that more than 50 % of the microbiota isolated from the carcasses, was mainly due to the presence of GNB (Table 3).

Diversity in GNB

Overall, 20 different species belonging to 10 families coming from air (n=15 pre-operative, n=15 operative), surfaces (n=6 pre-operative, n=16 operative) and carcasses (n=8) were identified (Table 4). Regarding the families, *Enterobacteriaceae* (n=22), especially *E. coli* (n=15) followed by *Serratia liquefaciens* (n=3), was the most represented one (Table 4). Major non-*Enterobacteriaceae* isolated families were *Pseudomonadaceae* (n=9) and *Moraxellaceae*, (n=9) with *Pseudomonas* and *Moraxella* as representatives, respectively. Additionally, minor families such as *Aeromonoadaceae, Burkholdeliaceae* and *Brucellaceae*, were also identified (Table 4).

Antimicrobial resistance

Out of the 60 isolates assayed, 37 (61.67%) were resistant to at least one antibiotic. As displayed in Table 4 and Supplementary Table S1, overall, 59.09% of the *Enterobacteriaceae* isolates presented resistance to AMP (n=13, MIC_{max} = 32 mg/L), 59.09 to % TRI (n=13, MIC_{max} = 16 mg/L), 45.05% to SMX (n=10, MIC_{max} = 512 mg/L), 40.91% to TET (n=9, MIC_{max} = 32 mg/L) and 22.72% to C (n=5, MIC_{max} = 64 mg/L). Two *E. coli* isolates, namely GN4 and GN7, were the only that displayed resistance to. CAZ (MIC_{max} = 8 mg/L) and to COL (MIC_{max} = 16 mg/L). Of note, one of the identified *S. liquefaciens*, namely GN21, was the only *Enterobacteriaceae*

Table 4. List of families, species, isolation sources, antibiotic resistance pattern and biofilm production of the Gram-negative isolates obtained in this study. For a complete list of MIC values for each antibiotic molecule tested, the reader is kindly referred to Table S1.

Isolate ID	Family	Species	Source	Sampling time	Room	Resistance Pattern	Biofilm production
GN1	*Enterobacteriaceae*	*Escherichia coli*	Air	PreOperative	Wet	AMP-SMX-TET-TRI	Weak
GN2		*Escherichia coli*	Air	PreOperative	Wet	AMP-SMX-TRI	Non producer
GN3		*Escherichia coli*	Air	PreOperative	Clean	AMP-C-SMX-TET-TRI	Weak
GN4		*Escherichia coli*	Air	PreOperative	Clean	AMP-CAZ-COL-TRI	Weak
GN5		*Escherichia coli*	Air	PreOperative	Clean	n.d.	Weak
GN6		*Escherichia coli*	Air	Operative	Wet	n.d.	Strong
GN7		*Escherichia coli*	Air	Operative	Wet	CAZ-COL-TRI	Moderate
GN8		*Escherichia coli*	Air	Operative	Clean	AMP-SMX-TET-TRI	Moderate
GN9		*Escherichia coli*	Air	Operative	Clean	AMP-SMX-TRI	Weak
GN10		*Escherichia coli*	Surface	Operative	Wet	n.d.	Weak
GN11		*Escherichia coli*	Surface	Operative	Clean	TRI	Strong
GN12		*Escherichia coli*	Surface	Operative	Clean	n.d.	Moderate
GN13		*Escherichia coli*	Surface	Operative	Clean	AMP-C-SMX-TET-TRI	Weak
GN14		*Escherichia coli*	Carcass	n.a.	n.a.	AMP-NA-SMX-TRI	Moderate
GN15		*Escherichia coli*	Carcass	n.a.	n.a.	TET	Weak
GN16		*Enterobacter gergoviae*	Surface	Operative	Wet	AMP	Moderate
GN17		*Pantoea agglomerans*	Air	PreOperative	Clean	n.d.	Moderate
GN18		*Pantoea agglomerans*	Air	Operative	Wet	AMP-SMX-TET-TRI	Moderate
GN19		*Serratia liquefaciens*	Air	Operative	Clean	AMP-C-SMX-TET-TRI	Moderate
GN20		*Serratia liquefaciens*	Carcass	n.a.	n.a.	AMP-TET	Weak
GN21		*Serratia liquefaciens*	Carcass	n.a.	n.a.	AMP-C-GEN-SMX-TET-TRI	Weak
GN22		*Klebsiella oxytoca*	Surface	Operative	Wet	n.d.	Non producer
GN23	*Morganellaceae*	*Morganella* spp.	Surface	Operative	Wet	AZI-COL	Weak
GN24	*Comamonaceae*	*Comamonas* spp.	Air	Operative	Wet	n.d.	Weak
GN25	*Moraxellaceae*	*Acinetobacter baumannii*	Air	Operative	Wet	n.d.	Strong
GN26		*Acinetobacter baumannii*	Surface	PreOperative	Wet	AMP-AZI-C	Strong
GN27		*Acinetobacter baumannii*	Surface	Operative	Wet	n.d.	Moderate
GN28		*Acinetobacter haemolyticus*	Air	PreOperative	Clean	n.d.	Weak
GN29		*Acinetobacter lwoffii*	Surface	Operative	Wet	COL	Moderate
GN30		*Acinetobacter* spp.	Air	PreOperative	Wet	n.d.	Weak
GN31		*Acinetobacter* spp.	Air	Operative	Wet	n.d.	Weak
GN32		*Moraxella* spp.	Air	PreOperative	Wet	n.d.	Non producer
GN33		*Moraxella* spp.	Air	PreOperative	Wet	n.d.	Weak
GN34		*Moraxella* spp.	Air	Operative	Clean	AMP-C	Strong
GN35		*Moraxella* spp.	Surface	Operative	Clean	n.d.	Strong
GN36		*Moraxella* spp.	Surface	Operative	Clean	n.d.	Strong
GN37		*Moraxella* spp.	Surface	Operative	Wet	AMP-CIP	Strong
GN38		*Moraxella* spp.	Carcass	n.a.	n.a.	n.d.	Strong
GN39	*Brucellaceae*	*Ochrobactrum anthropi*	Air	Operative	Clean	AMP	Weak
GN40		*Ochrobactrum anthropi*	Surface	PreOperative	Clean	n.d.	Moderate
GN41	*Burkholdeliaceae*	*Ralstonia pickettii*	Air	Operative	Wet	n.d.	Moderate
GN42		*Ralstonia pickettii*	Surface	PreOperative	Wet	n.d.	Weak
GN43		*Ralstonia pickettii*	Surface	PreOperative	Wet	n.d.	Weak
GN44	*Alcaligenaceae*	*Alcaligenes* spp.	Surface	Operative	Clean	n.d.	Strong
GN45	*Pseudomonadaceae*	*Pseudomonas fluorescens*	Air	PreOperative	Wet	AMP-AZI-CTX-C-COL-TIG	Strong
GN46		*Pseudomonas fluorescens*	Carcass	n.a.	n.a.	AMP-AZI-C-TET	Strong
GN47		*Pseudomonas orzyhabitans*	Air	PreOperative	Clean	AMP	Moderate
GN48		*Pseudomonas putida*	Air	Operative	Wet	AMP-C	Weak
GN49		*Pseudomonas putida*	Carcass	n.a.	n.a.	AMP-AZI-C	Strong
GN50		*Pseudomonas stutzeri*	Air	PreOperative	Wet	n.d.	Strong
GN51		*Pseudomonas stutzeri*	Air	Operative	Clean	AMP	Weak
GN52		*Pseudomonas stutzeri*	Surface	PreOperative	Wet	AMP-CIP	Strong
GN53		*Pseudomonas stutzeri*	Surface	Operative	Clean	AMP-CIP	Moderate
GN54	*Xanthomonadaceae*	*Stenotrophomonas maltophilia*	Carcass	n.a.	n.a.	AMP-CTX	Non producer
GN55	*Aeromononadaceae*	*Aeromonas hydrophila*	Air	PreOperative	Wet	AMP-CTX-C-TIG	Weak
GN56		*Aeromonas hydrophila*	Surface	Operative	Clean	C	Weak
GN57		*Aeromonas sobria*	Air	PreOperative	Clean	AMP-TET	Non producer
GN58		*Aeromonas sobria*	Air	Operative	Clean	AMP-TET	Moderate
GN59		*Aeromonas sobria*	Suface	PreOperative	Wet	AMP-TET-TIG	Weak
GN60		*Aeromonas* spp.	Surface	Operative	Wet	AMP	Weak

AMP: Ampicillin; AZI: Azitromycin; CTX: Cefotaxime; CAZ: Ceftazidime; C: Chloramphenicol; CIP: Ciprofloxacin; COL: Colistin; GENT: Gentamicin; NA: Nalidixic acid; SMX: Sulfamethoxazole; TET: Tetracycline; TIG: Tigecycline; TRI: Trimethroprim; n.a.: Not applicable; n.d.: Not detected.

member to display resistance to GEN (MIC = 4 mg/L). No resistance to AZI, CTX, CIP, MER and TIG was detected in any of the *Enterobacteriaceae* isolated.

Twelve of these isolates were classified as multidrug resistant (MDR), *i.e.* presenting resistance towards 3 or more antibiotic classes (Magiorakos *et al.*, 2012). Among *E. coli*, most MDR were isolated from air samples either from preoperative (isolates GN1, GN2, GN3 and GN4) and operative (isolates GN7, GN8 and GN9) sampling times. This MDR phenotype was also observed in *E. coli* from surfaces (isolate GN13) and carcass (isolate GN14). Other assayed *Enterobacteriaceae* members displaying the MDR phenotype were *S. liquefaciens* from air (isolate GN19) and carcass (isolate GN21) samples, and *Pantoea agglomerans* from air samples (isolate GN18) (Table 4).

Among non-*Enterobacteriaceae* members identified, the level of antimicrobial resistance was generally lower compared to *Enterobacteriaceae* with a 44.74% (n=17) presenting no resistance to any of the antibiotics (Table 4). In addition to this, 47.37% of the isolates presented resistance to AMP (n=18; MIC_{max} = 32 mg/L), followed by a 21.05% resistant to C (n=8; MIC_{max} = 64 mg/L), a 13.06% resistant to AZI (n=5; MIC_{max} = 64 mg/L), and a 10.53% resistant to TIG (MIC_{max} = 4 mg/L). Of note, most of these resistances were displayed by members of the genus *Pseudomonas* (Table 4, Supplementary Table S1). MDR was determined in 5 of the non-*Enterobacteriaceae* isolates assayed. Thus, the species displaying this phenotype were *Acinetobacter baumanii* (isolate GN26), *Pseuodomonas fluorescens* (isolates GN45 and GN46), *Pseudomonas putida* (isolate GN49), and *Aeromonas hydrophila* (isolate GN55) (Table 4).

Overall, maximum level of resistance was displayed by isolates *S. liquefaciens* G21 (carcass) and *P. fluorescens* GN45 (air) showing lack of susceptibility upon 6 out of the 15 molecules tested, representing 6 and 5 antibiotic classes, respectively (Table 4).

Biofilm formation

Most isolates (91.66%; n=55) were able to adhere to produce quantifiable biomass levels after 24 h of incubation. More specifically, 41.67% (n=25) were weak producers, 25% (n=15) were moderate producers and 25% (n=15) and were classified as strong producers (Table 4).

In the latter category, it is important to remark that the isolates were obtained both from environmental sources (*i.e.* air and surfaces) and from swine carcasses. At a species level, outcomes demonstrated a wide variability in terms of strong biofilm formers, showing that this phenotype is not limited to one particular species (Table 4). Specifically, most of them belonged to *Moraxella* spp. (n=5). However, this strong biofilm production was not concomitant with resistance towards the antibiotics tested (Table 4).

Isolates namely GN26 (*A. baumannii*), GN45, GN46 (*P. fluorescens*) and GN49 (*P. putida*) were categorised as MDR (Table 4). Statistical analysis (X^2 test) showed no correlation ($P>0.05$) between the biofilm forming capacity and MDR phenotype (results not shown).

Discussion

Bioaerosols represent a major factor of bacterial spread in food-related premises (EFSA, 2021; Jericho *et al.*, 2000). Consequently, knowing the aerobiology of a given environment would provide key information to avoid microorganism dissemination by including air dynamics in GHP, GMP, SSOP and HACCP schemes (EFSA, 2021; Haddrell and Thomas, 2017). In this line, our outcomes demonstrated that among pre-operative samples, highest TVC were found in the WR with mean values of 3.67±0.27 log CFU/m^3 that progressively diminished as the slaughter line advanced reaching minimum mean TVC of 2.19±0.11 log CFU/m^3 in the clean area which significantly increased ($P<0.05$) after the commencement of the abattoir activity (Table 1). The latter can be attributable to an increase or suspended water droplets loaded with microorganism clumps potentially mobilised by FBOs inside the premise (Haddrell and Thomas, 2017; Pearce *et al.*, 2006). Similar TVC values in WR and CR and the increase in aerial bacterial counts due to the plant activity have been previously reported by Pearce *et al.*, (2006) in an Irish pig abattoir although lower counts were obtained in pre-operative ones due to differences in clumping and physiological state of bacteria affecting their growth onto agar plates (Haddrell and Thomas, 2017; Okraszewska-Lasica *et al.*, 2014). Additionally, results showed a significant decrease of TVC between WR and CR indicating that, at the moment of sampling, physical barriers such as walls and doors and controlled ventilation systems, caused an interruption in the air flow in areas containing a higher bioburden thus impairing its spread (Pearce *et al*., 2006; Prendergast *et al.*, 2004).

Airborne Gram-negative bacteria (GNB) are commonly associated with animal skin, soil and manure (Okraszewska-Lasica *et al*., 2012) and disseminated within abattoirs by the incoming animals, suspended dust particles, and faecal matter present in workers' boots. (Cosenza-Sutton, 2004). A variety of members of *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* were mainly detected in air samples (Table 4). This is in line with the study performed by Cosenza-Sutton, (2004) in three pork abattoirs, concluding that these families are part of the normal microbiota of a swine abattoir. In the light of this, monitoring of aerosolised GNB could be a useful tool in HACCP schemes for hygienic status assessment in abattoirs in terms of contamination from faecal origin (Okraszewska-Lasica *et al.,* 2012). Of note, airborne GNB represented less than half of the TVC, indicating that, in this scenario, aerial microbiota may also harbour microorganisms other than GNB such as Gram-positive bacteria, fungi and yeasts as previously reported (Fernstrom and Goldblatt, 2013).

Regardless of the moment of sampling, recovered *Enterobacteriaceae* displayed a remarkable level of antimicrobial resistance to β-lactams, SMX, C, COL, TET and TRI (Table 4). Resistance to these antibiotics have been previously detected in *E. coli*, *Serratia* spp., *Pantoea* spp., *Klebsiella* spp., *Enterobacter* spp. and other *Enterobacteriaceae* recovered from samples in a Chinese abattoir (Li *et al.*, 2016). In a similar way, Amador *et al*. (2019) carried out an study dealing with the antimicrobial resistance of *Enterobacteriaceae* present in pork and beef slaughterhouses samples in Portugal demonstrating high levels of resistance to β-lactams, C, CIP, TET and sulphametoxazole-trimethoprim (SxT), being in line with our results. Plausible origins of these bacteria could be aerosolised wastewater containing manure considered as one of the major sources of antimicrobic-resistant *Enterobacteriaceae* in pig slaughterhouses (Homeier-Bachmann *et al.*, 2021; Savin *et al.,* 2020).

A remarkable number of airborne *Enterobacteriaceae* isolated were classified as MDR (Table 4) showing resistance towards AMP (MIC_{max} = 32 mg/L), SMX (MIC_{max} = 512 mg/L), TET (MIC_{max} = 32 mg/L) and TRI (MIC_{max} = 16 mg/L) somehow forming a sort of core-resistance pattern. This phenomenon has been previously described by Schwaiger *et al.* (2012) in pork samples where isolated *Enterobacteriaceae* showed common resistance to penicillins, tetracyclines and SxT, indicating not only a transmission and recirculation of genes associated to antimicrobial resistance but also that environmental

pressures (humidity, improper use of sanitizers, etc.) selecting for a bacterial subset are present (EFSA, 2021). Regarding this, recent data demonstrates that *E. coli* exposure to sub-lethal concentrations of disinfectants such as benzalkonium chloride or hydrogen peroxide, induces cross-resistance to AMP and C which is linked to modifications in the membrane composition and subsequent changes in cell surface charge, as well as the selection of mutant variants (Nordholt *et al*., 2021; Pereira *et al.,* 2021).

Moreover, five isolates were classified as strong biofilm formers (Table 4). Among them, isolate GN45 (*P. fluorescens*) was also considered as MDR. In abattoirs, MDR *Pseudomonas* spp. with outstanding capacity to form biofilms have been described (Lerma *et al.*, 2014). These structures consisting of bacteria embedded in a self-produced polymeric extracellular matrix providing species within high endurance to insults such as antimicrobials (Flemming, 2011). Consequently, isolate GN45 represent a potential hazard due to its antimicrobial resistance profile and the spreading capacity as airborne bacteria suspended into dust particles or water droplets, accumulating and adhering onto other surfaces generating new foci of contamination harbouring pathogenic and non-pathogenic bacteria that can be further transferred to carcasses (EFSA, 2021; Rodríguez-López *et al.*, 2020).

Among pre-operative samples, identified bacteria belonged to *Aeromonadaceae*, *Brucellaceae, Burkholdeliaceae*, *Moraxellaceae* and *Pseudomonadaceae* (Table 4), previously described as part of the microbiota in food-processing environments (Møretrø and Langsrud, 2017). Moreover, pre-operative samples give information about the surfaces' resident microbiota providing the first step for aggregation of further bacterial species coming from different sources (incoming animals, water, dust particles, etc.) during the normal activity of the plant (Møretrø and Langsrud, 2017). Among them, isolate GN26 (*A. baumannii*) is of especial relevance both because its adhesion capacity and its MDR characteristics (Table 4). Regarding this, a study carried out by Li *et al.* (2016) demonstrated that among *Acinetobacter* spp. isolated from pork at slaughter, high resistances to AMP and C were found and that more than 50% of them displayed resistance to multiple antibiotics. Similarly, Hrenovic *et al*. (2019) detected high resistance to AMI (MIC ≥ 256 mg/L) and reduced sensibility to carbapenems and AMP in *A. baumannii* isolated from swine manure. Considering the potential risk of these species, isolates displaying GN26 features should be carefully monitored due to its capability to adhere forming subsequent contamination foci and also becoming a potential reservoir of ARGs eventually transmitted into the food chain (EFSA, 2021).

Outcomes also demonstrated that not all bacteria isolated from surfaces in pre-operative samples were strong biofilm formers (Table 4). Therefore, the fact that one species is present in a given surface is not only dependent on the adhesion capacity of the bacterium itself but also that, in that particular spot, a lack of proper sanitation could be present. This highlights the importance of proper monitoring of environmental contamination for bacterial control carried out by FBOs and continuous verification of the SSOs efficacy or their modifications whenever needed.

Higher GNB counts concomitant with an increase in the bacterial diversity was observed among operative samples (Table 4). The latter was mainly due to species belonging to *Enterobacteriaceae* and *Moraxellaceae* found in both WR and CR (Table 4). Of note, among *Enterobacteriaceae* an isolate of *Klebsiella oxytoca* was found which is especially relevant since it is considered nowadays as an emerging pathogen causing nosocomial infections (Neog *et al.*, 2021). Moreover, among values showed that the more separated the locations were, the more different they were which was expected considering that sampled surfaces in the wet area (locations S1 and S2), are in more contact with the slaughtered animals compared with the locations in the clean one (locations S3 and S4) (Table 2). Previous published data described how members of these families have been associated with pigs (Abuoun *et al.,* 2017; Li *et al*., 2016), fact that can be linked to our results thus indicating that incoming pigs would be one of the main sources of contamination/recontamination in the sampled abattoir.

Conclusions

In this work, the presence of antimicrobial-resistant bacteria in the environmental of a high-throughput pig abattoir was demonstrated. This fact raises concerns in terms and assurance of safety and quality of meat products mainly in two respects: firstly, the diversity in the Gram-negative microbiota found and the levels of resistance, are indicative of a presumable higher burden of ARGs. These can be easily spread horizontally thus generating MDR variants, subsequently disseminated though air directly to foodstuffs or environmentally persist as biofilms. Secondly, it highlights once more the upmost importance for FBOs to follow a "One-Health" approach for the monitoring of hygienic conditions considering the different ecological niches in a given food-related scenario.

Therefore, it is of paramount importance that FBOs implement specific control measures regarding ARB colonisation and transmission in abattoirs and other related food-processing environments in order to avoid dissemination between animals, the environment and FBOs themselves. These objectives can be achieved through improvement of good hygienic practises during the normal activity of the plant as well as the biosecurity practises*, i.e.,* individual protection of personnel, monitoring of environmental contamination and efficacy of sanitation methodologies.

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