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Corresponding Author: Professor Jose L. Blanco, PhD, DVM

Corresponding Author's Institution: Facultad de Veterinaria. UCM.

First Author: Sergio Alvarez-Perez, PhD

Order of Authors: Sergio Alvarez-Perez, PhD; Jose L. Blanco, PhD, DVM; Rafael J Astorga, PhD, DVM; Jaime Gomez-Laguna, PhD, DVM; Belen Barrero-Dominguez; Angela Galan-Reaño; Celine Harmanus; Ed J Kuijper; Marta E Garcia, PhD, DVM

Abstract: The presence and genetic diversity of Clostridium difficile and C. perfringens along the slaughtering process of pigs reared in a free-range system was assessed. A total of 270 samples from trucks, lairage, slaughter line and quartering were analyzed, and recovered isolates were toxinotyped and genotyped. C. difficile and C. perfringens were retrieved from 14.4% and 12.6% of samples, respectively. The highest percentage of positive samples for C. difficile was detected in trucks (80%) whereas C. perfringens was more prevalent in cecal and colonic samples obtained in the slaughter line (85% and 45%, respectively). C. difficile isolates (n = 105) were classified into 17 PCR ribotypes (including 010, 078, and 126) and 95 AFLP genotypes. C. perfringens isolates (n = 85) belonged to toxinotypes A (94.1%) and C (5.9%) and were classified into 80 AFLP genotypes. The same genotypes of C. difficile and C. perfringens were isolated from different pigs and occasionally from environmental samples, suggesting a risk of contaminated meat products.

Madrid, 10th July 2018

Dear Editor of *Food Research International*,

We resubmit our manuscript entitled **'Distribution and tracking of *Clostridium difficile* and *Clostridium perfringens* in a free-range pig abattoir and processing plant'** to be considered for publication in your journal. In this new version of the manuscript, we have addressed all the comments from the reviewers. In particular, we have modified Figures 1 and 2 and their corresponding legends to clarify the meaning of the different codes shown on the tips of the dendrograms. This and other changes are explained in detail in the point-by-point response to the reviewers and can also be identified in the 'tracked changes' version of the manuscript.

We hope that you can consider now our manuscript adequate to be published in your prestigious journal.

Yours faithfully,

Prof. José L. Blanco, DVM, PhD

Department of Animal Health
Veterinary Faculty
Universidad Complutense
E-28040 Madrid, Spain
Phone: +34 91 394 3717 Fax: +34 91 394 3908
E-mail: jlblanco@ucm.es

ANSWER TO THE REVIEWERS

Response to the comments of Reviewer #1

The objective of the work was to investigate the presence and genetic diversity of Clostridium difficile and C. perfringens along the slaughtering process of pigs reared in a free range system. Generally, this is a novel study with well-writing, but there are some issues the authors should address.

We thank the Reviewer for these nice comments on our manuscript.

The authors should give more details on the environmental condition when sampling, e.g. temperature… And the hygiene condition should also be provided since it can affect the presence of bacteria.

Air temperature in the different rooms of the pig abattoir and processing plant sampled in this study ranged between 20 ºC and 25 ºC, except the quartering room with a temperature below 12ºC. This has been indicated in Material and Methods (lines 79-81).

On the other hand, as now indicated in the manuscript (see lines 78-79), all the facilities sampled in the study comply with the European Union, national and regional regulations on hygiene, food safety and animal welfare. We do not consider necessary to include further details about this aspect in the manuscript, but if the Editor and/or the Reviewer does we would be glad to provide them with such information.

Why to choose March as the sampling time? Please clarify.

Because most free-range pigs in Southern Spain are slaughtered between February and April, so we considered that the sampling date was just at the middle of the local peak season. This aspect has been indicated in lines 76-77.

The samples were stored at -70 ºC before bacterial isolation. Is this a standard protocol? Reference? And for how long?

This is the procedure that we and many other researcher groups follow for storage of samples to be analyzed for clostridial presence, especially when a high number of samples need to be handled. Please note that previous studies have demonstrated that storage temperature (4ºC and < 20ºC) and even multiple cycles of freezing (refrigeration)/thawing have minimal effects upon the viability of *C. difficile* spores (e.g. Freeman & Wilcox [J Clin Pathol. 2003, 56(2):126-8]).

Although the authors detected the presence of Clostridium difficile and C. perfringens by culturing methods, the numbers of these bacteria were unknown in difference samples, this could affect the risk assess of Clostridium difficile and C. perfringens.

Direct culturing of *C. difficile* and *C. perfringens* is unreliable for many different reasons: for example, low numbers of spores/vegetative cells may be present within a sample and these may be unevenly distributed (see a discussion on this topic in our previous paper Blanco et al. [Vet J. 2013, 197:694-8] and references listed therein). Accordingly, most researchers prefer to use enrichment protocols to retrieve *C. difficile* and *C. perfringens* from clinical and/or environmental samples. For that same reason, we used an enrichment protocol before plate culturing and did not try to enumerate the CFUs present in our samples. A brief mention to this aspect has been included in Materials and Methods (section 2.2, lines 106-109).

Response to the comments of Reviewer #2

Abstract

Line 24: abbreviation TLSQ necessary here?

Indeed, that abbreviation may not be needed in the abstract and, therefore, we have removed it (see line 24).

Line 28: please make clear that "cecal and colonic content" belong to slaughter line samples

Following the Reviewer's suggestion, we have rephrased that sentence as follows (lines 26-29): "The highest percentage of positive samples for *C. difficile* was detected in trucks (80%) whereas *C. perfringens* was more prevalent in cecal and colonic samples obtained in the slaughter line (85% and 45%, respectively)."

Material and methods.

It should be mentioned that spore selection in isolation procedures for C. perfringens will markedly reduce the number of isolates because most strains do not sporulate in the usually employed culture media.

We agree that vegetative forms of *C. difficile* and *C. perfringens* are eliminated by spore selection in absolute ethanol. However, most authors also perform ethanol shock after enrichment in broth culture so as to eliminate potential contaminations (see, e.g., Weese et al., 2010 [Anaerobe, 16:501-4]; Schneeberg et al., 2012 [Anaerobe, 18:484-8]; and Hussain et al., 2015 [Anaerobe, 36:9-13]). Furthermore, some studies have indicated that *C. difficile* strains of different PCR ribotypes can produce abundant spores within 24 h (see, e.g., Vohra and Poxton, 2011 [Microbiology 157:1343-53]). Therefore, we believe that our culturing procedures are adequate for the purposes of the present study.

Results.

Line 195-203 and Figure 1: C. difficile AFLP genotypes In Figure 1: To me it is not clear what the number after the ribotype (in red) is indicating.

I cannot follow how the AFLP genotypes (i.e. cd74, cd89) are named; they are also not depicted in Fig.1.

The codes in black shown next to ribotype designations correspond to isolate names. This has been clarified in the legend of Figure 1 and also in the dendrogram itself. Furthermore, we have included a brief mention in lines 202-203: *"AFLP-based fingerprinting grouped C. difficile isolates into 104 peak profiles and 95 distinct genotypes (**designated as cd1 to cd95;** Fig. 1)."*

Line 216-221 and Fig. 2: C. perfringens AFLP genotypes In Fig. 2: Again it is not clear what the number at the tip of the branches is indicating.

Correspondingly I cannot follow how the AFLP genotypes (i.e. cp55) are named; they are also not depicted in Fig. 2.

Again, the codes in black at the tip of branches refer to isolate names. This has been now clarified in the legend of Figure 2 and also in the dendrogram. Finally, a brief mention has been included in lines 224-225: *"AFLP-based fingerprinting of C. perfringens isolates yielded 85 different peak profiles and 80 distinct genotypes (**cp1 to cp80**; Fig. 2)."*

Discussion

Line 302: I suggest to replace "major threat" by "possible threat" given that C. perfringens type A is in general wide spread in the environment and is also part of the intestinal microbiota in animals and humans. Also to my knowledge complete absence of C. perfringens is not a general requirement for foods.

As suggested, we have changed *"major threat"* to *"possible threat"* in that line (now 310). Nevertheless, we should mention that, although toxinotypes C and D have been traditionally considered the main responsibles of human and animal disease, we have noticed in recent years at our institution an increase in the number of cases of animal diarrhea from which *C. perfringens* toxinotype A is isolated. Similarly, other authors have suggested that alfa toxin, which is more abundantly produced by toxinotype A, may be implicated in the enteric pathologies caused by this clostridial species.

Highlights

- Analysis of *C. difficile* (CD) and *C. perfringens* (CP) presence in a free-range pig abattoir.
- CD was mainly found in trucks, whereas CP was more prevalent in the slaughtering line.
- High diversity of AFLP genotypes was found among CD and CP isolates.
- The same CD and CP genotypes were found in slaughtered pigs and the environment.
- Some CD isolates belonged to epidemic ribotypes (e.g. 078 and 126).

Distribution and tracking of *Clostridium difficile* and *Clostridium perfringens* in a free-range pig abattoir and processing plant

Sergio Álvarez-Pérez[a], José L. Blanco[a,*], Rafael J. Astorga[b], Jaime Gómez-Laguna[c], Belén Barrero-Domínguez[b], Angela Galán-Relaño[b], Celine Harmanus[d], Ed Kuijper[d], Marta E. García[a]

[a] *Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Madrid, Spain*

[b] *Department of Animal Health, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain*

[c] *Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain*

[d] *Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands*

*** Corresponding author:**

Prof. José L. Blanco, PhD, DVM. Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid. Avda. Puerta de Hierro s/n, 28040 Madrid (Spain). Tel.: +34 91 394 3717. E-mail address: jlblanco@ucm.es

Abstract

The presence and genetic diversity of *Clostridium difficile* and *C. perfringens* along the slaughtering process of pigs reared in a free-range system was assessed. A total of 270 samples from trucks, lairage, slaughter line and quartering were analyzed, and recovered isolates were toxinotyped and genotyped. *C. difficile* and *C. perfringens* were retrieved from 14.4% and 12.6% of samples, respectively. The highest percentage of positive samples for *C. difficile* was detected in trucks (80%) whereas *C. perfringens* was more prevalent in cecal and colonic samples obtained in the slaughter line (85% and 45%, respectively). *C. difficile* isolates (n = 105) were classified into 17 PCR ribotypes (including 010, 078, and 126) and 95 AFLP genotypes. *C. perfringens* isolates (n = 85) belonged to toxinotypes A (94.1%) and C (5.9%) and were classified into 80 AFLP genotypes. The same genotypes of *C. difficile* and *C. perfringens* were isolated from different pigs and occasionally from environmental samples, suggesting a risk of contaminated meat products.

Keywords: Abattoir, *Clostridium difficile*, *Clostridium perfringens*, free-range pig, lairage, slaughter line

1. Introduction

Detection and tracking of microorganisms along the the food chain is of key importance to establish both pathogens' survival throughout a particular production chain and how these microorganisms may eventually reach the consumer (Duffy et al., 2008). Although virtually any food product can act as a reservoir of pathogenic microorganisms, meat and derivatives are frequently highlighted as important sources of human food-borne infection (Fosse et al., 2008; Nørrung and Buncic, 2008). Accordingly, farm-to-fork surveillance systems have been implemented for the most prevalent pathogens found in meat (Nørrung and Buncic, 2008).

The Gram-positive, spore-forming anaerobes *Clostridium difficile* and *C. perfringens* are frequent colonizers of the intestinal tract of diverse food animals, and particularly of pigs (Songer and Uzal, 2005). Both bacterial species have been found in the environment of pig abattoirs and in pork meat (Hall and Angelotti, 1965; Metcalf et al., 2010; Curry et al., 2012; Mooyottu et al., 2015; Wu et al., 2017). Nevertheless, while *C. perfringens* ranks among the most important agents of food-borne disease (Fosse et al., 2008; Butler et al., 2015), the classification of *C. difficile* as a pathogen causing food-borne outbreaks is still controversial (Warriner et al., 2017).

Although organic and eco-friendly pig rearing systems are gaining increased importance and popularity, most surveillance studies of *C. difficile* and *C. perfringens* prevalence in swine herds, abattoirs and pig carcasses published so far have focused on intensively-raised animals. However, Keessen et al. (2011) and Susick et al. (2012) found similar prevalence and strain types of *C. difficile* among conventional and outdoor, antimicrobial-free production systems. In a previous study, a high prevalence of the

epidemic *C. difficile* PCR ribotype 078 was detected in Iberian pigs reared in free-range systems in 'La Dehesa', a type of human-managed Mediterranean ecosystem where they feed on acorns and fresh grass, and only periparturient sows and pre-weaned (≤45-day-old) piglets are kept in closed facilities (Álvarez-Pérez et al., 2013).

In this study we determined the presence of *C. difficile* and *C. perfringens* in an abattoir and processing plant of free-range pigs, which have been previously identified as a common source of both clostridia (Álvarez-Perez et al., 2013; and unpublished observations). Additionally, bacterial isolates were toxinotyped and further characterized genetically to track possible sources of carcass contamination.

2. Material and methods

2.1. Sampling

Sampling was performed in March 2016 (middle of the local peak season for the slaughtering of free-range pigs) in an abattoir and processing plant located in southern Spain. All the facilities complied with the European Union, national and regional regulations on hygiene, food safety and animal welfare. Air temperature in the different rooms of the pig abattoir and processing plant sampled in this study ranged between 20 ºC and 25 ºC, except the quartering room with a temperature below 12ºC. Systematic cleaning and disinfection of the facilities is carried out following each slaughtering. In addition, in periods where no slaughtering is performed, more exhaustive and meticulous cleaning and disinfection protocols which include the dismantling of equipments are carried out.

Two different batches of animals, corresponding with batches at the beginning and at the end of the same working day (TLSQ1 and TLSQ2, respectively), were sampled to determine the prevalence of both clostridia species in Trucks, Lairage, Slaughter line and Quartering (TLSQ) (Hernández et al., 2013). The traceability of each pig was strictly followed along the abattoir, and samples were obtained in the following six stages of the production chain: i) trucks at their arrival (T1) and after cleaning and disinfection (T2) (floor, walls, ceiling, entrance ramps and cabin's mat); ii) lairage, prior entry of the pigs (cleaned and disinfected, L1) and just after departure to slaughter (dirty, L2); iii) ten pig carcasses per batch at six different stages (pre-scalding, S1; post-scalding, S2; post-flaming, S3; post-evisceration, S4; post-washing, S5; and, chilling, S6); iv) tonsils (To), cecal (Ce) and colonic (Co) contents; v) environmental samples from the slaughter line (ES) (scalding water, knives and saws) and from the quartering environment (EQ) (sterilization water, tables and knives); and vi) quartering samples (Q) (ham, shoulder and loin) (see details in Table 1). All samples were collected into sterile containers (for feces, tonsils, meat or water samples) or with sterile sponges into plastic bags (for samples from carcasses and surfaces), and transported to the laboratory, where they were stored at –70 ºC until analyzed.

2.2. Bacterial isolation and identification

As direct culturing of *C. difficile* and *C. perfringens* is unreliable (e.g. because of the low numbers of spores/vegetative cells that may be present within a sample and the uneven distribution of these; Blanco et al., 2013), isolation of these microorganisms from all sample types was performed by enrichment culturing. Briefly, sampling sponges were defrosted and cut into half, and the pieces were then introduced into 50-mL plastic tubes containing 15 mL of the enrichment broth used by Blanco et al. (2013)

or brain-heart infusion broth (BHI; TecLaim, Madrid, Spain), for enrichment of *C. difficile* and *C. perfringens*, respectively. After 7 days of incubation at 37 ºC (for *C. difficile*) or 72 h at 46 ºC (for *C. perfringens*) under anaerobic conditions, 2 mL of the liquid cultures were mixed with 2 mL of absolute ethanol (Panreac) and incubated for 1 h under agitation (200 rpm) at room temperature. Finally, the tubes were centrifuged at 1,520 *g* for 10 min, the supernatants were discarded and the precipitates collected using sterile cotton-tipped swabs and plated onto CLO agar (bioMérieux, Marcy l'Étoile, France) for selective culturing of *C. difficile*, and Brucella blood agar (bioMérieux) and tryptone sulfite neomycin agar (TSN; Laboratorios Conda, Madrid, Spain) for isolation of *C. perfringens*. Inoculated plates were incubated under anaerobic conditions for 48 h to 7 days at 37 ºC (for *C. difficile*) or 46 ºC (for *C. perfringens*).

Tonsils (5 g) and meat samples (ham, shoulder and loin, 5 g in total), obtained at quartering, were diluted in 15 mL of the aforementioned enrichment broths, mechanically homogenized for 2 min using a Seward 80 stomacher (Seward Medical, London, England) and further handled as described above. Swabs were introduced in the fecal samples, and then cultured into a 10 mL tube containing 5 ml of the enrichment broth for *C. difficile* or BHI for *C. perfringens*, and further handled as decribed above. Water samples (25 mL) were filtered through a 0.45-µm-pore-size membrane filter (Millipore Corporation, Billerica, MA, USA) using Microfil filtration funnels (Millipore) connected to a vacuum system. Filters were then washed with 20 mL of ethanol 70 % (v/v) and 20 mL of sterile distilled water, introduced in the sterile 50-mL polypropylene tubes containing 15 mL of BHI or *C. difficile* enrichment broth, and further handled as sponge and meat samples.

C. difficile isolates were identified by colony morphology, the typical odor of this microorganism and a positive PCR reaction for the species-specific internal fragment of the gene encoding for triose phosphate isomerase (*tpi*) (Lemee et al., 2004). Identification of isolates as *C. perfringens* was achieved by observing the typical double-zone hemolysis of this species when cultured on blood agar, formation of black colonies on TSN, Gram staining reaction and microscopic morphology.

2.3. Toxinotyping of isolates

For *C. difficile* isolates, expression of the genes which encode for toxin A and toxin B (*tcdA* and *tcdB*, respectively), and the two components of binary toxin (CDT) (*cdtA* and *cdtB*), was detected by PCR as previously reported (Álvarez-Pérez et al., 2009, 2015). The genes encoding for *C. pefringens* major toxins, enterotoxin and the consensus and atypical forms of β2 toxin (*cpb2*) were detected as described by Álvarez-Pérez et al. (2016, 2017a).

2.4. Ribotyping of C. difficile isolates

PCR ribotyping of *C. difficile* isolates was performed according to the high-resolution capillary gel-based electrophoresis method of Fawley et al. (2015). Ribotypes were designated according to the PHLS Anaerobic Reference Unit (Cardiff, UK) standard nomenclature and the Leiden-Leeds database (The Netherlands). Non-typeable isolates were also compared with the strain database at Leeds University (Dr. W. Falwey and Prof. M. Wilcox) that encompasses more than 600 different types.

2.5. Amplified Fragment Length Polymorphism (AFLP) typing

Genotyping of all *C. difficile* and *C. perfringens* isolates was performed by an AFLP method previously described (Álvarez-Pérez et al., 2017a,b). The products resulting from the selective amplification step were diluted 1/10 in nuclease-free water (Biotools, Madrid, Spain) and analyzed by capillary electrophoresis using the GeneScan 1200 LIZ size standard (Applied Biosystems, Madrid, Spain). All AFLP reactions were performed twice on different days for each strain.

2.6. Data analysis

Dendrograms of AFLP profiles obtained for *C. difficile* and *C. perfringens* isolates were created using Pearson's correlation coefficients and the unweighted-pair group method with arithmetic averages (UPGMA) clustering algorithm, as implemented in PAST v.3.11 (Hammer et al., 2001). Isolates clustering with ≥86% similarity were considered to belong to the same AFLP genotype (Killgore et al., 2008; Álvarez-Pérez et al., 2017a,b).

3. Results

3.1. Prevalence of C. difficile and C. perfringens

Clostridium difficile and *C. perfringens* were retrieved from 39 (14.4%) and 34 (12.6%) out of the 270 analyzed samples in total, respectively. Most culture-positive samples yielded only one *Clostridium* species, but eight samples (3% of total) yielded colonies of both *C. difficile* and *C. perfringens*. The distribution of positive samples per TLSQ assay and production stage is shown in Table 1. Overall, the highest percentage of positive samples for *C. difficile* was detected in the trucks (80%, considering T1 and T2) followed by the lairage stage (37.5%, L1 + L2), whereas *C. perfringens* was more prevalent in the slaughter line (16.7% of positive samples, considering all sample types

obtained at this stage) and, in particular, in the cecal and colonic content of sampled pigs (85% and 45%, respectively). The overall proportion of positive samples was higher in the TLSQ2 assay than in TLSQ1 (1.6 times, for both *C. difficile* and *C. perfringens*) and there was some variation in the distribution of both clostridia (Table 1).

3.2. Diversity of C. difficile isolates

A total of 105 *C. difficile* isolates (x ± S.D. = 2.7 ± 0.5 isolates per positive TLSQ sample) were selected from the original plate cultures for ribotyping and further characterization. About 72.4% of those isolates (76/105) could be classified into one of the already known PCR ribotypes: 078 (34 isolates), 572 (15), 110 (9), 126 (6), 202 (6), 010 (2), 013 (2) and 181 (2). The toxin profiles and other characteristics of these ribotypes are detailed in Table 2. The remaining 29 isolates (27.6% of total) belonged to nine unknown ribotypes, which will be hereafter referred to as U01 to U09 ('U' stands for 'unknown'; Table 2).

AFLP-based fingerprinting grouped *C. difficile* isolates into 104 peak profiles and 95 distinct genotypes (designated as cd1 to cd95; Fig. 1). All isolates belonging to ribotypes 078 and 126 (n = 40, in total) and to the toxigenic type U09 (n = 4) were included into two well-defined groups that clustered apart from the isolates of the other 14 PCR ribotypes (Fig. 1). Although eight AFLP genotypes included multiple isolates, only two out of these eight AFLP genotypes clustered isolates belonging to different PCR ribotypes (genotypes cd74 and cd89, both of which included two type 078 isolates and one U09 isolate). All samples from which multiple *C. difficile* isolates could be

recovered (38 in total) yielded two or more different AFLP types, and 23.7 % of these also yielded different PCR ribotypes.

3.3. Diversity of C. perfringens isolates

Eighty-five *C. perfringens* isolates (2.5 ± 0.8 isolates per positive culture of TLSQ samples) were selected for detailed characterization. Toxinotyping of these isolates revealed that 80 of them (94.1%) belonged to toxinotype A and only five isolates (5.9%) were of toxinotype C (Table 1). None of the isolates had the enterotoxin-encoding gene (*cpe*) but 20 type A isolates from diverse sample sources were positive for presence of an atypical form of the β2-encoding gene (*cpb2*), and other five type A isolates (three obtained from the tonsils of the same pig, one from the colonic content of another pig and the remaining from a truck's floor) were found to carry the consensus form of *cpb2* (Table 1).

AFLP-based fingerprinting of *C. perfringens* isolates yielded 85 different peak profiles and 80 distinct genotypes (cp1 to cp80; Fig. 2). Only four AFLP types grouped together two or more isolates (see details below) and all samples from which multiple isolates of *C. perfringens* could be obtained (29 in total) yielded two or more different AFLP types. Notably, one AFLP type (cp55) clustered together two toxinotype A and one toxinotype C isolates.

3.4. Distribution of C. difficile and C. perfringens genotypes along the production chain

Five out of the 17 PCR ribotypes of *C. difficile* (29.4%) were found in samples obtained at different steps of the pork production chain (Table 2): 078 (T, L, S and environmental samples), 110 (T and S), 572 and U01 (T, L and S) and U09 (L and S).

The tracking of individual AFLP genotypes of *C. difficile* and *C. perfringens* along the different sample sources investigated is shown in Fig. 3. Two *C. difficile* genotypes were found in multiple samples from the same TLSQ assay (cd38 from TLSQ1, and cd70 and cd79 from TLSQ2), and two additional genotypes were found in samples from both TLSQ1 and TLSQ2 (cd74 and cd89) and included isolates of PCR ribotypes 078 and U09 (Fig. 3). In addition, a same *C. perfringens* genotype (cp23) was retrieved from TLSQ2 samples obtained from the floor of a truck and the quartering of a carcass. However, while the truck isolate yielded a positive PCR result for presence of a consensus form of the *cpb2* gene, the isolate from the quartering sample was *cpb2*-negative (Fig. 3). No other AFLP genotype grouped together *C. perfringens* isolates from different sample sources but three AFLP types included isolates obtained from the cecal or colonic content of different pigs (cp12 and cp55, and cp33, respectively).

4. Discussion

Previous studies have demonstrated that *C. difficile* and *C. perfringens* are common environmental contaminants of abattoirs slaughtering intensively-raised pigs (Rho et al., 2001; Chan et al., 2012; Hawken et al., 2013; Rodriguez et al., 2013; Wu et al., 2017). However, much less is known about the prevalence and diversity of these two anaerobes in abattoirs dealing with pigs raised under free-range conditions (but see Susick et al., 2012).

In this study, we found that *C. difficile* and *C. perfringens* are widespread environmental contaminants in a free-range pig abattoir and processing plant. Both species were isolated from trucks (including cabin's mats which never came into direct

contact with animals) and lairage samples obtained after cleaning and disinfection, indicating that these procedures were not efficient to eliminate clostridial spores. A similar conclusion was reached by Hernández et al. (2013) in a survey for *Salmonella* spp. Despite these data may be biased due to the fact that a single abattoir was sampled, they highlight the potential risk of contamination by *C. difficile* and *C. perfringens* when exhaustive cleaning and disinfection protocols are not applied at every step from the transport of the animals to the lairage.

Detailed genetic characterization of the isolates obtained in this study showed a high genetic diversity for *C. difficile* and *C. perfringens* and revealed the presence of some particular strain types in both environmental samples and pig carcasses, which agrees with the observations of other authors (Hawken et al., 2013; Wu et al., 2017). Furthermore, high diversity of PCR ribotypes and AFLP was found even among isolates retrieved from a same sample, thus confirming the recommendation of examining multiple isolates from culture-positive clinical and environmental samples (Tanner et al., 2010; Álvarez-Pérez et al., 2016). Overall, these results agree with those obtained by Hernández et al. (2013) for *Salmonella* spp. but contrasts with the low genetic diversity detected for *Listeria monocytogenes* by López et al. (2008) in a different abattoir. Differences in the pig populations analyzed, sampling methods, target bacterial species and/or techniques used for molecular typing of isolates might account for these discrepancies.

Notably, we identified *C. difficile* PCR ribotypes which rank among the most prevalent in outbreaks of human disease, such as ribotypes 010, 078 and 126 (Davies et al., 2016). Interestingly, the PCR ribotypes 078 and 126 are close phylogenetic relatives (Stabler et

al., 2012; Schneeberg et al., 2013) that are frequently recovered from slaughtered animals and meat products (Metcalf et al., 2010; Curry et al., 2012; Hawken et al., 2013; Cho et al., 2015; Mooyottu et al., 2015; Wu et al., 2017). Moreover, high genetic relatedness between human and animal isolates of the 078/126 ribotype complex has been repeatedly reported (Bakker et al., 2010, Koene et al., 2012, Schneeberg et al., 2013; Knetsch et al., 2014; Álvarez-Pérez et al., 2017b). All of this has encouraged an ongoing discussion about the zoonotic and food-borne potential of the 078/126 lineage (Squire and Riley, 2013; Warriner et al., 2017). However, direct transmission of *C. difficile* (from animals to humans or vice versa) has not been yet demonstrated and the possibility of acquisition from a common environmental source cannot be excluded (Squire and Riley, 2013; Knetsch et al., 2014).

Regarding the toxigenic diversity of the isolates characterized in this study, most *C. difficile* isolates yielded a positive PCR result for the genes encoding toxins A, B and/or binary toxin, all of which are regarded as the main virulence factors of the species (Smits et al., 2016). Moreover, *C. perfringens* isolates were classified into toxinotypes A and C, both of which are common enteric pathogens of swine (Songer and Uzal, 2005). In addition, some *C. perfringens* isolates of diverse origins had the genes encoding for consensus or atypical forms of the β2 toxin, a plasmid-borne pore-forming toxin which may play a role in pathogenesis (Songer and Uzal, 2005; Uzal et al., 2014). However, regardless of their origin and AFLP-type, all *C. perfringens* isolates yielded a negative PCR result for the gene encoding enterotoxin CPE, which is the main toxin involved in food poisoning in humans (Songer and Uzal, 2005; Uzal et al., 2014). In any case, given the huge arsenal of additional toxins that *C. perfringens* strains can produce

(Uzal et al., 2014), the mere presence of this species in abattoirs and food-processing plants should be regarded as a possible threat to public health.

Finally, a 27.6% of the *C. difficile* isolates analyzed in this study belonged to previously unknown PCR ribotypes. Interestingly, one of these ribotypes, named U09, clustered with ribotype 078 and 126 isolates in the UPGMA dendrogram built from AFLP patterns and, as these two, included isolates with the genes encoding for toxins A, B and binary toxin. Thus, U09 could be regarded as a new 078/126-like ribotype and future studies should try to assess the prevalence and genetic and phenotypic characteristics of this novel strain type.

5. Conclusions

In conclusion, as previously observed for abattoirs slaughtering intensively-raised pigs, *C. difficile* and *C. perfringens* can be found in free-range pig abattoirs and processing plants. In addition, molecular tracking of individual genotypes revealed that, for both clostridia, the same strain types could be recovered from animal and environmental samples, highlighting the potential for cross contamination of free-range pig carcasses.

Declaration of interest

None.

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Figure legends

Figure 1. Dendrogram of AFLP profiles obtained for the *Clostridium difficile* isolates characterized in this study (*n* = 105). The dendrogram was created by unweighted pair group method with arithmetic mean (UPGMA) clustering using Pearson's correlation coefficients. Individual AFLP genotypes are distinguished at ≥86% similarity (red dotted vertical line). The origin of isolates is indicated at the tip of branches (see legend on the lower left corner), followed by PCR ribotype, isolate and AFLP type designations (shown in red, black and blue, respectively). The two clusters comprising all ribotype 078/126 and U09 isolates are indicated by a green background. Abbreviations in legend: T, trucks; L, lairage; S, slaughter line; Q, quartering; E, environment of the slaughter line and processing plant; 1, TLSQ1; 2, TLSQ2.

Figure 2. Dendrogram of AFLP profiles obtained for the *Clostridium perfringens* isolates characterized in this study (*n* = 85). The dendrogram was created by unweighted pair group method with arithmetic mean (UPGMA) clustering using Pearson's correlation coefficients. Individual AFLP genotypes are distinguished at ≥86% similarity (red dotted vertical line). The first column of colored squares at the tip of branches indicates the origin of isolates (see color legend on the lower left corner). Additionally, green- and violet-filled squares indicate toxinotype A and toxinotype C isolates, respectively, and the presence of the consensus or atypical form of the β2 toxin-encoding gene (*cpb2*) is indicated by filled and open circles, respectively. Alphanumeric codes refer to isolate and AFLP type designations (shown in black and blue, respectively). Abbreviations in legend: T, trucks; S, slaughter line; Q, quartering; 1, TLSQ1; 2, TLSQ2.

Figure 3. Tracking of individual AFLP genotypes of *Clostridium difficile* and *Clostridium perfringens* along the pork production chain. Detection of each genotype from the different sample sources is indicated by shaded boxes, which also include the PCR ribotype (for *C. difficile*) or toxin profile (for *C. perfringens*). Only genotypes detected in two or more sample sources are included. Abbreviations for sample sources: T1, trucks prior cleaning and disinfection; T2, trucks after cleaning and disinfection; L1, lairage prior entry of the pigs; L2, lairage after exit of the pigs; S1, pre-scalding; S2, post-scalding; S3, post-flaming; S4, post-evisceration; S5, post-washing; S6, chilling; To, tonsils; Ce, cecal contents; Co, colonic contents; Q, quartering samples (ham, shoulder and loin); ES, environment slaughter line (scalding water, knives and saws); EQ, environment quartering (sterilization water, tables and knives).

Tables

Table 1: Distribution of *Clostridium difficile* and *Clostridium perfringens* along the pig slaughtering process in the examined free-range pig abattoir and processing plant.

TLSQ assay[a]	Production stage	Sample[b] (*n*)	*C. difficile*			*C. perfringens*			Both clostridial species
			No. (%) of positive samples	No. isolates	Ribotypes (no. AFLP types)	No. (%) of positive samples	No. isolates	Toxinotypes[c] (no. AFLP types)	No. (%) of positive samples
TLSQ1	Trucks	T1 (5)	4 (80%)	11	010 (1), 078 (2), 126 (1), 572 (7)	0			0
		T2 (5)	2 (40%)	5	078 (2), 126 (3)	0			0
	Lairage	L1 (4)	0			0			0
		L2 (4)	2 (50%)	6	078 (3), U01 (1), U09 (2)	0			0
	Slaughter line	S1 (10)	2 (20%)	5	U02 (3), U07 (2)	0			0
		S2 (10)	0			0			0
		S3-S6 (40)	0			0			0
		To (10)	0			2 (20%)	4	A (1), A/*cpb2*+[c] (3)	0
		Ce (10)	2 (20%)	4	572 (2), U06 (2)	8 (80%)	22	A (9), A/*cpb2*+[a] (12), C (1)	2 (20%)
		Co (10)	2 (20%)	6	572 (3), U02 (3)	3 (30%)	8	A (5), A/*cpb2*+[a] (2)	0
	Quartering	Q (10)	0			0			0
	Environment	ES (7)	1 (14.3%)	3	078 (2)	0			0
		EQ (10)	0			0			0

	TOTAL	(135)	15 (11.1%)	40	010 (1), 078 (9), 126 (4), 572 (11), U01 (1), U02 (6), U06 (2), U07 (2), U09 (2)	13 (9.6%)	34	A (15), A/*cpb2*+[c] (3), A/*cpb2*+[a] (14), C (1)	2 (1.5%)
TLSQ2	Trucks	T1 (5)	5 (100%)	15	010 (1), 078 (6), 110 (2), 126 (2), U01 (2), U03 (1)	2 (40%)	2	A (2)	2 (40%)
		T2 (5)	5 (100%)	13	078 (8), U03 (3), U04 (1)	1 (20%)	2	A (2)	1 (20%)
	Lairage	L1 (4)	2 (50%)	4	078 (4)	0			0
		L2 (4)	2 (50%)	5	013 (2), 572 (3)	0			0
	Slaughter line	S1 (10)	7 (70%)	20	078 (5), 181 (2), 202 (6), U01 (2), U08 (3), U09 (2)	2 (20%)	2	A (2)	1 (10%)
		S2 (10)	1 (10%)	2	U05 (2)	0			0
		S3-S6 (40)	0			0			0
		To (10)	0			0			0
		Ce (10)	1 (10%)	3	110 (2)	9 (90%)	27	A (16), A/*cpb2*+[a] (5), C (4)	1 (10%)
		Co (10)	1 (10%)	3	110 (3)	6 (60%)	15	A (14), A/*cpb2*+[a] (1)	1 (10%)
	Quartering	Q (10)	0			1 (10%)	3	A (3)	0
	Environment	ES (7)	0			0			0
		EQ (10)	0			0			0
	TOTAL	(135)	24 (17.8%)	65	010 (1), 013 (2), 078 (20), 110 (7), 126 (2), 181 (2), 202 (6), 572 (3), U01 (4), U03 (4), U04 (1), U05 (2), U08 (3), U09 (2)	21 (15.6%)	51	A (39), A/*cpb2*+[a] (6), C (4)	6 (4.4%)
TLSQ1	Trucks	T1 (10)	9 (90%)	26	010 (2), 078 (8), 110 (2),	2 (20%)	2	A (2)	2 (20%)

+ TLSQ2					126 (3), 572 (7), U01 (2), U03 (1)				
		T2 (10)	7 (70%)	18	078 (10), 126 (3), U03 (3), U04 (1)	1 (10%)	2	A (2)	1 (10%)
	Lairage	L1 (8)	2 (25%)	4	078 (4)	0			0
		L2 (8)	4 (50%)	11	013 (2), 078 (3), 572 (3), U01 (1), U09 (2)	0			0
	Slaughter line	S1 (20)	9 (45%)	25	078 (5), 181 (2), 202 (6), U01 (2), U02 (3), U07 (2), U08 (3), U09 (2)	2 (10%)	2	A (2)	1 (5%)
		S2 (20)	1 (5%)	2	U05 (2)	0			0
		S3-S6 (80)	0			0			0
		To (20)	0			2 (10%)	4	A (1), A/*cpb2*+[c] (3)	0
		Ce (20)	3 (15%)	7	110 (2), 572 (2), U06 (2)	17 (85%)	49	A (25), A/*cpb2*+[a] (17), C (5)	3 (15%)
		Co (20)	3 (15%)	9	110 (3), 572 (3), U02 (3)	9 (45%)	23	A (19), A/*cpb2*+[a] (3)	1 (5%)
	Quartering	Q (20)	0			1 (5%)	3	A (3)	0
	Environment	ES (14)	1 (7.1%)	3	078 (2)	0			0
		EQ (20)	0			0			0
	TOTAL	(270)	39 (14.4%)	105	010 (2), 013 (2), 078 (29), 110 (7), 126 (6), 181 (2), 202 (6), 572 (14), U01 (5), U02 (6), U03 (4), U04 (1), U05 (2), U06 (2), U07 (2), U08 (3), U09 (4)	34 (12.6%)	85	A (54), A/*cpb2*+[c] (3), A/*cpb2*+[a] (20), C (5)	8 (3%)

[a] Two different batches of Iberian pigs, corresponding with the first and last batches allocated to the day of sampling (TLSQ1 and TLSQ2, respectively), were analyzed.

[b] T1, trucks prior cleaning and disinfection; T2, trucks after cleaning and disinfection; L1, lairage prior entry of the pigs; L2, lairage after exit of the pigs; S1, pre-scalding; S2, post-scalding; S3, post-flaming; S4, post-evisceration; S5, post-washing; S6, airing; To, tonsils; Ce, cecal contents; Co, colonic contents; Q, quartering samples (ham, shoulder and loin); ES, environment slaughter line (scalding water, knives and saws); EQ, environment quartering (sterilization water, tables and knives).

[c] A, toxinotype A; C, toxinotype C; *cpb2*+, positive PCR result for the consensus [c] or atypical [a] form of the β2 toxin-encoding gene.

Table 2: Toxin profiles and AFLP types of *Clostridium difficile* ribotypes identified in this study.

PCR ribotype	Toxin profile	No. isolates	No. AFLP types	No. samples[a]					
				Total	T	L	S	Q	E
010	A-B-CDT-	2	2	2 (5.1%)	2				
013	A+B+CDT-	2	2	1 (2.6%)		1			
078	A+B+CDT+	34	29	14 (35.9%)	8	3	2		1
110	A+B+CDT-	9	7	3 (7.7%)	1		2		
126	A+B+CDT+	6	6	3 (7.7%)	3				
181	A-B-CDT-	2	2	1 (2.6%)			1		
202	A+B+CDT-	6	6	2 (5.1%)			2		
572	A+B+CDT-	15	14	6 (15.4%)	3	1	2		
U01	A+B+CDT-	5	5	5 (12.8%)	2	1	2		
U02	A-B-CDT-	6	6	2 (5.1%)			2		
U03	A+B+CDT-	4	4	2 (5.1%)	2				
U04	A+B+CDT-	1	1	1 (2.6%)	1				
U05	A-B-CDT-	2	2	1 (2.6%)			1		
U06	A-B-CDT-	2	2	1 (2.6%)			1		
U07	A-B-CDT-	2	2	1 (2.6%)			1		
U08	A-B-CDT-	3	3	1 (2.6%)			1		
U09	A+B+CDT+	4	4	2 (5.1%)		1	1		

[a] T, trucks; L, lairage; S, slaughter line; Q, quartering; E, environment of the slaughter line and processing plant.

Distribution and tracking of *Clostridium difficile* and *Clostridium perfringens* in a free-range pig abattoir and processing plant

Sergio Álvarez-Pérez[a], José L. Blanco[a,*], Rafael J. Astorga[b], Jaime Gómez-Laguna[c], Belén Barrero-Domínguez[b], Angela Galán-Relaño[b], Celine Harmanus[d], Ed Kuijper[d], Marta E. García[a]

[a] *Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Madrid, Spain*

[b] *Department of Animal Health, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain*

[c] *Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain*

[d] *Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands*

*** Corresponding author:**

Prof. José L. Blanco, PhD, DVM. Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid. Avda. Puerta de Hierro s/n, 28040 Madrid (Spain). Tel.: +34 91 394 3717. E-mail address: jlblanco@ucm.es

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Abstract

The presence and genetic diversity of *Clostridium difficile* and *C. perfringens* along the slaughtering process of pigs reared in a free-range system was assessed. A total of 270 samples from trucks, lairage, slaughter line and quartering ~~(TLSQ)~~ were analyzed, and recovered isolates were toxinotyped and genotyped. *C. difficile* and *C. perfringens* were retrieved from 14.4% and 12.6% of samples, respectively. The highest percentage of positive samples for *C. difficile* was detected in trucks (80%) whereas *C. perfringens* was more prevalent in ~~the~~ cecal and colonic ~~content of animals~~samples obtained in the slaughter line (85% and 45%, respectively). *C. difficile* isolates (n = 105) were classified into 17 PCR ribotypes (including 010, 078, and 126) and 95 AFLP genotypes. *C. perfringens* isolates (n = 85) belonged to toxinotypes A (94.1%) and C (5.9%) and were classified into 80 AFLP genotypes. The same genotypes of *C. difficile* and *C. perfringens* were isolated from different pigs and occasionally from environmental samples, suggesting a risk of contaminated meat products.

Keywords: Abattoir, *Clostridium difficile*, *Clostridium perfringens*, free-range pig, lairage, slaughter line

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1. Introduction

Detection and tracking of microorganisms along the the food chain is of key importance to establish both pathogens' survival throughout a particular production chain and how these microorganisms may eventually reach the consumer (Duffy et al., 2008). Although virtually any food product can act as a reservoir of pathogenic microorganisms, meat and derivatives are frequently highlighted as important sources of human food-borne infection (Fosse et al., 2008; Nørrung and Buncic, 2008). Accordingly, farm-to-fork surveillance systems have been implemented for the most prevalent pathogens found in meat (Nørrung and Buncic, 2008).

The Gram-positive, spore-forming anaerobes *Clostridium difficile* and *C. perfringens* are frequent colonizers of the intestinal tract of diverse food animals, and particularly of pigs (Songer and Uzal, 2005). Both bacterial species have been found in the environment of pig abattoirs and in pork meat (Hall and Angelotti, 1965; Metcalf et al., 2010; Curry et al., 2012; Mooyottu et al., 2015; Wu et al., 2017). Nevertheless, while *C. perfringens* ranks among the most important agents of food-borne disease (Fosse et al., 2008; Butler et al., 2015), the classification of *C. difficile* as a pathogen causing food-borne outbreaks is still controversial (Warriner et al., 2017).

Although organic and eco-friendly pig rearing systems are gaining increased importance and popularity, most surveillance studies of *C. difficile* and *C. perfringens* prevalence in swine herds, abattoirs and pig carcasses published so far have focused on intensively-raised animals. However, Keessen et al. (2011) and Susick et al. (2012) found similar prevalence and strain types of *C. difficile* among conventional and outdoor, antimicrobial-free production systems. In a previous study, a high prevalence of the

epidemic *C. difficile* PCR ribotype 078 was detected in Iberian pigs reared in free-range systems in 'La Dehesa', a type of human-managed Mediterranean ecosystem where they feed on acorns and fresh grass, and only periparturient sows and pre-weaned (≤45-day-old) piglets are kept in closed facilities (Álvarez-Pérez et al., 2013).

In this study we determined the presence of *C. difficile* and *C. perfringens* in an abattoir and processing plant of free-range pigs, which have been previously identified as a common source of both clostridia (Álvarez-Perez et al., 2013; and unpublished observations). Additionally, bacterial isolates were toxinotyped and further characterized genetically to track possible sources of carcass contamination.

2. Material and methods

2.1. Sampling

Sampling was performed in March 2016 (middle of the local peak season for the slaughtering of free-range pigs) in ~~a pig~~an abattoir and processing plant located in southern Spain. All the facilities complied with the European Union, national and regional regulations on hygiene, food safety and animal welfare. Air temperature in the different rooms of the pig abattoir and processing plant sampled in this study ranged between 20 ºC and 25 ºC, except the quartering room with a temperature below 12ºC. ~~Air temperature in the different rooms that were sampled ranged between XX ºC and XX ºC.~~ Systematic cleaning and disinfection of the facilities is carried out following each slaughtering~~,~~. In addition, in periods where no slaughtering is performed, more exhaustive and meticulous cleaning and disinfection protocols which include the dismantling of equipments are carried out.

Two different batches of animals, corresponding with batches at the beginning and at the end of the same working day (TLSQ1 and TLSQ2, respectively), were sampled to determine the prevalence of both clostridia species in Trucks, Lairage, Slaughter line and Quartering (TLSQ) (Hernández et al., 2013). The traceability of each pig was strictly followed along the abattoir, and samples were obtained in the following six stages of the production chain: i) trucks at their arrival (T1) and after cleaning and disinfection (T2) (floor, walls, ceiling, entrance ramps and cabin's mat); ii) lairage, prior entry of the pigs (cleaned and disinfected, L1) and just after departure to slaughter (dirty, L2); iii) ten pig carcasses per batch at six different stages (pre-scalding, S1; post-scalding, S2; post-flaming, S3; post-evisceration, S4; post-washing, S5; and, chilling, S6); iv) tonsils (To), cecal (Ce) and colonic (Co) contents; v) environmental samples from the slaughter line (ES) (scalding water, knives and saws) and from the quartering environment (EQ) (sterilization water, tables and knives); and vi) quartering samples (Q) (ham, shoulder and loin) (see details in Table 1). All samples were collected into sterile containers (for feces, tonsils, meat or water samples) or with sterile sponges into plastic bags (for samples from carcasses and surfaces), and transported to the laboratory, where they were stored at –70 ºC until analyzed.

2.2. Bacterial isolation and identification

As direct culturing of *C. difficile* and *C. perfringens* is unreliable (e.g. because of the low numbers of spores/vegetative cells that may be present within a sample and the uneven distribution of these; Blanco et al., 2013), ~~Isolation~~ isolation of ~~*C. difficile* and *C. perfringens*~~these microorganisms from all sample types was performed by enrichment culturing. Briefly, sampling sponges were defrosted and cut into half, and the pieces were then introduced into 50-mL plastic tubes containing 15 mL of the

enrichment broth used by Blanco et al. (2013) or brain-heart infusion broth (BHI; TecLaim, Madrid, Spain), for enrichment of *C. difficile* and *C. perfringens*, respectively. After 7 days of incubation at 37 ºC (for *C. difficile*) or 72 h at 46 ºC (for *C. perfringens*) under anaerobic conditions, 2 mL of the liquid cultures were mixed with 2 mL of absolute ethanol (Panreac) and incubated for 1 h under agitation (200 rpm) at room temperature. Finally, the tubes were centrifuged at 1,520 *g* for 10 min, the supernatants were discarded and the precipitates collected using sterile cotton-tipped swabs and plated onto CLO agar (bioMérieux, Marcy l'Étoile, France) for selective culturing of *C. difficile*, and Brucella blood agar (bioMérieux) and tryptone sulfite neomycin agar (TSN; Laboratorios Conda, Madrid, Spain) for isolation of *C. perfringens*. Inoculated plates were incubated under anaerobic conditions for 48 h to 7 days at 37 ºC (for *C. difficile*) or 46 ºC (for *C. perfringens*).

Tonsils (5 g) and meat samples (ham, shoulder and loin, 5 g in total), obtained at quartering, were diluted in 15 mL of the aforementioned enrichment broths, mechanically homogenized for 2 min using a Seward 80 stomacher (Seward Medical, London, England) and further handled as described above. Swabs were introduced in the fecal samples, and then cultured into a 10 mL tube containing 5 ml of the enrichment broth for *C. difficile* or BHI for *C. perfringens*, and further handled as decribed above. Water samples (25 mL) were filtered through a 0.45-µm-pore-size membrane filter (Millipore Corporation, Billerica, MA, USA) using Microfil filtration funnels (Millipore) connected to a vacuum system. Filters were then washed with 20 mL of ethanol 70 % (v/v) and 20 mL of sterile distilled water, introduced in the sterile 50-mL polypropylene tubes containing 15 mL of BHI or *C. difficile* enrichment broth, and further handled as sponge and meat samples.

C. difficile isolates were identified by colony morphology, the typical odor of this microorganism and a positive PCR reaction for the species-specific internal fragment of the gene encoding for triose phosphate isomerase (*tpi*) (Lemee et al., 2004). Identification of isolates as *C. perfringens* was achieved by observing the typical double-zone hemolysis of this species when cultured on blood agar, formation of black colonies on TSN, Gram staining reaction and microscopic morphology.

2.3. Toxinotyping of isolates

For *C. difficile* isolates, expression of the genes which encode for toxin A and toxin B (*tcdA* and *tcdB*, respectively), and the two components of binary toxin (CDT) (*cdtA* and *cdtB*), was detected by PCR as previously reported (Álvarez-Pérez et al., 2009, 2015). The genes encoding for *C. pefringens* major toxins, enterotoxin and the consensus and atypical forms of β2 toxin (*cpb2*) were detected as described by Álvarez-Pérez et al. (2016, 2017a).

2.4. Ribotyping of C. difficile isolates

PCR ribotyping of *C. difficile* isolates was performed according to the high-resolution capillary gel-based electrophoresis method of Fawley et al. (2015). Ribotypes were designated according to the PHLS Anaerobic Reference Unit (Cardiff, UK) standard nomenclature and the Leiden-Leeds database (The Netherlands). Non-typeable isolates were also compared with the strain database at Leeds University (Dr. W. Falwey and Prof. M. Wilcox) that encompasses more than 600 different types.

2.5. Amplified Fragment Length Polymorphism (AFLP) typing

Genotyping of all *C. difficile* and *C. perfringens* isolates was performed by an AFLP method previously described (Álvarez-Pérez et al., 2017a,b). The products resulting from the selective amplification step were diluted 1/10 in nuclease-free water (Biotools, Madrid, Spain) and analyzed by capillary electrophoresis using the GeneScan 1200 LIZ size standard (Applied Biosystems, Madrid, Spain). All AFLP reactions were performed twice on different days for each strain.

2.6. Data analysis

Dendrograms of AFLP profiles obtained for *C. difficile* and *C. perfringens* isolates were created using Pearson's correlation coefficients and the unweighted-pair group method with arithmetic averages (UPGMA) clustering algorithm, as implemented in PAST v.3.11 (Hammer et al., 2001). Isolates clustering with ≥86% similarity were considered to belong to the same AFLP genotype (Killgore et al., 2008; Álvarez-Pérez et al., 2017a,b).

3. Results

3.1. Prevalence of C. difficile and C. perfringens

Clostridium difficile and *C. perfringens* were retrieved from 39 (14.4%) and 34 (12.6%) out of the 270 analyzed samples in total, respectively. Most culture-positive samples yielded only one *Clostridium* species, but eight samples (3% of total) yielded colonies of both *C. difficile* and *C. perfringens*. The distribution of positive samples per TLSQ assay and production stage is shown in Table 1. Overall, the highest percentage of positive samples for *C. difficile* was detected in the trucks (80%, considering T1 and T2) followed by the lairage stage (37.5%, L1 + L2), whereas *C. perfringens* was more prevalent in the slaughter line (16.7% of positive samples, considering all sample types

obtained at this stage) and, in particular, in the cecal and colonic content of sampled pigs (85% and 45%, respectively). The overall proportion of positive samples was higher in the TLSQ2 assay than in TLSQ1 (1.6 times, for both *C. difficile* and *C. perfringens*) and there was some variation in the distribution of both clostridia (Table 1).

3.2. Diversity of C. difficile isolates

A total of 105 *C. difficile* isolates (x ± S.D. = 2.7 ± 0.5 isolates per positive TLSQ sample) were selected from the original plate cultures for ribotyping and further characterization. About 72.4% of those isolates (76/105) could be classified into one of the already known PCR ribotypes: 078 (34 isolates), 572 (15), 110 (9), 126 (6), 202 (6), 010 (2), 013 (2) and 181 (2). The toxin profiles and other characteristics of these ribotypes are detailed in Table 2. The remaining 29 isolates (27.6% of total) belonged to nine unknown ribotypes, which will be hereafter referred to as U01 to U09 ('U' stands for 'unknown'; Table 2).

AFLP-based fingerprinting grouped *C. difficile* isolates into 104 peak profiles and 95 distinct genotypes (designated as cd1 to cd95; Fig. 1). All isolates belonging to ribotypes 078 and 126 (n = 40, in total) and to the toxigenic type U09 (n = 4) were included into two well-defined groups that clustered apart from the isolates of the other 14 PCR ribotypes (Fig. 1). Although eight AFLP genotypes included multiple isolates, only two out of these eight AFLP genotypes clustered isolates belonging to different PCR ribotypes (genotypes cd74 and cd89, both of which included two type 078 isolates and one U09 isolate). All samples from which multiple *C. difficile* isolates could be

recovered (38 in total) yielded two or more different AFLP types, and 23.7 % of these also yielded different PCR ribotypes.

3.3. Diversity of C. perfringens isolates

Eighty-five *C. perfringens* isolates (2.5 ± 0.8 isolates per positive culture of TLSQ samples) were selected for detailed characterization. Toxinotyping of these isolates revealed that 80 of them (94.1%) belonged to toxinotype A and only five isolates (5.9%) were of toxinotype C (Table 1). None of the isolates had the enterotoxin-encoding gene (*cpe*) but 20 type A isolates from diverse sample sources were positive for presence of an atypical form of the β2-encoding gene (*cpb2*), and other five type A isolates (three obtained from the tonsils of the same pig, one from the colonic content of another pig and the remaining from a truck's floor) were found to carry the consensus form of *cpb2* (Table 1).

AFLP-based fingerprinting of *C. perfringens* isolates yielded 85 different peak profiles and 80 distinct genotypes (cp1 to cp80; Fig. 2). Only four AFLP types grouped together two or more isolates (see details below) and all samples from which multiple isolates of *C. perfringens* could be obtained (29 in total) yielded two or more different AFLP types. Notably, one AFLP type (cp55) clustered together two toxinotype A and one toxinotype C isolates.

3.4. Distribution of C. difficile and C. perfringens genotypes along the production chain

Five out of the 17 PCR ribotypes of *C. difficile* (29.4%) were found in samples obtained at different steps of the pork production chain (Table 2): 078 (T, L, S and environmental samples), 110 (T and S), 572 and U01 (T, L and S) and U09 (L and S).

The tracking of individual AFLP genotypes of *C. difficile* and *C. perfringens* along the different sample sources investigated is shown in Fig. 3. Two *C. difficile* genotypes were found in multiple samples from the same TLSQ assay (cd38 from TLSQ1, and cd70 and cd79 from TLSQ2), and two additional genotypes were found in samples from both TLSQ1 and TLSQ2 (cd74 and cd89) and included isolates of PCR ribotypes 078 and U09 (Fig. 3). In addition, a same *C. perfringens* genotype (cp23) was retrieved from TLSQ2 samples obtained from the floor of a truck and the quartering of a carcass. However, while the truck isolate yielded a positive PCR result for presence of a consensus form of the *cpb2* gene, the isolate from the quartering sample was *cpb2*-negative (Fig. 3). No other AFLP genotype grouped together *C. perfringens* isolates from different sample sources but three AFLP types included isolates obtained from the cecal or colonic content of different pigs (cp12 and cp55, and cp33, respectively).

4. Discussion

Previous studies have demonstrated that *C. difficile* and *C. perfringens* are common environmental contaminants of abattoirs slaughtering intensively-raised pigs (Rho et al., 2001; Chan et al., 2012; Hawken et al., 2013; Rodriguez et al., 2013; Wu et al., 2017). However, much less is known about the prevalence and diversity of these two anaerobes in abattoirs dealing with pigs raised under free-range conditions (but see Susick et al., 2012).

In this study, we found that *C. difficile* and *C. perfringens* are widespread environmental contaminants in a free-range pig abattoir and processing plant. Both species were isolated from trucks (including cabin's mats which never came into direct

contact with animals) and lairage samples obtained after cleaning and disinfection, indicating that these procedures were not efficient to eliminate clostridial spores. A similar conclusion was reached by Hernández et al. (2013) in a survey for *Salmonella* spp. Despite these data may be biased due to the fact that a single abattoir was sampled, they highlight the potential risk of contamination by *C. difficile* and *C. perfringens* when exhaustive cleaning and disinfection protocols are not applied at every step from the transport of the animals to the lairage.

Detailed genetic characterization of the isolates obtained in this study showed a high genetic diversity for *C. difficile* and *C. perfringens* and revealed the presence of some particular strain types in both environmental samples and pig carcasses, which agrees with the observations of other authors (Hawken et al., 2013; Wu et al., 2017). Furthermore, high diversity of PCR ribotypes and AFLP was found even among isolates retrieved from a same sample, thus confirming the recommendation of examining multiple isolates from culture-positive clinical and environmental samples (Tanner et al., 2010; Álvarez-Pérez et al., 2016). Overall, these results agree with those obtained by Hernández et al. (2013) for *Salmonella* spp. but contrasts with the low genetic diversity detected for *Listeria monocytogenes* by López et al. (2008) in a different abattoir. Differences in the pig populations analyzed, sampling methods, target bacterial species and/or techniques used for molecular typing of isolates might account for these discrepancies.

Notably, we identified *C. difficile* PCR ribotypes which rank among the most prevalent in outbreaks of human disease, such as ribotypes 010, 078 and 126 (Davies et al., 2016). Interestingly, the PCR ribotypes 078 and 126 are close phylogenetic relatives (Stabler et

al., 2012; Schneeberg et al., 2013) that are frequently recovered from slaughtered animals and meat products (Metcalf et al., 2010; Curry et al., 2012; Hawken et al., 2013; Cho et al., 2015; Mooyottu et al., 2015; Wu et al., 2017). Moreover, high genetic relatedness between human and animal isolates of the 078/126 ribotype complex has been repeatedly reported (Bakker et al., 2010, Koene et al., 2012, Schneeberg et al., 2013; Knetsch et al., 2014; Álvarez-Pérez et al., 2017b). All of this has encouraged an ongoing discussion about the zoonotic and food-borne potential of the 078/126 lineage (Squire and Riley, 2013; Warriner et al., 2017). However, direct transmission of *C. difficile* (from animals to humans or vice versa) has not been yet demonstrated and the possibility of acquisition from a common environmental source cannot be excluded (Squire and Riley, 2013; Knetsch et al., 2014).

Regarding the toxigenic diversity of the isolates characterized in this study, most *C. difficile* isolates yielded a positive PCR result for the genes encoding toxins A, B and/or binary toxin, all of which are regarded as the main virulence factors of the species (Smits et al., 2016). Moreover, *C. perfringens* isolates were classified into toxinotypes A and C, both of which are common enteric pathogens of swine (Songer and Uzal, 2005). In addition, some *C. perfringens* isolates of diverse origins had the genes encoding for consensus or atypical forms of the β2 toxin, a plasmid-borne pore-forming toxin which may play a role in pathogenesis (Songer and Uzal, 2005; Uzal et al., 2014). However, regardless of their origin and AFLP-type, all *C. perfringens* isolates yielded a negative PCR result for the gene encoding enterotoxin CPE, which is the main toxin involved in food poisoning in humans (Songer and Uzal, 2005; Uzal et al., 2014). In any case, given the huge arsenal of additional toxins that *C. perfringens* strains can produce

(Uzal et al., 2014), the mere presence of this species in abattoirs and food-processing plants should be regarded as a ~~major~~ possible threat to public health.

Finally, a 27.6% of the *C. difficile* isolates analyzed in this study belonged to previously unknown PCR ribotypes. Interestingly, one of these ribotypes, named U09, clustered with ribotype 078 and 126 isolates in the UPGMA dendrogram built from AFLP patterns and, as these two, included isolates with the genes encoding for toxins A, B and binary toxin. Thus, U09 could be regarded as a new 078/126-like ribotype and future studies should try to assess the prevalence and genetic and phenotypic characteristics of this novel strain type.

5. Conclusions

In conclusion, as previously observed for abattoirs slaughtering intensively-raised pigs, *C. difficile* and *C. perfringens* can be found in free-range pig abattoirs and processing plants. In addition, molecular tracking of individual genotypes revealed that, for both clostridia, the same strain types could be recovered from animal and environmental samples, highlighting the potential for cross contamination of free-range pig carcasses.

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Declaration of interest

None.

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Figure legends

Figure 1. Dendrogram of AFLP profiles obtained for the *Clostridium difficile* isolates characterized in this study (*n* = 105). The dendrogram was created by unweighted pair group method with arithmetic mean (UPGMA) clustering using Pearson's correlation coefficients. Individual AFLP genotypes are distinguished at ≥86% similarity (red dotted vertical line). The ~~PCR ribotype and~~ origin of isolates is indicated at the tip of branches (see legend on the lower left corner), followed by PCR ribotype, isolate and AFLP type designations (shown in red, black and blue, respectively). The two clusters comprising all ribotype 078/126 and U09 isolates are indicated by a green background. Abbreviations in legend: T, trucks; L, lairage; S, slaughter line; Q, quartering; E, environment of the slaughter line and processing plant; 1, TLSQ1; 2, TLSQ2.

Figure 2. Dendrogram of AFLP profiles obtained for the *Clostridium perfringens* isolates characterized in this study (*n* = 85). The dendrogram was created by unweighted pair group method with arithmetic mean (UPGMA) clustering using Pearson's correlation coefficients. Individual AFLP genotypes are distinguished at ≥86% similarity (red dotted vertical line). The first column of colored squares at the tip of branches indicates the origin of isolates (see color legend on the lower left corner). Additionally, green- and violet-filled squares indicate toxinotype A and toxinotype C isolates, respectively, and the presence of the consensus or atypical form of the β2 toxin-encoding gene (*cpb2*) is indicated by filled and open circles, respectively. Alphanumeric codes refer to isolate and AFLP type designations (shown in black and blue, respectively). Abbreviations in legend: T, trucks; S, slaughter line; Q, quartering; 1, TLSQ1; 2, TLSQ2.

Figure 3. Tracking of individual AFLP genotypes of *Clostridium difficile* and *Clostridium perfringens* along the pork production chain. Detection of each genotype from the different sample sources is indicated by shaded boxes, which also include the PCR ribotype (for *C. difficile*) or toxin profile (for *C. perfringens*). Only genotypes detected in two or more sample sources are included. Abbreviations for sample sources: T1, trucks prior cleaning and disinfection; T2, trucks after cleaning and disinfection; L1, lairage prior entry of the pigs; L2, lairage after exit of the pigs; S1, pre-scalding; S2, post-scalding; S3, post-flaming; S4, post-evisceration; S5, post-washing; S6, chilling; To, tonsils; Ce, cecal contents; Co, colonic contents; Q, quartering samples (ham, shoulder and loin); ES, environment slaughter line (scalding water, knives and saws); EQ, environment quartering (sterilization water, tables and knives).

Tables

Table 1: Distribution of *Clostridium difficile* and *Clostridium perfringens* along the pig slaughtering process in the examined free-range pig abattoir and processing plant.

TLSQ assay[a]	Production stage	Sample[b] (*n*)	*C. difficile*			*C. perfringens*			Both clostridial species
			No. (%) of positive samples	No. isolates	Ribotypes (no. AFLP types)	No. (%) of positive samples	No. isolates	Toxinotypes[c] (no. AFLP types)	No. (%) of positive samples
TLSQ1	Trucks	T1 (5)	4 (80%)	11	010 (1), 078 (2), 126 (1), 572 (7)	0			0
		T2 (5)	2 (40%)	5	078 (2), 126 (3)	0			0
	Lairage	L1 (4)	0			0			0
		L2 (4)	2 (50%)	6	078 (3), U01 (1), U09 (2)	0			0
	Slaughter line	S1 (10)	2 (20%)	5	U02 (3), U07 (2)	0			0
		S2 (10)	0			0			0
		S3-S6 (40)	0			0			0
		To (10)	0			2 (20%)	4	A (1), A/*cpb2*+[c] (3)	0
		Ce (10)	2 (20%)	4	572 (2), U06 (2)	8 (80%)	22	A (9), A/*cpb2*+[a] (12), C (1)	2 (20%)
		Co (10)	2 (20%)	6	572 (3), U02 (3)	3 (30%)	8	A (5), A/*cpb2*+[a] (2)	0
	Quartering	Q (10)	0			0			0
	Environment	ES (7)	1 (14.3%)	3	078 (2)	0			0
		EQ (10)	0			0			0

	TOTAL	(135)	15 (11.1%)	40	010 (1), 078 (9), 126 (4), 572 (11), U01 (1), U02 (6), U06 (2), U07 (2), U09 (2)	13 (9.6%)	34	A (15), A/*cpb2*+[c] (3), A/*cpb2*+[a] (14), C (1)	2 (1.5%)
TLSQ2	Trucks	T1 (5)	5 (100%)	15	010 (1), 078 (6), 110 (2), 126 (2), U01 (2), U03 (1)	2 (40%)	2	A (2)	2 (40%)
		T2 (5)	5 (100%)	13	078 (8), U03 (3), U04 (1)	1 (20%)	2	A (2)	1 (20%)
	Lairage	L1 (4)	2 (50%)	4	078 (4)	0			0
		L2 (4)	2 (50%)	5	013 (2), 572 (3)	0			0
	Slaughter line	S1 (10)	7 (70%)	20	078 (5), 181 (2), 202 (6), U01 (2), U08 (3), U09 (2)	2 (20%)	2	A (2)	1 (10%)
		S2 (10)	1 (10%)	2	U05 (2)	0			0
		S3-S6 (40)	0			0			0
		To (10)	0			0			0
		Ce (10)	1 (10%)	3	110 (2)	9 (90%)	27	A (16), A/*cpb2*+[a] (5), C (4)	1 (10%)
		Co (10)	1 (10%)	3	110 (3)	6 (60%)	15	A (14), A/*cpb2*+[a] (1)	1 (10%)
	Quartering	Q (10)	0			1 (10%)	3	A (3)	0
	Environment	ES (7)	0			0			0
		EQ (10)	0			0			0
	TOTAL	(135)	24 (17.8%)	65	010 (1), 013 (2), 078 (20), 110 (7), 126 (2), 181 (2), 202 (6), 572 (3), U01 (4), U03 (4), U04 (1), U05 (2), U08 (3), U09 (2)	21 (15.6%)	51	A (39), A/*cpb2*+[a] (6), C (4)	6 (4.4%)
TLSQ1	Trucks	T1 (10)	9 (90%)	26	010 (2), 078 (8), 110 (2),	2 (20%)	2	A (2)	2 (20%)

+ TLSQ2					126 (3), 572 (7), U01 (2), U03 (1)				
		T2 (10)	7 (70%)	18	078 (10), 126 (3), U03 (3), U04 (1)	1 (10%)	2	A (2)	1 (10%)
	Lairage	L1 (8)	2 (25%)	4	078 (4)	0			0
		L2 (8)	4 (50%)	11	013 (2), 078 (3), 572 (3), U01 (1), U09 (2)	0			0
	Slaughter line	S1 (20)	9 (45%)	25	078 (5), 181 (2), 202 (6), U01 (2), U02 (3), U07 (2), U08 (3), U09 (2)	2 (10%)	2	A (2)	1 (5%)
		S2 (20)	1 (5%)	2	U05 (2)	0			0
		S3-S6 (80)	0			0			0
		To (20)	0			2 (10%)	4	A (1), A/*cpb2*+[c] (3)	0
		Ce (20)	3 (15%)	7	110 (2), 572 (2), U06 (2)	17 (85%)	49	A (25), A/*cpb2*+[a] (17), C (5)	3 (15%)
		Co (20)	3 (15%)	9	110 (3), 572 (3), U02 (3)	9 (45%)	23	A (19), A/*cpb2*+[a] (3)	1 (5%)
	Quartering	Q (20)	0			1 (5%)	3	A (3)	0
	Environment	ES (14)	1 (7.1%)	3	078 (2)	0			0
		EQ (20)	0			0			0
	TOTAL	(270)	39 (14.4%)	105	010 (2), 013 (2), 078 (29), 110 (7), 126 (6), 181 (2), 202 (6), 572 (14), U01 (5), U02 (6), U03 (4), U04 (1), U05 (2), U06 (2), U07 (2), U08 (3), U09 (4)	34 (12.6%)	85	A (54), A/*cpb2*+[c] (3), A/*cpb2*+[a] (20), C (5)	8 (3%)

[a] Two different batches of Iberian pigs, corresponding with the first and last batches allocated to the day of sampling (TLSQ1 and TLSQ2, respectively), were analyzed.

[b] T1, trucks prior cleaning and disinfection; T2, trucks after cleaning and disinfection; L1, lairage prior entry of the pigs; L2, lairage after exit of the pigs; S1, pre-scalding; S2, post-scalding; S3, post-flaming; S4, post-evisceration; S5, post-washing; S6, airing; To, tonsils; Ce, cecal contents; Co, colonic contents; Q, quartering samples (ham, shoulder and loin); ES, environment slaughter line (scalding water, knives and saws); EQ, environment quartering (sterilization water, tables and knives).

[c] A, toxinotype A; C, toxinotype C; *cpb2*+, positive PCR result for the consensus [c] or atypical [a] form of the β2 toxin-encoding gene.

Table 2: Toxin profiles and AFLP types of *Clostridium difficile* ribotypes identified in this study.

PCR ribotype	Toxin profile	No. isolates	No. AFLP types	No. samples[a]					
				Total	T	L	S	Q	E
010	A-B-CDT-	2	2	2 (5.1%)	2				
013	A+B+CDT-	2	2	1 (2.6%)		1			
078	A+B+CDT+	34	29	14 (35.9%)	8	3	2		1
110	A+B+CDT-	9	7	3 (7.7%)	1		2		
126	A+B+CDT+	6	6	3 (7.7%)	3				
181	A-B-CDT-	2	2	1 (2.6%)			1		
202	A+B+CDT-	6	6	2 (5.1%)			2		
572	A+B+CDT-	15	14	6 (15.4%)	3	1	2		
U01	A+B+CDT-	5	5	5 (12.8%)	2	1	2		
U02	A-B-CDT-	6	6	2 (5.1%)			2		
U03	A+B+CDT-	4	4	2 (5.1%)	2				
U04	A+B+CDT-	1	1	1 (2.6%)	1				
U05	A-B-CDT-	2	2	1 (2.6%)			1		
U06	A-B-CDT-	2	2	1 (2.6%)			1		
U07	A-B-CDT-	2	2	1 (2.6%)			1		
U08	A-B-CDT-	3	3	1 (2.6%)			1		
U09	A+B+CDT+	4	4	2 (5.1%)		1	1		

[a] T, trucks; L, lairage; S, slaughter line; Q, quartering; E, environment of the slaughter line and processing plant.

Figure 1

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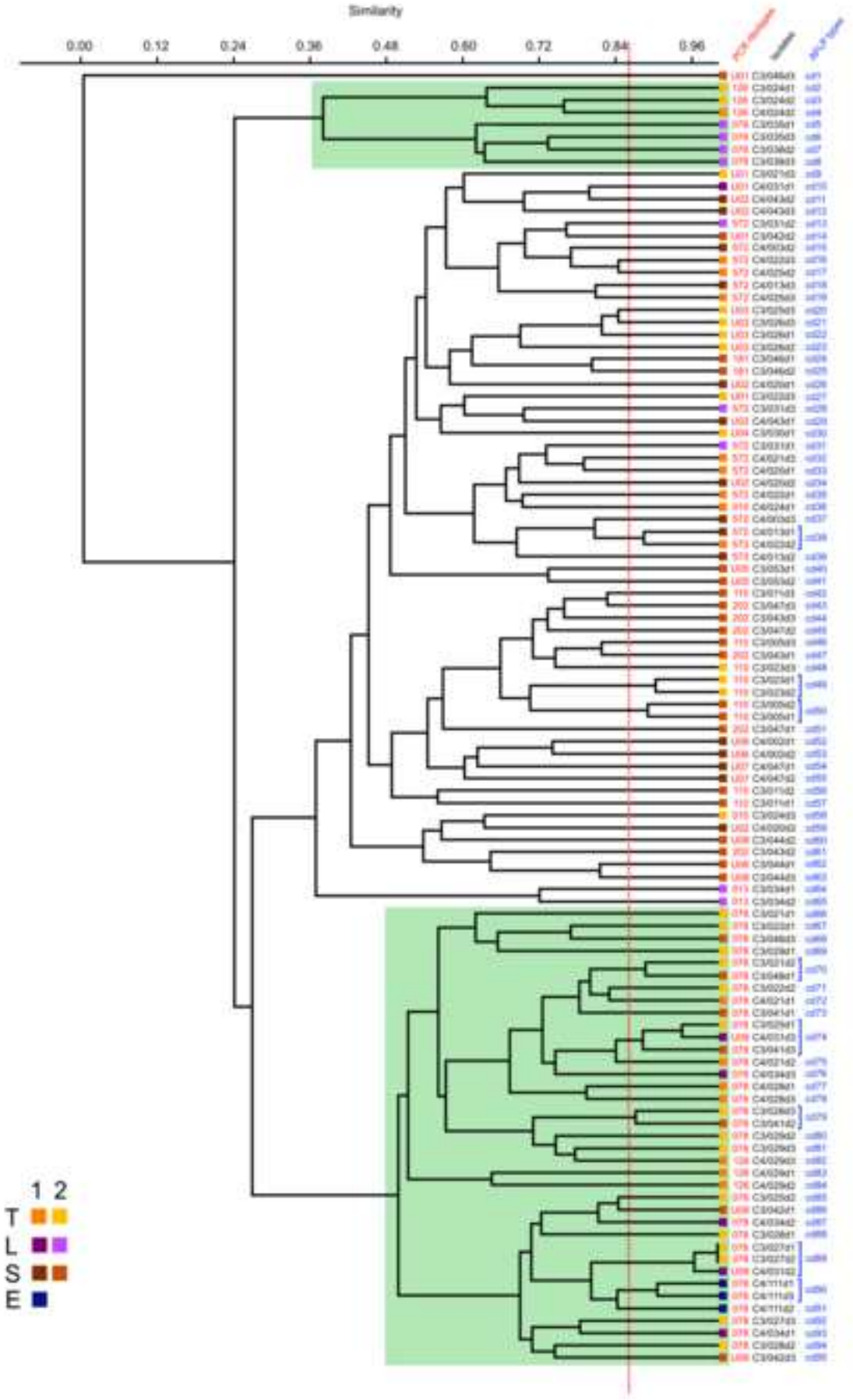

Figure 2

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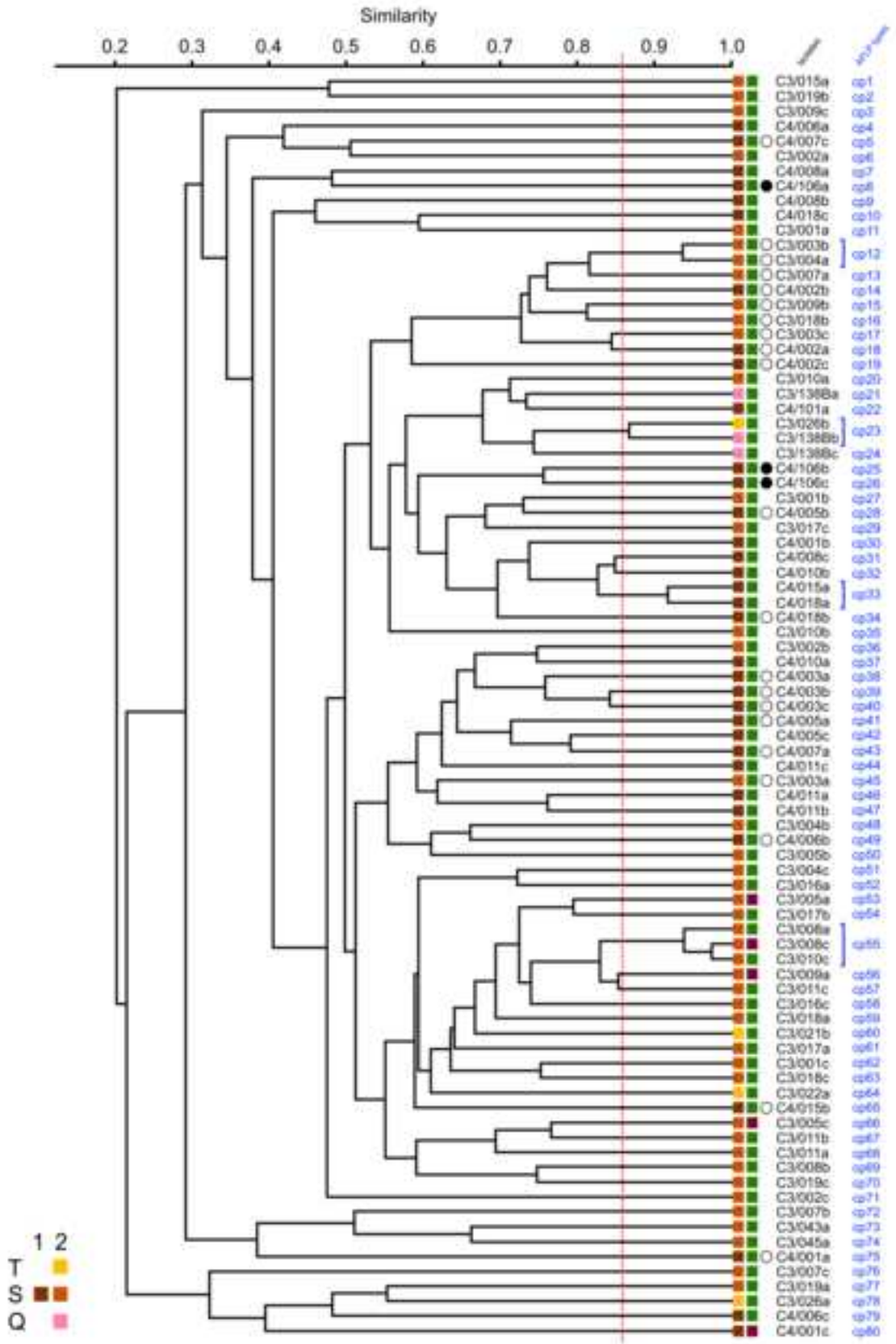

Figure 3

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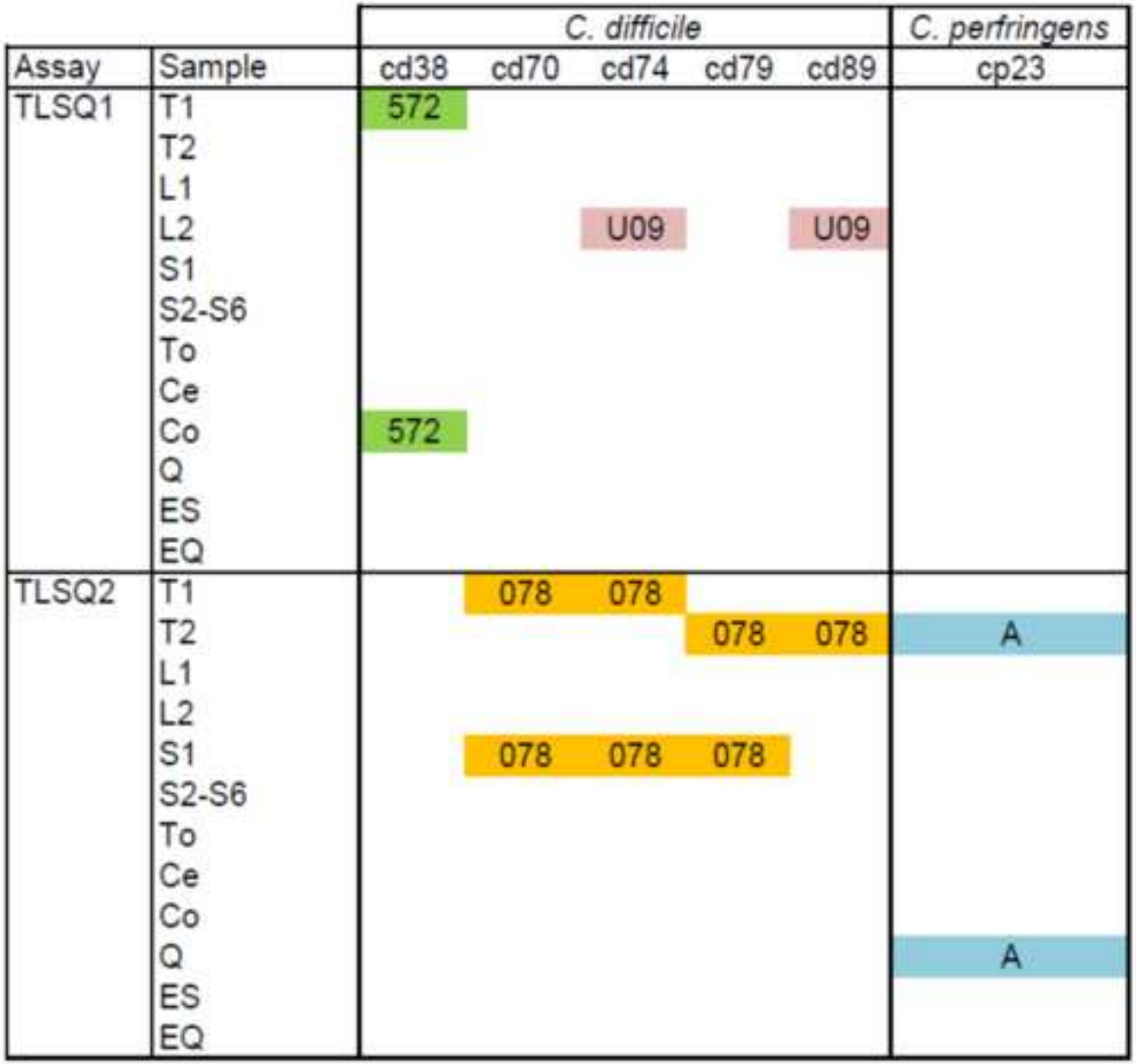

*Graphical Abstract

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