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Preliminary communication

ARE MEATS INDEED SOLD IN PORTUGAL WITHOUT *CLOSTRIDIOIDES DIFFICILE?*

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The incidence and severity of diarrhoea associated with *Clostridioides difficile* have been increasing exponentially. In 2014, an outbreak with the hypervirulent ribotype 027 strain was firstly reported in Portugal and, among others, this ribotype have been mainly isolated from animals and food. This study aimed to detect and quantify *C. difficile* from different meats sold in traditional commerce and hypermarkets in two different cities of Portugal, *Porto* and *Lisboa*.

Techniques of quantification and detection of *C. difficile* were performed, but absence of *C. difficile* in the 143 analysed samples indicates that, if present, the level of contamination should be very low (below 2 log CFU g^{-1}). Despite the lack of confirmed cases of foodborne diseases caused by *C. difficile*, the increased CDI incidence suggests that contaminated foods may contribute to *C. difficile*-acquired infections.

Keywords: *Clostridioides difficile*, detection, enumeration, meat, prevalence

Clostridioides difficile (formerly *Clostridium difficile*; Lawson et al., 2016) is an anaerobic Gram-positive spore-forming bacillus, and is found in both environment and intestinal microbiota of animals and humans (Donskey et al., 2015). Since 1974 and 1978 this microorganism has been associated with antibiotic-induced diarrhoea and pseudomembranous colitis, respectively (Tedesco et al., 1974; Bartlett et al., 1978). Incidence and severity of *C. difficile* infection (CDI) have been increasing exponentially all over the world in the past decade (Mulvey et al., 2010). Until recently, CDIs were believed to be almost exclusively nosocomial, associated with the use of antibiotics (altering the intestinal microbiota, enabling the proliferation and toxins segregation of *C. difficile*) and occurring mainly in immunocompromised and elderly patients (Rupnik et al., 2009). However, the infection is becoming increasingly common among low-risk individuals (young people and individuals without prior history of hospitalization or antibiotics exposure) (Kelly & LaMont, 2008). This has been explained by the existence of better detection methods, increased use of antibiotics/immunosuppressive agents, and further, by the emergence of virulent strains (Kelly & LaMont, 2008). Since 2003, *C. difficile* belonging to PCR-ribotype 027 or pulsotype NAP1 (North American Pulsotype 1) has been associated with large outbreaks with increased recurrence and mortality (Loo et al., 2005; McDonald et al., 2005). Limited information is available about CDI in Portugal; although in the few existing studies the strain

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involved was not identified, the authors have shown a significant increase in CDI cases, as well as mortality rates (Monteiro et al., 2008). In 2014, it was the first outbreak with the hypervirulent ribotype 027 strain (Oleastro et al., 2014); the authors were unable to associate its presence with the occurrence of other CDI cases in Portugal. This ribotype and others have been largely isolated from animals and food (Costa et al., 2012; Chai et al., 2015). Given the bacteria spores' nature and their presence in the intestinal tract of animals, it would be expected to find *C. difficile* in several foods (e.g. raw meats, vegetables, and seafood). In the USA, high prevalence rates have been reported for raw meats (>40% in beef, pork, and turkey samples), unlike in Europe (2.7% and 4.3% in chicken and ground beef/pork meat, respectively) (reviewed by Rodriguez et al., 2016). These differences may be due to different methodologies used. Chai and co-workers (2015) reported that in more than 55% of the inoculated samples of chopped beef, *C. difficile* was not recovered after enrichment in selective media. This means that values might be underestimated. Despite the lack of confirmed cases of foodborne diseases caused by *C. difficile*, the increased CDI incidence suggests that contaminated foods may contribute to *C. difficile*-acquired infections.

The objective of this study was to detect and quantify *C. difficile* from different meats sold in two different cities of Portugal, Porto and Lisboa.

1. Materials and methods

1.1. Sampling

One hundred and forty-three products were purchased in both traditional and hypermarket establishments: 60 samples of beef (calf, veal, and bovine), 20 samples of minced pork, 24 chicken samples (thighs, wing, neck, gizzards, and hamburgers), and 39 traditional sausages (*alheira*, fresh sausage, *paio*, ham, and chorizo). For the selection of meat samples purchased in the traditional commerce, preference was given to the already minced and exposed meats, while in hypermarkets the choice varied between pre-minced meats and meats which, from different suppliers, were packed in vacuum or modified atmosphere. In the case of traditional sausages, non-packaged products were also purchased, as well as products packaged under vacuum in a modified atmosphere or only with air, in order to maintain the heterogeneity of the samples. The transport of samples was carried out in portable thermal boxes. The samples were stored at 4 ºC for a maximum period of 24 h until analysis.

1.2. Detection method

Detection of *C. difficile* was performed using an alcohol shock treatment of the samples after their pre-enrichment (Rodriguez-Palacios et al., 2007; De Boer et al., 2011; Limbago et al., 2012). Aseptically and randomly, 10 g of each sample were placed in a stomacher bag and 20 ml of *Clostridium difficile* Moxalactam Norfloxacin broth (CDMN CM0601B, SR0173E, Oxoid, Hampshire, United Kingdom) and 7% (v/v) horse blood (Oxoid) were added for the pre-enrichment. Mixtures were homogenized in a stomacher for 2 min and incubated at 37 ºC for 7 days under anaerobic conditions. After incubation, 2 ml of the enriched samples were mixed with 2 ml of 96% (v/v) ethanol and homogenized every 15 min for 1 h. Then, each sample was centrifuged at 7000 r.p.m. for 10 min (Hettich Zentrifugen, Rotina 35R, Tuttlingen, Germany), and a loopful material from the pellet was streaked into *Clostridium difficile* Moxalactam Norfloxacin agar (CDMN agar). Plates were incubated at 37 ºC for

7 days under anaerobic conditions. After incubation, up to 2 suspected colonies (opaque, with a grey-white colour, swarming, and nonhemolytic) were sub-cultured and confirmed as described by LIMBAGO and co-workers (2012).

As control, one clinical isolate *C. difficile* U315639 (kindly provided by Hospital S. Marcos, Braga, Portugal) grown in Brain Heart Infusion broth (Biokar diagnostics, Beauvais, France) was diluted for concentrations ranged ~10^1 to 10^4 colony forming unit (CFU) ml^{-1}, and minced meat samples were inoculated with each culture. Each sample was treated as described above for detection of *C. difficile*, and after incubation, the typical colonies were counted and the CFU g^{-1} was calculated.

2. Results and discussion

Clinical isolate *C. difficile* U315639 inoculated in minced meat was recovered only from samples inoculated with concentrations equal or greater than 10^2 CFU ml^{-1}. With these results, it is possible to assume that its detection limit in minced meat was about 2 log CFU g^{-1} for the detection method after pre-enrichment and alcohol shock treatment. CDMN broth was firstly proposed by ASPINALL and HUTCHINSON (1992) for isolating *C. difficile* from faeces, and the authors concluded that their purposed culture media was significantly more productive when compared with other supplemented culture media.

From the 143 samples analysed, no *C. difficile* was found with the detection methodology used in this study. The absence of *C. difficile* indicates that, if present, the level of contamination of these 143 samples should be very low (below 2 log CFU g^{-1}). The low prevalence of *C. difficile* in meats and other food products is in line with other studies that reported low level and low occurrence of *C. difficile* in slaughter animals destined for food (RODRIGUEZ-PALACIOS et al., 2009; DE BOER et al., 2011; MOOYOTTU et al., 2015). In the study conducted by DE BOER and co-workers (2011), the authors analysed different meat samples for the presence of *C. difficile*, using the same detection method used in this study. After testing 500 samples, *C. difficile* was found in merely eight samples (one from lamb and seven from chicken meat). In the study of MOOYOTTU and co-workers (2015), the authors only found two positive samples for *C. difficile* out of 300 analysed. Similarly, no *C. difficile* was found in 150 samples of poultry meat and 80 samples of retail meat (beef, pork, chicken, and hamburger products) from the recent studies of ABDEL-GLIL and co-workers (2018) and PIRES and co-workers (2018), respectively.

This low prevalence of *C. difficile* should not be ignored, since the absence of standardized methodologies, from sampling to culture methods with higher sensitivities, could be masking their presence in several food products, altogether with the fact that the relationship between the dose and risk factors for CDI is still unknown.

Interestingly, other facts such as seasoning could be influencing this low prevalence, as shown by RODRIGUEZ-PALACIOS and co-workers (2009). In a previous study of these authors (RODRIGUEZ-PALACIOS et al., 2007), *C. difficile* was isolated from 12 out of 60 (20%) retail ground meat samples from a large area of Canada. In contrast, the prevalence observed for *C. difficile* in their other study (RODRIGUEZ-PALACIOS et al., 2007) varied from 1.4% to 2.3%, after 214 analysed meat samples. The authors argue that the low prevalence obtained should be due to reduced culture selectivity or a low number of spores present in the analysed samples and, comparing with the higher prevalence obtained in their first study, also suggested a possible seasonality, with higher prevalence in winter (RODRIGUEZ-PALACIOS et al., 2009).

3. Conclusions

Based on the methodology used, it is possible to affirm that at least 143 meat samples sold in Portugal did not have, or had a low number of *C. difficile*. However, the lack of standardized microbiological methods for the detection of this microorganism in foods should be highlighted.

In spite of the enormous attention this pathogen has been given, it is still urgent to define measures to limit its dissemination and, importantly, to determine whether *C. difficile* is really a foodborne pathogen.

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