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Prevalence and diversity of *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* in two free-range pig slaughterhouses

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ABSTRACT

Salmonella spp., *Campylobacter* spp. and *Listeria monocytogenes* have a significant impact on public health with slaughterhouses providing many opportunities for the proliferation of pathogenic bacteria. To evaluate the prevalence and diversity of these microorganisms along the free-range pork production chain, a total of 750 samples (5 samples/animal; 15 animals/farm; 5 farms/slaughterhouse) were collected from two slaughterhouses and analysed by specific ISO methodologies. *Salmonella* spp. (12.93%, CI_{95} 10.72–15.52%), *Campylobacter* spp. (17.17%, CI_{95} 13.00–21.74%) and *L. monocytogenes* (9.07%, CI_{95} 7.21–11.33%) were recovered at different stages of the production chain, with the highest prevalence detected in tonsils for *Salmonella* spp. (30.67%, CI_{95} 23.85–38.44%) and *L. monocytogenes* (39.33%, CI_{95} 31.87–47.32%) and in faeces for *Campylobacter* spp. (57.33%, CI_{95} 49.33–64.96%). Thirteen different *Salmonella* serotypes were detected with monophasic *Salmonella* Typhimurium as the most frequent one. *C. coli*, *C. jejuni* and *L. monocytogenes* serotype 4b and 1/2a were also identified. A significant higher prevalence of *Salmonella* spp. in total and from skin samples in slaughterhouse B than in slaughterhouse A was detected. In addition, a higher, although not significant, prevalence of the selected pathogens was observed in meat samples from slaughterhouse B with respect to slaughterhouse A (10.67% vs 0% for *Campylobacter* spp.; and 4% vs 0% for *Salmonella* spp. and *L. monocytogenes*). Our results highlight the risk of contamination of pork meat by the microorganisms under study and point out the importance of implementing specific control measures.

1. Introduction

Food-borne pathogens are of major health and economic significance in developed countries (Fosse, Seegers, & Magras, 2009; Fredriksson-Ahomaa, Gerhardt, & Stolle, 2009). To reduce transmission to humans, it is important to identify which animals and foodstuffs are the main sources of the causative agents. *Campylobacter* spp. and *Salmonella* spp. are the most frequently reported zoonoses in 2016 in the European Union with notification rates of 66.3 and 20.4 cases per 100,000 population, respectively (EFSA, 2017). Listeriosis caused by *Listeria monocytogenes* increased in 2016 in comparison with 2015 (0.47 cases per 100,000 population). Despite presenting a lower incidence than the other pathogens, listeriosis is the most harmful one in the elderly, pregnant woman and immunocompromised individuals, with the highest hospitalization and mortality rate since 2008 (EFSA, 2017).

Pigs can be asymptomatic carriers of *Salmonella enterica*, *Campylobacter* and *L. monocytogenes*, and these pathogens can be isolated from the intestinal tract and tonsils of pigs (Fredriksson-Ahomaa et al., 2009; Farzan, Friendship, Cook, & Pollari, 2010). These animals may be a source of contamination for other pigs and pork meat through the pork production chain (Argüello et al., 2013; Hellström et al., 2010; Hernández et al., 2013; Prencipe et al., 2012).

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Different risk factors have been associated with the presence of these pathogens at farm level (Hellström et al., 2010), just like the slaughtering process has been related to the spreading and final contamination of pork derived products (Argüello et al., 2013; Hernández et al., 2013). In this sense, the molecular tracking of isolates by different techniques, such as Pulsed Field Gel Electrophoresis (PFGE), along the pig production chain allows monitoring the potential cross contamination of meat by several food-borne pathogens (Prencipe et al., 2012; Argüello et al., 2013; Hernández et al., 2013).

To reduce the occurrence of this type of zoonosis, it is important to identify which stages of the pork production chain pose a major risk for bacterial transmission. Slaughterhouses in particular provide many opportunities for pathogenic bacteria proliferation with contamination arising from the air, animal hide, utensils, water and slaughter equipment during bleeding, polishing, splitting, scalding and forced chilling (Buncic & Sofos, 2012; Choi et al., 2013; Pearce et al., 2004, 2006). In this sense, a large number of animals for slaughter together with insufficient and/or irregular sanitation measures as well as post-harvest processing may be significant sources of bacterial introduction leading to carcass cross contamination (Delhalle et al., 2009; Hernández et al., 2013; Papadopoulou et al., 2012).

Nowadays there is growing interest for organic and eco-friendly pig rearing systems, but also for food quality and safety. Whereas free-range systems may allow a higher animal welfare status, food safety may be threatened by more limited biosecurity measures (Funk & Gebreyes, 2004). In a previous study we verified the risk of *Salmonella* spp. infection or recontamination prior and post slaughter and proposed control measures to reduce the final contamination of meat (Hernández et al., 2013). In this work the prevalence and diversity of *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* in two free-range pig slaughterhouses is analysed as well as the effectiveness of the control measures implemented in each slaughterhouse.

2. Materials and methods

2.1. Sample collection

Ten farms located in two different regions of southwestern Spain (Andalusia) were selected for this study based on their high *Salmonella* spp. seroprevalence (from 80.0% to 100.0% of individual seroprevalence; cut-off 40 OD%; SALMOTYPE® Pig Screen, Labor Diagnostik Leipzig, Leipzig, Germany). The distance among farms within each region was always lower than 30 km. Each farm had an average of 200 finisher pigs. All evaluated pigs (15 pigs/farm; 150 animals) were raised in free-range conditions, reared outdoors in sparse oak forests (dehesa) where they fed on acorns and grass and share natural resources with other wild and domestic animals. Sample collection was performed in two slaughterhouses (slaughterhouse A and slaughterhouse B, with five farms per slaughterhouse) from January to March 2013.

Only free-range pigs were slaughtered in both abattoirs, with an average of 1500 pigs/slaughtering, coinciding always with the timeframe from September to June next year. During this working period a systematic cleaning and disinfection protocol is carried out following each slaughtering. From June to September, wherein no slaughter was performed, more exhaustive and meticulous cleaning and disinfection protocols of the abattoir are carried out, including the dismantling of the equipments. In addition, more thorough cleaning strategies and sanitation processes including the routine sealing of the rectum before gut evisceration and intensification of hydroalcoholic disinfection protocol applied on the cutting surface at quartering in spray at approximate intervals of 4 h were performed in slaughterhouse A. Both slaughterhouses used gaseous asphyxiation to stun animals before bleeding, scalding of animals in a vertical scalding tunnel with a showered system with hot water at 60 ± 1 °C, dehairing with a dehairing machine and singeing with a blow lap. Pigs were slaughtered following Good Manufacturing Practices (GMP), Sanitation Standard Operating Procedures (SSOP) and Hazard Analysis Critical Control Point (HACCP) under veterinary supervision and the traceability throughout the slaughter was strictly followed. Five samples per animal were aseptically collected at different stages of the production chain and transported into sterile containers to laboratory as follows: i) post-stunning/pre-scalding skin sample using an abrasive sponge; ii) post-evisceration, ileocecal lymph nodes and iii) faeces; iv) tonsils samples; and v) quartering, a pool of meat samples from ham, loin and shoulder. According to previous studies, the results of tonsils, lymph nodes, skin and faeces were considered to be indicative of the pig infection status before the slaughter process (on the farm, during transport or in lairage) and meat samples results were considered to be informative about hygiene during the slaughter process (Swanenburg, Urlings, Snijders, Keuzenkamp, & van Knapen, 2001b). The choice of the ileocecal lymph nodes was carried out to allow detecting pigs in a carrier state (Argüello et al., 2013). Sterile dehydrated sponges were pre-moistened with 10 ml of sterile peptone water and the target surface (skin from shoulder, back and ham) was swabbed by using an overlapping S pattern to cover the entire surface. The surface of the complete tonsils and ileocecal lymph nodes was decontaminated using 100% ethanol and flaming to eliminate extrinsic bacteria. Then, to avoid the possible effect of non-homogeneous distribution of the evaluated bacteria throughout these samples, complete tonsils and ileocecal lymph nodes were individually cut into small pieces and mixed using sterile scalpels before analysis.

For each bacterial analysis one pre-moistened abrasive sponge, 1 g of tonsil homogenate, 1 g of ileocecal lymph nodes homogenate, 25 g of faeces and 25 g of meat homogenate were independently processed. In order to avoid an excessive slowing-down of the slaughter line, abrasive sponges for *Campylobacter* analysis were not collected.

2.2. Bacterial isolation and identification

All the isolates were isolated and identified according to specific ISO methodologies. When confirmed, pure cultures of each isolate were stored at - 80 °C on Microbank beads (Pro-Lab Diagnostics, Spain).

2.2.1. Salmonella isolation and typing

All samples were analysed by using specific ISO methodologies for the detection of *Salmonella* spp. (ISO 6579:2002). Sterile peptone water (Scharlab, Spain) was added to each sample at a 1:10 ratio and incubated at 37 °C for 24 h. Samples were homogenised in a Masticator Classic (IUL S.A, Spain) for 1 min at 1500 rpm before incubation. After incubation, pre-enriched culture was transferred to Rappaport-Vassiliadis Medium Semisolid Modified (MRSV) (Oxoid, UK) and incubated at 42 °C for 48 h. Isolates were cultured on Xylose lysine deoxycholate agar (XLD) and *Salmonella* Chromogenic agar base (Oxoid, UK) and incubated at 37 °C for 24 h. All presumptive *Salmonella* isolates were biochemically confirmed by lysine iron agar (Difco, Spain), Kligler's iron agar (Oxoid, UK) and motility indole ornithine agar (Difco, Spain).

Bacteria serotypes and phage types were determined by means of an agglutination technique using commercially available polyvalent and monovalent *Salmonella* antisera against O (somatic) and H (flagellar) antigens (BioRAD, Spain) and specific phage types to type *S.* Typhimurium and mST provided by the International