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Mapping the dominant microbial species diversity at expiration date of raw meat and processed meats from equine origin, an underexplored meat ecosystem, in the Belgian retail

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ABSTRACT

Although equine meats and their derived smoked or fermented products are popular in some regions of the world, they only form a minor fraction of the global meat consumption. The latter may explain why their associated bacterial communities have not received much attention. In the present study, 69 different samples of equine meats and meat products were investigated. The samples consisted of raw meat from horses (17 samples) and zebra (7), as well as non-fermented but smoked (24) and fermented (21) horse meat products. After purchase, all samples were stored at 4 °C and analysed at expiration date. Besides an estimation of the total microbial counts, specific attention was paid to the identification of lactic acid bacteria (LAB) and catalase-positive cocci, in particular the group of coagulase-negative staphylococci (CNS), involved, due to their technological relevance in view of the elaboration of meat products. Samples that were loosely wrapped in butcher paper instead of vacuum- or modified-atmosphere packages were also screened for pseudomonads and enterobacterial species. In total, 1567 bacterial isolates were collected, subjected to $(GTG)_5$-PCR fingerprinting of genomic DNA, and identified by multiple gene sequencing (based on the 16S rRNA, *pheS*, *rpoA*, *rpoB*, and/or *tuf* genes). Overall, the bacterial species diversity consisted mostly of LAB but was contingent on the type of product. Raw meat was dominated by *Carnobacterium divergens*, *Lactobacillus sakei*, *Latococcus piscium*, and *Leuconostoc gelidum*, with zebra meat being particularly rich in lactococci. Smoked and fermented horse meat products contained mostly *Lb. sakei* and, to a lesser degree, *Lactobacillus curvatus*. In addition, several catalase-positive cocci (mostly *Staphylococcus equorum*), *Anoxybacillus* sp., *Brevibacterium* sp., *Brochothrix thermosphacta*, and the enterobacterial species *Hafnia alvei* were found.

1. Introduction

Horse meat is part of the gastronomic and cultural heritage of many regions of Europe and Asia, albeit that it is often subjected to cultural (*e.g.*, in the Anglosphere) and religious (*e.g.*, in Jewish communities) taboos (Bell, 2015; Kelekna, 2009; Kesse et al., 2005; Zuckerman, 2013). Ritual slaughtering of horses was performed by some ancient cultures, including the ancient Chinese and several Celtic and Germanic tribes (Nam et al., 2010). Moreover, horse meat products are sometimes looked favourably upon, based on perceived health benefits compared to other meat types, due to the low cholesterol and fat contents and the high levels of proteins, omega-3 fatty acids, and iron (Lorenzo et al., 2014). The fact that methane production associated with horse meat production is lower than that for cattle may also lead to environmental benefits (Belaunzaran et al., 2015). From an ecological perspective, it has been argued that the consumption of horse meat may help to deal with the damaging overabundance of wild horses in the American West and in Australia (Chan, 2014; Enders, 2015). Nevertheless, horse meat only makes up a minor fraction of the world's meat production. In 2013, a total of 785,000 t was produced globally, whereas total meat production exceeded 310,000,000 t, corresponding with an average global horse meat supply of 0.10 kg *per capita* per year (Belaunzaran et al., 2015; FAO, 2018). In the European Union, several countries are known to produce and consume horse meat. In Belgium, horse meat consumption has historical roots and is still relatively widespread nowadays. The Belgian horse meat supply is estimated at 0.58 kg *per capita* per year. Products sold on the Belgian market include raw horse meat and non-fermented but smoked and/or fermented derivatives, which have a traditional image and importance. Besides horse meat, another type of equine meat can sometimes be found in the Belgian retail,

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namely zebra meat, albeit only as a restricted niche product (Anonymous, 2014).

The most popular meats and meat products worldwide, in particular beef, pork, and poultry, have often been subjected to analyses of their dominant microbial communities, in view of hygienic control (*e.g.*, Doulgeraki et al., 2012; Geeraerts et al., 2017, 2018; Vasilopoulos et al., 2008). In contrast, only little research has been done on less commonly consumed meats, such as goat, game, and equine meats (*e.g.*, Borilova et al., 2016; Russo et al., 2017). Fresh goat meat, for instance, contains lactic acid bacteria (LAB) and *Pseudomonas* spp. (Carrizosa et al., 2017). For equine products, the microbiota of raw foal meat packed under vacuum or modified atmosphere consists of different LAB species, besides some pseudomonads and enterobacterial species (Lorenzo and Gómez, 2012). In spontaneously acidified Belgian horse meat sausage ('Boulogne'), the dominant microorganisms consist of LAB species (mostly *Lactobacillus sakei*, besides *Leuconostoc* spp.) and coagulase-negative staphylococci (CNS) species (Janssens et al., 2012). Chinese fermented horse meat contains, in addition to the applied starter culture of *Lb. sakei* and *Staphylococcus xylosus*, several LAB species (*Enterococcus faecium*, *Lactobacillus plantarum,* and *Weissella hellenica*), *Staphylococcus carnosus*, *Enterobacter cloacae*, and *Pseudomonas* sp. (Lu et al., 2015). Although the use of starter cultures usually supresses bacterial species diversity during meat fermentation, the specificities of the processing environment can profoundly affect the pervasiveness of starter culture species in the end products. For instance, a too high or low pH may lead to the vanishing of *S. carnosus* (Stavropoulou et al., 2018) or *S. xylosus* (Ravyts et al., 2010), respectively. To the authors' knowledge, microbiological studies on Belgian horse meat products are scarce and little is known about the effects of starter culture application (Janssens et al., 2012).

The present study endeavours to obtain a better insight into the microbial species diversity associated with different types of equine meats and their derived products. Rather than to evaluate microbial community dynamics during production or as a result of different storage conditions in a specific product, the aim is to more broadly map the diversity in a broad selection of end products that are representative for Belgian retail. To this end, several samples of raw, non-fermented but smoked, and fermented products were collected and subjected to microbiological analysis at expiration date. Focus was on the total microbiota and, more specifically, on the LAB and CNS communities because of their potential technological importance. As such, both LAB and CNS may be of interest for the potential future development of starter cultures for appropriate meat fermentation (Ravyts et al., 2012), whereas LAB can also serve as bioprotective cultures for applications in non-fermented meat and meat products (Vasilopoulos et al., 2015).

2. Materials and methods

2.1. Sample acquisition

A total of 69 samples was purchased at three local supermarkets and six butcher shops in the agglomeration of Brussels between May 2015 and March 2017 (Tables 1–3; Table S1), being representative for conventional practice in Belgian retail. The samples consisted of raw meat derived from horse (17 non-frozen samples) and zebra (3 non-frozen and 4 frozen samples), as well as several processed horse meat products (24 non-fermented but smoked and 21 fermented samples, respectively). Of the raw horse meats, the samples purchased at the butcher shops (8) were loosely wrapped in paper, whereas the remaining horse and zebra meats were obtained from supermarkets as vacuum-packed products. More than half of the fermented (12 of 21 samples) and non-fermented, smoked (16 of 24 samples) horse meat products were stored under modified-atmosphere packaging (MAP) or vacuum and obtained from supermarkets, whereas the remaining ones were wrapped in butcher paper and purchased at the butcher shops. All MAP-, vacuum-, and paper-packaged samples were stored at 4 °C until the expiration date was reached. At expiration date (all supermarket samples; expiration date as stated on the label) or two days after acquisition (all butcher samples), all samples were checked for microbial growth and the pH was measured with an InoLab pH 7110 meter (WTW, Weilheim, Germany). All samples were verified for sensory defects based on visual inspection and odour evaluation.

2.2. General microbiological sample analysis

Dilution series of all equine product samples were made as described before (Geeraerts et al., 2017). Prior to a medium-strength mechanical treatment of 90 s with a Stomacher 400 (Seward, Worthington, UK), a mass of 10–20 g of equine meat was put in a stomacher bag and diluted ten times using a peptone physiological solution (0.85%, m/v, NaCl and 0.1%, m/v; peptone in ultrapure water). Dilution series were made and spread plating was carried out using brain heart infusion (BHI) agar medium (Oxoid, Basingstoke, Hampshire, UK) to estimate total microbial counts, de Man-Rogosa-Sharpe (MRS) agar medium (Oxoid) for presumable LAB, and mannitol salt-phenol red-agar (MSA; VWR International, Darmstadt, Germany) for presumable CNS. Dilution series of paper-packaged samples were plated on RAPID Enterobacteriaceae agar medium (Biorad, Marnes-La-Laquette, France) for presumable enterobacterial species and Pseudomonas agar base (PAB; Oxoid) with added Pseudomonas CFC supplement (Oxoid) for presumable pseudomonads. The BHI and MRS agar media were incubated at 22 °C for 120 h, MSA media were incubated at 30 °C for 96 h, and RAPID agar and PAB media were incubated at 25 °C for 5 days.

2.3. Isolation and identification of microbial species

Isolates were picked up randomly (10–30% of the total number of colonies) from the highest sample dilutions, transferred into the appropriate liquid media, and stored in cryovials, the media being supplemented with 25% (v/v) of glycerol, at −80 °C. DNA extraction and $(GTG)_5$-PCR fingerprinting of genomic DNA was done as described previously (Geeraerts et al., 2017). Briefly, BHI and MRS agar isolates were grown in BHI broth at 22 °C and MSA isolates at 30 °C for 24–48 h. Cell pellets were generated by a 5-min centrifugation step at 13,793 × *g* (Heraeus Biofuge 13, Thermo Fisher Scientific, Waltham, MA, USA), followed by a wash step with TES buffer (0.05 M Tris-base; 0.001 M EDTA; 0.2 M sucrose; pH 8.0), and a dual lysis step, first with a mixture of lysozyme (Merck, Darmstadt, Germany) and mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 60 min, and then with proteinase K (Macherey-Nagel, Düren, Germany) at 56 °C for 60 min. The extraction and purification of genomic DNA was performed using the NucleoSpin®96 Tissue kit (Macherey-Nagel), according to the manufacturer's instructions. The DNA concentrations were determined with a Nanodrop 2000 (Thermo Fisher Scientific), followed by $(GTG)_5$-PCR fingerprinting as described previously (Vasilopoulos et al., 2008). Bionumerics software (v. 5.10; Applied Maths, Sint-Martens-Latem, Belgium) was used for the classification and identification of the isolates, based on numerical cluster analysis of the $(GTG)_5$-PCR fingerprints obtained (Geeraerts et al., 2017). Representative isolates of the different clusters were identified by 16S rRNA gene sequencing (Vasilopoulos et al., 2008). The identity of the LAB stains was confirmed by sequencing of the *pheS* gene (De Bruyne et al., 2007; Snauwaert et al., 2013). Further identification to species level of *Staphylococcus* and *Kocuria* isolates was done by amplification of the *rpoB* and *tuf* genes using the rpoB-F (5'-AACCAATTCCGTATIGGTTT-3′) and rpoB-R (5'-CCGTCCCAAGTCATGAAAC-3′) primers (Drancourt and Raoult, 2002) and the tuf-F (5'-GCCAGTTGAGGACGTATTCT-3′) and tuf-R (5'-CCATTTCAGTACCTTCTGGTAA-3′) primers (Heikens et al., 2005), respectively. Secondary identification of the enterobacterial isolates was done by sequencing of the *rpoA* gene, using the rpoA_entro-L (5'-ATGCAGGGTTCTGTGACAGAG-3′) and rpoA_entro-R (5'-GGTGG-CCARTTTTCYAGGCGC-3′) primers (Kuhnert et al., 2009). The gene