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Traditional dry smoked fermented meat sausages: Characterization of autochthonous enterococci

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ABSTRACT

Enterococci are known members of the native microbiota in traditional dry smoked fermented sausages, but their exact role continues to be poorly understood. In the present study, the genetic diversity, technological features and overall safety were assessed for one-hundred and forty-seven enterococci isolated from meat-sausages produced in Portugal (Catalão, Chouriço-Preto, Linguiça, Paio and Salsichão). Fingerprinting analysis revealed high similarity levels between isolates obtained from distinct products, which associated with a high metabolic versatility and the ability to produce biofilms, points towards the persistence of specific strains in the manufacturing environment.

Although the meat-enterococci harbor antibiotic resistances and produce biofilms, a reduced number of virulence factors were detected. Overall, a low risk is most likely associated with the presence of enterococci in these food products, especially if we consider the absence of reports regarding foodborne infections connected with the consumption of traditional sausages. However, the selection of enterococci as putative starters should remain cautious, since these microorganisms harbor concerning levels of antibiotic resistances, which may, or may not, be spread to other bacteria.

1. Introduction

In Mediterranean countries many meat fermented foods are manufactured carrying out artisanal ways, without the use of starter cultures. The autochthone microbiota is mostly dominated by lactic acid bacteria (LAB), with the presence of enterococci being mainly attributed to their phenotypic features, such as low acidification ability, proteolytic/lipolytic activities and carbohydrate metabolism, which may contribute to improve the characteristics of fermented products (Hugas, Garriga, & Aymerich, 2003). However, over the years there have been reports on *Enterococcus* spp. recovered from several sources which carry virulence and antibiorresistance traits, unlike most LAB (Barbosa, Ferreira, & Teixeira, 2009; Belgacem et al., 2010; Ogier & Serror, 2008; Ribeiro et al., 2011; Semedo et al., 2003). In this context, the present study characterized enterococci collected from five dry smoked fermented meat-sausages, produced using artisanal procedures in a small-scale factory in Portugal. This research aimed to identify enterococcal features responsible for persistence in a manufacturing environment, as well as to assess for putative risk to the consumer.

2. Materials and methods

2.1. Sampling and microbial isolation

Five distinct fermented-sausages were collected in a small-factory located at Alentejo, Portugal (Catalão, Chouriço-preto, Linguiça, Salsichão and Paio). These products have natural casings, present ±3 cm in diameter and are submitted to a smoking period of 48–72 h at 18-24 °C, followed by a curing/drying period of 25–40 days at 9 °C. Bacterial isolation was performed over a five-month period (three samples from each sausage, per month) as described (Ribeiro and others, 2011), using Slanetz and Bartley agar

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(SB) and a variation of this media supplemented with 10 μg/ml of vancomycin (SB-van).

2.2. Molecular identification and genomic typing

Genus/species identification was performed according to (Ke et al., 1999) and (Jackson, Fedorka-cray, & Barrett, 2004). Genomic typing consisted in PCR-amplification using primers $(GTG)_5$ and OPC19 in independent reactions, according to (Svêc et al., 2005) and (Ribeiro et al., 2011), respectively. Primers and reagents were purchased from NZYTech Lda (Lisbon, Portugal). The genomic relatedness between the meat-enterococci was further assessed by Pulsed-Field-Gel-Electrophoresis (Turabelidze, Kotetishvili, Kreger, Morris, & Sulakvelidze, 2000). The BioNumerics software (version 6.6, Applied Maths, Kortrijk, Belgium) was used for data analysis, based on the evaluation of fingerprinting patterns, normalization of densitometric traces, calculation of Pearson correlation coefficient (for PCR-fingerprinting data) or DICE similarity coefficient (for PFGE patterns), and performance of cluster analysis by the unweighted pair group method with arithmetic mean algorithm (UPGMA).

2.3. Technological features

The isolate's enzymatic profiles were evaluated using API-ZYM galleries (BioMérieux, Marcy-L'Etoile, France), following the manufacturer's instructions.

2.4. Safety aspects

Protocols previously described were applied for the detection of enterococcal virulence factors (Carlos, Semedo-Lemsaddek, Barreto-Crespo, & Tenreiro, 2010; Eaton & Gasson, 2001; Nallapareddy et al., 2011; Qin, Singh, Weinstock, & Murray, 2000; Teng, Singh, Bourgogne, Zeng, & Murray, 2009). Biofilm-producing enterococci were analyzed according to (Extremina, Costa, Aguiar, Peixe, & Fonseca, 2011), for each assay three repetitions were performed. *E. faecalis* MMH594 was used as positive control for biofilm production (Laverde Gomez et al., 2011). Susceptibility to twenty antimicrobial agents (Oxoid Limited, Cambridge, United Kingdom) was also evaluated (CLSI, 2013). Minimal inhibitory concentrations (MICs) for vancomycin were determined using E-test strips (Oxoid Limited, Cambridge, United Kingdom) and vancomycin resistance genes, *van(A), van(B), van(C)* and *van(D)*, were detected according to Yean and others (Yean, Yin, Lalitha, & Ravichandran, 2007).

2.5. Statistical analysis

Fisher's exact test was calculated using *(fisher.test)* R software to test the statistical independence between the occurrence of virulence/antibiotic resistance and the source/species of the strains. When necessary, odds ratios (ORs) and 95% confidence intervals (CIs) were assessed.

3. Results and discussion

3.1. Isolation, identification and genomic diversity of the meat-enterococci

All products analyzed harbored enterococci, between 10^4 CFU/g for Catalão, Chouriço-preto and Linguiça and below 10^2 CFU/g for Salsichão and Paio; bacterial counts were equivalent for SBA and SBA-VAN. In Portugal, fermented meat-sausages can present high counts of *Enterococcus* spp., with reports ranging from 10^4 to 10^8 CFU/g (Barbosa and others, 2009), to 10^3–10^4 CFU/g (Ribeiro and others, 2011), which are more similar to our findings.

From a total of 147 isolates, 73 were identified as *E. faecalis*, 60 as *E. faecium* and 14 as *E. durans* (Table 1); species abundance was consistent with the literature (Barbosa and others, 2009; Hugas and others, 2003; Ribeiro and others, 2011). All but three *E. faecium* were recovered from the SBA-VAN medium. Previous reports suggest a vancomycin resistant/dependent phenotype to be mostly associated with this species (Cetinkaya and others, 2000). Comparison between fingerprint patterns allowed the selection of 75 strains, representing all genomic groups and meat-products, for additional characterization (data not shown).

Regarding PFGE, dendrograms were built based on macrorestriction-patterns obtained for each species (Fig. 1 for *E. faecalis* and data not shown for remaining enterococcal species). Results revealed high levels of similarity between isolates recovered from distinct meat-products, pointing towards the presence of specific strains in the raw materials and/or to their persistence in the manufacturing settings. Since all products were prepared using Iberian pork meat and manufacture in the same factory over a five-month period, these results correspond to the anticipated. However, in order to confirm the establishment of a particular *in house* microbiota a more detailed study, including sampling of machinery, workers and overall environment, over a longer time-period should be performed.

3.2. Technological features

The detected enzymatic activities are summarized in Table 2. Overall, results showed that meat-enterococci produce a wide spectrum of enzymes, mainly associated with lipid and protein metabolism, nutrients known to be the major components present in meat sausages. Thus, enterococcal metabolic versatility may be relevant for ripening and contribute to the production of characteristic organoleptic features (Belgacem and others, 2010; Sarantinopoulos and others, 2001).

3.3. Safety aspects

Results on the presence of virulence traits are summarized on Table 1. None of the strains was positive for the adhesin-coding gene *agg*, a relevant observation, since this protein mediates binding of donor cells to recipients, highly contributing to the acquisition and/or dissemination of virulence and resistance determinants; which significantly lowers the risk associated with these isolates. This safety assumption was further corroborated by the lack of positive results for β-hemolysis and genes *esp* and *cyl*, considered the most important enterococcal virulence factors. As for the remaining virulence determinants, minor percentages of below 30% were observed, similarly to previous studies (Barbosa et al., 2009; Ben Omar et al., 2004; Semedo et al., 2003). The exception was *gelE*, a gene detected on the majority of the isolates, this high incidence among food isolates had already been reported (Eaton & Gasson, 2001; Qin et al., 2000; Ribeiro et al., 2011; Semedo et al., 2003). In fact, gelatinase activity can present a dual role, as it can be considered either a technological or a virulence trait; the ability to produce this enzyme, capable of hydrolyzing gelatin, collagen, casein and other small biologically active peptides, can be an important advantage for meat-enterococci due to the usual presence of such components in the pork meat.

Regarding biofilm production, the isolates were allocated into four groups (Table 1): non-producer ($OD_{590nm} \leq 0.5$) for 9% of the isolates; weak producer ($0.5 < OD_{590nm} \leq 1.0$) to 28% of the enterococci; moderate biofilm producer ($1.0 < OD_{590nm} \leq 1.5$) to 25%; and strong producer ($1.5 < OD_{590nm} \leq 2.0$) to 37% meat-enterococci. Correlating species allocation with biofilm-forming