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Microbial, sensory and volatile changes during the anaerobic cold storage of *morcilla de Burgos* previously inoculated with *Weissella viridescens* and *Leuconostoc mesenteroides*

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ABSTRACT

Blood sausage, a widely consumed traditional product, would benefit from an increased commercial life. It is therefore pertinent to investigate the type, the evolution, and the behaviour of the Lactic Acid Bacteria responsible for their spoilage. This study aims to clarify the role played by *Weissella viridescens* and *Leuconostoc mesenteroides*, identified as their principal spoilage agents in vacuum-packaged *morcilla de Burgos*, through the study of microbiological, sensory, and volatile profile changes, following inoculation of the *morcilla*, both jointly and separately, with the two species. *L. mesenteroides* grew more rapidly and influenced the drop in pH, milky exudates and the sour smell, whereas *W. viridescens* influenced vacuum loss. With respect to volatile profiles, *L. mesenteroides* samples were richer in aldehydes (hexanal) and acids (acetic), on the contrary *W. viridescens* samples showed greater amounts of alcohols (ethanol) and ketones (acetoin and diacetyl). Both species inoculated together increased particular signs of *morcilla* spoilage.

1. Introduction

Lactic Acid Bacteria (LAB) dominate the population of spoilage bacteria on meat and meat products when left in cold storage under anaerobic conditions (Egan et al., 1983; Kitchell and Shaw, 1975). Product handling after cooking, coupled with slicing prior to packaging, recontaminates the cooked cured-meat products with *ca* 0.5–2 log CFU g^{-1} of total bacteria, mainly LAB (Holley, 1997; Samelis et al., 2000). The metabolic activity of LAB results in spoilage in the form of sourness, off-flavours, off-odours, milky exudates, slime production, swelling of the package through gas production and discoloration such as greening (Holzapfel, 1998; Samelis et al., 2000).

Blood sausages are traditional products, popular in many parts of the world although the ingredients vary between regions. No meat is added in this product, in contrast to other traditional cooked sausages such as *Cavourmas* in Greece (Arvanitoyannis et al., 2000) or German blood sausage in which offal is also added at times (Stiebing, 1990). *Morcella de Assar* in Portugal only includes fatty trimmings, blood and spices and is submitted to a short smoking period after the cooking process (Roseiro et al., 1998). Some other well-known examples are black pudding in Great Britain, *kaszanka* in Poland, *biroldo* in the San Francisco Bay area (USA) and *rellena* in Mexico and Colombia. However, despite their widespread production and consumption, these kinds of products have been overlooked from a scientific point of view, probably due to their local production and distribution patterns. Recently, increasing consumer demand for ethnic specialities has renewed interest in such products. Moreover, the European Union is more involved nowadays in the protection of high-quality traditional foods from specific regions or areas, which reflects a policy of supporting the inhabitants of rural areas and promoting regional products.

Morcilla de Burgos is a typical blood sausage from Spain. It is made from a mixture of onion, rice, animal fat (mainly lard), blood and spices. All these ingredients are mixed and stuffed in natural casings and then cooked in boiling water for 45–60 min. After this step, they are air cooled at room temperature and usually sold on without packaging to the consumer in local markets or are vacuum packaged by retailers. The shelf-life of vacuum-packaged *morcilla* is around 21 days and depends on initial contamination levels and storage conditions (Santos et al., 2005a). After this time, the presence of slime, vacuum loss and a sour odour and taste are appreciable. The preservation of this product is difficult due to its characteristically high content of fat and starch, as well as its high pH and a_w (Santos et al., 2003), which makes this product an interesting model for the study of spoilage in meat products in general.

Heterofermentative LAB contribute actively to the spoilage of *morcilla de Burgos*, whenever identified as the main microbial spoilage group involved in the spoilage, especially in vacuum and modified atmosphere packaging (Santos et al., 2001), in the same way as reported by many authors for different meat products (Blickstad and Molin, 1983; Borch et al., 1996; Franz and Von Holy, 1996; Korkeala and

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Mäkela, 1989; Korkeala and Björkroth, 1997; Von Holy et al., 1991). Previous studies have concluded that *W. viridescens*, *L. mesenteroides*, *L. carnosum* and *W. confusa* are the main LAB species in *morcilla de Burgos* (Diez et al., 2008b, 2009; Santos et al., 2005b). The typical sensory changes occurring in vacuum-packaged *morcilla* are swelling of the packs, development of drip, milky exudates, slime formation and souring, and the appearance of different types of product discoloration.

The objective of the present study is to clarify the role played by *Weissella viridescens* and *Leuconostoc mesenteroides*, identified as the principal agents in the spoilage of vacuum-packaged *morcilla de Burgos*, following inoculation of the product with the two species, both jointly and separately, and to analyse its microbiological, sensory and volatile profile changes.

2. Materials and methods

2.1. Characterisation and identification of the strains

Forty-three isolates of LAB were selected at random from previous studies performed on *morcilla de Burgos* (Diez et al., 2008a). The isolates came from vacuum-packaged *morcillas*, stored at 4 °C. Frozen strains were recovered through incubation at 30 °C, in MRS broth (oxoid) for 24 h. These isolates were cultured on MRS agar plates (oxoid) and incubated under anaerobic conditions with 6% CO_2, at 30 °C, for 2–3 days.

2.1.1 Ribotyping identification

Ribotyping and the resulting data were studied according to the procedure described by Santos et al. (2005b).

2.1.2 Phenotypic and biochemical characterisation

Phase-contrast microscopy was used to examine the cell morphology (Axioplan, Zeiss, Germany). Gas production from glucose and dextran production from sucrose were tested using the methods described by Schillinger and Lucke (1987). The production of both hydrogen peroxide and hydrogen sulphate was detected using the methods described, respectively, by Whittenbury (1964) and Shay and Egan (1981). Lactic acid production was studied by measuring the pH of the supernatant after centrifugation (5414R Eppendorf, Hamburg, Germany) at 12,000 rpm, at 4 °C for 5 min (from a culture of 24 h, with a Micro pH 2000 pH-metre (Crison, Barcelona, Spain)). To evaluate the growth at pH 3.9, 4.5 and 6.4, a volume of 0.1 ml from the different 24 h cultures was inoculated in sterile MRS broth, which had previously been adjusted with HCl concentrate at 37% (v/v). After incubation at 30 °C, over 3 days, the turbid tubes were considered positive. Carbohydrate fermentation was determined according to the method described by Schillinger and Lucke (1987) using the miniplate method described by Jayne Williams (1975), with the exception of using bromocresol purple as an indicator instead of chlorophenol red (Panreac) (Santos et al., 1998). The following carbohydrates were tested: D(+) galactose (Sigma, St. Louis, MO, USA), inulin (Sigma), maltose 1-hydrate (Panreac), D mannitol (Difco, Detroit, MI, USA), D (+) melezitose (Sigma) and finally, glucose (Panreac) and sterile water were used for positive and negative controls.

2.2. Preparation of morcilla de Burgos

One hundred and fifty-five *morcillas*, each approximately 200 g in weight, stuffed in natural beef casings prepared by the same producer, were selected for the different inoculation experiments. The formulation process in the factory included raw onion (54%), rice (17%), lard (17%), blood (8%), salt (1.8%) and a mixture of different spices that included paprika, black pepper and oregano (2.2%). The chopped onion and fat were mixed with the rice, salt, spices and blood and the sausage emulsion was stuffed into 35–45 mm natural beef casings that were preserved with salt and rinsed in clean water prior to use. No nitrite was included in the formulation. The blood sausages were then transferred to a cooking container and boiled in water at 95–96 °C for around 1 h. After cooking, the *morcillas* were air cooled at room temperature (8–10 °C). Samples were individually packaged in oxygen and water impermeable bags CN300 (Cryovac Grace S.A, Sealed Air Corporation, Barcelona, Spain) with an oxygen transmission rate of 15 $cm^3/m^2/24$ h/atm at 23 °C and 0% RH and a water vapour transmission rate of 2 $g/m^2/24$ h at 38 °C and 90% RH, in an EVT-7CD packaging machine (Tecnotrip, Tarrasa, Spain). In order to eliminate any possible initial bacterial load arising at the cooling stage, which might interfere with the inoculated strains, the *morcillas* selected for inoculation were pasteurised at 75 °C for 10 min; a treatment shown to be effective for that purpose in previous studies (Santos et al., 2007).

2.3. Preparation of the suspension of lactic culture

Two strains, one *W. viridescens* (dextran (+)) and one *L. mesenteroides* (dextran (−)) were selected among the 43 strains isolated for this and further use in inoculation studies. Two cultures of those bacteria were grown in 50 ml of MRS broth (Oxoid) for 24 h, at 30 °C, centrifuged (Eppendorf) at 7000 rpm, at 4 °C, for 15 min, washed three times and resuspended in 50 ml of Ringer's solution (Oxoid), reaching cellular concentrations of 10^9 cfu/ml. The bacterial suspension was diluted in Ringer's solution (Oxoid) at 1:10 prior to sample inoculation. In the case of the mixed inoculum of *W. viridescens* and *L. mesenteroides*, a mixture of the 1:10 bacterial single solutions was combined at equal parts.

2.4. Inoculation of the morcilla

On day 0, the one-hundred and fifty-five *morcillas* were divided up into 5 batches: control batch (C) made up of only vacuum-packaged *morcillas*, and the other batches, which were pasteurised as explained above, obtaining a pasteurised control batch (P), and 3 batches that were inoculated with 5 ml of *W. viridescens* (W), 5 ml of *L. mesenteroides*, and a 5 ml mixture of both strains (WL). The inoculum (cellular concentration of 10^8 cfu/ml) was carefully spread over the *morcilla* inside the pouches and then vacuum packaged. All samples were stored at 4 °C for 75 days and two *morcillas* from each batch were sampled throughout this period, to perform pH, microbiological, sensory, and volatile profile analyses. The experiment was duplicated.

2.5. pH measurement

Product pH was measured by blending 25 g of the product with 225 ml of Ringer's solution (Oxoid, Basingstoke, UK) for 2 min, as described by Diez et al. (2008a) and Santos et al. (2005a). Measurements were taken using a digital pH-meter Micro pH 2000 (Crison, Barcelona, Spain).

2.6. Microbiological analysis

A slice of 25 g of *morcilla* (casing included) was sterile weighted, diluted in 225 ml of Ringer solution (Oxoid, Basingstoke, UK), and homogenised for 120 s in a laboratory blender (Stomacher 400, Seward, London, UK), prior to the preparation of 1/10 serial dilutions for microbiological analysis. The following microbial parameters were determined: Total Viable Count (TVC) plated on PCA agar plates (Oxoid) and incubated at 30 °C, for 48 h; Lactic Acid Bacteria (LAB), grown in MRS agar (Biokar Diagnostics, Beauvais, France) and incubated anaerobically in 6% CO_2, at 30 °C, for 48 h; and finally, the production of dextran from sucrose was tested using the method described by Schillinger and Lucke (1987), which was plated on MRS agar with 5% of sucrose instead of glucose as sugar source (MRSD) and incubated at temperature of 30 °C, for 48 h. The pH and microbiological analyses were