SPOILAGE DYNAMICS OF *MORCILLA DE BURGOS* TREATED WITH HIGH HYDROSTATIC PRESSURE BY CULTURE-INDEPENDENT METHODS

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Introduction

Morcilla de Burgos is a typical blood sausage from Spain. Blood sausages are very popular and traditional meat products manufactured all around the world. However, besides their widespread production and consumption, this kind of products have been forgotten from the scientific point of view, probably because their production have been always related to small and craft producers selling mainly in local markets. Cooked meat products, as *morcilla de Burgos*, are liable to contamination during post-cooking handling, particularly during the chilling step, just before vacuum packaging. Consumer demands are currently driven towards more natural food and as a consequence producers and scientists are searching for more natural antimicrobial preservatives and new mild technologies such as high pressure treatments (HPP). HPP is a non-thermal mild technology of growing interest since pressures between 300 and 600 MPa inactivate yeasts, moulds and most vegetative bacteria, including most spoilage and pathogenic bacteria, but vitamins, colour and flavour remain largely unaffected (Hugas et al., 2002). The aim was to follow the evolution of the spoilage bacterial population in *morcilla de Burgos*, and evaluate their behavior when a HPP treatment was applied by denaturing gradient gel electrophoresis (DGGE), without the need of isolation and biochemical identification.

Materials and Methods

Samples and processing of the blood sausages: *Morcillas* stuffed in natural beef casings made by one producer were selected for the different preservation experiments. One sample of sausage batter was collected prior to stuffing. The blood sausages were then transferred to a cooking vessel and boiled in water at 95ºC for around 1 h. After cooking, *morcillas* were air cooled overnight at room temperature (8–10 ºC), and vacuum packaged. Blood sausages were subjected discontinuous hydrostatic pressurization unit Wave 6000/135 (NC Hyperbaric, Burgos, Spain), at 600 MPa for 10 min. The blood sausages were stored in a dark place at 4ºC. Samples of sausage batter prior to stuffing, HPP treated and not treated (control) blood sausages vacuum-packaged, were analyzed in triplicate at 0, 9, 14, 21, 28 and 35 days of storage at 4°C.

pH measurements: Potentiometric measurements of pH were made with a pin electrode of a pH meter (pH M82; Radiometer Copenhagen, Cecchinato, Italy) inserted directly into the sample.

Direct extraction of nucleic acids from blood sausages: samples (20g) were homogenized in 80 ml of salt solution (8 g/l NaCl) and 40 ml of solution were transferred into a 50 ml sterile tube. Big debris was allowed to deposit for 5 min, and 4 ml of supernatant was split into two 2-ml aliquots in tubes, one for DNA and one for RNA extraction. They were subjected to centrifugation at 4°C for 10 min at 14,000 x g to pellet the cells, which were subjected to DNA and RNA extraction. For DNA extraction, cells were resuspended in 150 µl of proteinase K buffer (50 mM Tris-HCl, 10 mM EDTA [pH 7.5], 0.5% [wt/vol] sodium dodecyl sulfate). Twenty-five microliters of proteinase K (25 mg/ml; [Sigma, Milan, Italy]) were added, and a 50°C treatment was performed for 1.5 h. After this step, 150 µl of 2x breaking buffer (4% [vol/vol] Triton X-100, 2% [wt/vol] sodium dodecyl sulfate, 200 mM NaCl, 20 mM Tris [pH 8], 2 mM EDTA [pH 8]) were added and the solution was moved into a screw cap tube containing 0.3 g of glass beads. Three hundred microliters of phenol-chloroform-isoamyl alcohol (25:24:1, pH 6.7; [Sigma]) were added and the tubes were subjected to three 30-s treatments at the maximum speed, with an interval of 10 s each, in a bead beater (Mini Bead Beader 8; Biospec Products, Inc., Bartlesville, Okla.). Then, 300 µl of TE were added (10 mM Tris [pH 8], 2 mM EDTA [pH 8]) and the DNA, in the acqueous phase was recovered and precipitated with ice-cold ethanol after centrifugation at 12,000 x g at 4°C for 10 min. While for the RNA 300 µl of phenol-chloroform (5:1, pH 4.7; [Sigma]) were added before the bead beater treatment. The DNA and RNA were collected at 14,000 x g at 4°C for 10 min, after addition of 1 ml ice-cold ethanol. Fifty microliters of sterile water were added, and a 30-min period at 45°C was used to facilitate the nucleic acid solubilization. One microliter of DNase-free RNase (Roche Diagnostics) and 1 µl of RNase-free DNase (Roche Diagnostics) were added to digest, respectively, RNA and DNA by incubation at 37°C for 1 h.

Reverse transcription step: Reverse transcription (RT)-PCR was performed with the RevertAidTM M-MuLV reverse transcriptase (Promega, Milan, Italy).

PCR protocol: Primers P1V1GC-P2V1 (Cocolin et al. 2001), were used for the amplification of the DNA extracted from the samples. The conditions used were as described by the authors.

DGGE analysis: All amplicons obtained by PCR amplification were separated in denaturing gradient from 40 to 60%. Gels were subjected to a constant voltage of 120 V for 4 h at 60°C, and after the electrophoresis, they were stained with SYBR Green and examined under UV illumination.
Sequencing of DGGE bands and sequence analysis: Blocks of polyacrylamide gels containing selected DGGE bands were punched by sterile pipette tips. The blocks were then transferred in 50 µl of sterile water, and the DNA of the bands was left to diffuse overnight at 4°C. Two microliters of the eluted DNA was used for the reamplification and DGGE run to confirm the presence of a single band with identical migration profile with respect to the one excised from the samples. After DGGE analysis, the PCR product was cloned into pGEM vector (Promega) and selected clones were tested again by PCR-DGGE to check the mobility of the insert. Only if the migration was again identical to the original band cut, the clone was sent for sequencing to a commercial facility (MWG Biotech, Germany). The Blast program was used for the analysis of the sequences obtained.
Statistical analyses: pH data were statistically analysed using ANOVA procedures. Data analyses were conducted using the statistical package Statgraphics Plus for Windows ver. 5.1.

Results and Discussion

Initial pH values were above 6.35 for the different samples. Control samples were characterized by a significant ($p<0.05$) drop, from pH above 6.20 (day 9) to pH just above 5.00 (day 21). In HPP samples, pH was over 6 during the first 28 days. pH decreased significantly ($p<0.05$), reaching values around 5.40, at the end of the study. Results confirm that the main spoilage bacteria in this product are species of heterofermentative LAB belonging to the genera Weissella and Leuconostoc as it was stated by Santos et al. (2005). Concerning the bacterial ecology as determined by DNA-DGGE profiles, species of *Weissella confusa*, *Weissella viridescens*, *Gamma proteobacterium* and *Leuconostoc lactis* were identified in sausage batter, HPP treated and non-treated samples, but *L. lactis* was only detected, as faint bands, at 0 and 9 days of storage in control samples and from 0 to 28 days in HPP treated samples. *Brochotrix thermosphacta* and *Leuconostoc mesenteroides* were only identified in control samples from 14 to 28 days and from 14 to 35 days of storage, respectively. The same species of bacteria were detected in the RNA-DGGE gels, however their presence and appearance were rather different. In control samples, *L. lactis* and *W. confusa* were detected in the sausage batter and at day zero and at day 9 a third band appeared, identified as *B. thermosphacta*. *L. mesenteroides* showed an intense band from day 14 till day 35. It is remarkable that this species expressed more band intensity when pH drops from 6.20 on day 9 till 5.59 on day 14. *W. viridescens* band appeared from day 21 onwards. In HPP treated samples, *L. mesenteroides* band did not appear and pH dropped from 6.30 on day 28 to 5.39 on day 35 coinciding with the appearance of the band of *W. viridescens*. *L. mesenteroides* was more sensitive to HPP, as reported by other products (Basak et al., 2002), *W. viridescens* showed more resistance to high pressure (Park et al., 2001). *L. lactis* showed a contrary behaviour to *L. mesenteroides* and it seemed more resistant to HPP treatment.

Conclusions

In HPP treated and non-treated product, the main spoilage bacteria were *W. viridescens, W. confusa, L. lactis* and *L. mesenteroides*, although with different prevalence. The HPP treatment was responsible for the change in the ecology of the product during storage. Concerning the RNA-DGGE profiles, *L. mesenteroides* in control samples, and *W. viridescens* in HPP treated samples, appeared more intense bands at the time when pH dropped in *morcilla*. In that sense, *W. viridescens* was able to recover in 35 days and carry out the typical spoilage of the product. *L. lac*tis was present in the product until the pH started to drop and this may reflect the high sensitivity to pH changes of this species. The combination of both methods DNA-DGGE and RNA-DGGE gives a more accurate picture of the spoilage dynamics in foods.

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