Title

Behavior and effect of combined starter cultures on microbiological and physicochemical characteristics of dry-cured ham

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BEHAVIOR AND EFFECT OF COMBINED STARTER CULTURES ON MICROBIOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF DRY-CURED HAM

Research Highlights

- The starter culture composed of LAB, moulds and yeast shows a potential interest for use in dry-cured ham production.
- The starter culture including fungal strains enhances some desirable aspects of dry cured-ham, such as the NPN contents.
- Higher fatty acid oxidation was described in dry-cured ham inoculated only with LAB.

Abstract

The behaviour of two combined starter cultures and their influence on the microbiological and physicochemical characteristics of dry-cured ham have been evaluated. Three lots of dry-cured hams have been tested during their processing (0, 9, 48, 74, 112, 142, 166 and 211 days). Lot1 had no added starter culture. To lot 2 a starter culture with *Penicillium chrysogenum, Penicillium digitatum, Penicillium nalgiovense, Debaryomyces hansenii*, *Lactobacillus plantarum, Lactobacillus acidophilus, Pediococcus pentosaceus* and *Micrococcus varians* was added and to lot 3 one with *L. plantarum, L. acidophilus, Pediococcus pentosaceus* and *Micrococcus varians*. The use of a selected starter culture based on a combination of lactic acid bacteria (LAB) and fungal strains with a demonstrated proteolytic activity such as *P. chrysogenum* and *D. hansenii* (lot 2) does not affect the main characteristics of dry-cured ham processing, even enhancing some desirable aspects, like its non-protein nitrogen contents. LAB strains are not significantly affected by combining them with fungal starter, and better counts are found with respect to lot control. A higher thiobarbituric acid reactive substances content was described in a lot inoculated only with LAB (lot 3). Potentially pathogenic microorganisms were not detected in any of the lots studied. The starter culture used in lot 2 shows a potential interest for use in dry-cured ham production.

Keywords: dry-cured ham, starter cultures, lactic acid bacteria (LAB), *Penicillium*, *Debaryomyces*.

Introduction

Dry-cured ham is currently a key feature of Spanish gastronomy and is among the traditional Spanish foods enjoying international renown (Toledano et al. 2011). Its increasing production and export capacity promote interest in all the quality factors implicated. Traditionally, its production process comprises several steps: preparation of pieces, salting, post-salting, ripening and aging.

Many factors influence the final quality of hams: their raw meat and processing conditions, among others. One main factor to consider is the role of microorganisms, taking them into account from a technological, sensorial and hygienic point of view. Several studies have previously addressed the importance of using starter cultures in dry-fermented meat products not only for safety or conformity reasons, but also for uniformity purposes (Talon et al 2008, Semedo-Lemsaddek et al 2016, Laranjo et al. 2017). However, not very much literature is available for this purpose on dry-cured hams. Despite starter cultures not being generally used in traditional Spanish dry-cured ham, some authors have suggested and/or revised their use to improve some of the characteristics of the product (Rodríguez et al. 2001, Sánchez-Molinero and Arnau 2008, Laranjo et al. 2017, Bosse et al. 2018). The transition from the empirical process to that controlled by the use of starter cultures is based on the importance of optimizing the process as well as preventing the growth of non-controlled strains which can spoil the ham. In fact, Martín et al. (2006) point out the importance of ham microorganisms, considering the typical cured product's taste as being the result of the combination of enzymes and microbial growth action. The addition of innocuous and highly adapted starter cultures guarantees the product's safety and its correct processing, inhibiting

spoiling and pathogenic microorganisms and contributing to the improvement of stability, sensorial quality and conservation of the pieces (Martín et al. 2000, Takeda et al 2017).

The use of bacterial starter cultures began with the addition of *Micrococcus* and *Staphylococcus* (Coagulase Negative Cocci - CNC) strains resulting in a faster colour, obtaining pH decrease, control of pathogenic microorganisms and a reduction in the economic requirements of the process, as well as making a positive contribution to sensorial characteristics (flavour and colour) of dry-cured ham (Rodríguez et al. 2001).

Using lactic acid bacteria (LAB) in cured meats is based on the success of their fermentation processes. These microorganisms produce acid which contribute to flavour as well as decreasing protein solubility and water retention capability, and improving the drying process. Although the role of microbial enzymes in protein degradation is currently accepted with a greater reluctance, LAB are endowed with proteolytic activity, mainly intracellular amino, di and tripeptidases (Fadda et al. 2010). *Lactobacillus plantarum* and *Pediococcus pentosaceus* are two of the main LAB cultures used as starter in meat products, as well as *Lactobacillus sakei, Lactobacillus curvatus* and *Pedicococcus acidilactici* (López et al. 2006). Also, LAB can be used as protective cultures in other cured meat products. Sánchez-Molinero and Arnau (2008) found that a starter culture (LAB - *L. sakei* and *P. pentosaceus*-, CNC and *Debaryomyces hansenii*) caused a reduction of mould growth and of the area of lean covered by oil drip.

As moulds are predominant on the surface of the product, it is coherent to consider the use of a fungal starter. Moulds have a great influence on volatile compound production in meat products (Marušić et al. 2011). However, uncontrolled mould growth on the surface of dry-cured meat products is causing significant quality problems. As some moulds are mycotoxigenic, their growth on the dry-cured meat products could also pose a serious

health risk. Those quality problems and potential health risks can be better handled if the types of moulds growing on the products are known (Asefa et al. 2009). Fungal strains such as *Penicillium nalgiovense* have been successfully tested in different meat products, even in hams. Also, they have been suggested for Iberian ham (Rodríguez et al. 2001). It is important to characterize the moulds in order to prevent the use of possible toxigenic strains (Battilani et al. 2007). Regarding yeasts, these have been used in combined starter cultures, together with CNC and LAB (Rodríguez et al. 2001). In fact, *D. hansenii* strains isolated from ham had been used as starter cultures for this product (Simoncini et al. 2015). These strains showed a high adaptation to the ham processing environment, remaining throught the production, and with a relevant aminopeptidase and proteolytic activity. At the same time, *D. hansenii* can inhibit some toxigenic fungal strains (Andrade et al. 2014, Peromingo et al. 2018).

Laranjo et al. (2017) reviewed the use of yeasts and moulds as starters. Yeasts can develop their activity during the first steps of the processing, when moulds have not yet been implemented. These authors review the potential advantages of using yeasts like *Debaryomyces*, and especially *D. hansenii* can be selected for the a_w conditions during the drying and ripening stages.

All these antecedents trigger the interest of testing the use of a combined starter culture, considering the possibility of mixing non-toxigenic moulds, yeasts, LAB and CNC.

The main goal of this work is to test the behaviour and dynamics of selected combined starter cultures and their influence on characteristic parameters of dry-cured ham during its manufacturing process.

Material and Methods

Samples

Fifteen Spanish dry cured hams were produced in a local manufacturing plant located in southern Spain. Their manufacture was carried out following the specifications of the Traditional Speciality Guaranteed (TSG) "Jamón Serrano", under controlled chamber conditions: Preparation-salting (1-4ºC and 75-85 % RH), post-salting (1-6ºC and 70-80 % RH), ripening and aging (slowly rising from 6ºC to 34ºC and from 80% RH to 60 % RH). Dry-cured hams had an initial weight of 10-11 Kg, and a fat thickness of 1-2 cm.

Samples were grouped in three lots (1, 2 and 3) with five samples each one. Lot 1 was produced without the addition of starter cultures. A starter culture integrated by *L. plantarum, Lactobacillus acidophilus, P. pentosaceus*, *Micrococcus varians, Penicillium chrysogenum, Penicillium digitatum, P. nalgiovense* and *D. hansenii* was added to lot 2. Lot 3 was inoculated with *L. plantarum, L. acidophilus, P. pentosaceus* and *M. varians.* Each sample was analysed in duplicate at the following production steps: Preparation-salting (0 days), after cleaning-brushing (9 days), post-salting A (48 days), post-salting B (74 days), ripening A (112 days), ripening B (142 days), ripening C (166 days) and aging (211 days).

Preparation and inoculation of starter cultures

In a previous study (Toledano et al 2011), eleven commercial LAB and mould strains were tested for proteolytic activity against pork myosin, with a view to their possible use as starter cultures. The strains showing the highest proteolytic activity were selected for the present study, specifically: *L. plantarum* L115 (Rhodia Ibérica, Madrid, Spain); *L. acidophilus* (Fargo 606 TM; Lab Amerex, Madrid, Spain); *P. pentosaceus* (Saga P TM; Lab Amerex, Madrid, Spain); *M. varians* (Saga P TM; Lab Amerex, Madrid, Spain); *P.*

digitatum (CECT 2954; Burjassot, Spain); *P. nalgiovense* LEM 50I (Rhodia Ibérica, Madrid, Spain); *P. chrysogenum* (Schneider TM; Schneider-Soprosal, Bloney-Vevey, Switzerland); and *D. hansenii* LEM 50I (Rhodia Ibérica, Madrid, Spain).

The commercial bacterial strains (*L. plantarum, P. pentosaceus, M. varians* and *L. acidophilus*) were diluted in sterile distilled water and prepared according to commercial instructions, with a final concentration of 10^9 CFU/ml. The commercial fungal strains (*P. nalgiovense*, *P. chrysogenum* and *D. hansenii*) were diluted in a saline solution and Tween 20 0.2%, following the manufacturer's instructions. *P. digitatum*, from a culture collection, was recovered on Potato Dextrose Agar (Merck, Darmstadt, Germany). After 5 days of incubation (25ºC), the spores were recovered adding saline solution (0.9% NaCl) and Tween 20 0.2%. This procedure was repeated until reaching $4 \cdot 10^7$ spores/ml. At day 0, the bacterial starters were inoculated in a volume of 1 ml in-depth and with a 12 cm sterile syringe, into ten different inoculation points equidistant from each other. Yeast and moulds starters were added to lot 2 after 74 days, spraying the surface of the samples.

Microbiological analyses

Ten grams of each sample were taken aseptically from *vastus medialis*, *gracilis* and *semimembranosus* area (about 25 cm^2), and by previously removing the surface area (2-3 mm thick), for the investigation of bacteria and without removing that area, for the investigation of moulds and yeasts. The 10 g were transferred to sterile pouches and homogenized for 2 min with 90 ml of sterile buffered peptone water 0.1% w/v (Oxoid, Unipath Ltd., Basingstoke, UK) as a diluent, using a Stomacher (Lab Blender, Model 4001, Seward Medical, London, UK). Appropriate dilutions of the sample homogenates were prepared and inoculated in growth media to estimate microbial counts.

The following microbiological parameters were determined: LAB, fungal biota (yeasts and moulds), CNC, Enterobacteriaceae and *Clostridium* spp. The possible occurrence of *Salmonella-Shigella* and *Listeria monocytogenes* was also investigated.

The LAB count was verified on MRS agar (Oxoid, Unipath Ltd., Basingstoke, UK) acidified to 5.40 and incubated in anaerobic jars at 30ºC for 72 h. The results were expressed as log CFU/g. The yeast and mould counts were verified on acidified PDA agar (Merck, Darmstadt, Germany). Plates were incubated at 25ºC for 72 h for yeast count and for 120 h for mould count. The results were expressed as log CFU/g.

CNC counts were verified on MSA agar (Oxoid, Unipath Ltd., Basingstoke, UK) and incubated at 37ºC for 72 h. The results were expressed as log CFU/g.

Total Enterobacteriaceae counts were verified on VRBD agar (Oxoid, Unipath Ltd., Basingstoke, UK) incubated at 35-37ºC for 24 hours. The results were expressed as log CFU/g.

To detect *Salmonella-Shigella* and *L. monocytogenes*, 25g of each ham were aseptically sampled. For the determination of *Salmonella-Shigella* the ISO 6579:2002 method was employed. In the case of *L. monocytogenes,* the ISO 11290-2 method was used. The *Clostridium* spp. were counted on sulphite polymyxin sulphadiazine agar (Merck, Darmstadt, Germany) at 45ºC for 48 h under anaerobic conditions. The results were expressed as log CFU/g.

Physicochemical analyses

Fifty grams of each sample were taken from the *vastus medialis, gracilis* and *semimembranosus* area, were minced to obtain a homogeneous sample and placed in clean and dry containers at 4ºC until the time of analysis.

For pH determinations, a digital pH meter (SENTRON 1001 pH, Roden, The Netherlands) was used. Moisture content was measured using the procedure no. 950.46 (AOAC, 1990). Chloride content was determined as chloride concentration following Bandeira et al. (1990). The non- protein nitrogen (NPN) was analyzed using the procedure described by Bandeira et al. (1990). Thiobarbituric acid reactive substances (TBARS) was determined in accordance with Tarladgis et al. (1964). Finally, ash content was measured after incinerating 10 g of sample in an oven at 550ºC for 14 hours.

Statistical analysis

The normal distribution of the data was evaluated using the Kolmogorov-Smirnov test. Parametric statistics were used in the event of $p > 0.05$, applying a one factor analysis of variance (ANOVA) for the effect of the starter culture. In the case of populations with a non-normal distribution ($p<0.05$), the non-parametric Kruskal-Wallis test was made to analyse differences between groups.

Results and Discussion

Microbiological parameters

Table 1 shows, for each lot, the counts of the microbiological parameters throughout the different manufacturing steps of the hams studied.

LAB show an increase in their counts in the cleaning-brushing step (maximum 6.96 log CFU/g in lot 2), to later decrease in post-salting steps. This decrease was delayed in lots with an added starter culture. After this, an important increase starts to reach maximum numbers (about 7 log CFU/g) in ripening A for the lots with starter cultures added (lots 2 and 3). These results are coherent with those reported by Hernández and Huerta (1993), where LAB increased their counts just after the first step, to decrease after 30 days mainly due to a_w reduction. Sánchez (2005) also reported significant higher LAB

counts in dry-cured ham previously inoculated with a LAB starter, as was desired, but with slightly lower final counts than ours. Also, Sánchez-Molinero and Arnau (2008), using a combined starter culture (LAB, CNC and *D. hansenii*), found low LAB counts (under 1 log CFU/g) in muscle at the end of the processing. In our case, sampling was not only carry out from the surface of the products. With a similar sampling, our final counts agree with those of Hernández and Huerta (1993). Our lot 1 shows irregular LAB counts, demonstrating that the process was not enhanced with a starter culture, with lower LAB counts during the process. The statistical analysis certainly shows differences ($P \leq 0.05$) for LAB counts between the two lots with added starter cultures and the control. However, no differences are found between lots 2 and 3. The importance and contribution of starter cultures is to enhance the increase and maintenance of LAB counts throughout the processing, with positive consequences for the safety of the product. In fact, Takeda et al. (2017), consider that what is required of the LAB is a rapid growth, making the pH drop, thus preventing contamination by microorganisms that may spoil the product, given that, in our case, there is no sterilization process during its manufacture.

Regarding CNC, their counts increased after post-salting A in all the lots. Up to that moment (about 50 days), their numbers were similar to those of the preparation step. These results agree with those of Arnau et al. (1987) and Sánchez (2005). While lot 1 (control) reached its maximum count in the post-salting step, lots 2 and 3 reached their maximum numbers in ripening B (7.41 log CFU/g for lot 2, and 8.42 log CFU/g for lot 3). In all the cases this important increase occurred on the last days of post-salting and first days of ripening, in coherence with the work of Hernández and Huerta (1993), Vilar et al. (2000) and Sánchez (2005). Also, lot 1 maximum counts are higher than those obtained in the other lots, probably due to CNC not being good competitors and because,

in that lot, as there were no starter cultures, they could grow better. From the ripening stage on, CNC counts generally decrease to values of around 4-5 log CFU/g. Rodríguez et al. (1998) found similar behaviour during ripening and aging steps, mainly due to the a_w reduction. Sánchez-Molinero and Arnau (2008) reported similar counts to ours at 120 days of processing, although final counts were lower (in their case processing reached 310 days). In our opinion, and although similarly to LAB discussion, CNC counts from ripening A are generally higher than those of lot 1, and a_w values are low enough to cause a homogenization in final counts. This fact is coherent, especially in the final steps, with Sánchez (2005), who pointed out significant differences between inoculated (CNC, LAB and *D. hansenii*) and non-inoculated samples (the decrease in the post-salting step was higher than in our work). However, from the statistical point of view, we cannot consider as being significant ($P>0.05$) the differences between lots as far as CNC is concerned, although there are evident ones between the different processing steps ($P\leq0.001$).

Ockerman et al. (2000) inoculated a fungal culture (*P. chrysogenum*) into a meat substratum and found that, similarly to our work, it influenced the total mesophilic aerobic bacteria count but not the CNC counts. As a general consideration, CNC counts are higher, in comparison with other microorganisms, during ham processing (Martín et al. 2000).

Vilar et al. (2000), in cured ham, affirm that all the microbial groups (with the exception of enterococci and enterobacteria) reach their maximum counts after ripening to gradually decrease up to the final steps. The fall in water activity seems to be decisive in the decrease in the counts during the ripening stage, as a consequence of the loss of moisture in the pieces. As far as LAB and CNC counts are concerned, our results agree with that consideration.

The fungal starter was added to lot 2 at day 74 (post-salting B). After this, lot 2 reached its maximum (4.27 log CFU/g) during ripening A. Up to that moment, all the lots behaved similarly (based on wild population) with no significant differences. Also, the evolution in mould counts up to the final of the process trends to homogenize the lots' counts due to the values of some physicochemical parameters (mainly pH and a_w). Martín (1999) reported that the optimal conditions for inoculating *P. chrysogenum* into cured ham to be on the final days of post-salting and first days of ripening, which is coherent with our design and the behaviour of the fungal strains during the first ripening step. Also, in lots 1 and 3 (with no fungal starter added), the wild mould population increased during this step. Certainly, Hernández and Huerta (1993) found their maximum counts for cured ham during ripening, and a later decrease in them, and that behaviour was also reported by Arnau et al. (1987). Regarding the final steps, Martín (1999) describes higher fungal counts in a lot inoculated with *P. chrysogenum* and *D. hansenii* than in a non-inoculated lot at 7 months, although after 12 months the counts were similar. In our work, and from the statistical view point, there are no differences ($P>0.05$) for the evolution of mould counts between the three lots. However, it is true that the maximum reached corresponds to the lot with a fungal starter which can be used to control the mould population with respect to the wild strains. Obviously, the different processing steps seem to be significant in influencing these counts. ($P\leq0.001$). Acosta et al. (2009) confirmed the inhibitory activity of *P. chrysogenum* against reference toxigenic moulds.

Similarly to mould, yeast growth is mainly favoured during the final post-salting phase and the first ripening period. During aging, a new increase is shown, as reported by Núñez et al. (1996), identifying yeasts as being the predominant microorganisms in the last phases of Iberian ham processing. *D. hansenii* is the most frequently detected yeast

species (> 99% of cases) in this type of ham. Furthermore, *D. hansenii* has shown lipolytic activity at 4ºC and a_w 0.87. These characteristics are seen to be of interest in the processing of meat products with a long maturation period (Rodríguez et al. 2001). These authors consider that the combined inoculation of *P. chrysogenum* and *D. hansenii* into the ham surface improves the myofibrillar protein hydrolysis, increasing the presence of free amino acids. Taking into account these considerations, it has been appropriate to include it as a yeast starter in our work (lot 2).

The behaviour of the wild populations before inoculation (at day 74) is one of the points most divergent in the available references, mainly due to their variability in environments, raw material and ingredients, and, obviously, in the different types of ham. In our case, before salting, the counts reached around 3.3 log CFU/g, higher than those reported by Sánchez (2005), with 0.6 log CFU/g from non Iberian cured ham. On the other hand, Rodríguez et al. (2001) detected 5 log CFU/g in Iberian ham in the same processing step. In all the lots in our work, the yeast population displayed an important increase, especially enhanced by the competitive physicochemical conditions in post-salting A compared to other microorganisms.

Regarding the performance of the yeast counts after the inoculation of the fungal starter in lot 2, they reach their maximum number (8.03 log CFU/g) after this inoculation. But, in general, the yeast counts were homogeneous, with an increase occurring during aging, that was more noticeable in lot 2 (5.83 log CFU/g). Sánchez (2005) obtained lower counts during the whole processing after the inoculation of a LAB plus *D. hansenii* starter. According to Rodríguez et al. (2001), the increase in temperature at the end of post-salting and during ripening favoured the highest yeast counts in cured ham (around 6-7 log CFU/g). Sánchez (2005), at the end of the ripening step, obtained 6.1 log CFU/g,

and these results were also similar for lots inoculated and non-inoculated with *D. hansenii*. However, Martín (1999) obtained important differences after 7 months of processing (4 log ucf/g for a non-inoculated lot versus 8 log CFU/g for an inoculated one). After 12 months of processing differences were slighter (5 log CFU/g versus 6 log CFU/g). Also, for these authors, yeasts were predominant in the central processing steps. Regarding other types of ham, Simoncini et al. (2007) also consider the possibilities of other species different from *Debaryomyces* spp. in the sensorial and hygienic characteristics of Italian cured ham, in which these authors determined high yeast counts.

Regarding contaminating microorganisms, it is always useful to use indicators to check the hygienic characteristics and possibilities of the processing, and the influence of starter microorganisms on the physicochemical parameters and competitive substrata. At the beginning of the process, enterobacteria counts of around 2 log CFU/g were found. These counts gradually decreased until they disappeared during the post-salting step. This behaviour, that confirms the hygienic conditions of the processing of this type of product, is in agreement with that found by Vilar et al. (2000). When the pH is approximately 5 and a_w around 0.940, enterobacteria cannot survive.

Neither during the processing of the samples studied, nor in any of the lots, were *Salmonella-Shigella, L. monocytogenes* and *Clostridium perfringens* detected. A correct processing and the use of starter cultures have given similar results in different works (Rodríguez et al. 2001). During salting and post-salting a_w decreases and the temperature is below 5ºC, conditions which make microbial growth difficult, especially for mesophilic species such as *Salmonella* spp. or clostridia. Another influential factor is NaCl, that develops a selective environment for halo-tolerant psychrotrophic microorganisms such as many CNC (micrococci, among others). This practice carried out

in salting and post-salting contributes to the absence of pathogen microorganisms (Sánchez, 2005).

Physicochemical parameters

Table 2 shows, for each lot, the evolution of the physicochemical parameters throughout the different manufacturing steps of the hams studied.

Its pH develops similarly throughout the processing in all the lots. According to Arnau et al. (1998), the pH decreases during salting due to a loss of phosphates and alkaline compounds, and salt absorption. In their opinion, the pH in cured ham ranges from 5.6 to 6.2, with a general trend towards a slight increase. Our data agree with their considerations, as well as for the modest increase during the post-salting step. This increase is higher in lot 1 (control) than in those inoculated with starter cultures. In fact, a slighter increase is reported for the lot with LAB starter. These results agree with those reported by Sánchez (2005), who considers the influence on the pH of the LAB growth, that ferments the surface sugars during the first drying steps. In fact, Sánchez (2005) and Kim et al. (2016) point out that the highest pH values are reported on lots not inoculated with LAB. In our work, significant differences ($p< 0.05$) were found between the pH of lot 3 (with only LAB added) and pH of lots 1 and 2. In lot 1 (control) we found the highest increase in the pH throughout the processing (0.61), while lot 3 showed the lowest increase (0.44).

No significant differences ($p<0.05$) were found for moisture between the different lots. As expected, moisture decreases throughout ham processing due to water loss. All the lots behaved similarly, which agrees with the data reported by Marušić et al. (2011). These authors described moisture values ranging from 37 to 45% after 14 months. The moisture losses in ham occur almost exclusively in loin parts, where water is present. The

fat coating acts as a barrier making desiccation more difficult. In our work, the final product's moisture is around 20-28%. In particular, lot 2 (with fungal strains added) has the highest value (27.75%), agreeing with the higher yeast and mould counts. This could be because of the superficial microclimate created by these microorganisms, regulating the moisture loss of the product.

Also chloride performed similarly in all the lots studied, and no significant differences ($p<0.05$) were found between lots. The maximum value was obtained during salting-brushing, as expected. Later, an important decrease during post-salting is described, remaining at similar values up to the end of the processing. According to Toldrá (2008), after salting, the amount of salt is very high in the outer muscles but low inside the ham. Towards the 4-5 month of the process, the salt tends to equalize, but this profile is reversed (higher concentration in the deep muscles) toward the end of the process. Our results agree with this consideration.

According to Rodríguez et al. (2001), NPN increases throughout the maturation process because of protein degradation. Proteolysis has a great influence on the quality characteristics of dry cured ham, as it is an important source of flavour compounds, such as free aminoacids and small peptides (Pérez-Palacios et al. 2010). Our samples started with a low content in NPN, which decreased to a minimum value (about 0.1%) during the cleaning-brushing step in lots 2 and 3 (previously inoculated with a LAB culture) and in post-salting A for lot 1 (0.07%). In this step, the LAB activity had started to influence the protein degradation in lots 2 and 3. This effect agrees with that found by Casaburi et al. (2008) for sausages previously inoculated with *Lactobacillus* spp. and *S. xylosus* as starter cultures. From this step on, the protein degradation behaviour of the different lots shows an increasing trend up to the end of the process. This increase is especially

perceptible during ripening, which agrees with the works of Martín (1999), Pérez-Palacios et al. (2010), among others. These authors point out that salt concentration and temperature have a marked influence on protein degradation during dry-cured ham processing. Protein degradation is intensified by high temperatures and low salt concentrations, with a marked proteolysis increase during aging, when the temperature increases again (Rodríguez et al., 2001). Most of the oligopeptides and free aminoacids increase during the last steps of the processing (Sforza et al. 2006). Regarding the role of moulds, during ripening, they reach their highest development, showing a greater proteolytic activity during this step. In fact, Rodríguez et al. (1998) observed the important proteolytic activity of *P. chysogenum* in dry-cured ham. Also, Martín (1999) suggests the *P. chysogenum* and *D. hansenii* starter cultures being applied at the end of post-salting and at the beginning of the ripening steps.

In the present work, the highest values are obtained for lot 2. The differences between the evolution of this lot and lots 1 and 3 appear to be significant ($p<0.05$). From the post-salting step onwards, lot 2 show higher NPN values, with the greatest differences in the final steps of ripening and aging. Thus, lot 2 (which includes moulds and *D. hansenii*) had the highest values of NPN during the phases when the moulds grew more profusely, in accordance with the work of Martín (1999). An adequate fungal biota for dry-cured ham can enhance the protein hydrolysis, increasing the presence of free amino acids. The possibilities of *P. chrysogenum* as an NPN enhancer were previously described by Ockerman et al. (2000), and its proteolytic capacity was reported by Rodríguez et al. (1998). Martín et al. (2004) pointed out the need to research the impact of a selected fungal biota on the production of the volatile compounds and sensorial characteristics of dry-cured ham.

TBARS content describes fatty acid oxidation caused by lipolysis. The first steps show values of under 0.001%. Starting from the ripening A step (112 days) the TBARS content increases to reach maximum values (between 0.29 and 0.33%) in ripening B or C. Also, Martín (1999) concludes that lipolytic activity is greater during ripening. In our work, lot 2 was seen to have lower TBARS percentages during the processing than lots 1 and 3. These differences were significant (p<0.05). Martín et al. (2003) observed that *P. chrysogenum* isolated from dry-cured ham caused a decrease in compounds produced by lipid oxidation in sliced loin; however, the compounds derived from proteolytic activity increased. Martín (1999), also in hams, researched the effect of *P. chysogenum* and *D. hansenii* on volatile compounds derived from lipid oxidation. They concluded that those compounds were at a higher proportion in hams with a six-month maturation and not inoculated with fungal starter, which seems to inhibit the aldehydes, ketones or alcohol production and transformation. Most volatile compounds detected in cured meat products are derived from lipid oxidation. However, they have very different profiles depending on the product itself. For dry-cured ham there are a larger number and higher level of compounds derived from protein and lipid degradation, because of the long processing time, sometimes up to 24 months.

Regarding ashes, no significant differences (p<0.05) were found between the different lots. However, there were differences between the different steps of the processing, as expected. The maximum values occurred during the cleaning-brushing step (up to 19-20% for lots 2 and 3), coinciding with the highest NaCl levels. Later, the values decrease to remain stable (4-6%) from the post-salting steps onwards.

Conclusions

The use of a selected starter culture based on a combination of LAB and fungal strains with a demonstrated proteolytic activity, such as *P. chrysogenum* and *D. hansenii,* enhance some desirable aspects in ham, like its NPN contents. LAB strains are not significantly affected by combining it with a fungal starter, and better counts are found with respect to the control lot. A higher TBARS content was described in lots inoculated only with LAB. *Salmonella* spp*., Shigella* spp*., L. monocytogenes* and *C. perfringens* were not detected in any of the lots studied. The ham processing hinders their occurrence and the addition of starter cultures strengthens this fact. The starter culture used in lot 2 shows a potential interest for its use in dry-cured ham production.

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Table 1. Evolution of microbial counts [1] (log CFU/g) in inoculated dry-cured ham at different stages.

Stage		Preparation-salting	Cleaning-brushing	Post-salting A	Post-salting B	Ripening A	Ripening B	Ripening C	Aging
Days		0	9	48	74	112	142	166	211
Lactic acid bacteria	Lot 1[x]	3.60±0.61[a]	5.05±0.36[b]	3.46±0.46[a]	5.66±0.43[bc]	4.58±0.24[bc]	3.60±0.27[a]	3.26±0.33[a]	3.35±0.27[a]
	Lot 2[y]	3.95±0.14[a]	6.96±0.39[b]	5.64±1.07[c]	5.76±0.50[c]	7.00±0.35[b]	6.90±0.81[b]	4.19±0.38[a]	4.49±0.64[a]
	Lot 3[y]	3.38±0.16[a]	6.95±0.89[b]	6.62±0.62[bc]	5.32±0.84[c]	7.02±0.88[b]	6.39±0.79[b]	4.33±0.40[ac]	3.29±0.33[a]
Coagulase-negative cocci	Lot 1[x]	4.05±0.23[a]	4.02±0.35[a]	3.65±0.61[a]	8.90±0.56[b]	7.38±0.64[c]	6.83±0.66[c]	5.10±0.44[d]	5.12±0.72[d]
	Lot 2[x]	3.85±0.37[a]	4.08±0.25[a]	4.37±0.79[a]	6.69±0.61[b]	6.67±0.82[b]	7.41±0.30[c]	5.77±0.48[a]	5.14±0.82[a]
	Lot 3[x]	4.29±0.38[a]	4.09±0.60[a]	4.39±0.79[a]	6.88±0.86[b]	7.30±0.84[b]	8.42±0.78[c]	6.93±0.90[b]	4.47±0.47[a]
Yeasts	Lot 1[x]	3.36±0.22[a]	3.27±0.21[a]	6.08±0.81[b]	7.03±0.57[c]	7.52±0.23[c]	6.03±0.57[b]	4.25±0.62[a]	4.73±0.46[a]
	Lot 2[x]	3.78±0.17[a]	3.70±0.59[a]	6.60±0.75[b]	8.03±0.62[c]	7.52±0.54[c]	6.77±0.38[bc]	4.12±0.66[a]	5.83±0.70[b]
	Lot 3[x]	3.24±0.31[a]	3.41±0.27[a]	6.90±0.56[b]	7.50±0.88[b]	7.29±0.46[b]	6.44±0.40[b]	4.87±0.79[c]	4.75±0.33[c]
Moulds	Lot 1[x]	< 1.00[a]	2.00±1.20[a]	2.15±1.27[a]	1.78±1.11[a]	3.86±0.84[b]	2.38±1.38[ab]	2.60±1.48[ab]	1.75±0.83[a]
	Lot 2[x]	< 1.00[a]	1.78±0.21[a]	< 1.00[a]	2.42±0.38[b]	3.47±0.39[c]	2.53±0.43[b]	1.86±0.33[ab]	3.42±0.97[c]
	Lot 3[x]	< 1.00[a]	1.78±1.10[a]	1.30±0.89[a]	2.48±1.15[b]	4.27±0.94[c]	3.42±1.47[c]	0.78±0.66[a]	3.38±1.02[c]
Enterobacteriaceae	Lot 1[x]	2.61±0.66[a]	1.58±0.24[b]	< 1.00[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]
	Lot 2[x]	2.54±0.50[a]	1.87±0.48[b]	0.30±0.45[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]	< 1.00[c]
	Lot 3[x]	2.25±0.36[a]	1.87±0.45[b]	0.60±0.55[c]	< 1.00[d]	< 1.00[d]	< 1.00[d]	< 1.00[d]	< 1.00[d]

[1] mean of n=5

[x-y] Lots with different superscript in the same column differ significantly ($p < 0.05$).

[a-d] Means with different superscript in the same row differ significantly ($p < 0.05$).

Table 2. Evolution of physicochemical parameters[1] in inoculated dry-cured ham at different stages.

Stage		Preparation-salting	Cleaning-brushing	Post-salting A	Post-salting B	Ripening A	Ripening B	Ripening C	Aging
Days		0	9	48	74	112	142	166	211
pH	Lot 1[x]	5.552±0.206[a]	5.378±0.073[a]	5.624±0.085[ab]	5.698±0.077[bc]	5.826±0.064[c]	5.992±0.078[d]	5.824±0.164[c]	5.888±0.061[cd]
	Lot 2[x]	5.514±0.159[a]	5.442±0.113[a]	5.580±0.032[a]	5.644±0.038[ab]	5.814±0.129[bc]	5.528±0.336[a]	5.672±0.332[bc]	5.94±0.377[c]
	Lot 3[y]	5.692±0.268[a]	5.584±0.072[a]	5.618±0.093[a]	5.752±0.025[ab]	5.820±0.131[b]	5.892±0.154[bc]	6.006±0.168[c]	6.028±0.031[c]
Moisture	Lot 1[x]	66.487±3.450[a]	37.305±3.937[b]	30.199±6.045[bc]	27.472±2.952[c]	27.529±6.678[c]	30.478±4.092[bc]	22.113±1.215[d]	22.851±3.106[d]
(%)	Lot 2[x]	67.155±4.457[a]	48.109±4.528[b]	33.153±3.782[cd]	33.983±3.214[d]	32.130±4.165[cd]	29.851±7.565[cd]	17.918±5.332[e]	27.715±3.868[c]
	Lot 3[x]	67.172±3.927[a]	54.699±1.460[b]	34.427±2.284[c]	24.368±4.012[d]	30.501±5.943[cd]	20.223±3.861[de]	17.167±5.279[e]	20.372±1.655[de]
Chloride	Lot 1[x]	0.291±0.159[a]	11.526±0.058[b]	3.534±0.600[c]	3.094±0.139[c]	1.679±0.507[d]	2.215±0.415[ef]	1.861±0.507[de]	2.504±0.368[f]
(%)	Lot 2[x]	0.198±0.172[a]	11.572±0.026[b]	3.569±0.127[c]	2.920±0.66[d]	1.874±0.234[e]	2.020±0.432[e]	1.382±0.403[f]	3.009±0.271[d]
	Lot 3[x]	0.105±0.026[a]	10.909±0.747[b]	3.974±0.714[c]	3.302±0.209[d]	2.081±0.428[e]	1.996±0.364[e]	2.029±0.433[e]	2.447±0.268[e]
NPN	Lot 1[x]	0.323±0.083[ab]	0.163±0.054[a]	0.075±0.027[a]	0.401±0.275[b]	0.407±0.210[b]	0.582±0.101[b]	1.044±0.421[c]	1.326±0.347[c]
(%)	Lot 2[y]	0.288±0.098[a]	0.099±0.029[a]	0.193±0.052[a]	0.462±0.056[ab]	0.484±0.158[ab]	0.686±0.090[b]	1.335±0.339[c]	1.844±0.287[d]
	Lot 3[x]	0.156±0.053[a]	0.123±0.067[a]	0.160±0.038[a]	0.245±0.048[ab]	0.332±0.216[b]	0.784±0.200[c]	0.987±0.121[d]	1.495±0.221[e]
TBARS	Lot 1[x]	0.00141±0.00007[a]	0.00007±0.00001[a]	0.00039±0.00005[a]	0.00073±0.00006[a]	0.00083±0.00016[a]	0.30694±0.01685[b]	0.28094±0.01409[c]	0.27009±0.01302[c]
(%)	Lot 2[y]	0.00026±0.00015[a]	0.00008±0.00002[a]	0.00037±0.00003[a]	0.00069±0.00006[a]	0.00079±0.00016[a]	0.20242±0.01745[b]	0.29083±0.03007[c]	0.25743±0.03766[d]
	Lot 3[x]	0.00024±0.00009[a]	0.00005±0.00001[a]	0.00045±0.00007[a]	0.00062±0.00012[a]	0.00081±0.00012[a]	0.30445±0.3985[b]	0.33038±0.01757[c]	0.27924±0.01604[d]
Ash	Lot 1[x]	2.642±0.396[a]	13.684±2.144[b]	6.492±0.553[c]	6.061±0.603[c]	4.231±0.845[d]	5.336±0.375[c]	4.676±0.814[d]	4.354±0.262[d]
(%)	Lot 2[x]	2.984±0.235[a]	19.038±2.217[b]	6.555±0.200[c]	4.179±0.255[d]	5.013±0.460[d]	5.275±0.561[cd]	4.151±0.483[d]	5.688±0.948[c]
	Lot 3[x]	2.764±0.336[a]	20.182±3.170[b]	9.205±0.386[c]	5.641±0.475[d]	4.814±0.869[d]	4.870±0.662[d]	4.228±0.849[d]	4.552±0.481[d]

[1] mean of n=5

[x-y] Lots with different superscript in the same column differ significantly ($p < 0.05$).

[a-f] Means with different superscript in the same row differ significantly ($p < 0.05$).