D. hansenii may be decreasing the amount of some free amino acids while increasing other different ones, as it has been reported for a different strain of this yeast incubated with sarcoplasmic proteins [49]. Thus, the final balance observed on pork loins for *D. hansenii* seemed to be negligible [42].

Dry-cured hams were also inoculated with the selected strains of *P. chrysogenum* and *D. hansenii*. After 6 months of ripening, hydrolysis of some proteins, including H-meromyosin, was higher in inoculated samples than in controls with the naturally occurring population (Fig. 3). No difference in protein hydrolysis was observed after 12 months of ripening, which was attributed to the proteolytic effect of the usual microflora in control samples. However, higher levels of free Asp, Glu, and Ser were observed in hams inoculated with *P. chrysogenum* and *D. hansenii*. Thus, the micro-organisms present on the surface of dry-cured ham may exert a decisive influence on proteolysis during ripening. When the normal microbial population includes proteolytic organisms, inoculation of selected organisms may assure an early set up of this activity.

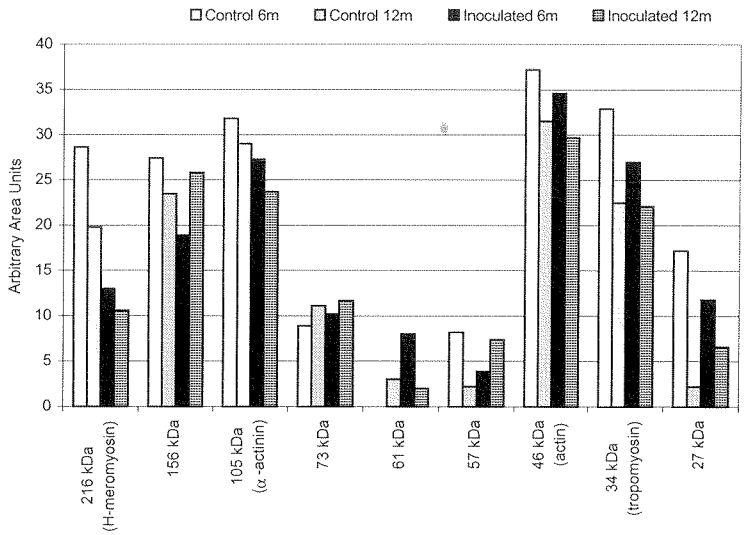

Figure 3. Myofibrillar proteins in control and inoculated (*P. chrysogenum* Pg222 and *D. hansenii* Dh345) dry-cured hams at 6 months and at the end of ripening (12 months). Adapted from ref. 79.

However, an excess of proteolysis may result in a high concentration of peptides and free amino acids, sometimes so excessive that they may impair the typical flavor of dry-cured ham by exaggerating the bitter and metallic taste [50]. In addition, an excess of proteolysis results in a poor firmness associated with low ratings by panelists and consumers [51]. This is unlike to happen in dry-cured ham for several reasons. First, the microorganisms selected for their high proteolytic effect are commonly present on normal products, and there is no texture defect attributed to these micro-organisms. Also, these questions arose for ground products, where tissue structure offers very little contribution to texture. No negative effect was observed when selected proteolytic

micro-organisms were assayed in dry-cured ham [52]. In addition, the use of these organisms just at a surface level restricts drawbacks to the outer layer of the exposed muscle surface. A different comment would deserve the use of micro-organisms active against proteins not hydrolyzed during ripening or manipulated to increase their activity on proteins.

On the other hand, proteolysis could be promoted using purified bacterial [53] and fungal proteases [54] of strains isolated from dry-cured ham. The protease EPg222 from *P. chrysogenum* Pg222 has shown a high hydrolytic activity against myofibrillar proteins [54]. This protease was active from 10 to 30°C, from 0 to 3 M NaCl, and in a pH range from 5 to 7. This enzyme can be of great interest to increase proteolysis on dry-cured ham, since most of the ripening process takes place within that range of ecological conditions [4], remaining active during the processing of dry-cured ham.

Volatile compounds

The proteolytic effect of fungi may influence the sensorial characteristics of the ripened product by their direct effect of amino acids on flavor. However, the contribution to sensorial characteristics includes formation of volatile compounds responsible for the distinct flavor of dry-cured ham. There is a number of different components and chemical reactions responsible for volatile compounds in meat products that have been reviewed in previous chapters. Most of such agents must play an identical role in dry-cured ham, but very little work has been published on the specific contribution of fungi to this product. Growth of *Penicillium camembertii* and *P. aurantiogriseum* in a culture medium with meat components has shown a positive impact on flavor [55]. Perhaps there is just one aspect that deserves being considered singularly here, i.e. the role of fungi in the special conditions of surface/volume ratio and for a product with several months of ripening time.

From the different groups of volatile compounds found in dry-cured meat products, some like linear aldehydes are generally recognized as products of lipid auto-oxidation. A few groups, like branched carboxylic acids and esters, are associated only to microbial metabolism. However, other compounds can derive from both autolytic reactions and microbial metabolism. Some of them come typically from lipids (aliphatic alcohols and linear carboxylic acids) and others from amino acids (branched aldehydes and pyrazines). One pending question on dry-cured hams is to know the contribution of molds to the latter group of compounds, even though the contribution of *P. aurantiogriseum* to some of these compounds in fermented sausages has been reported [56].

Given that dry-cured ham is an extremely complex, slow, and expensive system to work with, sterile pork loins ripened under aseptic condition have been also used to study the effect of *P. chrysogenum* and *D. hansenii* on volatile compounds [57]. This system showed that some hydrocarbons increased with incubation time, but only minor (in terms of detector response) compounds showed significant differences [57]. Linear aldehydes were lower in inoculated samples, particularly with *P. chrysogenum* (Fig. 4). Given that n-aldehydes are attributed to lipid oxidation [58], the microbial catalase activity may explain the lower values in inoculated samples [57].

Ketones and aliphatic alcohols showing significant differences were also obtained at lower mean values with *P. chrysogenum* (Fig. 4). On the other hand, branched aldehydes

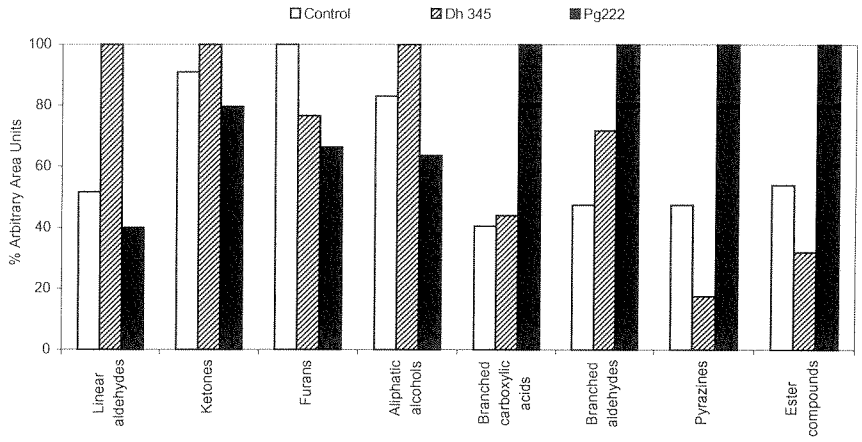

Figure 4. Selected volatile compounds of pork loins inoculated with *P. chrysogenum* Pg222 and *D. hansenii* Dh345 ripened for 106 days in sterile conditions. Adapted from ref. 57. Data are referred to the highest value within each group of volatile compounds.

(*i.e.* 2-methyl propanal, 2-, and 3-methyl butanal) and branched carboxylic acids (*i.e.* 2-methyl propionic, 2-, and 3-methyl butanoic acids) have been found at higher levels in loins inoculated with *P. chrysogenum* (Fig. 4). These compounds may be formed from valine, leucine, and isoleucine by non-enzymatic Strecker degradation [59,60,61]. Therefore, the proteolytic activity reported for this microorganism yielding free amino acids may be essential for the generation of these aldehydes via Strecker degradation. Nonetheless, the concentration of valine, leucine, and isoleucine did not correlate to the branched aldehydes [57]. For this, *P. chrysogenum* may play an additional role on generation of the above branched compounds, perhaps through deamination and decarboxylation, as it has been proposed for different micro-organisms [62,63].

Similarly, pyrazines showed higher amounts in pork inoculated with *P. chrysogenum* (Fig. 4), and they can be formed from amino acids through either Maillard reactions [61] or microbial metabolism [64,65,66]. The main contribution of *P. chrysogenum* to pyrazine synthesis can be due to the increase in free amino acids [42], but direct synthesis should be also considered.

The possible contribution of *D. hansenii* is not that clear. It does not seem to exert a decisive influence on any of these compounds, at least more than *P. chrysogenum*, except for decane, cyclohexanol and cyclohexanone [57].

Given the positive correlation of flavor in dry-cured meat products with pyrazines [36] and branched aldehydes [62,67,68,69,70], the selection of fungal starter cultures for dry-cured ham is of great interest.

However, a preliminary work to test the effect of *P. chrysogenum* and *D. hansenii* on the volatile compounds of dry-cured ham did not fully confirm the expected results. Lower concentrations of linear aldehydes, linear alcohols and ketones were found at 6 months of ripening time in inoculated hams (unpublished data). This confirmed the role of tested micro-organisms in the restriction of lipid auto-oxidation. However, none of the volatile compounds derived from amino acids showed higher values in inoculated than in

control hams [52]. This was explained by a higher activity of the microbial population naturally occurring in control samples.

Selection of fungi for starter cultures

In addition to toxicological evaluation, several aspects should be considered in the selection of molds and yeasts as starter cultures for dry-cured ham. First, appropriate substrates to screen candidate strains must be chosen. Caseinate agar is a common medium to test microbial cultures for proteolytic activity [71]. Given that most proteolytic enzymes are substrate specific [72], screening for proteolytic activity for meat products should be carried out with meat proteins. In addition, the proteins suffering the main changes during ripening should be considered. Sarcoplasmic proteins do not bear drastic changes during dry-cured ham processing [4]. Myofibrillar proteins undertake major changes during dry-cured ham processing [4]. For this, the latter are a better substrate to select micro-organisms on the basis of proteolytic activity. Myofibrillar proteins have been used for this purpose, as it has been discussed under the header of "proteolytic activity" in this chapter. Myosin has been added to culture medium to screen for microbial proteolysis [39]. This test offers the advantage of using a single protein. For a more complete screening of the proteins affected, myofibrillar proteins [54] or myofibrils [73] have also been incorporated to culture media.

A similar approach can be followed to evaluate lipolytic activity with trioleine or pork fat as appropriate substrates for molds and yeasts [32]. Screening tests must consider not only specific meat substrates, but also the environment offered by dry-cured ham. In contrast to other foods, no heat treatment can be applied to reduce unwanted organisms to acceptable levels. The long ripening time and the ineffective control of microbial contamination makes that only organisms capable to thrive in the ham succeed to colonize the product. Screening tests should be carried out trying to simulate the conditions of temperature, a_w and NaCl concentration found on hams at the aimed stages of the ripening process. To test molds and yeasts for proteolytic activity, the conditions found at drying and cellar stages (20°C, 5% NaCl), have been used [39].

Selected fungi aimed for dry-cured ham should be further tested in meat pieces of low surface/volume ratio to know the effect on surface and deep tissues.

The proteolytic activity of fungi on meat tissue has been evaluated by SDS-PAGE and HPLC [39,74,75]. However, these methods are expensive and time-consuming. Capillary gel electrophoresis (CGE) allows detecting changes in both sarcoplasmic and myofibrillar proteins in 1-2 h analysis, taking just a small sample [41]. In addition, capillary zone electrophoresis (CZE) can be used to detect bulk changes in sarcoplasmic proteins, free amino acids and peptides derived from proteolysis [41]. These methods allow a rapid and non-destructive evaluation of fungal proteolytic activity.

Finally, the use of starter cultures requires establishing the appropriate time for inoculation, to ensure a proper development over the natural fungal population. To know the environmental conditions that favor the selected strains it is necessary to know their relative growth to other common competitors. For this, the fungal strains can be cultured individually or mixed within different combinations of a_w and temperatures. This model was followed to compare growth rates of non toxigenic *P. chrysogenum* and toxigenic isolates of *P. commune*, *Eurotium repens* and *E. herbariorum* using a meat extract based broth with 5% NaCl at different temperatures and a_w values [76]. The results showed

that *P. chrysogenum* grew faster than the toxigenic strains at 25°C and 0.85 a_w. Given that these conditions are found at the surface of the hams at the beginning of drying stage, the time suggested to inoculate the selected strains was the end of post-salting [76].

Future work

There is enough evidence proving the ability of fungi to contribute to the quality of dry-cured ham. Nonetheless, further work is still needed. Recent works offer a deep insight on microbial production of volatile compounds in meat products [77,78], but the specific substrates and metabolic pathways followed by fungi in dry-cured ham are not known. Studies with individual meat compounds to elucidate the pathways leading to a lower lipid oxidation and increased amino acid catabolism are required. Also a very limited knowledge of the fungal ecology of dry-cured ham is available. There is much work needed to know the effect of the environmental conditions and microbial interactions for modeling fungal growth in dry-cured meat products. In addition, the production of antifungal peptides by molds offers a new perspective for this field. The use of purified proteases for accelerating dry-cured ham ripening requires a suitable way to distribute enzymes to deep tissues. Construction of enzyme-overproducing yeasts would solve most of the queries associated to objectionable molds. For this, the gene encoding the mold protease has to be sequenced and a suitable vector for appropriated yeast should be developed. Finally, a better understanding of the microbial metabolism of amino acids and the interactions of different micro-organisms is essential to design genetically modified strains with a higher flavor potential.

References

1. Lücke, F.L. (1986). Fleischwirtsch. 66: 1505-1509.
2. Núñez, F., Rodríguez, M.M., Córdoba, J.J., Bermúdez, M.E., and Asensio, M.A. 1996a, Int. J. Food Microbiol., 29, 271.
3. Núñez, F., Rodríguez, M.M., Bermúdez, M.E., Córdoba, J.J., and Asensio, M.A. 1996b, Int. J. Food Microbiol., 32, 185.
4. Córdoba, J.J., Antequera, T., Ventanas, J., López, C., García, C., Asensio, M.A. 1994, Meat Sci., 37, 217.
5. Córdoba, J.J., Antequera, T., García, C., Ventanas, J., López, C., Asensio, M.A. 1994, J. Agric. Food Chem., 42, 2296.
6. Kemp, J.D., Langlois, B.E., and Johnson, A.E. 1982, J. Food Prot., 45, 244.
7. Langlois, B.E., Kemp, J.D., and Johnson, A.E. 1982, J. Food Prot., 45, 271.
8. Silla, H., Molina, I., Flores, J., and Silvestre, D. 1989, Fleischwirtsch., 69, 1128.
9. Carrascosa, A.V., Cornejo, I., and Marín, M.E. 1992, Fleischwirtsch., 72, 1008.
10. Monte, E., Villanueva, J.R., and Domínguez, A. 1986, Int. J. Food Microbiol., 3, 355.
11. Huerta, T., Querol, A., and Hernández-Haba, J. 1988, Microbiologie-Aliments-Nutrition, 6, 227.
12. Molina, I., Silla, H., Flores, J., and Monzo, J.L. 1990, Fleischwirtsch. Int., 2, 47.
13. Saldanha da Gama, A., Malfeito, M., and Loureiro, V.1997, Int. J. Food Microbiol., 37, 201.
14. Comi, G., and Cantoni, C. 1983, Ind. Alimentari, 22, 102.
15. Deak, T. 1991, Adv. Appl. Microbiol., 36, 179.
16. Fleet, G. 1992, Crit. Rev. Biotechnol., 12, 1.
17. Leistner, L., and Ayres, J.C. 1968, Fleischwirtsch., 1, 62.
18. Strzelecki, E., Lillard, H.S., and Ayres, J.C. 1969, Appl. Microbiol., 18, 938

19. Sutic, M., Ayres, J.C., and Koehler, P.E. 1972, Appl. Microbiol., 23, 656.
20. Wu, M.T., Ayres, J.C., and Koehler, P.E. 1974, Appl. Microbiol., 27, 427.
21. Wu, M.T., Ayres, J.C., and Koehler, P.E. 1974, Appl. Microbiol., 28, 1094.
22. Dragoni, I., Marino, C., and Cantoni, C. 1980, Ind. Alimentari, 19, 405.
23. Spotti, E., Mutti, P., and Campanini, M. 1989, Ind. Conserve, 64, 110.
24. Peintner, U., Geiger, J., and Poeder, R. 2000, J. Food Prot., 63, 1399.
25. Huerta, T., Sanchis, V., Hernández, J., and Hernández, E. 1987, Microbiologie-Aliments-Nutrition, 5, 247.
26. Rojas, F.J., Jodral, M., Gosálvez, F., and Pozo, R. 1991, Int. J. Food Microbiol., 13, 249.
27. Núñez, F., Díaz, M.C., Rodríguez, M., Aranda, E., Martín, A., and Asensio, M.A. 2000, J. Food Prot. 63, 231
28. Sosa, M.J., Córdoba, J.J., Díaz C., Rodríguez, M., Bermúdez, E., Asensio, M.A., and Núñez, F. 2002, J. Food Prot., In press.
29. Halls, N.A., and Ayres, J.C. 1973, Appl. Microbiol. 26, 636.
30. Escher, F.E., Koehler, P.E., and Ayres, J.C. 1973, Appl. Microbiol. 26, 27.
31. Motilva, M.J., Toldra, F., Nieto, P., and Flores, J.,1993, Food Chem., 48, 121.
32. Sørensen, B.B.1997, Int. J. Food Microbiol., 34, 187.
33. Selgas, M.D., Casas, C., Toledo, V.M., García, M.L. 1999, Eur. Food Res. Technol., 209, 360.
34. Molina, I., Nieto, P., Flores, J., Silla, H., and Bermell, S. 1991, Fleischwirtsch. Int., 71, 906.
35. Toldrá, F., Aristoy, M.C., Part, C., Cerveró, C., Rico, E., Motilva, M.J., and Flores, J. 1992, J. Food Sci., 816.
36. Flores, M., Aristoy, M.C., and Toldrá, F. 1997, Zeitschrift für Lebensmittel Untersuchung und Forschung A, 205, 343.
37. Flores, M., Aristoy, M.C., and Toldrá, F. 1998, J. Agric. Food Chem., 46, 4982.
38. Rodríguez, M.M., Núñez, F., Córdoba, J.J., Sanabria, C., Bermúdez, M.E., and Asensio, M.A. 1994, Int. J. Food Microbiol., 24, 329.
39. Rodríguez, M.M., Núñez, F., Córdoba, J.J., Bermúdez, M.E., and Asensio, M.A. 1998, J. Appl. Microbiol., 85, 905.
40. García-Ruiz, A., Núñez, M.D., Cabezudo, M.D., Sciences des Aliments, 21, 161.
41. Martín, A., Córdoba, J.J., Rodríguez, M.M., Núñez, F., and Asensio, M.A. 2001, J. Appl. Microbiol., 90, 163.
42. Martín, A., Asensio, M.A., Bermúdez, M.E., Córdoba, M.G., Aranda, E., and Córdoba, J.J. 2002, Meat Sci. In press.
43. Sentandreu, M.A., and Toldrá, F. 2000, J. Agric. Food Chem., 48, 5014.
44. Díaz, O., Fernández, M., García de Fernando, G. D., de la Hoz, L., and Ordóñez, J. A. 1992, Proc. 38th ICoMST, Clermont-Ferrand (France) p. 779
45. Díaz, O., Fernández, M., García de Fernando, G. D., de la Hoz, L., and Ordóñez, J. A. 1997, Meat Sci., 46, 115.
46. Ansorena, D., Zapelena, M. J., Astiasarán, I., and Bello, J. 1998, Meat Sci., 10, 37.
47. Zapelena, M. J., Astiasarán, I., and Bello, J. 1999, Meat Sci., 52, 403.
48. Toldrá, F., Sanz, Y. and Flores, M. 2001, Meat Science and Applications. Y.H. Hui, W.K. Nip, R.W. Rogers and O.A. Young (Ed.) Marcel Dekker, New York, 538.
49. Santos, N.N., Santos-Mendoça, R.C., Sanz, Y., Bolumar, T., Aristoy, M.C., and Toldrá, F. 2001, Int. J. Food Microbiol., 68, 199.
50. Virgili, R., Parolari, G., Scivazzappa, C., Bordini, C., and Volta, R. 1995, Ind. Conserve, 70, 21.
51. Parolari, G., G., Virgili, R., and Scivazzappa, C. 1994, Meat Sci., 38, 117.
52. Martín, A., Ruiz, J., Núñez, F., Córdoba, J.J., and Asensio, M.A. 2000, Eurocarne, Suplemento Jamón Curado, 55.
53. Sanz, Y., and Toldrá, F. 2001, Appl. Environ. Microbiol., 67, 1815.

54. Benito, M.J., Rodríguez, M., Núñez, F., Asensio, M.A., Bermúdez, E., and Córdoba, J.J. 2002, Appl. Environm. Microbiol. Submitted.
55. Ockerman, H.W., Céspedes, F.J., and León-Crespo, F. 2000, J. Muscle Food, 11, 247.
56. Bruna, J.M., Hierro, E.M., de la Hoz, L., Mottram, D.S., Fernández, M., and Ordóñez, J.A. 2001, Meat Sci., 97.
57. Martín, A., Córdoba, J.J., Benito, M.J., Aranda, E., and Asensio, M.A. 2002, Int. J. Food Microbiol. Submitted.
58. Belizt, H.-D., and Grosch, W. 1999, Food chemistry. Springer Verlag. Berlin.
59. García, C., Berdagué, J.J., Antequera, T., López-Bote, C., Córdoba, J.J., and Ventanas, J. 1991, Food Chem., 41, 23.
60. Barbieri, G., Bolzoni, L., Parolari, G., Virgili, R., Buttini, R., Careri, M., Mangia, A. 1992, J. Agric. Food Chem., 40, 2389.
61. Ventanas, J., Córdoba, J.J., Antequera, T., García, C., López-Bote, C., Asensio M.A. 1992, J. Food Sci. 57, 813.
62. Hinrichsen, L.L., and Pedersen, S.B. 1995, J. Agric. Food Chem., 43, 2932.
63. Jacobsen, T., and Hinrichsen, L. 1997, Food Chem. 60, 409.
64. Janssens, L., De Pooter, H.L., Schamp, N.M., and Vandamme, E.J. 1992, Process Biochemistry 27, 125.
65. Tressl, R., Helak, E., Kersten, E., and Nittka, C. 1993, Recent developments in flavor and fragance chemistry. VCH Publishers, New York. pp. 167-181.
66. Magan, N., and Evans, P. 2000, Journal of Stored Products Research 36, 319.
67. Berdagué, J. L., Montel, P., Montel, M.C., and Talon, R. 1993, Meat Sci., 35, 275.
68. Stahnke, L.H. 1995, Meat Sci., 41, 193.
69. Montel, M.C., Reitz J., Talon, R., Berdagué, J. L., and Rousset-Akrim, S. 1996, Food Microbiol., 13, 489.
70. Ruiz, J., Ventanas, J., Cava, R., Andrés, A., and García, C. 1999, Meat Sci. 52, 19.
71. Atlas, R.M. 1993, Handbook ofmicrobiological media, L.C. Parks (Ed.), CRC Press, Boca Raton, 180.
72. Sikes, A., and Maxcy, R.B. 1979, J. Food Sci., 44, 1228.
73. Fransen, N.G., O'Connell, M.B., and Arendt, E.K. 1997, Int. J. Food Microbiol., 36, 235.
74. Molina, I., and Toldrá, F. 1992, J. Food Sci., 57, 1308.
75. LeBlanc, E.L., Singh, S., and LeBlanc, R.J. 1994, J. Food Sci., 59, 1267.
76. Martín, A., Córdoba, J.J., Rodríguez, M.M., Aranda, E., Díaz, M.C., and Asensio, M.A. 1998, 11th Spanish National Congress of Food Microbiology. Pamplona, Spain, 185.
77. Demeyer, D., Raemaekers, M., Rizzo, A., Holck, A., De Smedt, A., ten Brink, B., Hagen, B., Montel, C., Zanardi, E., Murbrekk, E., Leroy, F., Vandendriessche, F., Lorentsen, K., Venema, K., Sunesen, L., Stahnke, L., De Vuyst, L., Talon, R., Chizzolini, R., and Eerola, S. 2000, Foos Res. Int., 33, 171.
78. Ordóñez, J. A., Hierro, E.M., Bruna, J.M., and de la Hoz. L. 1999, Crit. Rev. Food Sci. Nutr. 39, 329.
79. Martín, A. (2000). Contribution of fungal population to the development of desirable sensorial characteristics in dry-cured ham. (in Spanish). Doctoral Thesis. University of Extremadura.