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International Journal of Food Microbiology 84 (2003) 327–338

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

# Effect of *Penicillium chryso genum* and *Debaryomyces hansenii* on the volatile compounds during controlled ripening of pork loins

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Received 3 December 2001; received in revised form 9 August 2002; accepted 5 October 2002

**Abstract**

During ripening of meat products such as dry-cured ham, the moulds and yeasts that proliferate on the surface may contribute to flavour development. However, their contribution to volatile components of dry-cured meat products is not known. One strain each of *Penicillium chrysogenum* and *Debaryomyces hansenii*, selected from dry-cured ham by their proteolytic activity, were tested to determine their effect on the volatile compounds during ripening. Sterile pork loins were inoculated and ripened for 106 days. Volatile compounds collected with a Solid Phase Micro-Extraction (SPME) fibre were analysed by GC/MS. Inoculation of pork loins with *P. chrysogenum* lead to a decrease in compounds attributed to lipid oxidation and to an increase of compounds derived from free amino acids. Inoculation with *D. hansenii* seemed to favour the formation of complex alcohols.
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*Keywords:* *Penicillium chrysogenum*; *Debaryomyces hansenii*; Volatile compounds; Dry-cured ham

## 1. Introduction

The role of microorganisms in the generation of volatile compounds is well documented for semi-dry and dry-fermented sausages (Berdagué et al., 1993; Stahnke, 1994, 1995; Montel et al., 1996; Zapelena et al., 1999; Bruna et al., 2001). In these products, ethyl esters, methyl aldehydes, methyl ketones, and other volatile compounds have been attributed to staphylococci (Stahnke, 1995; Montel et al., 1996). However, little information is available on the contribution of microorganisms to the formation of volatile compounds in dry-cured ham. It has been suggested that microorganisms, particularly micrococci, may improve Parma ham flavour (Hinrichsen and Pedersen, 1995), but their counts appear to be too low for most of the ripening time to play a significant role on flavour formation.

Fungi show high surface counts on dry-cured ham of long ripening time with *Penicillium*, *Eurotium*, and *Debaryomyces* being the genera most widely present (Monte et al., 1986; Huerta et al., 1987; Núñez et al.,

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0168-1605/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1605(02)00474-9

1996a,b). Several strains of *Penicillium chrysogenum* and *Debaryomyces hansenii* isolated from dry-cured ham have proved to be proteolytic against myofibrillar proteins, yielding peptides and free amino acids (Rodríguez et al., 1998; Martín et al., 2001, 2002). Production of methyl ketones, secondary alcohols, and other volatile compounds by fungi in different foods is well documented (Karahadian et al., 1985; Kinderlerer, 1989; Börjesson et al., 1990; González de Llano et al., 1990), including dry-fermented sausages (Bruna et al., 2001). Nonetheless, the contribution of these microorganisms to volatile compounds on non-fermented dry-cured meat products is not known. *Penicillium aurantiogriseum* and *Penicillium camemberti* have shown a positive impact on flavour in a culture medium added with meat components (Ockerman et al., 2000). However, the responsible compounds were not determined.

Given that dry-cured ham is very complex and is time demanding, system for studying the contribution of microorganisms to flavour, appropriate controlled systems must be used. Sterile pork loins ripened under aseptic conditions have been used (Martín et al., 2002) to study the proteolytic activity of the strains of *P. chrysogenum* and *D. hansenii* used here. That work has shown that *P. chrysogenum* increases proteolysis, yielding free amino acids, such as valine, leucine, isoleucine, lysine, and arginine. The latter may undergo secondary reactions leading to volatile compounds contributing to desirable flavour attributes of dry-cured ham (Ruiz et al., 1999).

The aim of this work has been to investigate the effect of *P. chrysogenum* and *D. hansenii* on the volatile compounds during controlled pork ripening, to know the role of such microorganisms on dry-cured meat products.

## 2. Materials and methods

### 2.1. Microbial isolates

Strains Pg222 of *P. chrysogenum* and Dh345 of *D. hansenii*, selected from Iberian dry-cured ham (Núñez et al., 1996a,b), were used. Both microorganisms were grown individually on malt extract agar (Oxoid, Unipath, Basingstoke, UK) for 5–7 days. To obtain the inoculum, 3 ml of sterile water were added to the grown culture media, the spores and colonies were gently rubbed, and the suspension taken for inoculation of the loins.

### 2.2. Controlled pork loin ripening

Four loins were removed from carcasses 15 min after slaughter. The external surface was sterilised by searing and the burnt tissues were removed down to a depth of about 5 mm (Martín et al., 2002). Every sterile loin was placed in a presterilised orthogonal receptacle made of methacrylate and salted at 5% w/w with sterile NaCl. Next, loins were inoculated on the surface with approximately $10^6$ cfu/cm$^2$ of either *P. chrysogenum*, *D. hansenii*, or both microorganisms, using the fourth as a control. Then, the orthogonal receptacles were placed in a ripening chamber CC-3-1 (Kowell, Navarra, Spain) and ripened under controlled conditions. This assay was carried out in triplicate. In the three assays, temperature increased from 5 to 20 °C, while relative humidity decreased from 80% to 60% during the first 47 days, then both were kept constant for 30 days, next were taken to 15 °C and 70% relative humidity in 5 days, and kept unchanged for 24 days (Martín et al., 2002). At 59 and 106 days of ripening time, triplicate samples of c.a. 300 g each from every batch were taken, down to a depth of about 5 mm. Microbial counts were tested on Malt extract agar (Oxoid) to check for proper growth.

### 2.3. Extraction of volatile compounds

Portions of loin samples were vacuum packaged and stored at −80 °C until analysis. Frozen samples were minced and 1 g was weighed into a 10-ml headspace vial (Hewlett-Packard, Palo Alto, CA, USA) and sealed with a PTFE butyl septum (Perkin-Elmer, Foster City, CA, USA) in an aluminium cap. Volatile compounds were extracted by Solid Phase Micro-Extraction technique (SPME) (Ruiz et al., 1998) with a 10 mm long, 100 μm thick fibre coated with poly-dimethylsiloxane (Supelco, Bellefonte, PA, USA). Prior to collection of volatiles, the fibre was preconditioned at 220 °C for 50 min at the GC injection port. The SPME fibre was inserted into the headspace vial through the septum and exposed to headspace for 30 min at 40 °C in a water bath.

### 2.4. Gas chromatography/mass spectrometry (GC/MS) analyses

GC/MS analyses were performed using a Hewlett-Packard 5890 S II gas chromatograph coupled with a Hewlett-Packard 5971A ion-trap mass spectrometer. A 5% phenyl-95% dimethyl polysiloxane column ($50 \times 0.32$ mm ID, 1.05 μm film thickness; Hewlett-Packard) was used for the separation of volatile compounds. The carrier gas was helium. The injection port was in a splitless mode. The SPME fibre was kept in the injection port at 220 °C during the whole chromatographic run. The temperature program was isothermal for 15 min at 35 °C, next increased to 150 °C at 4 °C min$^{-1}$, and then to 250 °C at 20 °C min$^{-1}$. To calculate the Kovats index of the compounds, *n*-alkanes (Sigma R-8769) were run under the same conditions. The GC/MS transfer line temperature was 280 °C. The mass spectrometer was operated in the electron impact mode, with an electron energy of 70 eV, a multiplier voltage of 1650 V and a rate of 1 scan s$^{-1}$ over a range of $m/z$ 40–300 for data collection. The NIST/EPA/NIH mass spectral library and Kovats indexes were used to identify the volatile compounds.

### 2.5. Statistical analysis

Statistical analysis of data was carried out by one-way analysis of variance, and means were separated by Newman–Keuls test using a StatGraphics software package from Statistical Graphics (Rockville, MD, USA). The statistical analysis was performed on results expressed in arbitrary units of peak area.

## 3. Results and discussion

Over 150 volatile compounds were identified in all four batches in the meat-based model system (Table 1), including aliphatic, branched, and aromatic hydrocarbons, aldehydes, furans, carboxylic acids, alcohols, aromatic alcohols, ketones, pyrroles, pyrazines, sulfur, ester and ether compounds. In some batches, several of the above-mentioned volatile compounds were detected at trace levels. Most of the identified compounds have been reported in meat products such as dry-cured ham (Berdagué et al., 1991; García et al., 1991; Barbieri et al., 1992; Buscailhon et al., 1993; Hinrichsen and Pedersen, 1995; Ruiz et al., 1998, 1999; Flores et al., 1998).

Some aliphatic, branched, and aromatic hydrocarbons increased with ripening time in all batches ($P_{time}$ 0.05–0.001), with no significant difference due to the microorganisms inoculated (Table 2). Decane, cyclohexane, and ethylbenzene were either detected only in inoculated samples, or showed some correlation to inoculation ($P_{inoc} < 0.05$). Other branched hydrocarbons found at low (Table 2) or trace amounts (Table 1) were detected mainly in samples inoculated with *D. hansenii*. However, the low amounts of the latter mean that only a minimal contribution to flavour can be expected. The negligible contribution of *P. chrysogenum* or *D. hansenii* ($P_{inoc} > 0.05$) supports that lipid oxidation plays a decisive role for hydrocarbons formation in dry-cured meat products (García et al., 1991).

The linear aldehydes heptanal, octanal, and nonanal showed a decreasing trend with ripening time ($P_{time} < 0.05–0.001$). In addition, heptanal and octanal were lower in inoculated samples ($P_{inoc}$ 0.01), particularly with *P. chrysogenum* (Table 3). Given that the above aldehydes are formed by oxidation of unsaturated fatty acids (Belizt and Grosch, 1999), the catalase activity of the inoculated microorganisms may contribute to decrease linear aldehydes by retarding autoxidation due to decomposition of hydrogen peroxide.

The branched aldehydes 2-methyl propanal, 2- and 3-methyl butanal increased by the second sampling time, mainly in samples inoculated with *P. chrysogenum* (Table 3). These compounds may be formed from amino acids, particularly valine, leucine, and isoleucine, by non-enzymatic Strecker degradation (García et al., 1991; Barbieri et al., 1992; Ventanas et al., 1992), but also through deamination and decarboxylation by different microorganisms (Hinrichsen and Pedersen, 1995; Jacobsen and Hinrichsen, 1997). Therefore, the great increase in free amino acids observed from 59 to 106 days of ripening time in these samples (Núñez et al., 1996b) may be essential for these branched aldehydes to raise. On the other hand, the concentration of valine, leucine, and isoleucine (Martín et al., 2002) does not directly relate to 2-methylpropanal, 2-, and 3-methylbutanal (Table 3). For this,

Table 1
Volatile compounds from dry-cured loins using Solid Phase Micro-Extraction technique (SPME) CG/MS

| Compound | Peak Number[a] | Reliability of identification[b] | Compound | Peak Number[a] | Reliability of identification[b] |
|---|---|---|---|---|---|
| *Aliphatic hydrocarbons* | | | *Aldehydes* | | |
| Pentane | 5 | A | 2-Methylpropanal | 12 | A |
| Hexane | 22 | A | Butanal[c] | 21 | A |
| Cyclohexane | 30 | A | 3-Methylbutanal | 29 | A |
| 1-Heptene[c] | 37 | A | 2-Methylbutanal | 31 | A |
| Heptane | 40 | A | Pentanal[c] | 39 | A |
| Octane | 63 | A | Hexanal | 64 | A |
| Decane | 104 | A | Heptanal | 87 | A |
| Dodecane[c] | 133 | A | 3-Methylthiopropanal[c] | 88 | A |
| Tridecane | 140 | A | 2-Heptanal (Z)[c] | 92 | A |
| 1-Tetradecene[c] | 149 | A | Benzaldehyde | 94 | A |
| Tetradecane | 150 | A | Octanal | 103 | A |
| 1-Pentadecene | 153 | A | Bencenacetaldehyde | 110 | A |
| Pentadecane | 154 | A | Nonanal | 123 | A |
| Hexadecane | 157 | B | Decanal[c] | 134 | A |
| Heptadecane | 160 | B | 2-Undecenal[c] | 146 | A |
| 1-Heptadecene | 161 | B | Hexadecanal | 163 | B |
| *Branched hydrocarbons* | | | *Carboxylic acids* | | |
| 2-Methylbutane | 3 | B | Acetic acid | 23 | A |
| Cyclopropyl 1-butine | 11 | A | Propionic acid | 38 | A |
| 2,3-Methylbutane | 13 | A | 2-Methyl propionic acid | 54 | A |
| 2-Methylpentane | 15 | A | Butanoic acid | 58 | A |
| 3-Methylpentane | 16 | A | 3-Methyl butanoic acid | 70 | A |
| 4-Methylpentane[c] | 21 | A | 2-Methyl butanoic acid | 71 | A |
| 2,3-Dimethyl 1,3-pentadiene | 42 | A | Hexanoic acid | 95 | A |
| 2-Ethyl 1-hexene[c] | 65 | A | 2-Ethyl hexanoic acid[c] | 121 | B |
| 5-Methyl decane[c] | 111 | A | Octanoic acid | 128 | A |
| 4-Methyl decane | 112 | B | Benzoic acid[c] | 129 | A |
| 4(1,1-Dimethylethyl) cyclohexene[c] | 148 | B | Benzeneacetic acid[c] | 136 | A |
| | | | Nonanoic acid | 137 | A |
| *Aromatic hydrocarbons* | | | Decanoic acid[c] | 145 | A |
| Benzene[c] | 32 | A | Tetradecanoic acid | 162 | B |
| Toluene | 57 | A | Hexadecanoic acid | 164 | B |
| Ethylbenzene | 74 | A | | | |
| 1,3-Dimethylbenzene | 78 | A | *Aliphatic alcohols* | | |
| 1,4-Dimethylbenzene | 79 | A | Ethanol | 2 | B |
| 1,2-Dimethylbenzene[c] | 85 | A | 1-Propanol | 6 | A |
| Decahydro cis naphtalene[c] | 124 | B | 2-Methyl 1-propanol | 27 | A |
| Decahydro 2methyl naphthalene[c] | 126 | B | 1-Penten-3-ol | 34 | A |
| Naphthalene | 135 | B | 3-Methyl 3-buten-1-ol | 46 | B |
| Nonylbenzene | 156 | B | 3-Methyl 1-butanol | 47 | A |
| | | | 2-Methyl 1-butanol | 48 | A |
| | | | Pentanol | 55 | A |
| | | | Butane-2,3-diol | 59 | A |
| | | | 2-Hexanol[c] | 62 | A |
| | | | 1-Hexanol | 75 | A |

[a] Peak number in chromatogram of dry-cured loins.

[b] The reliability of the identification or structural proposal is indicated by the following symbols: A: mass spectrum and retention time identical to those of an authentic sample. B: mass spectrum consistent with spectra found in NIST, EPA, NDH library. C: tentative identification by mass spectrum.

[c] The components occured in small or trace amounts.

| Compound | Peak Number[a] | Reliability of identification[b] |
|---|---|---|
| Cyclohexanol | 81 | A |
| 1-Octen-3-ol | 96 | A |
| 2-Ethyl hexanol | 107 | A |
| Octanol | 116 | A |
| Nonanol[c] | 130 | A |
| *Aromatic alcohols* | | |
| Phenol[c] | 99 | A |
| 2-Methyl phenol | 117 | A |
| Ethanol benzene | 125 | A |
| *Ketones* | | |
| 2-Propanone | 4 | A |
| 2,3-Butadione (diacetyl) | 18 | A |
| 2-Butanone | 24 | A |
| 1-Hydroxy-2-propanone[c] | 33 | A |
| 2-Pentanone | 35 | A |
| 2-Butanol-3-one (acetoin) | 44 | A |
| 4-Methyl-2-pentanone[c] | 49 | B |
| 3-Methyl-2-pentanone | 52 | A |
| 3-Hexanone[c] | 60 | A |
| 2-Hexanone | 61 | A |
| 4-Heptanone | 77 | A |
| 2-Heptanone | 82 | A |
| Cyclohexanone | 84 | A |
| 2,3-Octen-2-dione[c] | 97 | A |
| 2-Octanone | 100 | A |
| 3-Octen-2-one[c] | 108 | A |
| 2-Nonanone | 119 | A |
| 2-Decanone[c] | 132 | A |
| Branched cyclohexanone[c] | 152 | C |
| *Furans* | | |
| Tetrahydro furane | 28 | A |
| 2-Furanmethanol[c] | 73 | B |
| Dihydro-2(3H)-furanone | 90 | C |
| 4,5,5-Trimethyl-2(5H)-furanone[c] | 91 | B |
| 2-pentylfuran | 101 | A |
| 5-Ethyldihydro-2(3H)-furanone | 113 | A |
| Dihydro-5-pentyl-2(3H)-furanone[c] | 147 | B |
| Hexyldihydro-2(3H)-furanone | 155 | C |
| *Pyrazines* | | |
| Methylpyrazine | 69 | A |
| 2,5-Dimethylpyrazine | 89 | A |
| Trimethylpyrazine | 105 | B |
| 2-Etenyl-4-methylpyrazine[c] | 106 | B |
| 2-Ethyl-3,5-dimethylpyrazine | 118 | B |
| Tetramethylpyrazine | 120 | B |
| 2-Alyl-3-methylpyrazine[c] | 122 | B |
| 2-Butyl-3,5-dimethylpyrazine[c] | 142 | B |
| *Sulfur compounds* | | |
| Methanethiol | 1 | B |
| Dimethyl sulfide | 7 | A |
| Thiourea[c] | 9 | A |
| Carbon disulfide | 10 | A |
| Dimethyl disulfide | 51 | A |
| Benzothiazol | 138 | A |
| Benzene thiobis | 159 | B |
| *Ester compounds* | | |
| Ethyl acetate | 25 | A |
| Ethyl 3-methylbutanoate | 72 | A |
| Ethyl methylpentanoate | 83 | A |
| Ethyl hexanoate | 102 | A |
| Dimethyl 1,2-benzenodicarboxilate | 114 | C |
| Diethyl benzenocarboxilate | 158 | B |
| 1,2,3-Propanotriil hexanoate[c] | 185 | C |
| *Ether compounds* | | |
| Methoxy 2-methypropane[c] | 17 | B |
| 1-Methoxy 3-methylbutane | 36 | A |
| 1,1-Dimethoxy etane | 67 | C |
| Methoxy cyclohexane | 76 | B |
| *Other compounds* | | |
| Dichoromethane | 8 | B |
| Trichloromethane[c] | 41 | A |
| *n*-Ethylene ethenamine[c] | 45 | B |
| Pyridine | 53 | B |
| Tetrachloromethane[c] | 66 | A |
| Hexanenitrile[c] | 80 | B |
| 3-Ethyl pyridine[c] | 93 | A |
| Benzenamine | 99 | A |
| *n*-Isopentylidene isopentylamine[c] | 109 | C |
| 2-Methylbenzenenitrile[c] | 127 | C |
| 2-Piperidione | 131 | B |
| Benzamide[c] | 144 | B |

Table 2
Selected volatile hydrocarbons of dry-cured pork loins inoculated with tested microorganisms (Arbitrary Area Units)

| Compound | 59 days of ripening time | | | | 106 days of ripening time | | | | $P^{a}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control[b] | Dh345 | Dh345 + Pg222 | Pg222 | Control | Dh345 | Dh345 + Pg222 | Pg222 | inoc | time |
| *Aliphatic hydrocarbons* | | | | | | | | | | |
| Pentane | 48 | 155 | 113 | 72 | 367 | 305 | 309 | 140 | | +++[c] |
| Hexane | 38 | 15 | 262 | 145 | 115 | 122 | 57 | 129 | | |
| Heptane | 72 | 160 | 81 | 96 | 339 | 240 | 367 | 46 | | |
| Octane | 144 | 280 | 125 | 132 | 331 | 186 | 470 | 115 | | |
| Decane | n.d.$^{2}$[d,e] | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | $18^{1}$ | n.d.$^{2}$ | $7^{1,2}$ | | ++ |
| Tridecane | 4 | 3 | 1 | 2 | 7 | 10 | 8 | 7 | | +++ |
| Tetradecane | 4 | 1 | 1 | 1 | 5 | 11 | 9 | 6 | | +++ |
| Pentadecane | 9 | 9 | 10 | 3 | 17 | 30 | 31 | 24 | | ++ |
| Hexadecane | 119 | n.d. | n.d. | n.d. | 15 | 16 | 13 | 26 | | |
| Heptadecane | 90 | n.d. | 20 | 20 | 51 | 29 | 38 | 66 | | |
| Cyclohexane | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | $7^{2}$ | $4^{2}$ | $35^{1}$ | | + |
| 1-Pentadecene | 25 | 6 | 2 | n.d. | 15 | 16 | 22 | 14 | | |
| 1-Heptadecene | n.d. | n.d. | n.d. | n.d. | 8 | 17 | 24 | 9 | | ++ |
| *Branched hydrocarbons* | | | | | | | | | | |
| 2-Methylbutane | n.d. | n.d. | tr.[f] | n.d. | n.d. | n.d. | n.d. | 0.3 | | |
| Cyclopropyl 1-butine | n.d. | n.d. | 5 | 1 | 16 | n.d. | 26 | 1 | | + |
| 2,3-Dimethylbutane | n.d. | n.d. | n.d. | n.d. | 22 | 29 | n.d. | n.d. | | |
| 2-Methylpentane | 3 | 4 | 30 | 6 | 44 | 70 | 47 | 58 | | +++ |
| 3-Methylpentane | n.d. | n.d. | 0.1 | n.d. | n.d. | n.d. | n.d. | n.d. | | |
| 2,3-Dimethyl 1,3-pentadiene | $13^{1}$ | $6^{1,2}$ | $4^{1,2}$ | $4^{1,2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | | +++ |
| 4-Methyldecane | n.d. | n.d. | n.d. | n.d. | n.d. | 0.1 | n.d. | n.d. | | |
| *Aromatic hydrocarbons* | | | | | | | | | | |
| Toluene | 103 | 134 | 123 | 102 | 243 | 304 | 272 | 269 | | +++ |
| Ethylbenzene | $3^{2}$ | $5^{1,2}$ | $9^{1,2}$ | $5^{1,2}$ | $7^{1,2}$ | n.d.$^{2}$ | $8^{1,2}$ | $21^{1}$ | + | |
| 1,3-Dimethylbenzene | 24 | 22 | 16 | 6 | 38 | 44 | 27 | 29 | | ++ |
| 1,4-Dimethylbenzene | 3 | n.d. | n.d. | n.d. | 12 | 29 | 17 | 23 | | +++ |
| Naphtalene | 2 | 2 | 9 | 3 | 2 | 7 | 10 | 10 | | |
| Nonylbenzene | n.d. | n.d. | n.d. | n.d. | 15 | 17 | 24 | 12 | | +++ |

[a] $P_{inoc}$: $P$ value due to the microorganisms inoculated. $P_{time}$: $P$ value due to incubation time.
[b] Control: uninoculated sterile batch. Dh345: batch inoculated with *D. hansenii* Dh345. Dh345 + Pg222: batch inoculated with both *D. hansenii* Dh345 and *P. chrysogenum* Pg222. Pg222: batch inoculated with *P. chrysogenum* Pg222.
[c] $P$ values: + ($P < 0.05$); ++ ($P < 0.01$); +++ ($P < 0.001$).
[d] n.d.: not detected.
[e] Values with different numbers as superscript are significantly different ($P < 0.05$).
[f] tr.: trace amounts.

the microorganisms tested have to play an additional role on formation of branched aldehydes. Any direct contribution to these branched aldehydes would be of great interest for selection of starter cultures, since such compounds are associated with flavour of dry-cured meat products (Berdagué et al., 1993; Stahnke, 1995; Montel et al., 1996; Hinrichsen and Pedersen, 1995; Ruiz et al., 1999).

Butane-2,3-diol, 1-hexanol, octanol, and 1-octen-3-ol were lower in inoculated samples ($P_{inoc} < 0.05$–0.001), similar to linear aldehydes (Table 3). Other linear alcohols, such as 1-penten-3-ol and pentanol, showed an increasing trend with incubation time ($P_{time} < 0.05$), but poorly correlated to inoculation ($P_{inoc} > 0.05$). These compounds may be derived from aldehydes that originated from linolenic acid (Shahidi et al., 1986). In spite that some of the above compounds, such as 1-octen-3-ol, have been reported as metabolites of *Aspergillus* and *Penicillium* spp. (Janssens et al., 1992; Jacobsen and

Table 3
Selected volatile carbonyls, carboxylic acids and alcohols of dry-cured pork loins inoculated with tested microorganisms (Arbitrary Area Units)

| Compound | 59 days of ripening time | | | | 106 days of ripening time | | | | P[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control[b] | Dh345 | Dh345 + Pg222 | Pg222 | Control | Dh345 | Dh345 + Pg222 | Pg222 | inoc | time |
| *Aldehydes* | | | | | | | | | | |
| 2-Methylpropanal | n.d.$^{2}$[c,d] | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | 36$^{1}$ | 36$^{1}$ | | ++[e] |
| 3-Methylbutanal | 3$^{2}$ | 8$^{2}$ | 27$^{2}$ | 13$^{2}$ | 49$^{1,2}$ | 67$^{1,2}$ | 83$^{1}$ | 82$^{1}$ | | +++ |
| 2-Methylbutanal | 3$^{2}$ | 7$^{2}$ | 14$^{2}$ | 8$^{2}$ | 37$^{1,2}$ | 63$^{1}$ | 62$^{1}$ | 63$^{1}$ | | +++ |
| Hexanal | 118 | 93 | 85 | 9 | 15 | 134 | 16 | 34 | | |
| Heptanal | 43$^{1}$ | 25$^{2}$ | 16$^{2,3}$ | 15$^{2,3}$ | n.d.$^{3}$ | n.d.$^{3}$ | n.d.$^{3}$ | n.d.$^{3}$ | ++ | +++ |
| Benzaldehyde | 66 | 41 | 23 | 9 | 43 | 51 | 30 | 47 | | |
| Octanal | 40$^{1}$ | 23$^{1,2}$ | 11$^{1,2}$ | 13$^{1,2}$ | 27$^{1,2}$ | 8$^{1,2}$ | 11$^{1,2}$ | n.d.$^{2}$ | ++ | + |
| Benceneacetaldehyde | 3 | 8 | 8 | 6 | 15 | 19 | 9 | 7 | | |
| Nonanal | 76 | 56 | 35 | 43 | 29 | 40 | 40 | 21 | | ++ |
| Hexadecanal | 29 | 8 | n.d. | n.d. | 8 | 13 | 2 | 4 | | |
| *Carboxylic acids* | | | | | | | | | | |
| Acetic acid | n.d. | n.d. | n.d. | n.d. | n.d. | 166 | 102 | 66 | | + |
| Propionic acid | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | 3$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | 7$^{1,2}$ | 69$^{1}$ | ++ | + |
| 2-Methyl propionic acid | 4 | 6 | n.d. | 7 | 45 | 19 | 40 | 79 | | ++ |
| Butanoic acid | 3 | n.d. | n.d. | n.d. | 17 | 23 | n.d. | n.d. | | |
| 3-Methyl butanoic acid | 15$^{2}$ | 79$^{2}$ | 4$^{2}$ | 40$^{2}$ | 160$^{2}$ | 204$^{2}$ | 242$^{2}$ | 490$^{1}$ | | +++ |
| 2-Methyl butanoic acid | 10$^{2}$ | 10$^{2}$ | n.d.$^{2}$ | 24$^{2}$ | 112$^{1,2}$ | 116$^{1,2}$ | 129$^{1,2}$ | 254$^{1}$ | | +++ |
| Hexanoic acid | 20 | 1 | 1 | 1 | n.d. | 26 | 33 | n.d. | | |
| Octanoic acid | 6 | 1 | 1 | 1 | 2 | 5 | 6 | 3 | | |
| Nonanoic acid | 5 | 3 | 3 | 5 | 2 | 3 | 7 | 12 | | |
| Tetradecanoic acid | n.d. | n.d. | 61 | 10 | n.d. | 15 | 14 | 68 | | |
| Hexadecanoic acid | n.d. | n.d. | n.d. | 16 | 14 | 18 | 24 | 65 | | ++ |
| *Aliphatic alcohols* | | | | | | | | | | |
| Ethanol | 473 | 448 | 644 | 602 | 307 | 335 | 322 | 245 | | ++ |
| 1-Propanol | 3 | 1 | 15 | 23 | 18 | 40 | 25 | n.d. | | |
| 2-Methyl 1-propanol | 1 | 1 | 5 | 4 | 6 | n.d. | n.d. | 16 | | |
| 1-Penten-3-ol | n.d. | 6 | 2 | 1 | 22 | 23 | 17 | 11 | | ++ |
| 3-Methyl 3-buten-1-ol | 1 | 5 | n.d. | 1 | 12 | n.d. | 6 | 8 | | + |
| 3-Methyl 1-butanol | 17 | 32 | 30 | 39 | 79 | 74 | 49 | 71 | | +++ |
| 2-Methyl 1-butanol | 7 | 21 | 18 | 19 | 29 | 37 | 16 | 28 | | + |
| Pentanol | 19 | 15 | 14 | 9 | 17 | 25 | 35 | 30 | | ++ |
| Butane-2,3-diol | 17$^{1}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | + | |
| 1-Hexanol | 138$^{1}$ | 44$^{2}$ | 26$^{2}$ | 11$^{2}$ | 68$^{1,2}$ | 68$^{1,2}$ | 40$^{2}$ | 28$^{2}$ | +++ | |
| Cyclohexanol | 113$^{2}$ | 185$^{2}$ | 161$^{2}$ | 189$^{2}$ | 351$^{1,2}$ | 559$^{1}$ | 345$^{1,2}$ | 292$^{1,2}$ | | +++ |
| 1-Octen-3-ol | 176 | 104 | 28 | 22 | 102 | 57 | 110 | 41 | ++ | |
| Octanol | 25 | 14 | 11 | 8 | 15 | 10 | 3 | 8 | + | |
| 2-Ethyl hexanol | 26 | 39 | 16 | 23 | 30 | 44 | 34 | 32 | | |
| *Aromatic alcohols* | | | | | | | | | | |
| Ethanol benzene | 18$^{2}$ | 337$^{1}$ | 36$^{2}$ | 7$^{2}$ | 245$^{1,2}$ | 169$^{1,2}$ | 165$^{1,2}$ | 65$^{2}$ | ++ | |
| 2-Methyl phenol | 3 | 3 | 3 | 3 | 9 | 16 | 3 | 8 | | ++ |
| *Ketones* | | | | | | | | | | |
| 2-Propanone | 85 | 8 | 114 | 87 | 23 | 59 | 73 | 94 | | |
| 2,3-Butadione (diacetyl) | 108$^{1}$ | 1$^{2}$ | 6$^{2}$ | 6$^{2}$ | 14$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | +++ | + |
| 2-Butanone | 230 | 75 | 93 | 108 | 109 | 104 | 89 | 99 | | |
| 2-Pentanone | 6 | 16 | 19 | 15 | 17 | 23 | 5 | 8 | | |
| 2-Butanol-3-one (acetoin) | 2.9 | tr.[f] | 0.1 | 0.2 | 0.4 | n.d. | n.d. | 0.1 | | |

(continued on next page)

Table 3 (*continued*)

| Compound | 59 days of ripening time | | | | 106 days of ripening time | | | | $P^a$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control[b] | Dh345 | Dh345 + Pg222 | Pg222 | Control | Dh345 | Dh345 + Pg222 | Pg222 | inoc | time |
| *Ketones* | | | | | | | | | | |
| 3-Methyl 2-pentanone | n.d. | n.d. | n.d. | n.d. | 0.4 | n.d. | 0.1 | n.d. | | |
| 2-Hexanone | 8 | 29 | 20 | 4 | 10 | 10 | n.d. | 4 | | |
| 4-Heptanone | n.d. | n.d. | 24 | 20 | n.d. | 9 | 9 | n.d. | | |
| 2-Heptanone | 28 | 32 | 24 | 30 | 59 | n.d. | 32 | 17 | | |
| Cyclohexanone | 74[2] | 133[2] | 109[2] | 162[2] | 383[1,2] | 471[1] | 431[1] | 300[1,2] | | +++ |
| 2-Octanone | 9 | 5 | 5 | 4 | 15 | 17 | 15 | 30 | | |

[a] $P_{inoc}$: $P$ value due to the microorganisms inoculated. $P_{time}$: $P$ value due to incubation time.
[b] Control: uninoculated sterile batch. Dh345: batch inoculated with *D. hansenii* Dh345. Dh345 + Pg222: batch inoculated with both *D. hansenii* Dh345 and *P. chrysogenum* Pg222. Pg222: batch inoculated with *P. chrysogenum* Pg222.
[c] n.d.: not detected.
[d] Values with different numbers as superscript are significantly different ($P < 0.05$).
[e] $P$ values: + ($P < 0.05$); ++ ($P < 0.01$); +++ ($P < 0.001$).
[f] tr.: trace amounts.

Hinrichsen, 1997), they were formed on a different substrate i.e. on grains and wheat bread. Branched alcohols 3-methyl 3-buten-1-ol, 3-, and 2-methyl 1-butanol showed an increasing trend with incubation time ($P_{time} < 0.05–0.001$), with no observed effect due to the microorganism inoculated ($P_{inoc} > 0.05$). Therefore, linear and branched alcohols in dry-cured pork seem to derive from autolytic breakdown rather than from microbial metabolism. However, some of these branched alcohols have been found at higher levels in mould-inoculated sausages (Bruna et al., 2001). Ethanol benzene seems to be the only alcohol favoured by *D. hansenii*, which could be related to the low level of ethylbenzene found in the samples ripened with the yeast for 106 days (Table 2).

Cyclohexanol and cyclohexanone were the most outstanding alcohols and ketones in the samples. Both increased during ripening, particularly in samples inoculated with *D. hansenii* (Table 3). These two cyclic compounds have been detected in dry-cured meat products (Barbieri et al., 1992; Janssens et al., 1992; Berdagué et al., 1993; Stahnke, 1994; Ruiz et al., 1999), but in a lower proportion to other volatile compounds.

Diacetyl (2,3 butadione) was detected at higher levels in control samples, and it was not even detected in inoculated samples after 106 days. Branched ketones (Table 1) were detected only at trace amounts, and never from samples inoculated only with *D. hansenii* (Table 3).

Carboxylic acids showing significative differences ($P < 0.05$) reached the highest level in samples with *P. chrysogenum* at the second sampling time (Table 3). Propionic acid was detected only in samples inoculated with *P. chrysogenum*, particularly at the last sampling time. Free fatty acids can be originated from lipids by microbial lipases (Zalacaín et al., 1997) and from amino acids by fermentation (Gottschalk, 1986). The branched carboxylic acids found, i.e. 2-methyl propionic, 3-, and 2-methyl butanoic acids, increased with incubation time ($P_{time} < 0.01$), showing the highest mean values for samples inoculated with Pg222 (Table 3). This implies that *P. chrysogenum* mould may also contribute to increase the concentration of these compounds in dry-cured meat products, as it has been

Notes to Table 4:
[a] $P_{inoc}$: $P$ value due to the microorganisms inoculated. $P_{time}$: $P$ value due to incubation time.
[b] Control: uninoculated sterile batch. Dh345: batch inoculated with *D. hansenii* Dh345. Dh345 + Pg222: batch inoculated with both *D. hansenii* Dh345 and *P. chrysogenum* Pg222. Pg222: batch inoculated with *P. chrysogenum* Pg222.
[c] $P$ values: + ($P < 0.05$); ++ ($P < 0.01$); +++ ($P < 0.001$).
[d] tr.: trace amounts.
[e] n.d.: not detected.
[f] Values with different numbers as superscript are significantly different ($P < 0.05$).

Table 4
Other selected volatile compounds of dry-cured pork loins inoculated with tested microorganisms (Arbitrary Area Units)

| Compound | 59 days of ripening time | | | | 106 days of ripening time | | | | $P$[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control[b] | Dh345 | Dh345 + Pg222 | Pg222 | Control | Dh345 | Dh345 + Pg222 | Pg222 | inoc | time |
| *Furans* | | | | | | | | | | |
| Tetrahydro furane | 6 | 20 | 13 | 17 | 29 | 55 | 32 | 24 | | +[c] |
| Dihydro 2(3H)-furanone | tr.[d] | n.d.[e] | n.d. | n.d. | n.d. | 1.3 | 0.1 | n.d. | | |
| 2-Pentylfuran | 30 | 19 | 9 | 11 | 50 | 61 | 58 | 26 | | +++ |
| 5-Ethyldihydro-2(3H)-furanone | 5 | 4 | 4 | 3 | 2 | 11 | 17 | 9 | | |
| Hexyldihydro-2(3H)-furanone | n.d. | n.d. | 31 | n.d. | n.d. | n.d. | 2 | 44 | | |
| 2-Nonanone | 3 | 4 | 24 | 10 | n.d. | 13 | 15 | n.d. | | |
| *Pyrrols* | | | | | | | | | | |
| 1-Methyl-1H-pyrrole | n.d. | n.d. | 0.1 | tr. | n.d. | n.d. | n.d. | 0.1 | | |
| 1-Ethyl-1H-pyrrole | n.d. | n.d. | 0.1 | n.d. | n.d. | n.d. | n.d. | n.d. | | |
| 1-Pentyl-1H-pyrrole | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.1 | | |
| Amine pyrrolidine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.1 | | |
| H-indole | n.d. | n.d. | 0.3 | n.d. | n.d. | n.d. | tr. | 0.1 | | |
| *Pyrazines* | | | | | | | | | | |
| Methylpyrazine | n.d. | 1 | 4 | 4 | 21 | 43 | 27 | 37 | | +++ |
| 2,5-Dimethylpyrazine | $3^{2}$[f] | $6^{2}$ | $20^{2}$ | $77^{2}$ | $491^{1,2}$ | $135^{1,2}$ | $449^{1,2}$ | $1054^{1}$ | | +++ |
| Trimethylpyrazine | $2^{2}$ | $2^{2}$ | $16^{2}$ | $19^{2}$ | $75^{1,2}$ | $38^{2}$ | $46^{2}$ | $146^{1}$ | | +++ |
| 2-Ethyl-3,5-dimethylpyrazine | $1^{2}$ | n.d.$^{2}$ | $3^{2}$ | $3^{2}$ | $11^{2}$ | $15^{2}$ | $12^{2}$ | $47^{1}$ | ++ | +++ |
| Tetramethylpyrazine | $1^{2}$ | $2^{2}$ | $1^{2}$ | $1^{2}$ | $26^{1}$ | n.d.$^{2}$ | n.d.$^{2}$ | $30^{1}$ | +++ | +++ |
| *Sulfur compounds* | | | | | | | | | | |
| Methanethiol | n.d. | n.d. | 96 | 99 | 122 | 29 | 63 | 131 | | |
| Dimethyl sulfide | n.d. | 3 | 6 | 17 | 17 | 21 | 43 | n.d. | | |
| Carbon disulfide | 20 | 69 | 79 | 68 | 119 | 177 | 76 | 44 | | |
| Dimethyl disulfide | 2 | n.d. | 84 | 10 | 88 | 29 | 86 | 29 | | |
| Benzothiazol | $5^{2}$ | $6^{2}$ | $3^{2}$ | $5^{2}$ | $10^{2}$ | $23^{1,2}$ | $25^{1,2}$ | $41^{1}$ | ++ | +++ |
| Benzene thiobis | n.d.$^{2}$ | $6^{1,2}$ | n.d.$^{2}$ | n.d.$^{2}$ | $25^{1}$ | $13^{1,2}$ | $10^{1,2}$ | $8^{1,2}$ | | +++ |
| *Ester compounds* | | | | | | | | | | |
| Ethyl acetate | 128 | n.d. | n.d. | 2 | n.d. | n.d. | n.d. | n.d. | | |
| Ethyl 3-methylbutanoate | $4^{1}$ | $2^{1,2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | | + |
| Ethyl methylpentanoate | $5^{1}$ | $1^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | ++ | ++ |
| Ethyl hexanoate | $12^{1}$ | n.d.$^{2}$ | $4^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | + | ++ |
| Diethyl benzenodicarboxilate | n.d. | 24 | 13 | n.d. | 22 | n.d. | 8 | 31 | | |
| Bis (dimethyl) 1,2-benzenodicarboxilate | n.d. | n.d. | 13 | 18 | 5 | 16 | 12 | 19 | | |
| *Ether compounds* | | | | | | | | | | |
| 1-Methoxy 3-methylbutane | 1 | 2 | 24 | 25 | n.d. | 8 | n.d. | 12 | | |
| 1,1-Dimethoxy etane | n.d. | n.d. | 13 | n.d. | n.d. | n.d. | 52 | 13 | | |
| Methoxy cyclohexane | n.d. | n.d. | 5 | 12 | n.d. | n.d. | n.d. | n.d. | | ++ |
| *Miscellaneous compounds* | | | | | | | | | | |
| Dichloromethane | 3 | 3 | 3 | 5 | 34 | 22 | 42 | 38 | | +++ |
| Pyridine | 2 | 8 | 1 | 5 | 9 | 2 | 28 | 41 | | + |
| Benzenamine | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | n.d.$^{2}$ | $42^{1}$ | $41^{1}$ | | + |
| 2-Piperidione | n.d. | n.d. | 39 | n.d. | n.d. | n.d. | 9 | 39 | | |

shown for *Penicillium nalgiovense* on meat extract (Jacobsen and Hinrichsen, 1997).

Neither the main furans (Table 4) nor those detected at trace levels (Table 1) showed significative differences due to the microbial strain. Only 2-pentylfuran seems to increase with ripening time ($P_{time} < 0.01$). Several furan derivatives have been described among the autoxidation products of linoleic and linolenic acids, including 2-pentylfuran (Belizt and Grosch, 1999).

Pyrroles were detected only at low amounts, and only in samples inoculated with *P. chrysogenum* (Table 4). Several pyrrole derivatives have been obtained by Maillard reaction of an amino acid with 2 acylfuran (Belizt and Grosch, 1999). Most pyrroles reach detectable levels in samples inoculated with *P. chrysogenum* and ripened for 106 days. In such samples, both proteolysis (Martín et al., 2002) and lipid auto-oxidation may have reached an extension sufficient to support pyrrole synthesis. However, the fact that pyrroles were also found in samples inoculated only with *D. hansenii* and ripened just for 59 days suggests that microorganisms might play an additional role that deserves further research.

All pyrazines detected increased with incubation time ($P_{time} < 0.001$), reaching for 2,5-dimethylpyrazine the maximum detector response obtained in this assay. For those showing significant differences, the highest mean value was obtained in the samples inoculated with *P. chrysogenum* (Table 4). These compounds can be formed from amino acids through either Maillard reactions (Belizt and Grosch, 1999) or microbial metabolism (Janssens et al., 1992; Tressl et al., 1993; Magan and Evans, 2000). The former has been suggested to take place in dry-cured ham (Ventanas et al., 1992). The microorganisms inoculated, particularly *P. chrysogenum*, may contribute to pyrazine synthesis by the increase in free amino acids reported by Martín et al. (2002), but no direct synthesis can be inferred from the present results. In any case, the contribution of microorganisms to pyrazines formation is of interest, due to their positive correlation with desirable aroma of dry-cured ham (Flores et al., 1997).

As seen in Table 4, some sulfur compounds, like benzothiazol and benzene thiobis, also increased with incubation time ($P_{time} < 0.001$). Benzothiazol content correlates to the inoculated microorganism too ($P_{inoc} < 0.01$), being significantly higher with *P. chrysogenum* than in control samples. The origin of many sulfur compounds is associated with the production of methanethiol by microbial cultures (Spinnler et al., 2001). Methanethiol is believed to result from the degradation of L-methionine by L-methionine-γ-demethiolase or an aminotrasferase of microbial origin (Spinnler et al., 2001). In fact, methanethiol was detected first in samples inoculated with *P. chrysogenum* (Table 4). Even though sulfur compounds provide a whole range of characteristic aromatic notes including "garlic", some of them have a meat broth-like aroma at very low levels and are incorporated as flavouring agents in synthetic meat flavours (Baines and Mlotkiewicz, 1984).

Esters and ethers are either not detected in the last sampling time or not significantly higher than

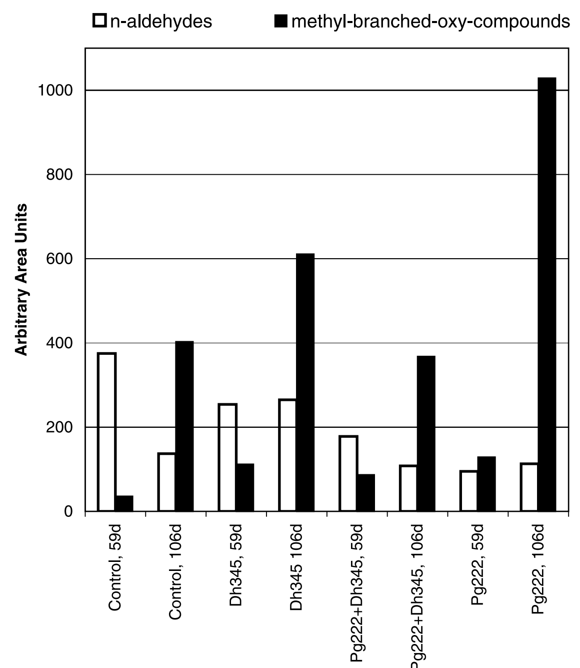


Fig. 1. Accumulated area of *n*-aldehydes and methyl branched oxy-compounds in control and inoculated dry-cured pork loins. Control: uninoculated sterile batch. Dh345: batch inoculated with *D. hansenii* Dh345. Dh345 + Pg222: batch inoculated with both *D. hansenii* Dh345 and *P. chrysogenum* Pg222. Pg222: batch inoculated with *P. chrysogenum* Pg222. 59d: sampling at 59 days or ripening time. 106d: sampling at 106 days or ripening time.

in the first sampling (Table 4). Esters, particularly ethyl acetate, has been found at higher concentrations in dry-fermented sausages ripened with *P. aurantiogriseum* in the same study, but a decisive role was attributed to bacteria present (Bruna et al., 2001). Other compounds, such as dichloromethane, pyridine, and benzenamine have been detected only in small amounts and tend to increase with incubation time as seen in Table 4 ($P_{time} < 0.05-0.001$).

From all these groups of compounds, *n*-aldehydes and methyl branched oxy-compounds seemed to be good indicators of the development of volatile compounds in dry-cured ham (Hinrichsen and Pedersen, 1995). The former is associated to secondary autoxidation products, and the latter to other more complex reactions. The results described here, show lower figures for *n*-aldehydes in both *P. chrysogenum* Pg222 inoculated batches than in control samples at each sampling time (Fig. 1). On the other hand, methyl branched oxy-compounds, mainly 3-methy butanal, 3-, and 2-methyl butanoic acid, reached the highest figures in the batch inoculated with *P. chrysogenum* Pg222. Thus, the ratio of these two groups of compounds may be useful to evaluate the role of microorganisms on volatile compounds of dry-cured meat products.

To conclude, the inoculation of *P. chrysogenum* Pg222 to pork loin decreased compounds derived from the lipid oxidation, but increased some carboxylic acids, branched aldehydes, and pyrazines derived from amino acids catabolism. In addition, *D. hansenni* 345 contributes to higher levels of cyclic and aromatic alcohols. All these changes may differ quite remarkably in the presence of other microorganisms, due not only to different growth kinetics, but also to variations in the substrates available and further reactions among microbial metabolites. However, given that the volatile compounds generated by these pure cultures are of interest to flavour in dry-cured meat products, these microorganisms may play a key role in products of long ripening time.

## Acknowledgements

The authors thank Jorge Ruiz (Food Technology, University of Extremadura) for helpful discussions. This work is part of the project ALI98-0253 founded by the Spanish Comisión Interministerial de Ciencia y Tecnología. A. Martín was the beneficiary of a predoctoral scholarship from the Spanish Ministerio de Educación y Cultura.

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