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Dynamics and characterization of yeasts during ripening of typical Italian dry-cured ham

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Abstract

The evolution of the yeast population during manufacturing and ripening of dry-cured Parma ham was investigated. Contamination levels ranged from 10^5 to 10^7 cfu/g on muscle surface, 10^4 to 10^6 cfu/g on covering fat and exceeded 10^7 cfu/g on spreadable fat mince (''sugna'').

Two hundred and sixty one yeast isolates underwent identification test, showing that the predominant species of yeast population during the whole maturing process were *Debaryomyces hansenii*, *Candida zeylanoides, Debaryomyces maramus*, and to a lesser extent, *Candida famata* and *Hyphopichia burtonii*. The species *Candida catenulata, Candida guilliermondii, Candida edax* and other genera like *Cryptococcus* and *Wingea* were occasionally found.

The yeast counts and species distribution changed according to the stage of processing and to the ham sampling location. At the end of the cold phase, the washing procedure was effective in lowering the yeast count in muscle and fat surface layers, but during the next ageing stages, yeast colonization of unskinned ham muscle increased again, though species distribution changed if compared to previous manufacturing phases.

The ripening steps taken into account from the end of the cold phase to the final outcome, were always characterized by more than one yeast species, suggesting that yeasts other than *Debaryomyces* spp. could play a remarkable role on the sensory and safety properties of typical Italian dry-cured ham.

Keywords: Yeast species; Dry-cured ham; Processing phases; *Hyphopichia burtonii*

1. Introduction

Surface layers of dry-cured hams harbour a typical microbial flora composed of bacteria, yeasts and filamentous fungi (Huerta et al., 1988). Previous studies reported that staphylococci and micrococci may not be recovered at the end of maturation of the Iberian dry-cured ham, whereas yeasts can be the predominant microorganisms (Núñez et al., 1996a). On the ham surface, yeast presence is witnessed by the so-called ''white powder''; when conditions of a_w (0.85–0.92), temperature (10–28 °C) and NaCl amount favourable to growth occur, yeasts outnumber bacteria and form a film on the whole ham (Comi and Cantoni, 1983). Comi and Cantoni (1983) analysed Parma dry-cured ham where *Debaryomyces hansenii, Torulopsis candida* and *Torulopsis famata* proved to be the predominant species. Studies on the Spanish raw ham evidenced that the profile of yeast population greatly changed during processing. *Candida zeylanoides* was the main species at the fresh stage (more than 90% of isolates), but *D. hansenii* dominated the yeast population after post salting (Núñez et al., 1996a).

Recently, several studies revealed yeast contribution to the sensory characteristics of dry-cured meat products thanks to their proteolytic and lipolytic activity (Rodríguez et al., 1998; Sørensen, 1997) and their role in volatile compounds generation (Bolumar et al., 2006; Flores et al., 2004). Durá et al. (2004) found that the addition of *Debaryomyces* spp. as a starter culture could partly modify the flavour pattern of dry-cured sausages by means of an enhanced degradation of branched-chain amino acids.

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Yeast colonization on the surface of dry-cured meat products could also play an important role against pathogenic microorganisms which may cause health problems to the consumer, such as *Staphylococcus aureus* (Metaxopoulos et al., 1996). Moreover, the detoxifying ability of some yeasts in foods and wine contaminated by mycotoxins is well known (Beajoui et al., 2004; Cecchini et al., 2006) while in substrates like barley and grapes the presence of selected epiphytic yeasts showed a potential biocontrol of ochratoxigenic moulds (Ramakrishna et al., 1996; Bleve et al., 2006).

The aim of this work was to quantify and characterize the yeast wild population on the surface of Parma hams during the main steps of the ripening period, to evaluate the influence of the current manufacturing practices over the dynamics of Parma dry-cured ham yeasts.

2. Materials and methods

2.1. Ham processing

Forty hams were processed according to Parma Ham Consortium Regulation (1992) in two plants, to take into account the product variability due to modifications occurring in processing procedures.

After the cold period (salting and resting), the hams were labelled to be identified up to the end of the manufacturing period and analysed at established processing steps (Table 1) from the end of resting (last phase of the overall cold period protracted for 3–4 months) to the final outcome (12-month old dry-cured hams). As a rule, at the beginning of the ripening phase, raw hams were washed to remove the dry salts and the microbial film grown on product surface during salting and resting. After washing, hams underwent a drying step, which was one week long (T = 15–18 °C; RH = 60–65%).

After the first maturing period, corresponding to 6/7 months of processing, the hams were smeared with a spreadable mince made up of pork fat, called "sugna", to prevent excessive drying of lean meat (Parolari, 1996). In one plant the spreadable mince was made only of pork fat and salt while in the other one rice flour was also added. Then, the labelled hams were placed in the ageing room up to the end of processing (ageing phase).

2.2. Environmental data recording

Air temperature and % relative humidity (RH) values were continuously collected in processing rooms and recorded every 15 min with a thermo hygrometer Data Logger (ESCORT Data Logging Systems Ltd.). Data were downloaded with the ESCORT program for Windows (version 1.61.06). Environmental data were averaged over the measures given by two Data Logger, placed in each established processing location.

2.3. Ham sampling for microbiological and chemical analyses

Four hams were microbiologically and chemically analysed at the end of resting and drying periods, whereas, during the next ripening steps (Table 1), 8 hams were sampled to remedy the low homogeneity of environmental conditions inside maturing rooms.

Muscle sampling: surface samples, approximately 2 mm thickness, were aseptically removed from three different areas of the lean surface (Fig. 1), and collected together in a sterile bag. The samples were weighed and diluted 1:3 (w/w) with peptone physiological solution (PPS) composed of 8.5 g NaCl (Carlo Erba Reagenti S.p.a., Milan, Italy); 1 g tryptone (Oxoid Ltd., Basingstokes, UK) and 1000 ml distilled water. They were homogenized using a Stomacher (pbi International, Milan, Italy) for 2 min.

Fat sampling: surface samples, approximately 2 mm in thickness, were aseptically removed from three different areas of the covering fat (Fig. 1), collected and appropriately diluted 1:3 (w/w) with PPS, adding 1% (w/w) Tween 80 (Liofilchem, Roseto degli Abruzzi, Italy) and homogenized using a Stomacher (pbi International) for 2 min.

The spreadable fat mince ("sugna") was collected and kept in sterile boxes. Samples of 10 g were diluted 1:10

Table 1
Scheme of dry-cured ham processing

Process stage		No. of assayed ham	Processing time	Environmental conditions	
				Temperature (°C) (±std. dev.)	% Relative humidity (±std. dev.)
Cold period (CP)	Salting	/	25 days	1.5±0.4	85.0±2.5
	Resting	4	65–100 days	2.5±0.2	69.9±1.4
Washing					
Drying (D)		4	7 days	16.0±1.0	67.4±3.7
Maturing	1st step (M1)	8	3 months	15.0±0.8	59.4±2.1
	2nd step (M2)	8		15.9±0.5	61.6±2.8
Fat application					
Ageing	1st step (A1)	8	5 months	16.6±0.5	60.5±1.3
	2nd step (A2)	8		16.2±1.7	61.0±7.4

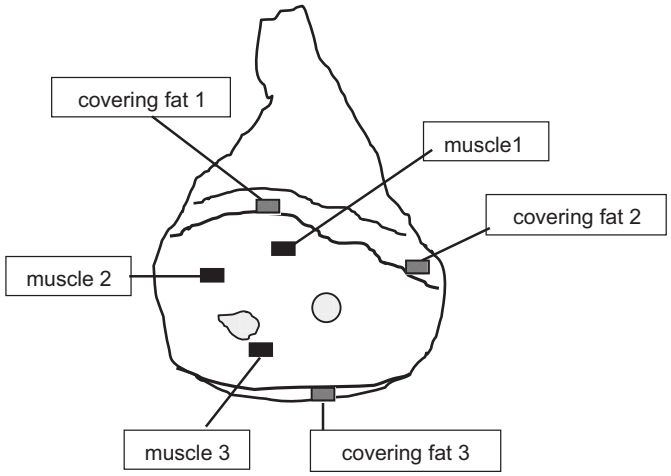


Fig. 1. Sampling areas on dry-cured ham surface.

(w/w) with PPS, adding 10 g of Tween 80. In order to obtain a better dispersion and homogenization of the diluted sample, sterile glass beads (∅ 3–4 mm) were added and manually mixed for 15 min.

For each sample, serial dilutions were made in PPS and 0.1 ml of the appropriate dilution was spread-plate inoculated onto dichloran-glycerol agar plates (DG18, Oxoid). Yeast counts were obtained after incubation at 25 °C for 4–5 days.

2.4. Yeast identification and characterization

Twenty per cent of the colonies were selected from the plates corresponding to the highest dilutions on DG18 plates and purified by streaking on malt extract agar (MEA, Oxoid). Pure cultures were maintained at room temperature on MEA plates and at −80 °C on malt extract broth (MEB, Oxoid) containing 15% glycerol (w/v).

Isolates were identified to the species level using the Biolog system (Biolog Inc., Hayward CA, USA) following the manufacturer's instructions for YT MicroPlate™. Additional tests included the study of colony and cell morphology on MEA, production of ascospores on modified Gorodkowa Agar, fermentation of glucose and assimilation of nitrate (Kreger-Van Rij, 1987).

Proteolytic activity was evaluated streaking pure cultures of yeasts onto a culture medium composed of 40 g/l gelatine (Oxoid) and 24 g/l tryptone glucose extract agar (Oxoid). Plates were incubated up to 21 days at 25 °C and gelatine liquefaction was assessed after flooding plates with 8–10 ml of a mercuric chloride solution composed of 15 g mercuric chloride (Sigma Inc., St. Louis, MO, USA), 20 ml HCl 37% (Carlo Erba) and 100 ml distilled water (Harrigan and McCance, 1976). Unhydrolysed gelatine formed a white opaque precipitate with the reagent, while hydrolysed gelatine appeared as a clear zone.

Lipolytic activity was evaluated by measuring the clear halos in a culture medium composed of 5 g/l tributyrin (Sigma), 5 g/l Tween 80 (Liofilchem) and 24 g/l tryptone glucose extract agar (Oxoid), after incubation at 25 °C for 7 days (Saldanha-da-Gama et al., 1997).

2.5. Physical and chemical analysis

Physical and chemical analyses were performed on 5 mm thick samples, taken from the muscle surface; moisture and sodium chloride content (g/100 g muscle) was analysed according to standard AOAC methods (AOAC, 2002). Water activity (a_w) was measured at 25 °C with the a_w recorder AquaLab, series 3, Model TE (Decagon Devices, Inc., Pullman, USA), in accordance with ISO/FDIS 21807 (2004).

2.6. Statistical analysis

The chemical–physical and microbiological data were submitted to statistical evaluation with SPSS software package, version 12.5 (SPSS, Bologna, Italy, 2003).

One-way analysis of variance was run to compare the dry-cured ham composition and the yeast populations corresponding to different manufacturing stage, and the least significant difference (LSD) *t*-test was used to statistically separate means ($P<0.05$).

3. Results and discussion

3.1. Ham and yeast population changes during processing

The environmental conditions of the dry-curing process are reported in Table 1.

At the beginning of the cold phase (salting), high %RH must be set inside processing rooms (80–90%) to allow NaCl solving and diffusing from outer to inner ham layers, but during the next resting, drying and maturing phases %RH values gradually decrease to favour ham dehydration. The changes in environment %RH and temperature together with the product modifications during ripening, influence a_w and salt amount of ham surface rind (5 mm thickness). The effects of processing steps were evaluated with reference to surface composition and yeast counts in ham muscle, covering fat and "sugna" (sampled during ageing after fat application). The results are reported in Table 2.

Before fat application, a_w of ham surface lowered as a consequence of ham drying; the effect of dehydration prevailed over the concomitant salt reduction to achieve the a_w decrease. After fattening, the observed sharp increase in moisture and surface a_w values above 0.90 was due to the rehydration of muscle outer layers.

Changes in a_w and NaCl during dry-cured ham ripening fell in the range reported in Table 2; the ability to grow on ham outer layer at these a_w and salt values is a tool of differentiation among yeasts during ham processing (Núñez et al., 1996a).

The manufacturing procedures significantly influenced the number of yeasts on the ham muscle surface. During

Table 2
Effect of the process stage on chemical and microbiological data

	Process stage[1]						*P*-value[2]
	CP	D	M1	M2	A1	A2	
Muscle surface							
Water activity (a_w)	0.906[a]	0.884[b]	0.874[b]	0.875[b]	0.909[a]	0.911[a]	0.000
NaCl[3]	4.00[a]	3.74[a,b]	3.08[b]	3.13[b]	3.38[a,b]	3.34[a,b]	0.067
Moisture[3]	38.5[b]	34.7[c]	29.6[d]	29.8[d]	36.1[c]	40.7[a]	0.000
Yeasts (log cfu/g)	6.72[a]	5.25[b]	5.68[b]	6.57[a]	6.58[a]	6.57[a]	0.005
Covering fat							
Yeasts (log cfu/g)	6.06[a]	4.32[a,b]	4.88[a,b]	4.24[b]	4.72[a,b]	4.61[a,b]	0.332
Spreadable fat mince							
Yeasts (log cfu/g)					7.28	7.46	0.123

Different letters in the same row mean significant differences among processing stages ($P<0.05$ multiple paired t-test according to the LSD method).
[1]CP, cold period; D, drying; M1, 1st step of maturing (5 months of ripening); M2, 2nd step of maturing (6/7 months of ripening); A1, 1st step of ageing (1 month after fat application); A2, 2nd step of ageing (12 months of ripening).
[2]*P*-value was reported to test the main effect (processing stage) significance.
[3]Expressed in g per 100 g muscle.

the whole investigated period, the yeast population ranged between 10^5 and 10^7 cfu/g. The yeast counts found in the muscle surface and in the covering fat at the end of the cold phase decreased as a consequence of the washing procedure. A period of about 2 months was needed to achieve the previous initial counts in the environmental (temperature, %RH, oxygen availability) and product conditions (%NaCl, a_w and available nutrients) typical of the ripening phase. During the next manufacturing steps, the yeast population on muscle surface remained unchanged up to the end of ageing (12 months).

On average, in ham adipose tissue the yeast population reached lower levels than in muscle surface and fat mince (Table 2). Covering fat in dry-cured ham has low moisture (2–6%), low salt (0.3–1.3%) and low protein (1–8%) content (Toscani et al., 2003); yeasts can break down lipids and the presence of nutrients like free fatty acids, can explain the growth of a yeast population in ham fat portion. Since appearance and colour of the typical Parma ham external fat are among its main features, Parma Ham Consortium Regulation (1992) established an allowed range of fat composition to eliminate fat rich in poly-unsaturated fatty acids more prone to yellowness and rancidity. The yeasts growing on the ham fat surface may exert an antioxidative effect due to their oxygen-scavenging activities, by depleting oxygen from fat surface and destroying peroxides, thus inhibiting the impairment of colour and odour during the fat protracted exposure to air at room temperature.

The highest yeast count was found in "sugna", both after application and at the end of ageing. Population values exceeded those of ham muscle surface (Table 2). "Sugna" is a mince of fat (75–90%), salt (1–3%), moisture (5–7%) and, eventually, rice flour (0–30%). Available nutrients in "sugna" are free fatty acids, starch, proteins and sugars generated by starch hydrolysis. Since analyses made in fresh fat mince before application on ham showed yeast contamination between 10^3 and 10^4 cfu/g (results not shown), a positive contribution from the close and protracted contact between fat mince and ham muscle may be inferred, in terms of available nutrients and a_w.

Yeast counts in "sugna" samples containing rice flour were similar to those where only pork fat was present (see the Section 2.1), ranging from 7.39–7.43 to 7.16–7.50 log cfu/g, respectively.

3.2. Identification and characterization of the isolates

The 261 yeast isolates from dry-cured Parma hams during ripening were identified at the species level using the Biolog system. This identification system has been previously used with good results for the identification of environmental and clinically important yeasts (McGinnis et al., 1996) as well as of yeasts isolated from cheese (Heard et al., 2000) and other foods and beverages (Praphailong et al., 1997). In this study, only 20 of the 261 strains isolated were not identified, neither at the genus level.

The distribution of yeast strains among the species isolated from dry-cured ham is reported in Table 3, and displayed according to sampling location (muscle, fat and "sugna"). In accordance with other authors (Comi and Cantoni, 1983; Huerta et al., 1988; Núñez et al., 1996a), the predominant species were *D. hansenii* (30% of isolates), *C. zeylanoides* (23%) and *Debaryomyces maramus* (21%), while *Candida famata* (6%) and *Hyphopichia burtonii* (3%) were less often isolated. Together, these species represent the 83% of the overall yeasts isolated during dry-cured ham maturing process. Table 3 reports the extracellular lipolytic and proteolytic properties of the identified yeast strains. Most strains (96%) showed lipolytic activity on tributyrin. This positive lipolytic activity was strain-related and not influenced by sampling

Table 3
Number of isolates for each species of yeast isolated from muscle surface, fat and fat mince (“sugna”) and lytic activities on tributyrin and gelatine

	Muscle	Fat	Fat mince	Total (%)	Lipolysis[a]	Proteolysis[a]
Candida zeylanoides	33	18	8	59 (23)	57	0
Candida famata	11	4	2	17 (6)	17	1
Debaryomyces hansenii	37	22	19	78 (30)	74	10
Debaryomyces maramus	24	27	5	56 (21)	53	8
Hyphopichia burtonii	3	—	5	8 (3)	8	8
Others[b]	10	8	5	23 (9)	23	2
Not identified	8	8	4	20 (8)	NT	NT
Total	126	87	48	261 (100)	232	29

NT: not tested.
[a]Number of strains giving positive results.
[b]Other species of yeasts included in Table 4.

Table 4
List of yeast species included on the entry “others” in Table 3

Other species	Number of isolates
Candida guillermondii	3
Candida catenulata	4
Candida nitratophila	1
Candida edax	3
Candida versatilis	1
Candida vanderwaltii	2
Candida santamariae	1
Cryptococcus albidus	1
Cryptococcus luteolus	1
Debaryomyces castelli	1
Pichia carsonii	2
Saccharomyces kluiverii	1
Wingea robertsiae	2
Total	23

location (fat or muscle), according to Saldanha-da-Gama et al. (1997). The extracellular lipolytic activity may contribute to flavour development during ripening process by means of the release of aroma precursors such as long-chain free fatty acids. Oleic and palmitic acids were the predominant fatty acids produced by *D. hansenii* and its anamorphic form *C. famata* isolated from southern Italy traditional sausages if assayed for lipolytic activity on pork fat (Gardini et al., 2001).

In this study, a few strains of *D. hansenii* and *D. maramus* and one strain of *C. famata* showed positive extracellular proteolytic activity, while gelatine was strongly hydrolysed by all *H. burtonii* strains (Table 3).

Twenty-three strains among the yeasts isolated were identified belonging to 13 different species and are reported in Table 4. These yeasts were occasionally isolated during ham processing and represented less than 10% of the isolates. *Pichia carsonii* and *Cryptococcus albidus* were also isolated from Iberian hams (Molina et al., 1990; Núñez et al., 1996a); a strain of *Candida* (*Pichia*) *guillermondii,* also recovered from Portuguese pork-based products (Saldanha-da-Gama et al., 1997) showed the ability to decrease, in vitro, ochratoxin A amount (Virgili et al., 2004). *Candida catenulata* isolated from covering fat showed a high lipolytic activity in accordance with other authors (Corbo et al., 2001; Romano et al., 2001).

The profile of yeast populations greatly changed during processing as shown in Fig. 2a–c. *C. zeylanoides*, *D. hansenii* and *D. maramus* were isolated throughout the whole processing, being *C. zeylanoides* present at a remarkable percentage in the early processing stages (after the cold resting and the drying step) but still not negligible at the end of ageing. These data partly differ from those reported by Núñez et al. (1996a) since in aged Italian typical hams, *Debaryomyces* spp, though present at a high percentage at the end of ageing, represented only 50–60% of the total yeast strains recovered, whereas in Iberian hams more than 99% of isolates belonged to *D. hansenii*.

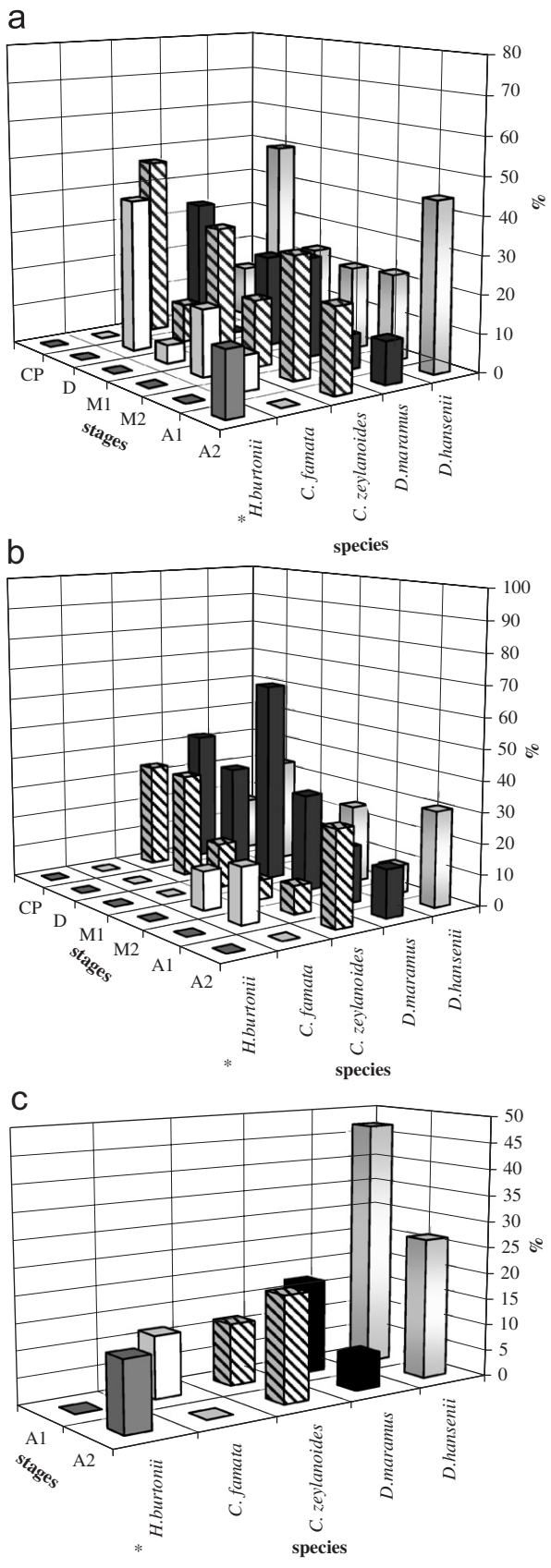
At the end of drying, at least 99% of yeasts on the ham surface were identified as *D. hansenii* and *C. famata*: as displayed in Table 2, at this step, one week after ham washing, a significant reduction of the yeast population was found, causing a possible selection in favour of yeast strains with high tolerance to dry-cured ham substrate properties (a_w, sodium chloride, temperature, available nutrients).

Since the washing step is not mandatory in typical ham processing, dry-cured hams achieved without washing might display a different trend as to wild yeast dynamics. During subsequent phases M1 and M2, other species besides *D. hansenii* grew on the muscle outer layer (Fig. 2a). As reported in Table 2, a sharp a_w and moisture increase occurred in the muscle surface at process stage A2, but neither yeast count nor species distribution were found to be strongly affected by these modifications in muscle substrate.

The yeast species of fat mince partly reflected the muscle surface profile showing a higher percentage of strains belonging to genera *Debaryomyces* (more than 30% of isolates) and a constant presence of *C. zeylanoides*.

C. famata was sporadically present throughout the processing of Parma ham, but it was not isolated at the end of ageing.

The dominating yeasts that develop on the Parma ham surface during the whole ripening period, i.e. *Debaryomyces* and *Candida* spp., appear to be similar to those described by other authors for similar products: the most noticeable difference from other kinds of dry-cured hams relies on the distribution and prevalence of yeast species during processing steps. As to these species, the high yeast counts (mainly in "sugna" and ham unskinned muscle) and the contemporary presence of different yeast species from cold resting to final product is more probably due to the peculiar manufacturing of the typical Italian dry-cured ham allowing several yeast species to grow at assayed ripening phases, than to considerable differences in substrates like muscle and fat.



In the last processing stage A2, a difference on yeast population from other kinds of dry-cured hams (Molina et al., 1990; Núñez et al., 1996a; Huerta et al., 1988) was due to the presence of the species *H. burtonii,* referred to as "yeast-like fungi". This species was isolated in hams from one manufacturing unit at the last stage of ripening, both from "sugna" and muscle surface, and it represented about 20% of the total strains isolated and identified at this processing stage.

H. burtonii was identified in accordance with both the classical method for moulds identification proposed by Pitt and Hocking (1997) and the YT MicroPlate^{TM} system (Biolog). Barnett et al. (2000) reported it between the yeast species as *Pichia burtonii*, thelemorphic form of *Candida chodatii*. This species is typical of cereals and derivates, especially of ready-made bread (Spiker, 1986) and it was just among the yeast species most frequently isolated from Italian cured hams (30% of isolates) by Comi and Cantoni (1983) and reported with the obsolete name *Trichosporon variabile*. The colonization could originate from the presence of rice flour (30–35%) mixed with the spreadable pork fat used in one of the manufacturing units. *H. burtonii* achieved very high counts in "sugna" ($>10^6$ cfu/g) and in ham muscle ($>10^5$ cfu/g). The interest in this species is connected with its ability to compete with undesirable moulds as reported for airtight-stored cereal grains, since *H. burtonii* showed inhibitory activity against the germination of spores of *Penicillium verrucosum* and the production of ochratoxin A (Ramakrihna et al., 1996) and vs. the growth of *Penicillium roquefortii* (Druvefors and Schnürer, 2005) when its count exceeded 10^7 cfu/g.

In case of dry-cured ham, the ability of yeasts to grow to high population levels (10^6–10^8 cfu/g) in ham muscle and "sugna" could be competitive against microorganisms growing in the ecological system of dry-cured ham ripening, i.e. moulds, for essential environmental factors like nutrients or space. Nutritional competition (Björnberg and Schnürer, 1993; Druvefors and Schnürer, 2005;

Fig. 2. Incidence of yeasts on muscle surface (a), covering fat (b) and fat mince "sugna" (c): distribution and percentages of the different species at the main stages of processing (CP = cold period; D = drying; M1 = first step of maturing (5 months of ripening); M2 = second step of maturing (6/7 months of ripening); A1 = first step of ageing (1 month after fat application); A2 = the second step of ageing (12 months of ripening)). *H. burtonii* was always isolated from dry-cured hams of one manufacturing plant.

Petersson and Schnürer, 1998) and mycocin production (Santos and Marquina, 2004) are reported to play a fundamental role in yeast–mould interactions, thus affecting mould secondary metabolism, including mycotoxin production. The presence of toxigenic moulds in the air of dry-curing plants (Núñez et al., 1996b; Spotti et al., 1989) and in some dry-cured meat products together with the detection of ochratoxin A in dry-cured ham (Pietri et al., 2006) can make yeasts eligible as possible biocontrol agents in dry-cured meat products.

Moreover, the interest in yeasts also takes into account their possible contribution to product flavour; dry-cured ham (muscle and fat) is a source of peptides, free amino acids and free fatty acids released by endogenous and exogenous proteolytic and lipolytic enzymes (Virgili et al., 1999; Motilva et al., 1993). Yeast catabolic activities against free amino acids and fatty acids to generate volatile compounds during dry-cured ham ripening (Martín et al., 2006) may be an additional requirement to select yeasts for dry-cured ham colonization and flavour improvement.

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