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POSTER

House-mycobiota and use of biocompetitors in a *Culatello* manufacturing plant

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SUMMARY

One artisanal plant manufacturing *Culatello* (a typical Italian dry-cured meat product) was sampled on summer and winter to assess the mycobiota occurring in the air of the ripening rooms and on product, with special attention given to undesired moulds and potential mycotoxin producers. Among the house-mycobiota, fungal strains potentially producing ochratoxin A were sporadically isolated and resulted the least prevailing species collected from *culatelli*, while fungal strains producing unpleasant spots on the casings were massively found in the first steps of the ageing process, even if their presence proved to diminish at the end of the ageing time. For this reason, a study concerning the use of fungal autochthonous strains as biocompetitors was carried out, in order to find out a possible solution to the settlement of certain undesired moulds on meat derivatives. Autochthonous *Eurotium* and *Penicillium* strains were selected and inoculated on some culatelli, to act as biocompetitors over *Sporendonema* spp. naturally contaminating products. Despite its fluctuating concentration, the undesired mould gave samples a spotted, flame-red appearance throughout the ageing process, so it can be stated that use of our autochthonous biocompetitors do not represent an effective counter-action against moulds producing unpleasant spots on meat products.

Micobiota autoctono e uso di biocompetitori in un impianto per la produzione di *Culatello*

Parole chiave aggiuntive

Prodotti carnei.
Eurotium.
Penicillium.
Biocompetizione.
Muffe.

SOMMARIO

Un impianto artigianale che produce *Culatello* (un prodotto carneo stagionato tipico italiano) è stato sottoposto a campionamento in inverno ed estate per valutare il micobiota presente nell'aria degli ambienti di stagionatura e sui prodotti, con particolare attenzione alle muffe indesiderate e alle potenziali produttrici di micotossine. Tra il micobiota caratteristico, i ceppi fungini potenzialmente produttori di ocratossina A sono stati rilevati sporadicamente e sono risultati quelli meno prevalenti tra le specie isolate da culatelli. Al contrario, i ceppi fungini responsabili di colorazioni anomale sui budelli sui budelli sono stati rilevati in quantità massive nei primi step del processo di stagionatura, pur essendo la loro presenza in diminuzione verso la fine di tale processo. Per tale ragione, è stato condotto uno studio relativo all'impiego di ceppi fungini autoctoni quali biocompetitori, al fine di trovare una soluzione allo sviluppo di certuni ceppi fungini sui prodotti carnei stagionati. Ceppi autoctoni di *Aspergillus* e *Penicillium* sono stati selezionati ed inoculati su alcuni *culatelli* per agire come biocompetitori nei confronti di ceppi di *Sporendonema* naturalmente presenti sui prodotti testati. Nonostante la sua fluttuante concentrazione, la muffa sgradita è riuscita ad impartire ai prodotti inoculati una chiazzatura color *rosso fuoco* nel corso della stagionatura. Per tale motivo, l'uso di biocompetitori autoctoni non ha rappresentato un'effettiva azione di contrasto nei confronti di muffe in grado di produrre colorazioni anomale sui prodotti carnei.

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INTRODUCTION

In Europe, meat products have a long tradition originating from curing and ripening techniques developed by the Mediterranean populations of the Roman Empire (Comi et al. 2005). Nowadays, in Mediterranean countries a lot of traditional dry-cured and ripened meats are still being produced, Italy being one of the leading countries with more than 40 DOP and IGP meat products (ASSICA, 2017).

Among them, Culatello is a typical DOP meat (European Commission, 1996) obtained from muscles of the back and of the inner thigh of pork. The climatic

conditions of the production area (Parma), characterized by the presence of a dense fog in autumn and winter months, are peculiar to this territory and are strictly connected to the preparation of this meat product. Culatello is made from a piece of meat encased in a pig bladder and the production process involves both a salting phase (similar to that of dry-cured hams) and a maturing phase (typical of fermented sausages).

For all these typical meats, the peculiar thermal and hygrometric conditions applied to the production process of most European meat products quickly lead to the development of a specific surface mycobiota (Berni, 2015). In aged meats such as dry-cured hams, surface moulding is not favoured by the addition of selected fungal starter on product surface, as often occurs in ripened meats such as salami. On aged products, an accidental contamination of the product surface by airborne Fungi is more frequent and could cause adverse effects such as the formation of unsightly colored spots, unpleasant off-flavors or highly toxic substances (Scaramuzza et al. 2015). An anesthetic appearance is often due to growth of dematiaceous genera such as Scopulariopsis, Cladosporium or of alophilic moulds such as Sporendonema. The formation of toxic compounds is always caused by the settlement of ochratoxigenic species such as Penicillium nordicum and Aspergillus westerdijkiae. The control of these microorganisms is nowadays based on chemical prevention measures (i.e. periodical fumigation treatments or use of gaseous ozone in ageing or ripening rooms), but undesirable moulds proved to recur on product surface after treatments were carried out. For this reason, the microbiological approach is starting to be tested as an alternative to classical prevention methods (Simoncini et al. 2014, Spotti et al. 2009, Ferrara et al. 2016, Berni et al. 2017). Based on this, a screening of the house-mycobiota in a Culatello manufacturing plant has been carried out, in order to highlight the potential problems connected to fungal settlement on this product. Moreover, a biocompetition test has been tempted, in order to assess the effectiveness of some autochthonous moulds isolated on Culatello as biocontrol agents over undesired moulds such as Sporendonema casei which tends to give products a characteristic flame-red colored appearance.

MATERIAL AND METHODS

Air sampling

Air samples were collected from a single point in three ageing rooms by means of a SAS super 100™ air sampler (International PBI, Milan, Italy). The sampler was located about 1.0 m from the centre of each room and 1.0 m above the floor. The volume of analysed air was 10 L/sample. For each sampling, 60-mm RODAC plates were used with three different culture media: (i) Malt Extract Agar (MEA) added with 0.01% chlorotetracycline; (ii) Dichloran Glycerol Agar (DG18); (iii) Lab-Lemco Medium (LLM), a selective medium for detection of *Sporendonema casei* (Berni *et al*., 2014) for a total of 9 samples collected on winter and 9 samples collected on summer. MEA and DG18 plates were incubated at 25°C up to seven days; LLM plates were incubated at 15°C up to 14 days. Contamination was estimated according to Most Probable Number (MPN) of Colony Forming Units (CFU) and expressed as log CFU/m^3 air. All powdered media were provided by OXOID (Cambridge, UK).

Product sampling

Nineteen and fifteen culatelli from different batches were sampled in winter and summer, respectively. A 20-cm^2 surface from each *culatello* was scraped with a sterile swab previously soaked in a sterile Tween80 water solution (0.1%, w/v). Swabs were then spread in: (i) Malt Extract Agar added with 0.01% chlorotetracycline; (ii) Dichloran Glycerol Agar, and incubated at 25°C up to seven days. All powdered media were provided by OXOID (Cambridge, UK).

Identification of fungal species

On the basis of their morphological and physiological characteristics, fungal colonies isolated by means of both air- and product sampling were identified at a species level (Scaramuzza et al. 2015).

Test on natural substrate

Four fungal strains were selected among the Filamentous Fungi detected during product sampling and used for the biocompetition trials: *Aspergillus pseudoglaucus (≡Eurotium repens)* SSICA 1312; *Aspergillus tonophilus (≡Eurotium tonophilum)* SSICA 1212; *Penicillium brevicompactum* SSICA 28212; *Penicillium griseofulvum* SSICA 27212; *Sporendonema casei* SSICA 1112.

A spore suspension containing *A. pseudoglaucus, A. tonophilus,* and *P. brevicompactum* in equal amounts was diluted in mineral water at a concentration of 5.75 log CFU/ml and used to inoculate ten *culatelli* by dipping them in the spore suspension. Ten non-inoculated *culatelli* were also used as a control. All samples were dried and aged, according to *Culatello* Product Specification (http://www.qualigeo.eu/en/prodotto-qualigeo/culatello-di-zibello-dop/). Fungal growth was checked at the beginning of the maturing period and every six months. Data were presented as mean values ± Standard Deviation (SD) and the statistical significance between the means was determined with the Fisher's test (LSD) at 0.05 level.

RESULTS

Air sampling

A total of 684 fungal isolates were collected. Mean contamination was equal to 4.15 log CFU/m^3 in winter and to 4.26 Log CFU/m^3 in summer. Most of the species isolated in the environments were also found on the product surface, the exception being *Aspergillus alliaceus, Aspergillus fumigatus, Cladosporium cladosporioides, Epicoccum nigrum, Penicillium olsonii* and *Penicillium brevicompactum*. In both season, aspergilli with *Eurotium*-type ascomata proved to prevail, totalling 48.4% of the fungal isolates. They were followed by penicilli (30.9%), asexual aspergilli (16.5%), and *Sporendonema casei* **(Table I)**. With regard to summer and winter samplings, fungal species proved to recur

Table I. Fungal occurrence (%) in the air of the manufacturing plant (Occorrenza di specie fungine (%) nell'aria dell'impianto produttivo).

Fungal Species	Occurrence (%)			
	w	s	w+s (sl)	w+s (gl)
Aspergillus alliaceus	3.21	0.81	1.90	
Aspergillus candidus	11.21	4.57	7.60	
Aspergillus fumigatus	0.64	0.00	0.29	16.52
Aspergillus taichungensis	0.64	1.08	0.88	
Aspergillus versicolor	1.60	9.41	5.85	
**Aspergillus glaucus (≡Eurotium herbariorum)*	17.94	15.86	16.81	
**Aspergillus tonophilus (≡Eurotium tonophilum)*	17.31	18.82	18.13	48.39
**Aspergillus pseudoglaucus (≡Eurotium repens)*	10.26	16.13	13.45	
Cladosporium cladosporioides	0.00	0.27	0.15	0.15
Epicoccum nigrum	0.00	0.27	0.15	0.15
Penicillium brevicompactum	7.06	6.45	6.73	
Penicillium chrysogenum	12.82	10.22	11.40	
Penicillium nalgiovense	1.28	1.08	1.17	
Penicillium nordicum	0.64	0.00	0.29	30.85
Penicillium griseofulvum	0.00	1.34	0.73	
Penicillium olsonii	10.26	9.41	9.80	
Penicillium solitum	0.00	1.34	0.73	
Scopulariopsis candida	0.32	0.27	0.29	0.29
Sporendonema casei	4.81	2.69	3.65	3.65
TOTAL	100.0	100.0	100.0	100.0

w: winter; s: summer. sl: percentage of each single species; gl: percentage of single genera.
*Since the recent synonymization of the teleomorph-based genus *Eurotium* with *Aspergillus* by the International Commission on *Penicillium* and *Aspergillus* (http://www.aspergilluspenicillium.org/index.php/single-name-nomenclature/88-single-names/105-aspergillus-options), species formerly included in the genus *Eurotium* are displayed with their *Aspergillus* name, while their teleomorph is in round brackets [e.g. *Aspergillus glaucus* (≡ *Eurotium herbariorum*)].

in similar percentages, the presence of the least occurring species (*A. fumigatus, C. cladosporioides, E. nigrum, P. nordicum, P. griseofulvum, P. solitum* and *S. candida*) being so sporadic that almost all were collected either on summer or on winter.

Product sampling

A total of 119 fungal isolates were collected. Predominant mycobiota consisted of both sexual and asexual *Aspergillus spp.*, which represented the most of the fungal population detected, totalling 72.3% of the fungal isolates. Among these, *Aspergillus candidus* was the most occurring species (16.0%). A minor percentage of isolates belonged to *Penicillium* (14.3%) and to *Sporendonema casei* (9.2%), while the incidence of fungal isolates identified as *Scopulariopsis* was very low, being 4.2% of the total **(Table II)**. With regard to summer and winter samplings, fungal species were detected in similar percentages within each of the most occurring fungal genera, the exception being *P. solitum* and *P. griseofulvum* (isolated just on summer samples), or *P. nordicum* (found just on winter *culatelli*). These negligible differences cannot be attributed to the different physiological characteristics of the single species, but they rather could depend on the variability occurring among *culatelli* that were collected in different points of three diverse ageing rooms and could therefore be subjected to different thermos-hygrometric parameters.

Test on natural substrate

Among the strains found in the manufacturing plant, *Aspergillus pseudoglaucus, Aspergillus tonophilus* and *P. brevicompactum* were selected by means of preliminary tests on synthetic media (unpublished results) to be used on natural substrate, since their combination was thought to be suitable to possibly contrast *S. casei* throughout all the ageing process.

Average concentration of the inoculated strains and of *S. casei* throughout the ageing process is reported in **Table III**. With regard to total mycetical population, a significant difference ($P<0.05$) was observed only for non-inoculated samples after 30 and 180 days of the ageing process. With regard to *S. casei*, though it was not detected in the first part of maturing process, after 180 and 360 days it reached concentrations that were significantly higher ($P<0.05$) than those of *P. brevicompactum*, but significantly lower ($P<0.05$) than those of

Table II. Fungal occurrence (%) on *culatelli* (Source: Scaramuzza *et al.*, 2015) (Occorrenza di specie fungine (%) su culatelli (Fonte: Scaramuzza et al., 2015).

Fungal Species	Occurrence (%)			
	w	s	w+s (sl)	w+s (gl)
Aspergillus candidus	17.33	13.64	15.97	
Aspergillus taichungensis	1.33	2.27	1.68	31.10
Aspergillus versicolor	12.00	15.91	13.45	
**Aspergillus glaucus (≡Eurotium herbariorum)*	17.34	6.82	13.45	
**Aspergillus tonophilus (≡Eurotium tonophilum)*	16.00	9.09	13.45	41.19
**Aspergillus pseudoglaucus (≡Eurotium repens)*	13.34	15.91	14.29	
Penicillium chrysogenum	2.67	2.27	2.52	
Penicillium griseofulvum	0.00	6.82	2.52	
Penicillium nalgiovense	2.67	4.55	3.36	14.28
Penicillium nordicum	2.67	0.00	1.68	
Penicillium solitum	0.00	11.36	4.20	
Scopulariopsis candida	4.00	4.55	4.20	4.20
Sporendonema casei	10.67	6.82	9.24	9.24
TOTAL	100.0	100.0	100.0	100.0

w: winter; s: summer. sl: total percentage of each single species; gl: total percentage of single genera.
*Since the recent synonymization of the teleomorph-based genus *Eurotium* with *Aspergillus* by the International Commission on *Penicillium* and *Aspergillus* (http://www.aspergilluspenicillium.org/index.php/single-name-nomenclature/88-single-names/105-aspergillus-options), species formerly included in the genus *Eurotium* are displayed with their Aspergillus name, while their teleomorph is in round brackets [e.g. *Aspergillus glaucus (≡ Eurotium herbariorum)].*

aspergilli with *Eurotium*-type ascomata on both inoculated and non-inoculated samples.

DISCUSSION

With regard to air- and product-sampling, the prevailing genus was *Aspergillus* whose presence was equal to 64.9% in the air of the manufacturing plant and to 72.3% on *culatelli*, if both sexual and asexual species were considered. In particular, in both seasons a prevalence of sexual (*Eurotium*) aspergilli was observed, their presence amounting to 48.4% and 41.2% of the fungal isolates detected in the air and on the products, respectively. This could be explained by the fact that *culatelli* are subjected to a shorter aging and tend to quickly dehydrate, so low aw values (up to 0.89) on their surface are rapidly reached, thus favoring the growth of xerophilic rather than xerotolerant species. This could also be the reason why a small number of species adapted to high aw were identified early in the process, but tended to be not encountered in the final part of the process (our unpublished results).

The partial correspondence between air- and product-mycobiota seemed to indicate a natural selection of those strains (mainly belonging to *Aspergillus, Eurotium* or *Penicillium* genera) which had better adapted to the thermo-hygrometric conditions applied, as frequently observed in meat derivatives. The fact that a minor number of fungal strains were massively present in the air, but were not detected on products is probably due to the peculiar thermo-hygrometric conditions of the rooms and to the surface a_w values of *culatelli*, both establishing prohibitive conditions for their germination and growth or causing their progressive inactivation (Diaferia et al. 1996).

Among the potentially toxigenic species, only *P. nordicum* was found. Since its low occurrence, its presence can be considered sporadic and did not represent a worrying risk for consumers' health.

Among the undesired species, *S. casei* proved to be widespread on different lots of *culatelli*, independently of the ageing time. Its growth on the contaminated samples started from the string used to tie the products and resulted strongly evident also at an unaided eye due to the production of flame red-coloured spots throughout all the casing. Fortunately, it proved not to be ochratoxigenic (Berni et al. 2014) and its intense-coloured conidiation proved to soften at the end of the maturing process.

With regard to tests on natural substrate, almost all aspergilli and penicilli tested for their growth ability in preliminary tests on synthetic media seemed good autochthonous fungal starters and their use was then thought suitable to possibly contrast *S. casei* throughout all the ageing process. Unfortunately, despite a partial competition occurred on *culatelli*, *S. casei* gave both inoculated and non-inoculated samples a spotted, flame-red appearance throughout the ageing process, so it can be stated that use of our autochthonous biocompetitors was not an effective counter-action

Table III. Average concentration (log CFU/cm²) of fungal strains throughout the ageing process (Concentrazione media (log ufc/cm²) di ceppi fungini nel corso del processo di stagionatura).

Fungal species	Inoculated Samples - Fungal Count (log CFU/cm²)					
	30 days		180 days		360 days	
	Mean Value	SD	Mean Value	SD	Mean Value	SD
A. pseudoglaucus	4.99	0.91	4.90[a]	0.17	5.07[a]	0.21
A. tonophilus	4.00	0.10	5.23[a]	0.40	5.27[a]	0.15
P. brevicompactum	< 2.00	nd	< 2.00[b]	nd	< 2.00[b]	nd
S. casei	< 2.00	nd	4.24[c]	0.21	3.52[c]	0.08
Total Population	5.66[A]	0.35	6.04[A]	0.22	5.65[A]	0.15
Fungal species	Non-inoculated Samples - Fungal Count (log CFU/cm²)					
	30 days		180 days		360 days	
	Mean Value	SD	Mean Value	SD	Mean Value	SD
A. pseudoglaucus	3.15	1.74	4.80[a]	0.85	3.70[a]	0.10
A. tonophilus	2.10	0.10	4.49[a]	0.50	3.73[a]	0.25
P. brevicompactum	< 2.00	nd	< 2.00[b]	nd	< 2.00[b]	nd
S. casei	< 2.00	nd	3.46[c]	0.41	3.40[c]	0.10
Total Population	4.24[B]	1.23	5.87[A]	0.32	4.80[A]	0.10

For values printed in italics, fungal concentration was under the limit of detection (e.g., < 2.00 log CFU/cm² means that fungal concentration was inferior to 100 CFU/cm²).
Different superscript letters for each mean indicate significant differences among fungal strain counts (small letters for values within the same column) or total fungal counts (capital letters for values within the same row).

against moulds producing unpleasant spots on meat derivatives.

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