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Compositional Changes in Surface Mycoflora During Ripening of Naturally Fermented Sausages

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ABSTRACT

Changes in the composition of the surface mycoflora during ripening of naturally fermented sausages were examined. The samples were collected from small-scale production plants in Northern Italy. In the first part of the ripening process yeast dominated the mycoflora and constituted more than 95% (colony forming units [CFU]). After 2 weeks' ripening, yeast and molds were present in equal quantities. The molds continued to increase in numbers and at the end of processing the result was a more than 95% dominance. The genus *Penicillium* dominated the mycoflora at the end of the ripening process. *Penicillium nalgiovense*, a species frequently used as a starter culture, constituted 50% of the molds and was found to occur naturally in the environment. Four species, *Penicillium olsonii, Penicillium spathulatum, Penicillium oxalicum* and *Penicillium capsulatum*, that have not been isolated from this environment before constituted, respectively 15%, 5%, 3%, and about 1% of the mycoflora. Also, *Penicillium* species that are known as potential producers of mycotoxins were isolated; e.g., *Penicillium verrucosum* and *Penicillium commune* constituted 5 and 3% of the mycoflora. It was shown that six out of nine isolates of *P. verrucosum* produced ochratoxin A and one isolate produced citrinin. One isolate of *P. commune* was examined and shown to produce cyclopiazonic acid. A large number, 53, of *Penicillium nalgiovense* isolates were examined, but no known mycotoxins were shown to be produced after growth on synthetic agar media.

Key words: Composition, mycoflora, fermented sausages

Traditionally, fermented sausages are spontaneously colonized by the house flora. The composition and development of the mycoflora depend on the nature of the sausage product, the processing time, and the ripening conditions (*2,18*). Normally, the mycoflora is dominated by *Penicillium* spp. (*16,20,27*), but dominating species belonging to the genera *Aspergillus* and *Scopulariopsis* have also been reported (*15,17,19,26*).

The taxonomy of the genera *Penicillium* and *Aspergillus* is difficult (*10*). The complex taxonomy, especially of the genus *Penicillium*, entails numerous possibilities of misinterpretation, and besides several taxonomic systems exist: e.g., it is generally accepted that isolates previously identified as *Penicillium verrucosum* var. *cyclopium* (*Penicillium cyclopium*) represent the species *Penicillium aurantiogriseum, Penicillium commune, Penicillium crustosum, Penicillium polonicum, Penicillium solitum* and *Penicillium verrucosum* (*4,10,13,24,25*).

A species-specific identification of the mycoflora is important, because some molds produce mycotoxins and thereby represent a health hazard. Leistner and Eckardt (*20*) found that 40 to 60% of the *Penicillium* strains isolated from Italian and Hungarian sausages produced mycotoxins. Mycotoxin production has also been detected in meat products. For instance, the following mycotoxins produced by *Penicillium* molds have been found in skinned sausages: brevianamide A, citreoviridin, citrinin, cyclopiazonic acid, fumitremorgin B, griseofulvin, ochratoxin A, rugulosin and verrucologen (*8*).

There is a growing interest in using well-characterized starter cultures for surface treatment of meat products. This is to avoid undesirable molds that may produce mycotoxins, antibiotics, result in an off-flavor or cause discoloration. Furthermore, a more uniform product with respect to flavor, aroma and color can be obtained. In developing new starter cultures the first step will be to determine which mold species prevail on fermented sausages. These molds are well adapted to the sausage environment. This gives them an advantage in the competition with other molds. The dominating species of the mycoflora are probably the same species that contribute most to the typical appearance, flavor and aroma. Therefore, new starter cultures should be selected from the environment where they will be used.

In this investigation, changes in the composition of the mycoflora during processing of naturally fermented sausages was examined. Samples were collected from small-scale production plants in Northern Italy. In developing new starter cultures it is not only of interest to know which mold species are present in the house flora and are well adapted to the environment; it is also of interest to know when spontaneous colonization takes place, e.g., when in the fermentation process it is of importance to have a competitive and fast-growing starter culture, since even prevalent species may be regarded as contaminants because of their appearance and possible production of mycotoxins and antibiotics.

MATERIALS AND METHODS

Isolation and identification of the mycoflora

Sampling. Sampling took place at seven small-scale production plants in Northern Italy (Faenza, Lavezzola, Montebelluna, Parma, Treviso, Venezia). Sausage samples were taken in the production rooms and transferred to sterile plastic bags. At each production plant, samples were taken during the first week of ripening and, additionally, at two more advanced stages of the drying process (a maximum of 8 weeks). Air samples were collected by leaving open petri dishes with DG18 (Oxoid) for 10 to 15 min in the production rooms.

Isolation. The mycoflora was isolated from the sausages by aseptic removal of the casings, using a sterile scalpel and tweezers. The casings were transferred into sterile plastic bags, 150 ml dilution medium was added and the sample was homogenized for 2 min using a Stomacher™ (Colworth, 400). Suitable dilutions were made before plating.

Identification. The mycoflora was identified according to Samson and van Reenen-Hoekstra (*24*), Pitt (*22*) and Frisvad and Filtenborg (*12*). Only penicillia were all identified to species level; yeast was not so identified.

Media and growth conditions. The mycoflora was isolated from DG18. For identification, the molds were inoculated on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), yeast extract-sucrose agar (YES), Czapek yeast extract sucrose 20% agar (CY20S), and creatine-sucrose agar (CREA). For formulations of the media see Samson and van Reenen-Hoekstra (*24*) and Samson and Pitt (*23*). The fungi were incubated at 25°C for 7 days.

Chromatographic examination of mycotoxin production

Fungi. Fifty-three strains of *P. nalgiovense*, 9 strains of *P. verrucosum* and 1 strain of *P. commune* isolated from sausages were examined for mycotoxin production.

Media and growth conditions. CYA, MEA, YES and oatmeal agar (OA) were used. For formulations see Samson and van Reenen-Hoekstra (*24*) and Samson and Pitt (*23*). The fungi were incubated at 25°C for 14 days.

Analyses for mycotoxins. Isolates were examined for mycotoxin production by use of the agar-plug methods of Filtenborg and Frisvad (*5*), Filtenborg, Frisvad and Svendsen (*6*), and Frisvad and Thrane (*14*), based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (*14*).

RESULTS AND DISCUSSION

The compositional changes in the mycoflora during processing were evaluated as a whole for the seven production plants, as it was not possible to get sausage samples from exactly the same stages of production. In the first part of the processing, yeast was the predominating microorganism, constituting more than 95% (colony-forming units) of the mycoflora. At this stage molds were present only in limited numbers. As the ripening process proceeded, molds became competitive and after two weeks' ripening, the number of yeast and molds balanced. Later in the drying process, e.g., after a production time of 4 to 8 weeks, the molds dominated completely and constituted more than 95% of the mycoflora. The change in composition of the mycoflora is probably caused by alterations in the environmental conditions, since molds, in general, are more tolerant to low water activity than yeast. Also, the growth of yeast may be influenced by secondary metabolites produced by molds.

Casado, Borrás and Aguilar (*2*) examined the mycoflora on cured Spanish ham and also found that molds were present at low levels in the beginning of the ripening process, whereas yeast was recovered with high frequency throughout the process.

The limited number of molds present at the beginning of the process shows that it takes some time (1 to 3 weeks) for the spontaneous flora to get established. The yeast may delay the colonization. Thus, it is important to use a starter culture that grows fast and covers the surface before the spontaneous flora gets established. This will reduce the risk of contamination, especially if the starter culture is a good competitor. As the process progresses, the growth conditions will change. It is therefore important to use a starter culture that is not only "strong" in the beginning but throughout the process.

The air samples collected in the production rooms represent the house flora. The mycoflora on the surface of the sausages comprises those species in the house flora that are well adapted to the sausage environment (substrate, water activity, temperature, etc.). Qualitatively a good agreement was observed between air samples and the mycoflora isolated from the sausages. However, three species, *Penicillium expansum, Aspergillus ochraceus* and *Aspergillus niger* were found only in air samples. These species have previously been isolated from fermented sausages (*3,15,16*). No yeast was demonstrated in the air samples.

The composition of the mycoflora at the end of processing is given in Table 1. The predominating genus was *Penicillium*, constituting 96% of the mycoflora. The remaining 4% belonged to the genera *Aspergillus, Cladosporium, Eurotium, Mucor, Wallemia* and yeast. *Penicillium nalgiovense* was the predominating species, constituting 50% of the mycoflora. The *P. nalgiovense* strains isolated belonged to different types of this species that produce white, turquoise, or green conidia. As *P. nalgiovense* constituted 50% of the mycoflora, it must be well adapted to the growth conditions and play an important role in the development of the characteristic sausage flavor and aroma. It is stressed that these examinations were of naturally fermented sausages only, and that the high frequency of *P. nalgiovense* is not caused by its use as starter culture. Thus, *P. nalgiovense* occurs naturally in the environment.

Four species, *P. olsonii, P. spathulatum, P. oxalicum* and *P. capsulatum*, that have not been isolated from this environment before, constituted respectively 15%, 5%, 3%, and about 1% of the mycoflora (Table 1). Like *P. nalgiovense*, these species, especially *P. olsonii*, appeared well adapted to the environment and their enzymatic activity may influence the final product. *Pencillium olsonii* may originate from the content of spices as it has previously been isolated from parsley and other herbs (*12*). The species *Penicillium chrysogenum, P. verrucosum, P. oxalicum* and *P. commune* constituted 10%, 5%, 3%, and 3% of the mycoflora, respectively (Table 1). Together with other species present, they are potential producers of mycotoxins. The species *P. nalgiovense, P. olsonii, P. spathulatum, P. solitum* and *P. capsulatum* do not produce any known mycotoxins. However, these species may potentially produce mycotoxins that are unknown or not yet examined. Still, more than 20% of the isolated mycoflora are potential producers of mycotoxins (Table 1).

TABLE 1. *Composition of mycoflora isolated at the end of processing and potential mycotoxin production.*

Fungi	Percent of Mycoflora	Potential Mycotoxins[a,b]
Penicillium nalgiovense	50	NK
P. olsonii	15	NK
P. chrysogenum	10	CG, EA, PE, RQ
P. verrucosum (Chemotypes I and II)	5	CT, OA
P. spathulatum	3	NK
P. solitum	3	NK
P. oxalicum	3	OX, RQ, SA
P. commune	3	CY, IC, RU
P. viridicatum	4	CN, PA, VF, VI, XA
P. polonicum		PA, VE, VF
P. brevicompactum		BD, MA, PO
P. capsulatum		NK
P. crustosum		CN, IC, PN, RQ, TE
Aspergillus candidus	4	CD, TP, XT
Aspergillus spp.		
Cladosporium spp.		
Eurotium repens		PY
E. rubrum		PY
Mucor spp.		
Wallemia sebi		WA, WM
Yeast spp.		

[a] BD = botryodiploidin, CD = candidulin, CG = chrysogine, CN = cyclopenin, CT = citrinin, CY = cyclopiazonic acid, EA = emodic acid, IC = isofumigaclavine A, MA = mycophenolic acid, NK = none known, OA = ochratoxin A, OX = oxaline, PA = penicillic acid, PE = penicillin(s), PN = penitrem A, PO = pebrolides, PY = physcion, RQ = roquefortine C, RU = rugulovasine A, SA = secalonic acid A, TE = terrestric acid, TP = terphenyllin, VE = verrucosidin, VF = verrucofortine, VI = viomellein, WA = walleminol A, WM = wallemia A and B, XA = xanthomegnin, XT = xanthoascin.

[b] References: *1,7,9,11,12,13,28.*

The ability to produce mycotoxins was tested for sausage isolates of the species *P. commune, P. nalgiovense* and *P. verrucosum*. The single strain of *P. commune* in the examination produced cyclopiazonic acid. Out of 53 tested *P. nalgiovense* strains, none produced any known mycotoxins. Nine strains of *P. verrucosum* were examined. One strain produced citrinin and six strains produced ochratoxin A and B. Ochratoxin A, which is known to have pathological effects on kidney and liver, was detected in high quantities. It should be emphasized that only *P. verrucosum* isolates of chemotype II produce the mycotoxin citrinin (*12*). Normally only chemotype I is found on meat, and this chemotype is common on meat and cheese, whereas chemotype II is common on cereals. Another problem with *P. verrucosum* isolates recovered from sausages is that some of them produced white conidia, making it impossible to see the contamination directly on the sausages. Generally, mold species regarded as contaminants produce colored conidia.

The mycotoxins produced by the isolated strains of *P. verrucosum* and *P. commune* may also be produced in sausages. Consequently, the presence of these mycotoxin-producing molds, plus other potential mycotoxin producers in the house flora represent a health hazard, as mycotoxin production may take place under the given environmental conditions.

The species *P. nalgiovense* constituted 50% of the mycoflora at the end of processing and occurs naturally in the environment. Although none of the examined isolates demonstrated ability to produce any known mycotoxins, it has been reported in the literature that isolates of the species *P. nalgiovense* can produce secondary metabolites toxic to brine-shrimp larvae, mice and rats (*8,21*). Whether these metabolites are produced in sausages is not known, and even if produced they may not be stable. Still, there are more advantages than disadvantages in using a starter culture. The naturally fermented sausages have a mycoflora of unknown composition that varies between production plants and is affected by the season. This makes it difficult to control production (relative humidity and water activity) and to produce a uniform product with respect to flavor, aroma, appearance and texture. Another problem is that the house flora is dominated by species of the genus *Penicillium*; many of these are potential producers of mycotoxins. The use of a starter culture makes it possible to produce a uniform product at the optimal conditions. When selecting a starter culture, a nontoxic isolate that is neither pathogenic nor antibiotic-producing is preferable. However, a technologically suitable starter culture that may be toxigenic can be a recommended alternative to the house flora. Being a potential producer of mycotoxin, the starter culture may not produce mycotoxin in the given environment, or the mycotoxin may not be stable in the product.

Growth and mycotoxin production do not always take place simultaneously. Temperature and water activity interact strongly in mycotoxin production (*13*). That is, the minimal water activity necessary for mycotoxin production is generally higher than it is for growth (*13*). Thus, with a thorough knowledge of the identity and properties of the starter culture, its growth and mycotoxin production, if any, can be controlled technologically by adjustment of the relative humidity and temperature during processing. *Penicillium camemberti* is an example of a mycotoxin-producing species used as a starter culture, especially in cheese production but also for fermentation of sausages. *Penicillium camemberti* produces the mycotoxin cyclopiazonic acid; according to Leistner (*18*), it is produced in cheese in significant quantities only at high temperatures. When the storage temperature was kept around 8°C no cyclopiazonic acid was found in the cheese within a month, but at 25°C the mycotoxin was detected after 5 days (*18*). Thus, even though *P. camemberti* is a known producer of the mycotoxin cyclopiazonic acid, it is an accepted and widespread starter culture, but some constraints on temperature should be fulfilled when it is used.

Starter cultures are used to avoid the presence of undesirable molds, that may produce mycotoxins or antibiotics, and to obtain a more uniform product with respect to flavor, aroma and color. In conclusion, a starter culture for fermentation of sausages should be well adapted to the sausage and a good competitor under the given environmental conditions, because a large number of different molds have demonstrated a capability to colonize the sur-

face of the sausages. Since *P. nalgiovense* occurs naturally and constitutes a large part of the mycoflora, this species seems to be a rational choice for a starter culture. The reported results show distinct compositional changes in the mycoflora during ripening of naturally fermented sausages. The mycoflora changed from a yeast-dominated to a mold-dominated flora. The numbers of yeast and molds are balanced after two weeks' ripening. To avoid growth of undesirable molds the starter culture must cover the surface within a week, before establishment of the spontaneous flora. Also it is of primary importance that the natural house flora be suppressed by keeping hygienic conditions at a high standard.

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