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Meat Science 65 (2003) 935–948

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Review

Mould starter cultures for dry sausages—selection, application and effects

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Received 21 August 2002; accepted 15 October 2002

Abstract

The use of moulds on sausage surfaces can lead to both desirable and undesirable effects. The pursued effects are mainly related to successful production or consumer appeal. The undesirable effects are usually connected to growth of undesirable moulds. Most importantly, moulds may produce highly toxic secondary metabolites and β-lactams. Inoculations of sausages with moulds were traditionally done with the indigenous flora of the processing plants, the so-called houseflora, which was mainly composed of penicillia and aspergilli. The gradual shift in sausage production from small local producers to large-scale factories and increasing awareness of the risks for consumer safety has paved the way for industrialised procution of mould starter cultures. The industrialized starter cultures are carefully selected among hundreds of candidates going through multi-stage concepts including several analytical and biochemical investigations. While the technological aspects of mould inoculations are fairly resolved, its influence on prodution of secondary metabolites is yet emerging. Moulds produce enzymes for the degredation of lipid- and protein-matter, but studies show that the proteolytic and lipolytic capabilities differs significantly both between strains and is highly dependent on media, pH and temperature. Only few studies have addressed the aroma impact of mould growth on sausages, but information from related areas like milk based systems or sausage models still gives a quite clear picture of which components are important and their possible routes of information. One example is that methyl ketones play significant roles in the flavour of Blue cheeses. Here the presence and production of methyl ketones is ascribed to the β-oxidation activities of moulds and therefore investigations in sausages flavour have successfully focused on confirming this relationship and its aroma influence. Sausage producers are interested in using the established knowledge on aroma formation in sausages for improving aroma and texture but also for shortening ripening periods or even expanding shelf life. Addition of concentrates of enzymes have yielded some positive results but it is clear that the use of enzymes as additives in sausages production is not straightforward. To ensure optimal consumer safety starter cultures should be applied to achieve maximal controll over the mould population. Starter strains should under no circumstances show pathogenic or toxigenic signs in neither chemical or biological test. Additionally they should not be able to produce antibiotics.

Keywords: Mould; Starter culture; Sausage; Aroma; Mycotoxins

Contents

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doi:10.1016/S0309-1740(02)00281-4

1. Introduction

In Europe, moulds are used for processing of meat and cheese products. The use of mould in meat processing is concentrated especially in Southern European regions like Italy, Spain, France, Hungary and Southern Germany. Typical products are ham, Speck, Bündnerfleisch, coppa and dry-cured sausages (Steinkraus, 1996).

Mould fermented sausages were first mentioned around 1730 in Italy, and in 1835 two Italian butchers introduced production of mould fermented sausages in Hungary (Leistner, 1986a).

The use of moulds on sausage surfaces can lead to both desirable and undesirable effects. The pursued effects are: the typical flavour and taste mediated by lactate oxidation, proteolysis, degradation of amino acids, lipolysis, β-oxidation (Grazia, Romano, Bagni, Roggiani, & Guglielmi, 1986; Leistner, 1984; Lücke, 1997), protection against spontaneous colonisation with unwanted moulds, yeasts and bacteria (Lücke & Hechelmann, 1987), the delay of rancidity and stabilisation of colour through catalase activity, oxygen consumption and protection against light (Bacus, 1986; Bruna, Hierro, de la Hoz, Mottram, Fernandez, & Ordóñez, 2001; Lücke & Hechelmann, 1987),- the characteristic white or greyish appearance of the mycelium and conidia (Lücke, 1997), the reduced risk of development of a dry edge and reduced water loss due to slower water evaporation (Lücke, 1997), easy skin peeling (Grazia et al., 1986).

The undesirable effects are usually connected to growth of undesirable moulds. Most importantly, moulds may produce highly toxic secondary metabolites, mycotoxins, which besides their acute toxic effects often also have long termed toxinogenic, carcinogenic, haemorrhagic or liver degenerative effects (Samson, Hoekstra, Frisvad, & Filtenborg, 1995). Moulds may produce green, brown or black spots that are non-acceptable to most consumers, other moulds may have negative impact on flavour and taste. Finally many food-associated penicillia possess abilities for producing penicillin (Andersen, 1994), which might increase risk of allergy towards this antibiotic if ingested (Bremmelgaard, 1998).

Inoculations of sausages with moulds were traditionally done with the indigenous flora of the processing plants, the so-called houseflora, which was mainly composed of penicillia, aspergilli or *Scopulariopsis* (Horwitz & Wehner, 1977; Jirkovski & Galgóczy, 1966; Leistner & Eckart, 1981). The gradual shift in sausage production from small local producers to large-scale factories and increasing awareness of the risks for consumer safety has paved the way for industrialised production of mould starter cultures. The first toxicologically and technologically suitable mould starter culture for meat products, a *P. nalgiovense* was selected by Mintzlaff and Leistner (1972) and later made commercially available as ''Edelschimmel Kulmbach''. Today all major starter culture suppliers offer moulds as part of their product range.

Application of commercial moulds to sausage surfaces improves primarily the safety towards mycotoxin production. Additionally sausage producers achieve more consistent flavour, taste, drying rate, and a more uniform appearance.

2. Spontaneous mould flora, mould starter selection and genetic modification

The spontaneously colonising houseflora, vary in composition from each production site, region and country. In seven small scale production plants in Northern Italy Andersen (1995) found that penicillia comprised more than 95% of the mycoflora at the end of processing. *P. nalgiovense* accounted for 50%, *P. olsonii* for 15%, *P. chrysogenum* for 10% and *P. verrucosum* for 5%. On 55 sausages from small scale North Italian producers 30 were dominated by *P. verrucosum* var. *cyclopium* and five were dominated by *P. chrysogenum*, and out of 20 industrially produced sausages 15 were dominated by *Aspergillus candidus* and five were dominated by *P. chrysogenum* (Grazia et al., 1986). Quite differently Jirkovski and Galgóczy (1966) found that out of 59 perfectly ripened Hungarian winter salamis *Scopulariopsis brevicaulis* var.*alba* were found on 47 salamis (80%), *Penicillium camenberti* on 36 (61%), *Penicillium commune* on 35 (59%) and *Scopulariopsis brevicaulis* on 12 (20%), but it should be noted, that these sausages were lightly smoked before inoculation.

Fink-Gremmels and Leistner (1990) applied a multistage concept for selection of suitable starter cultures on 112 strains of *P. nalgiovense* isolated from mould-fermented meats and other sources. Only two strains proved toxic after the initial thin layer chromatography of chloroform extracts of cultures grown on malt extract agar when compared with 27 known *Penicillium* toxins. One produced cyclopiazonic acid, the other produced roquefortine. Forty-eight per cent of the remaining strains were evaluated as toxic in brine shrimp and mouse tests, and of the rest only 40% produced the acceptable white colonies. This left 14 strains for a salami application test, which reduced the practically applicable strains to eight. Re-examination for toxigenicity by oral dosing of mice and a multiple-dose-study in rats finally reduced the number of acceptable strains to five. A similar scheme for selection and evaluation of mould starter cultures was suggested by Rotheneder, Gareis, and Rödel (1996).

Hwang, Vogel, and Hammes (1993a, 1993b) began their selection among 166 isolates from sausages by discarding 128 isolates because of their technological insufficiency regarding colour and growth. Thereafter three biological test systems were applied (Artemia salina, chicken embryo and human cell culture) which reduced the acceptable cultures to seven strains of *P. chrysogenum* and two strains of *P. nalgiovense*. The performance of these strains was evaluated on sausages for competitiveness, resistance to abrasion, height and density of mycelium, growth on sausages with different casing materials and sensory impressions resulting in only two strains being suitable as starter cultures—one *P. chrysogenum* and one *P. nalgiovense*.

It is unclear whether *P. chrysogenum* is suitable as starter culture. On one hand Hwang et al., (1993a) found seven toxicologically safe isolates, and Lücke and Hechelmann (1987) mention a suitable starter isolated by Leistner, Hechelman, and Trapper but not made commercially available. On the other hand El-Banna, Fink-Gremmels, and Leistner (1987) found that 85% of 123 isolates of *P. chrysogenum* produced roquefortine C, and only one isolate passed all test for mycotoxins, antibiotics and toxicity toward brine shrimps and mice. Additionally growth above 15 °C stimulated green conidia formation, which is technologically unwanted. Also Frisvad and Filtenborg (1983) reported consistent production of roquefortine C by *P. chrysogenum*. At least it would be advisable to test the seven strains found by Hwang et al. (1993a) for their production of roquefortine C. Commercially marketed strains of *P. chrysogenum* from French producers were said to have been mutated and selected to yield white mycelia and conidia and to have higher temperature optimum (25–26 °C) than *P. nalgiovense* (22–23 °C) (Lücke & Hechelmann, 1987). However Leistner (1990) stated that unpublished reidentification in 1985 showed that all French starter cultures belonged to the species *P. nalgiovense*.

According to Lücke (1997) *Aspergillus* spp. can only compete with penicillia at relatively high storage temperature and low water activity, as found during aging of dry Mediterranean style hams, which are unlikely used during sausage ripening. This was supported by Incze, Mihályi, and Frank (1976) who found that if drying temperatures were at 15 °C or below *Scopulariopsis* and penicillia outgrow aspergilli on Hungarian salami. Andersen (1995) found that only a few percent of the houseflora on North Italian sausages belonged to aspergilli. A market research (Sunesen, 1999) revealed that in Europe at least nine companies supplies around 30 different mould starter cultures, all belonging to the genus *Penicillium*. Often the product declarations only stated ''Penicillium spores'', but on 15 out of 20 products with specification of species *P. nalgiovense* was declared. Three companies supplied *P. candidum* and two supplied *P. chrysogenum*.

3. Genetic modification

If a certain feature of any microorganism cannot be found by selecting among the natural reservoir of diversity, basically to types of genetic changes can be applied—mutation or genetic engineering. Mutations often lead to other genetic changes than the desired one. These secondary mutations may result in new and unknown negative side effects. Therefore mutated strains have to be evaluated carefully for technological and toxicological aspects. Geisen, Glenn, and Leistner (1989) stated that improvement of fungal starter strains

could be achieved through recombinant DNA technology or protoplast (cell without cell wall) fusion, and documented how formation and regeneration of protoplasts could be achieved though hydrolytic enzymes and enzyme mixtures like chitinase, β-gluconase, snail gut enzymes or a combination of Novozyme 234 and β-glucuronidase. Further Geisen and Leistner (1989) described the successful transformation of *P. nalgiovense* with the dominant selectable marker *amdS* gene from *A. nidulans*. This was an important step in the isolation and subsequent analysis of regulation of genes in *P. nalgiovense* since no definite mutant strain was available for the incorporation of foreign DNA by a complementing marker. With this method the lysostaphin gene was introduced to *P. nalgiovense*, which afterwards could inhibit *S. aureus* (Geisen, Lücke, & Kröckel, 1991). In effect bacteriocin production was expanded from the usual lactic acid bacteria base to include mould starter cultures. Examples like this could help assuring microbiologically safer products in the future. Gene multiplication and other genetic alterations may also lead to increased production of proteases, lipases, catalases, nitrate reductases, etc. (Leistner, Geisen, & Böckle, 1991). Besides legal regulation, the only limitation seems to be consumer acceptance, which in recent years clearly have lacked in spite of the evident positive possibilities. If instead unwanted features are present in a given microorganism, another helpful technique is gene disruption. This technique may inactivate undesired genes like those responsible for mycotoxin or penicillin formation (Geisen, 1993).

4. Application

Commercially available mould starter cultures are supplied as either concentrated hypertonic liquid suspensions or freeze-dried powder. According to Andersen (1994) freshly harvested spores gives faster colonisation on sausage surfaces, but this advantage must be compared to the shelf life of liquid cultures ranging from 6 weeks to 6 months at 0–5 °C, and the freeze dried powders storable for up to 2 years below −18 °C (Jessen, 1995). Freeze-dried powders are recommended to resuscitate for several hours before use to allow faster colonisation, but solutions should be prepared daily, since spores in suspension rapidly die out. After dilution cultures are applied either by spraying with or dipping in solutions of approximately 10^6 colony forming units (CFU)/ml water (Jessen, 1995)

Andersen (1994) found that by dipping highest CFU/cm^2 numbers were transferred to the casings at the beginning of a batch. Using spore concentrations of 10^5, 10^6 and 10^7 CFU/ml resulted in application of approximately 2×10^3, 2×10^4 and 2×10^5 CFU/cm^2. This means that only 1/50 of the concentration of starterculture (spores/ml) is applied on the sausages in an upright position (spores/cm^2). Addition of sugar or protein to the suspensions did not increase transfer rate, and no notable difference was observed between natural casing and cellulose fibre casings. Growth capacity measured as mycelial density was significantly better for natural casings especially in the beginning of the growth period (Andersen, 1994). Roncales, Aguilera, Beltran, Jaime, and Peiro, (1991) found deeper and larger colonisation on natural casing than on collagen casing, and they demonstrated that while the CFU/cm^2 was 100–1000 initially, it increased during the 10 first days to between 10^6 and 10^7. Toledo, Selgas, Casas, Ordóñes, and Garcia (1997) also found colonization most intense on natural casings when compared with collagen casings.

During initial processing the humidity immediately above the sausage surface should be considered carefully. If cold sausages are brought to warm and moist drying rooms, condensing water might rinse away conidia. During the fermentation period the combination of air humidity and air speed should be observed. If water activity on casing surface drops below 0.80 neither germination nor growth will take place (Samson et al., 1995).

Light smoking of sausages before inoculation is occasionally conducted in Hungary (Lücke, 1997), in that case moulds inoculation should be retarded until the cyclic smoke compounds toxic to moulds have evaporated. Depending on the combination of mould starter culture and drying conditions moulds will start colonisation of the sausage casing between 3 days and several weeks after inoculation (Leistner, 1986a, 1986b). Spores of *P. roqueforti* developed germ tubes within 12–15 h in a rich medium containing carbon and nitrogen sources at pH 6.5 and 26 °C (Fan, Hwang, & Kinsella, 1976). Hereafter the tubes grew approximately 0.5 mm per day. Since moulds breakdown lactate and release ammonia from protein and thereby rises pH, care should be taken, that pathogenic bacteria like *Staphylococcus aureus* are previously inhibited by a suitable pH drop. Rödel, Stiebing, and Kröckel (1993) documented the pH-increasing effects of mould inoculation, especially in sausages with small diameters, and suggested, that additional sugar might be added to allow lactobacilli to decrease pH sufficiently in small-diameter-sausages. Those observations were supported by Grazia et al. (1986), who also detected a pH increase caused by moulds. The pH-increase was highest near the edge but mycelium was found even in the innermost parts of sausages.

Filtenborg and Frisvad (1988) stated that moulds normally are not considered as allergic, but for employees in industrial environments with high spore concentrations in the air allergic alveolitis have been reported for *P. roqueforti*, *P. camemberti*, *P. commune*, *P. nalgiovense* and *P. chysogenum*. The normal procedure of brushing

or blowing conidia off the sausage before or during packaging expose workers to heavy loads of conidia and severely endanger the working environment.

5. Aroma formation by mould starter cultures

Several authors have described a large range of volatile compounds found in fermented sausages with mould covering (Mateo & Zumalacárregui, 1996; Meynier, Novelli, Chizzolini, Zanardi, & Gandemer, 1998; Schmidt & Berger, 1998; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2002; Sunesen, Dorigoni, Zanardi, & Stahnke, 2001). But very few authors comment the influence of the mould growth on the composition of volatiles. Other studies have compared the effect of inoculating sausages with different moulds. Hwang et al. (1993a, 1993b) focused on technological aspects, but included sensory assessments of mould flavour and external appearance. These revealed only limited differences between 6 *Penicillium chrysogenum*, one *P. nalgiovense* and one unspecified *Penicillium* strain on their contribution to taste and aroma. Singh and Dincho (1994) compared two *Penicillium camemberti* strains with a *P. nalgiovense*. They found no differences on colour, consistency and overall acceptability, but the *camemberti* strains were superior on flavour and taste. Geisen et al. (1991) described unpublished results where 11 strains of *P. nalgiovense* were compared and showed marked differences in their ability to influence sausage aroma, but no qualitative descriptions were presented. Garcia, Casas, Toledo, and Selgas (2001) compared one *Mucor*, one commercial *Penicillium nalgiovense* and two unspecified *Penicillium* strains by their effect on aroma, colour, texture, taste, external appearance and overall acceptability in experimental batches of fermented sausages. A correlation between proteolytic and lipolytic activity of moulds and overall acceptability was shown. While the commercial *P. nalgiovense* had superior external appearance, it had lowest enzymatic activity and was evaluated lowest on the other sensory parameters. Hence, the authors proposed the other strains to be incorporated into commercial starter cultures.

5.1. Primary and secondary metabolites

Mould metabolites are as other microbial metabolites categorised into primary and secondary. Primary metabolites are amino acids, vitamins, nucleic acids, and intermediates from the citric acid cycle, etc. (Hwang, 1991), most of them important in the essential growth processes in all moulds. Secondary metabolites are much more restricted in their distribution among mould species, usually they are produced by only 1–15 species (Frisvad, 1989). This may be utilised in the taxonomic classification of the species as demonstrated by Frisvad and Filtenborg (1983). The secondary metabolites are ecologically important in chemical signalling between mould species (Wicklow, 1988), and the reason for formation of many secondary metabolites by moulds should be sought in their co-evolution with insects since they have co-existed 100 million years longer with insects than mammals (Dowd, 1992). Therefore mycotoxins probably serve primarily as chemical defence system against predatory insects. Volatile compounds may act as repellents in this defence system, and may additionally affect or even inhibit competitive fungal growth and sporulation (Wicklow, 1988). Using volatile compounds to interact with surroundings increases the operative range of the compounds significantly compared to the slow diffusion found in substrates that moulds normally colonise. Carbon dioxide is the mould metabolite most often reported as affecting growth and metabolism of other microorganisms (Hutchinson, 1973), but secondary volatile metabolites are also thought to affect mycological interactions (Andersen, 1994).

5.2. Enzymatic protein degradation and lipolysis

The influence of moulds on sausage aroma is beside their antioxidative effect mainly their ability to produce enzymes for the degradation of lipid- and protein-matter. According to Geisen et al. (1991) both exolipases and exoproteases are primarily produced in cases of shortage of nutrients in the mould-microenvironment, but are also produced in lower amounts in the nutritive sausage environment. The studies of this area in sausages are scattered, and not nearly as detailed as those performed in cheese.

5.2.1. Protein degradation

Geisen (1993) found positive correlation between the aroma intensity in mould-ripened sausages and the degree of proteolysis. The higher proteolytic abilities among 11 strains of *P. nalgiovense* the more intense aroma formation. Additionally electrophoretic investigations of the protein composition revealed differences in the band pattern, which was taken as an indication of the proteases influence on aroma. Trigueros, Garcia, Casas, Ordóñes, and Selgas (1995) compared the proteolytic activity of nine *Penicillium* strains, one *Mucor* and one *Aspergillus oryzae* against both myofibrillar and sarcoplasmic proteins in two different media. The study showed, that the proteolytic capability of the strains differed up to 30-fold and were highly dependent on media, pH and temperature. Toledo et al. (1997) further compared some of the same strains plus a commercial *P. nalgiovense* strain on experimental sausage batches. These authors found that the commercial culture performed best in providing a uniform exterior, while inoculation with the strains termed *Penicillium* 3, 6 and 7, respectively produced significantly higher amounts of

both water soluble nitrogen, non protein nitrogen and phosphotungsten-acid-soluble nitrogen. The amount of free amino acids doubled and tippled in control batches of artificial and natural casings, respectively. These increases were thought to reflect the activity of exoproteases, though it could not be concluded whether they were of microbial of indigenous meat origin. In inoculated sausages its noteworthy that the strains *Penicillium* 3 and 6 were thought to be able to decarboxylate free amino acids, a feature normally ascribed to bacteria (Toledo et al., 1997).

According to Lücke (1997) amino acids and nitrogen containing compounds add only to sausage taste, not to the sausage aroma. This statement was built on the observation that only few volatile nitrogen containing compounds have been identified in fermented sausages. However the deamination of amino acids by mould is thought to be responsible for the ammonia smell in sausage ripening rooms in Southern Europe (Bacus, 1986). Grazia et al. (1986) also addressed nitrogen metabolism in sausages, but in inoculated experimental sausages with nine different *Penicillium* strains and one *Aspergillus* strain, they only found significant increases in amount of ammonia and water-soluble nitrogen after fermentation in a few strains, while for other strains the amounts had decreased. Recently Bruna, Ordóñez, Fernandez, Herranz, and de la Hoz (2001) found that *Penicillium aurantiogriseum* and and a cell free extract of the same strain increased the concentration of ammonia in sausages with approximately 40 and 20% respectively. Concurrently the concentrations of free amino acids increased 50%. In the outer parts of sausages amines are formed concurrently with ammonia, but though moulds are probably responsible (Lücke, 1997) and though biogenic amines can cause allergies, concentrations found in mould-fermented sausages are much lower than in fish, and therefore should only pose a very limited health risk.

5.2.2. Lipolysis

Only few studies have addressed the contribution of moulds to lipolysis in fermented sausages. Iwai and Tsujisaka (1984) investigated lipases from *Aspergillus niger*, *Rhizopus delemar*, *Geotrichum candidum* and *Penicillium cyclopium* and found marked differences in both production and action of the lipases. Production of lipases is strain specific, depending on substrate, pH and temperature, but the activities of lipases also depend on these factors. In substrates lipases from different moulds exert different specificity towards fatty acids and even positional specificity towards the ester bonds in triglycerides. On pork fat Trigueros et al. (1995) showed that the lipolytic activity was very scarce when moulds were previously grown on malt extract agar, whereas previous growth on an enrichment media yielded lipolytically active mould strains. The results also documented that the fungal lipases predominantly hydrolysed triglycerides containing short-chain fatty acids, but the strains investigated were also able to hydrolyse pork fat. The strains with highest activities were studied further by Selgas, Casas, Toledo, and García (1999) in a series of sausages experiments. The studies showed that the above mentioned strains *Penicillium* 3 and 6 not only possessed high proteolytic abilities, but these strains also mediated high levels of free fatty acids (1.8 and 2.2 g/100 g dry matter) compared with control samples (0.5 g/100 g dry matter). The other strains, all selected for their lipolytic capabilities in vitro did not show significant activities in sausages. The study on *P. aurantiogriseum* by Bruna, Ordóñez, et al. (2001) showed that while concentrations of free fatty acids increased 2–3 fold during a 22-day ripening period when sausages where surface inoculated, concentrations only increased slightly when cell free extracts were added. Accordingly lipolysis is partly dependant on live mould. According to Iwai and Tsujisaka (1984) lipolysis occurs at the oil–water interface, and therefore the velocity of the hydrolysis of ester-bonds is a function of the surface of lipid substrate. In sausages this would mean that if sufficient lipases were present, lipolysis and aroma-formation would increase with the degree of mincing since this would increase the contact area of lipid and protein particles, but no literature have documented this.

5.3. Enzymatic aroma formation

As mentioned above, only few studies have addressed the aroma impact of mould growth on sausages specifically, but information from related areas like milk-based systems or sausage models still gives a quite clear picture of which compounds are important and their possible routes of formation. The above described degradation of protein and lipid matter is often a prerequisite for further enzymatic metabolisation leading to formation of aroma compounds.

5.3.1. Methyl ketones

The presence and production of methyl ketones in moulded sausages is often ascribed to the β-oxidation activities of moulds. This assumption is probably mainly based on intensive experimentation with spores and mycelium of *P. roqueforti* conducted in milk-based systems, reviewed by Hawke (1966), Kinsella and Hwang (1976) and Grosch (1982). Methyl ketones play significant roles in the flavour of Blue cheeses, and also seem important for fermented sausage flavour (Berdagué et al., 1993). However, in the studies of fermented sausages only little attention has been devoted to possible pathways for production of methyl ketones. Therefore knowledge from moulded cheeses has to be interpreted into the fermented sausage contexts. Okumura and Kinsella (1985) found that *P. camemberti*

produced 60% of its carbonyl compounds on a milk-based medium as methyl ketones, and that the majority of moulds from the *Penicillium* and *Aspergillus* genera were able of producing methyl ketones, including *P. nalgiovense*.

Starkle (1924) was the first to report of moulds being able to convert fatty acids to methyl ketones. Subsequent work has shown that in *P. camemberti* methyl ketones are products by β-oxidation of fatty acids liberated from triglycerides as schematically outlined in Fig. 1. The oxidation presumably proceeds along the lines of mammalian β-oxidation until the formation of a ketoacyl-CoA complex (Stryer, 1988). But apparently the mammalian split of β-ketoacyl CoA by thiolase into acetyl CoA and an acyl CoA with two less carbon atoms, is substituted by deacylation by thiohydrolase into a β-keto-acid. The following decarboxylase produces 2-alkanones so rapidly that this has been suggested as the driving force for the abstraction from the normal β-oxidation (Kinsella & Hwang, 1976), see Fig. 2.

β-Ketooctanoyl-CoA is the preferred substrate for the deacylation reaction, but all short or medium chain fatty acids are deacylated, and long-chain fatty acids may also yield 2-alkanones after successive cycles of β-oxidation (Okumura & Kinsella, 1985). Reduction of 2-alkanones to 2-alkanols proceeds easily in the presence of *P. camemberti*, and may serve both as minimising the toxic effects of methyl ketones, but also a method for regeneration of the oxidised nucleotides NAD and NADP (Kinsella & Hwang, 1976).

- Fatty acid + ATP $\rightarrow$ acyl adenylat + pyrophosphat
- acyl adenylat + CoA $\rightarrow$ acyl-CoA + AMP
- acyl-CoA + FAD $\rightarrow$ enoyl-CoA + FADH
- enoyl-CoA + H_2O $\rightarrow$ L-hydroxyacyl-CoA
- L-hydroxyacyl-CoA + NAD^+ $\rightarrow$ ketoacyl-CoA + NADH + H^+

Fig. 1. The first steps in fatty acid oxidation in moulds. ATP = Adenosine triphosphate, CoA = coenzyme A, AMP = adenosine monophosphate, FAD = flavin-adenine dinucleotide, NAD = nicotineamide-adenine dinucleotide.

Hwang, Lee, and Kinsella (1976) demonstrated that the relative specific activity of β-ketododecanoyl decarboxylase increased when spores germinated, and again as spores grew into mycelium. But mycelium is more sensitive to the toxic effects of fatty acids and spores may therefore still play a significant role in methyl ketone formation when fatty acids are present in concentrations above the 1 mM optimum for mycelium (Kinsella & Hwang, 1976). When the concentration of fatty acid to mycelium is low the work of Lawrence and Hawke (1968) showed that complete oxidation to CO_2 prevailed, while increasing the ratio increased methyl ketone production. Production of 2-heptanone and 2-nonanone can also be increased by addition of glucose and L-leucine to spore suspensions with semi synthetic triglycerides (Demyttenaere, Koninckx, & Meersman, 1996). Methyl ketones may also be produced from β-keto-alkanoic acids esterified in triglycerides undergoing hydrolysis and decarboxylation to yield methyl ketones (Kochhar, 1993). The concentration of β-keto-alkanoic acids in milk-fat is 0.045%. This pathway seems to be the explanation for slowly increasing levels of methyl ketones found in Cheddar cheese, as opposed to high levels found in early stages of mould-ripened cheeses.

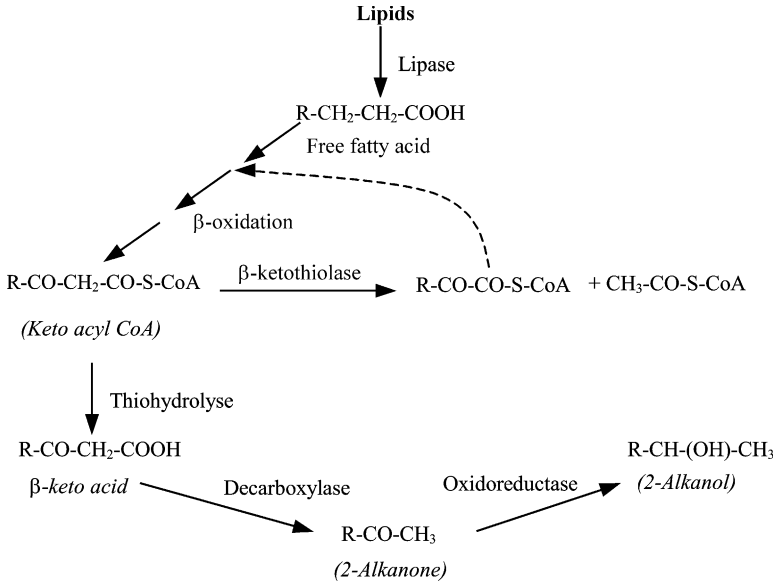

Fig. 2. Formation of 2-alkonones and 2-alkonols from lipids. Reproduction from (Grosch, 1982).

5.3.2. 2-Butanone

Some studies indicate that 2-butanone is produced differently in sausages than the longer methyl ketones. Namely by degradation of diacetyl and acetoin via 2,3-butanediol (Margalith, 1981). It has been shown in moulded Italian sausages that 2-butanone disappeared from a level of 50 ng octane equivalents/g during 40 days of maturation. Whereas methyl ketones from C_5 to C_9 all increased from levels around 100 ng up to as high as 8250 and 4133 ng for 2-heptanone and 2-nonanone respectively (Sunesen et al., 2001). Bruna, Hierro, de la Hoz, Mottram, Fernández, and Ordóñez, (2001) found 2-butanone to be present in control samples at 3.2 ng/g sausage, while sausage covered with *P. aurantiogriseum* contain no 2-butanone. On the other hand, in French sausages 2-butanone increased from 0.2 to 6.8 ng nonane equivalents/g during the 55 days of production (Croizet, Denoyer, Tran, & Berdagué, 1992). Likewise, concentrations of 2-butanone increased in control samples of meat and fat sausages models, but increases were significantly higher with *P. nalgiovense* inoculation (Sunesen, Trihaas, & Stahnke, submitted for publication-b). Production of 2-butanone also characterised *P. nalgiovense* in Petri dishes (Jacobsen & Hinrichsen, 1998) and model sausages (Sunesen, Trihaas, & Stahnke, submitted for publication-a).

5.3.3. Methyl ketones from 2-pentanone to 2-tridecanone

Over 50 days of production of commercial Italian moulded sausages concentrations of 2-pentanone increased from 101 to 267 ng octane equivalents/g, 2-heptanone increased from 211 to 8250 ng octane equivalents/g, 2-octanone increased from 67 to 690 ng octane equivalents/g and 2-nonanone increased from 75 to 4122 ng octane equivalents/g (Sunesen et al., 2001). In French moulded sausages 2-pentanone increased from 0.7 nonane equivalents/g to 2.8 ng, while 2-heptanone concentrations remained around 0.4 ng during the 55 days of production (Croizet et al., 1992).

Several reports of mould degradation of methyl ketones are published. Fan et al. (1976) report that 2-heptanone is degraded by *P. roqueforti* when spores or mycelium is present in high concentrations in a potassium phosphate buffer. Yagi, Kawaguchi, Hatano, Fukui, and Fukui (1990) showed that *P. decumbens* produced 119 mg methyl ketones (C_7, C_9, C_{11} and C_{13}) from 100 ml of 1% palm-kernel oil medium during the first 4 days of cultivation, but afterward metabolised these again, leaving a total of only 16 mg after 10 days. Likewise *P. camemberti* and *P. caseifulvum* growing in liquid cream media produced large amounts of methyl ketones from 2-butanone to 2-undecanone during the first 10 days of growth. During the next 5 days of growth, were mycelia concentrations were high, the same methyl ketones disappeared (Larsen, 1998a). In Spanish sausages significantly lower concentrations of 2-pentanone and 2-heptanone were found in mould covered samples than in control samples (Bruna, Gierro et al., 2001). This study is the only one describing decreases in concentrations of methyl ketones longer than 2-butanone, and no comments were made on the degree of mould growth on the sausage surface.

5.3.4. Aliphatic eight-carbon compounds

Volatile compounds produced by moulds have been used as an important tool in fungal taxonomy (Larsen, 1998b) because each strain consistently produces a certain set of volatiles on every well defined media. Changing the media will often be reflected in either quantitative or qualitative changes in the composition of volatiles. Moulds are often reported to produce a number of aliphatic eight-carbon compounds. Among these is 1-octene-3-ol which in edible fungi have been shown to originate from linoleic acid (C18:2) and be biosynthesised through two enzyme-catalysed reactions (Wurzenberger & Grosch, 1982). Even if actual data for enzymatic production in moulds have not been reported, the pathway is regarded as parallel to that for mushrooms (Karahadian, Josephson, & Lindsay, 1985). Production has been found to begin simultaneously with depletion of glucose for *P. caseicolum* on a synthetic medium (Spinnler, Grosjean, & Bouvier, 1992) coinciding with increases in concentration of vinyl benzene (styrene), another secondary metabolite. In sausages Bruna, Hierro et al. (2001) found that *P. aurantiogriseum* growth correlated with higher concentrations of 1-octen-3-ol, while the addition of a cell free extract did not show this correlation. The flavour of 1-octen-3-ol characterises mushrooms, and at the same time constituted respectively, 78 and 82.5% of all flavour compounds extracted from the mushrooms *Agaricus bisporus* and *Boletus edulis* (Kaminski, Stawicki, & Wasowicz, 1974). The same authors showed that 1-octen-3-ol was also produced effectively by a number of *Penicillium* species along with 3-octanone, 3-octanol, 1-octanol and 2-octen-1-ol on sterile coarse wheat meal (Kaminski et al., 1974). Moulds growing on oatmeal agar and producing musty aroma revealed strong correlation to high concentrations of 1-octen-3-ol, dimethyl-disulfide and 2-methylisoborneol (Börjessen, Stöllman, & Schnürer, 1993), but also compounds like geosmin and 3-octanone contribute to mould-produced musty aroma. On potato dextrose agar *P. camemberti* and *P. caseicolum* also produced large amounts of 1-octen-3-ol, but also other eight-carbon compounds such as 1,5-octadien-3-ol, 1,5-octadien-3-one, 3-octanol and 3-octanone (Karahadian et al., 1985). Those compounds were described as mushroom- or green-plant-like, while 2-methylisoborneol strongly contributed to the overall musty/

mouldy aroma together with 2-methoxy-3-isopropylpyrazine. Harris, Karahadian, and Lindsay (1986) also found 1-octen-3-ol to contribute to the overall musty aroma together with other eight-carbon compounds when *P. roqueforti* grew on agar and bread. But in this study 2-methylisoborneal was described as giving an old-spoiling fruit or cellar-like mustiness.

5.3.5. Aroma compounds from amino acid degradation

On meat extract-, glucose-, peptone and maize extract-media *P. nalgiovense* consistently produced 1-octanol, 3-octanone, 2-butanone, 2-methyl-propanol, 3-methyl-butanal, 3-methyl-butanol and 2-methyl-butanol (Jacobsen & Hinrichsen, 1998). The last four compounds are normally ascribed to bacterial amino acid metabolism. Resent results by Sunesen et al. (submitted for publication-b) showed that on meat agar media *P. nalgiovense* is capable also of increasing concentrations 2-methyl-propanol and 2-methyl-propanal. On inoculated meat media these authors found lower concentrations of 2-methyl-butanal, 3-methyl-butanal and 3-methyl-butanol than on uninoculated meat media, indicating consumption rather than production by *P. nalgiovense*. In sausages Bruna, Hierro et al. (2001) found that high concentrations of 2-methylpropanal and 2- and 3-methylbutanal were correlated to surface growth of *P. aurantiogriseum*. The authors stated two reason; higher amino acid substrate concentrations due to increased proteolysis near the sausage edge, and the L-amino oxidase activity of moulds. Stahnke (2000) identified a characteristic popcorn odour in mould fermented sausages as 2-acetyl-1-pyrroline, and found significantly higher concentrations of this compound near the edges. It was suggested, that moulds were responsible for conversion of proline often found in sausage collagen casings to 2-acetyl-1-pyrroline.

6. Concentrated fungal enzymes as additives

Fungi are particularly valuable for industrial enzyme production among microorganisms because most of their enzymes are extra-cellular, which makes isolation of the enzymes much easier (Iwai & Tsujisaka, 1984). Additionally sausage producers are interested in using enzymes for shortening ripening periods, improving aroma and texture or even expanding shelf live. Several studies have been published within this field, and though some positive results have emerged it is clear that the use of enzymes as additives in sausages production is not straightforward.

Díaz, Fernández, García de Fernando, de la Hoz, and Ordóñez (1997) added aspartylprotease from *Aspergillus oryzae*, Pronase E from *Streptomyces griseus* and papain to accelerate sausage maturation. They found increasing effect by increasing the amounts of proteases ranging from almost no effect to sausages with excessive softness and high levels of non-protein nitrogen, free amino acids and amines. Similar results were obtained by using pancreatic lipases, which led to increased concentrations of free fatty acids (Fernández, de la Hoz, Díaz, Cambero, & Ordóñez, 1995a, 1995b). However in both cases the authors found only slight improvements in the flavors produced, and therefore concluded that either improved bacterial starter cultures, or other enzymes capable of the further degradation of free amino acids or free fatty acids into volatiles compounds had to be used.

Zapelena, Astiasarán, and Bello (1999) found that adding a protease from *Aspergillus oryzae* to sausages had two significant effects: (1) it lowered pH, probably because the products of protease activity can increase the metabolic activity of bacterial starter cultures and thereby lower pH and (2) it increased the amount of free amino acids. Though some effects in the sensory assessment were statistical significant, it was thought unlikely that they would have been detected by consumers. Zalacain, Zapelena, Peña, Astiasarán, and Bello (1999) tested three levels of the lipase Palatase from *Rhizomucor miehei* and found significant increases were generated for free fatty acids, juiciness and pleasant taste by this addition, but the overall acceptability was not affected. (Bruna, Fernández, Hierro, de la Hoz & Ordóñez, 1999; Bruna, Fernández, Hierro, Herranz, Ordóñez, & de la Hoz, 1999) tried to further degrade the accumulated free amino acids and free fatty acids by adding both the protease Pronase E and extracts of *Mucor racemosus*. The fungal extract supposedly contained both lipases and proteases, but more importantly for aroma formation also the deaminases, transaminases and dehydrogenases that could participate in free amino acid catabolism and improvement of sausage flavour and aroma. Indeed the addition of mould extract increased concentrations of branched aldehydes (2- and 3-methylbutanal, 2-methylpropanal) and their corresponding alcohols. But sensory results for sausages added Pronase E and those added both Pronase E and fungal extract could not be differentiated. The best sensory results were obtained with surface inoculation of the *Mucor* strain combined with addition of the enzymatic extract. The results were later repeated with extracts of *Penicillium aurantiogriseum* and Pronase E. Again sausages with both extract and Pronase E contained highest amounts of the volatiles free amino acid breakdown compounds (Bruna, Fernandez, Hierro, Ordóñez, & de la Hoz, 2000). Several of the earlier-mentioned authors faced decreased textural cohesiveness with addition of proteases. The gel formation properties of proteins may be the upper limit to the use of enzymes in sausages.

7. Unwanted secondary metabolites

7.1. Mycotoxins

A significant part of the secondary metabolites produced by moulds must be regarded as mycotoxins. Mycotoxins are normally defined as those secondary metabolites that in small concentrations are toxic to vertebrates and other animals when introduced via a natural route (Samson et al., 1995). According to this definition antibiotics are not mycotoxins and hence are discussed separately later. When food shows signs of mould growth, there is a possibility of mycotoxin presence, but the mycotoxin formation is influenced by substrate composition, temperature, water activity, pH, etc. (Leistner, 1986b). Therefore it is impossible to conclude backwards that mycotoxins are present because moulds are present. Mycotoxins are a heterogeneous group of mainly nonpolar, low-molecular-weight and chemically rather stable molecules, therefore removal of spores and mycelia does not ensure toxicological safe products. According to Leistner (1984) mycotoxins in meat products are found in the first 5 mm below the surface. Scheuer (1995) only found verrucosidin in *P. aurantiogriseum* mycelium and casing of sausage, after inoculation and normal sausage ripening with potent verrucosidin producing strains. Diffusion of mycotoxin into the outer 2 mm sausage could not be detected. The biological effect of mycotoxins must be divided into two. The dose-dependant effects, for instance specific damage to liver or kidneys, is not likely to occur in foods as threshold value are very high compared to actually found levels. Effects for which no threshold value can be defined, like carcinogenic, immunologic (suppression of the immune system) and sensitising (allergy causing) effects, are of much more relevance in the context of human food ingestion (Fink-Gremmels, El-Banna, & Leistner, 1988). Here the carry over of mycotoxins accumulated in animal feed to animal tissue, or from animal tissue to human food should be noticed, this effect may increase ingestion considerably as shown for Aflatoxine A, Ochratoxin and other compounds (Leistner & Eckardt, 1981).

Strains of penicillia are the most frequently isolated mould on both "naturally" and intentionally inoculated sausages. Out of 793 tested Hungarian salamis ripened approximately for 2 months at 10–13 °C and 80–85% relative humidity none were found mycotoxin-positive in a chicken embryo test or chemical tests for aflatoxins, sterigmatocystin, penicillic acid, ochratoxin, patulin and citrinin (Incze et al., 1976). Even inoculation with potent aflatoxine and sterigmatocystin builders like *Aspergillus flavus*, *A. parasiticus* and *A. versicolor* yielded mycotoxin positive results, and therefore the ripening parameters were regarded as safe protectors towards mycotoxins. Even though the same ripening parameters were not followed, the work of Leistner and Eckardt (1981) points in a more concerning direction. The authors showed that 75% of the penicillia isolated from "houseflora" of commercial sausages are potential mycotoxin producers. Further, out of 15 mycotoxins investigated and produced in culture media 10 were also produced in meat products. Likewise Leistner (1984) describes to investigation of 1481 *Penicillium* isolates from various foods and feeds. Considering both chemical and biological assays 1166 isolates (79%) were regarded as toxigenic. One part of the review described investigations of 48 Hungarian sausages, 89 Italian sausages and 38 West German sausages. Toxigenic isolates of *P. verrucosum* var. *verrucosum* and *P. verrucosum* var. *cyclopium* constituted 77% of Hungarian sausages, while *P. verrucosum* var. *cyclopium* and *P. chrysogenum* was the predominant species among the 66% of toxigenic moulds on Italian sausages. The West German sausages were dominated by *P. nalgiovense* which was found non-toxic, but still *P. verrucosum* var. *cyclopium* was detected on 21% of the sausages, this strain is capable of producing cyclopiazonic acid. These studies demonstrate the tight connection between control over species and strain population on sausage surface and consumer safety. To ensure optimal consumer safety starter cultures should be applied to achieve maximal control over the mould population. Starter strains should under no circumstances shown pathogenic or toxigenic signs in neither chemical nor biological test. Additionally they should not be able to produce antibiotics, as discussed in detail below. Even though analysis standards for mycotoxins have been established, the increasing international trade with agricultural commodities highlights the need for harmonization on mycotoxin levels and methodology (Trucksess, 1998).

7.1.1. P. nalgiovense

Leistner and Eckardt (1981) investigated 38 *P. nalgiovense* strains, no strains produced any of the 20 mycotoxins tested chemically, but 14 strains showed toxicity in brine-shrimp- or chicken-embryo-tests. Only two studies described specific mycotoxins produced by *P. nalgiovense*. El-Banna, Pitt, and Leistner (1987) who claims to be the first to report on cyclopiazonic acid production, found this in one of 113 strains investigated. Fink-Gremmels and Leistner (1990) investigated 112 strains, and found one producer of cyclopiazonic acid and one producer of roquefortine, but immediately state that these strains could be taxonomically misinterpretated, and perhaps did not belong to the species *P. nalgiovense*. The authors mentioned no overlap in the isolates of these two studies.

Andersen (1994) found three secondary metabolites produced among 64 *P. nalgiovense* isolates. Chrysogine a metabolite also produced by the closely related *P.*

chrysogenum was produced by 96%, nalgiolaxin and nalgiovensin was produced by 9%. The last two compounds display orange colours and may be responsible for the characteristic orange reverse colour of *P. nalgiovense* grown on yeast extract-sucrose (YES) media. No known mycotoxins were found. Larsen and Breinholt (1999) described a range of isocoumarins detected in *P. nalgiovense* including dipodazine, dichlorodiaportin, diaportinol, diaportinic acid, citreoisocoumarin, 6-methyl-citreoisocoumarin but made not conclusions towards possible toxicity.

7.1.2. P. chrysogenum

As discussed earlier, controversy on the suitability of *P. chrysogenum* as starter cultures exist. While Hwang et al. (1983a, 1983b) and (Lücke & Hechelmann, 1987) described suitable strains, El-Banna et al., (1987) and Frisvad and Filtenborg (1983) found very high risk of either roquefortine C or biological toxic substance production by *P. chrysogenum*. According to Samson et al. (1995) some strains of *P. chrysogenum* also produce meleagrin. Whether antibiotics are considered mycotoxins or not, they are unwanted in food, and since most *P. chrysogenum* strains are strong producers of substances in this class like penicillin and negapillin (Samson et al., 1995), its use as starter culture should be based on thorough investigations.

7.1.3. P. camemberti

P. camemberti is commonly used in white cheese production, but all naturally occurring strains produce cyclopiazonic acid (Leistner, 1990). Among 20,000 mutants Geisen, Glenn, and Leistner (1990) only found two mutants that did not produce this mycotoxin. Cyclopiazonic acid was also found in meat products (Leistner, 1984) although in low amounts.

7.1.4. P. verrucosum

Whereas most contaminant mould strains are easily recognized on sausages due to abnormal colours, *P. verrucosum* like starter cultures produce a dense white mycelium and white spores. The neurotoxin verrucosidin produced by *Penicillium aurantiogriseum* (synonym *P. verrucosum* var.*cyclopium*) was found by Scheuer (1995) in mycelium of one out of four *P. aurantiogriseum* applied to fermented sausages. It was also found in the casing but not in the outer 2 mm of the sausage with this strain. Notably three out of the four strains produced verrucosidin in meat extract media. Some strains of *P. verrucosum* are reported to produce ochratoxins, a group of seven related isocoumarin derivatives linked to phenylalanine. Ochratoxin A, the most commonly encountered, is nephrotoxic, carcinogenic and immunotoxic (Pestka, 1995). Production on meat has not been reported, but accumulation in pig meat tissue under both experimental and natural conditions has been described after their ingestion of contaminated feed (Pestka, 1995). Samson et al. (1995) described citrinin production as less important than ochratoxin A in cases of porcine nephrotoxicity.

7.1.5. P. roqueforti

Cheese produced with *P. roqueforti* contained roquefortine C in ppm levels (Scott, 1981). Roquefortine C is consistently produced on agar media, but also mycophenolic acid is produced by 50–80% of isolates (Frisvad & Filtenborg, 1983). The same authors showed production of patulin, an undefined PR-toxin and penicillic acid.

7.2. Antibiotics and antagonistic substances

Increasing evidence for production of antibiotics in moulded cured meats has accumulated over the years. Ciegler, Mintzlaff, Weisleder, and Leistner (1972) found that 10% of 346 *Penicillum* cultures were capable of producing penicillic acid. Inoculation of five penicillin producing strains to sausages did not produced measurable amounts of penicillic acid. Penicillic acid added to sausages as positive control disappeared within 24 h, which seemed to be caused by reaction with amino acids. Two in vitro studies of P. *nalgiovense* isolates used as starter cultures or isolated from sausages confirmed production of penicillin for eight strains (Färber & Geisen, 1994) and 41 strains (Andersen & Frisvad, 1994), respectively. Notably the first study showed no penicillin production by a commercial *P. camemberti* strain. The latest work in this field demonstrated in situ production of penicillin in moulded dry cured sausages (Laich, Fierro, Cardoza, & Martin 1999). Five *P. nalgiovense* strains, including one commercial, produced penicillin in vitro. On sausages in early stages of ripening and coinciding with mould colonisation they detected 1.25 μg penicillin/cm^2 in casing, 0.66 μg/cm^2 in the outer 4 mm of sausage, 0.08 μg/cm^2 in depth 4–8 mm but nothing in the sausage core. In finished sausages they were not able to detect penicillin, and suggested that the prolonged exposure to acid pH may have caused inactivation. Although fungal β-lactam production could be helpful for insensitive starter cultures to assert dominance during colonisation, this feature is clearly unacceptable due to concern for cross-resistance in harmful bacteria and development of allergy by consumers.

Both in Petri dishes and in sausages it has been shown, that some strains of *P. nalgiovense* is able to inhibit *Listeria monocytogenes* (Glenn, Geisen, & Leistner, 1989). Within 21 days of ripening this inhibitory *P. nalgiovense* showed 100–400 fold faster reduction in *L. monocytogenes* in sausage edges than another *P. nalgiovense* without this property. The molecular nature of this antagonistic property was not specified, but con-

sidering the high pH often found for mould fermented sausages, selection of starters with this property could be one way to improve mould starter cultures in the future.

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