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Review

On the safety of *Mortierella alpina* for the production of food ingredients, such as arachidonic acid

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Abstract

Mortierella alpina is the most efficient production organism for arachidonic acid (AA) presently known. Since AA is being developed as a food ingredient, and since *M. alpina* has no history of use for such applications, we have undertaken this safety evaluation. *M. alpina* is a common soil fungus, to which humans are frequently exposed. The production strains are non-pathogenic and do not form potentially allergenic spores under production conditions. Moreover, there are no reliable reports in the literature connecting the species with disease or allergenic responses. No production of mycotoxins was observed, in line with the absence of literature reports describing such products, and with the results of toxicological tests. On solid growth media the strains showed antibiotic activity against Gram-positive bacteria. In submerged culture, which is used for AA production, no significant antibiotic activity was found. We conclude that *M. alpina* in general, and the AA production strains CBS 168.95 and CBS 169.95 in particular, should be considered safe for the submerged production of food ingredients. © 1997 Elsevier Science B.V.

Keywords: Safety; Filamentous fungus; *Mortierella alpina*; Arachidonic acid; PUFA; Single cell oil

1. Introduction

This paper addresses the safety of the use of the fungus *Mortierella alpina*, for the production of arachidonic acid (AA) for human nutrition. This comprises a literature review of the pathogenicity and toxigenicity of *M. alpina* and related species, and experimental tests for the pathogenic and toxigenic potential of production strains.

AA is a polyunsaturated fatty acid (PUFA), containing 20 carbon atoms and 4 double bonds [(all-*cis*)-5,8,11,14-eicosatetraenoic acid]. It is an important structural component of the lipids in the neural system, and it serves as the biosynthetic

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Table 1
Scope of safety assessment

(1) Taxonomy	The evaluation of the relevance of published data requires a taxonomy that is (1) stable and (2) phylogenetically sound. Furthermore, the taxonomic description of the producing strains under consideration should be unequivocal.
(2) Pathogenicity/ allergenicity	One needs to consider the pathogenic and allergenic potential of the producing organisms and related organisms. In the case of the production of arachidonic acid, where the producing organism is not present in the product, this is primarily relevant to the safety of the production personnel, and to the risk upon accidental release of the organism into the environment.
(3) Toxigenicity	In the case of moulds, many of which are able to produce persisting mycotoxins, this is the most critical safety aspect, since these toxins may be co-isolated with the intended product as impurities.
(4) Antibiotic by-products	One needs to consider whether by-products, other than the well-characterized mycotoxins and compounds that produce a demonstrable toxic response, could pose a risk to consumer, production workers or the environment.

precursor of several classes of biologically active molecules (German et al., 1996). It can be synthesized in the human body from linoleic acid (an essential fatty acid), but there is accumulating evidence that the rate of synthesis does not always satisfy the demand. AA can be obtained from the diet—being present in sources such as meat, fish and eggs—but the concentrations are low. Moreover, in specific cases these sources may not be suitable, as is the case for infant nutrition and parenteral nutrition, and for strict vegetarians. As a consequence there is an increasing interest in the production of a high-potency AA oil by fermentation for specific dietetic applications.

It is generally believed that filamentous fungi are the most promising sources for commercial production of AA (Radwan, 1991). In the past it has proved difficult to commercialize microbial lipid products (Ratledge, 1993). The specialty character of AA, and the absence of competing plant-derived products, leads to the conclusion that this may be the first fungal lipid product to meet with long-term commercial success, provided that the fungal source is regarded as safe.

From the literature it appears, that the filamentous fungus *M. alpina* is the most productive species (Bajpai and Bajpai, 1992; Eroshin et al., 1996). This mould is one of the so-called oleaginous fungi (Ratledge, 1993), capable of accumulating large amounts of lipid in their cells, which is a first prerequisite for efficient AA production. Moreover, of all the fungi described thus far, selected strains of this species have been reported to contain the highest proportion of AA in the lipid, up to 70% in solid state culture (Totani et al., 1987). Extraction of the mycelium after submerged fermentation yields a triglyceride oil, typically containing 30–50% AA (Ward, 1995).

Species of the genus *Mortierella* have not been used extensively for food production, but a number of examples exist. It has been mentioned that various *Mortierella* species are good producers of fats, oils and fatty acids that may be used as food and feed ingredients (Bigelis, 1991). This application is comparable to the production of γ-linolenic acid with *Mucor javanicus*, which has been exploited commercially in the 1980s. The ATCC list of Industrial Fungi (Jong et al., 1994) mentions *Mortierella* species as producers of several food-related products (apart from lipids), such as enzymes (α-galactosidase and lipase) and β-carotene. Most of these examples concern the species *M. isabellina*, *M. ramanniana* and *M. vinacaea*, which are currently no longer considered to be valid members of the genus *Mortierella* (see Section 2). On the other hand, related fungi within the Mucorales have a long track-record as a source of commercial food enzymes.

Considering that there is little experience with the use of *Mortierella* for food ingredients, it seems appropriate to consider the safety aspects of the organism in some detail. This safety assessment comprises the issues listed in Table 1. This 'organism-oriented' approach to the safety issue

should be used (1) to assess the risk to production workers and the environment and (2) as an addition to the safety studies performed on the products derived from the organism.

2. Taxonomy

The genus *Mortierella* is presently classified as a member of the family Mortierellaceae within the order of the Mucorales, class Zygomycetes (Hesseltine and Ellis, 1973). It is expected that this taxonomy will be changed in the near future, when the *Mortierella* group will be elevated to the rank of a separate order within the Zygomycetes: the Mortierellales (Gams, 1995).

The genus *Mortierella* is divided into 'sections' of particularly related species (Domsch et al., 1980). The AA producing species *M. alpina* is a member of the 'section' *Alpina*, along with *M. horticola*. Synonyms for *M. alpina*, that may be used in older literature, are *M. renispora*, *M. thaxteri*, *M. monospora* and *M. acuminata*. The genus *Mortierella* contains one recognized pathogen, the thermophilic species *M. wolfii* (Di Menna et al., 1972), which is a member of another section. Finally, it is worth mentioning that a group of species, *M. vinacaea*, *M. ramanniana*, *M. nana* and *M. isabellina* have long been suspected to be invalidly contained in the genus *Mortierella* (Domsch et al., 1980). Indeed, in due course these species will be placed in a separate genus *Umbelopsis*, and even in a separate family (Gams, 1995).

Gist-brocades' AA producing strains DS 30340 and DS 30341 have been deposited at the Centraalbureau voor Schimmelcultures (CBS, Baarn, The Netherlands) as CBS 168.95 and 169.95, respectively. These strains have been identified as belonging to the species *M. alpina* by Dr W. Gams of the CBS (Samson, 1995a).

3. Pathogenicity

3.1. The Mucorales

In general, members of the Mucorales have been associated with Mucormycosis (also: Hyphomycosis, Phycomycosis or Zygomycosis). In its most malign form, this is a fatal disease of the respiratory and gastrointestinal tract, which can proceed quite fast. The disease becomes fatal once the fungal mycelium reaches a vital organ, such as the brain or the liver. This disease is the result of an opportunistic infection, which means that the development of the disease is dependent on predisposition of the patient. Classic risk groups are uncontrolled diabetics and immunocompromised patients. The pathogenic agents are the spores of common saprophytic fungi, that are omnipresent in the environment (Emmons, 1962; Edwards, 1983; Scholer et al., 1983; Hall and Larsh, 1985; Mooney and Wanger, 1993; Nussbaum and Hall, 1994). Thus, exposure to these infective agents occurs frequently (Greer and Rogers, 1985; Hall and Larsh, 1985; Gollard et al., 1994; Nussbaum and Hall, 1994). Nevertheless, the disease is rare.

Infection with members of the Mucorales is easily distinguished histologically from other fungi. However, identification of family, genus or species is usually not possible (Scholer et al., 1983). As a consequence, one cannot rely on published identifications of specific species, unless the experimental procedures are disclosed. In general, all strains capable of growing at 37°C should be regarded as potentially pathogenic (Emmons, 1964; Greer and Rogers, 1985; Reiss, 1986), whereas strains that are unable to grow at body temperatures should be regarded as safe with respect to this disease (Edwards, 1983; Scholer et al., 1983). Indeed, this has been shown experimentally for various Mucorales: strains unable to grow at body temperature, were non-pathogenic to rabbits after nasal infection with spores (Reinhardt et al., 1970).

3.2. The Mortierellaceae

The Mortierellaceae are ubiquitous saprophytic fungi, which are easily and frequently isolated from soil. The pathogenic potential of the genus seems to be quite low. In a recent review on

uncommon mycoses in AIDS patients (Cunliffe and Denning, 1995), a number of Mucorales are mentioned, but not *Mortierella*.

In fact, the only reliable reports concern the species *M. wolfii*, isolated from mucormycosis-samples in cattle. As discussed above, this is connected with the ability of this species to grow at high temperatures, as opposed to the other *Mortierella* species.

3.2.1. Mortierella wolfii

M. wolfii is a well-known pathogen of cattle. The natural habitat of the organism is probably the soil in tropical areas. In the temperate zone, this species may be found in thermophilic environments, such as overheated, spoiled silage (Austwick, 1976).

The question, then, is how to discriminate this opportunistic pathogen from the other species of the genus. Although the morphological traits that define *M. wolfii* are quite characteristic, the determination requires a detailed morphological study (Seviour et al., 1987), including sporulation (MacDonald and Corbel, 1981; Johnson et al., 1990), undertaken by an experienced mycologist. This kind of investigation has usually not been performed in the reports found in the literature. In this connection, it is relevant that all isolates described in the literature have been isolated at temperatures of 35°C or higher (Carter et al., 1973). This again emphasizes that the most straightforward way to assess the pathogenic potential of a fungus is to determine its temperature tolerance, and that one should not rely too much on species determinations mentioned in the literature.

3.2.2. Mortierella alpina

All strains found in the culture collections, including the AA producing strains CBS 168.95 and CBS 169.95, have originally been isolated from soil samples, without association with animal material (Centraalbureau voor Schimmelcultures, 1987; Jong and Gantt, 1987). There is one exception: strain CBS 396.91 has been isolated from the air bladder of juvenile fish, so that association with pathogenesis of cold-blooded animals cannot be excluded entirely. However, the environmental risk of the release of biomass may be regarded as exceptionally low: *M. alpina* is a commonly isolated fungus of the soil of temperate climates, so that exposure to the spores of this organism occurs frequently.

In view of the safety of the production personnel, the pathogenicity towards warm-blooded animals is of particular importance. To assess the pathogenic potential of the species in a systematic way, all strains classified as *M. alpina* in the collection of the CBS were tested for their optimum and maximum growth temperatures. None of the strains, including some tropical isolates, showed growth at 36°C. The two AA production strains, CBS 168.95 and CBS 169.95, showed optimal growth at 21–24°C, whereas the maximum was at 30°C (Samson, 1995b). It is concluded that the species *M. alpina* in general, and the two AA production strains in particular, show no pathogenic potential towards warm-blooded animals.

3.2.3. References to Mortierella species in connection to suspected pathogenicity

As mentioned above, *M. wolfii* is the only currently recognized pathogen of the genus. This is due to the fact that only for this species the association of the organism with the disease has been proven conclusively. Nevertheless, there are reports, in the older literature, describing the isolation of fungi classified as *Mortierella* from humans or other warm-blooded animals with pathological symptoms, although these reports are few, and have never been followed up with more recent work. The cases are compiled in Table 2.

Today, none of the species mentioned is accepted as pathogenic (Edwards, 1983; Scholer et al., 1983) and it is believed that these reports represent either misclassifications, or identity with *M. wolfii* (Edwards, 1983; Scholer et al., 1983; Kwon-Chung and Bennett, 1992). In this connection it is striking that all experimental reports describing mycosis by species other than *M. wolfii* are older than 1968, which implies that they predate the characterization of *M. wolfii* as a pathogen in 1973. In this respect it must be realized that prior to this characterization, isolates of this or-

Table 2
Literature references for suspected pathogenicity of *Mortierella* species other than *M. wolfii*

Species description	Source	Reference
Mortierella sp.	Abortion in cows Abortion/pneumonia in cow Histological sections of cow	Van Ulsen, 1955 Munday, 1967 Harcourt and Thompson, 1969
M. alpina	Liver lesions in calves	Smith, 1966
M. hygrophila (nom. inv.) currently: *M. hyalina*	Lung mycosis in birds	Hörter and Hunsteger, 1962
M. mycetomi (nom. inv.)	Subcutaneous lesion in man	Nicolau and Evolceanu, 1947
M. niveo-luteum (nom. inv.)	Probably erroneous reference to *M. niveo-velutina*	Emmons, 1964
M. niveo-velutina (nom. inv.)	Ulcer of the leg in man	Ciferri and Ashford, 1929
M. polycephala	Lung mycosis in cow	Scholz and Meyer, 1965
M. zychae	Abortion/pneumonia in cow	Smith, 1966, 1968

ganism would be designated as *Mortierella sp.*, or would be classified within 'known' species (Ainsworth and Austwick, 1959; Cordes and Shortridge, 1968).

Three of the cases mentioned above deserve closer attention in view of the specific application in mind—the production of food ingredients with *M. alpina*: the two reports involving Man, and the one case mentioning *M. alpina*. With respect to Man, there are two reports, describing isolates from skin infections. The first report (Ciferri and Ashford, 1929) describes a subcutaneous infection by an organism described as *Mortierella niveo-velutina*, resembling the lesions caused by *Basidiobolus ranarum* (Kwon-Chung and Bennett, 1992). The other report (Nicolau and Evolceanu, 1947) describes a fungus isolated from an ulcer of the leg, which is atypical for mucormycosis, as *Mortierella mycetomi*. This species name is probably a mistranslation from *Madurella mycetomi* (W. Gams, personal communication). In view of the fact that in neither case the fungus isolated has been adequately described or determined according to accepted procedures, and since the strains were classified in taxa that have not been used since, there is no factual basis connecting these reports with strains currently classified as *Mortierella* (Edwards, 1983; Scholer et al., 1983)

With respect to *M. alpina* there is a report by Smith (1966), who isolated a fungus he considered to be *M. alpina* from liver lesions of slaughtered calves. He indicated that this was the first time that this organism was implied in disease, and it has remained the only report up to date. The publication is in fact not a research paper, but a letter asking for more material to investigate, and it does not give experimental detail. It seems never to have been followed up, although the author has published on mycotic infections later (Smith, 1968; Davey et al., 1973). Scholer et al. (1983) make the following comment concerning this report in their 'Annotated List of Species Not Accepted as Pathogenic': '*Mortierella alpina* and *Mort. zychae* (Smith, 1966); these fungi were probably *Mort. wolfii*.'

In conclusion, there are no reliable reports—reliable in the sense that they should be published in peer-reviewed journals, and describe the experimental procedures—that connect any species of *Mortierella*, other than *M. wolfii*, with the habitat of the warm-blooded animal. Furthermore, the ecological characteristics of the non-*wolfii* Mortierellas—isolation from soil, frequent exposure, no reliable reports of pathogenesis, temperature maximum below body temperature—lead to the conclusion that these fungi have an extremely low pathogenic potential towards warm-blooded animals (Lelieveld et al., 1995).

Despite this, the review literature contains a number of entries describing the genus *Mortierella* as pathogenic. Invariably, these reviews refer to the cases described in Table 2 (Hutter, 1959; Smith, 1968; Lyon et al., 1979; Reiss, 1986), refer to *M. wolfii* (Davey et al., 1973; Rippon, 1982; Knudtson and Kirkbride, 1992), or do not give reference at all (Emmons, 1964; Fleisher, 1974; Lehrer, 1980; Greer and Rogers, 1985; Hall and Larsh, 1985; Mooney and Wanger, 1993). We conclude that there is no scientific basis connecting these entries with *M. alpina*.

4. Allergenicity

It is well-known that fungal spores can be allergenic (Collins and Grange, 1990). There are no indications that the spores of *Mortierella* species pose a particular risk in this respect. Therefore, the standard safety procedures for working with sporulating fungi should be observed. Moreover, we have never observed sporulation in liquid culture in a range of *Mortierella* species, while the ability to sporulate on solid media varied strongly between species, and between strains of the same species (S.T.W. Tuinder and H. Streekstra, unpublished results). Generally, in industrial practice, solid media are used only in strain conservation, which is executed on small scale in specialized laboratories, whereas the production process is completely submerged. This is also the case for AA production with *M. alpina*. Thus, exposure to allergenic spores is all but impossible, and this applies both to production personnel and to consumers of the final product.

5. Toxigenicity

Filamentous fungi are able to produce a large number of secondary metabolites, which may show antagonistic effects towards various living organisms. The term 'mycotoxins' is usually restricted to those secondary metabolites that are toxic to vertebrates at low concentrations when introduced via a natural route (Frisvad and Thrane, 1995).

In general, the Mucorales are regarded as weakly toxigenic (Rippon, 1982). Toxigenicity has been described for some species of the genera *Rhizopus* and *Mucor*, and *Mortierella wolfii* (Reiss, 1993). But according to the definition given above, the only species known to produce a mycotoxin is *Rhizopus microsporus*, and the actual occurrence of mycotoxicosis due to Mucorales has never been documented (Frisvad and Thrane, 1995). On the other hand, Teuscher and Lindequist (1988) mention that many Mucorales are toxin producers, but that the active substances are generally not known. This implies that these 'toxins' are not identical with the well-characterized mycotoxins, and that their presence was inferred from antagonistic action of culture fractions in test systems. This situation—the production of antagonistic substances by species of related taxonomic groups—exists for all fungi, including species commonly used for the production of food-ingredients, such as *Aspergillus* (Barbesgaard et al., 1992) and *Trichoderma* (Nevalainen et al., 1994).

We have found no reference to any specific toxin production by members of the genus *Mortierella* in the review literature (e.g. Wyllie and Morehouse, 1977; Cole and Cox, 1981; Tu, 1992). Also a search in the primary literature was without results, with the exception of the pathogenic species *M. wolfii*, which excretes a water-soluble heat-labile trypsin-labile nephrotoxin (Davey et al., 1973; Corbel and Eades, 1991). Reiss (1993) reported toxicity of water extracts of *M. ramanniana* mycelium towards pea seedlings and tobacco plants, but not to brine shrimp larvae. However, as mentioned in Section 2, this species is currently no longer regarded as specifically related to the genus *Mortierella*. For *M. alpina*, there is an old report on antagonistic action of the culture filtrate towards wheat seedlings (Manozzi Torini, 1932, cited in Domsch et al., 1980).

The demonstration of an antagonistic effect as such does not yet imply that specific toxins have been formed, since numerous components of a culture filtrate may be responsible for such antagonism (for instance products of primary metabolism, such as organic acids). With that in mind, and on the basis of the modest effects

described, we conclude that there is no evidence in the literature to suggest that species of *Mortierella* have a strong toxigenic potential, as compared to other fungi used for food products, or that specific (myco)toxins are produced. Of course this must be shown experimentally for the actual strains used in production.

In this respect, experimental assessment of the toxicological safety of the final product is the most important test. At least two parties involved in the commercialization of AA production with *M. alpina* have performed toxicological studies (Boswell et al., 1996; Hempenius et al., in press). Both studies concluded that there were no treatment-related adverse effects, in mutagenicity tests and in acute and sub-acute (28 days) oral toxicity tests. A more elaborate study has been performed (a sub-chronic 90 days toxicity test preceded by *in utero* exposure), but the results have not been published yet (Gist-brocades, unpublished results). In addition, oil derived from *M. alpina* has been used in physiological studies in rats (Huang and Craig-Schmidt, 1996), human adults (Innis and Hansen, 1996) and infants (Foreman-van Drongelen et al., 1996; G. Hornstra, personal communication). These studies, which employed AA dosages at the physiological level, showed clear physiological effects of the AA supplementation, but did not show adverse effects on health parameters.

In addition to these studies on the final product, we have conducted a study into the potential of *M. alpina* for the production of known mycotoxins, according to the method of Filtenborg et al. (1995). Strain CBS 168.95 and CBS 169.95 were cultivated on solid media, expected to be most suitable for secondary metabolite production by *Mortierella* strains: Yeast Extract Sucrose agar, Oatmeal agar, Potato Sucrose agar and Rice Meal agar (Samson et al., 1995). Subsequently, the contents of each plate were combined, and the biomass was extracted with $CHCl_3$:CH_3OH (2:1) and ethyl acetate, subsequently, by the method of Frisvad and Thrane (1987). The extracts were analyzed by HPLC with gradient elution, using diode array detection to obtain UV spectra of the eluted compounds, according to Frisvad and Thrane (1993). The retention index and the UV spectrum were used to compare the eluted compounds with a data library for known mycotoxins (Frisvad and Thrane, 1987, 1993).

When tested via this method, most species of fungi produce a large number of secondary metabolites, usually 50–100 (Filtenborg et al., 1995). The two *M. alpina* strains also produced several metabolites, but none of these was identical to one of the known mycotoxins (Cole and Cox, 1981; Smith and Moss, 1985; Frisvad and Thrane, 1987, 1993).

It must be emphasized that this kind of test serves to assess the potential of organism to produce known dangerous compounds. We conclude that these production strains do not show such a potential. This gives additional robustness to the, independent, conclusion that the product derived from this organism has been found to be safe in toxicological testing.

6. Antibiotic action

Again, detection of antibiotic action, defined here as an antagonistic effect towards the growth of microorganisms, should not be confused with the production of specific secondary metabolites (antibiotics). Within the genus *Mortierella*, the only known antibiotic is fusidic acid (or ramycin), produced by some strains of *M. ramanniana*. However, as mentioned before, this species is not regarded as a valid member of the genus *Mortierella*. For *M. alpina*, antifungal activity has been reported (Domsch, 1960; Maciejowska, 1962, cited in Domsch et al., 1980), but this has never been characterized further.

We undertook a screening for the antibiotic effects of the AA producing strains *M. alpina* CBS 168.95 and CBS 169.95 towards Gram-positive and Gram-negative bacteria and towards yeast and mould (see Table 3). We compared the activity of *M. alpina* with that of a commercial fungal rennet-producing strain of *Mucor miehei* and a low-penicillin-producing strain of *Penicillium chrysogenum* as negative and positive controls, respectively.

We used two methods for this screening: one as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1992) for the testing of food samples for antibiotic activity, and one adapted from the test for secondary metabolites, as described above. In the first method, 40 μl of liquid culture supernatant of the producing organism was spotted on a sterile filter paper disk (Φ 7 mm), which was placed on the surface of an agar medium seeded with the target organism, and the degree of growth inhibition was assessed by measuring the zone of no-growth extending from the paper disk after 7 days of incubation.

In the second method, the producing organism was grown on agar plates, an agar plug (Φ 7 mm) was excised, placed on a plate culture of the target organism, and the growth inhibition was measured as in the first method. In this case the incubation period was extended to 14 days, because some transient growth inhibition was observed, especially with the positive control *P. chrysogenum*. The longer incubation period gave inhibition patterns consistent with the known action spectrum of penicillin, in contrast to the shorter period. In the JECFA protocol, the target organisms are grown in TSB-agar plates. This was modified for the yeast and the mould, which were grown on Yeast Extract Peptone Glucose agar, in the case of the mould after sporulation on Potato Dextrose Agar plates. A further modification was required for the *B. circulans* strain, which grew poorly on TSB medium. Instead, we used Brain Heart Infusion for this target organism.

Table 3
Strains used for testing of antibiotic activity

Organism	Strain number	Remarks
Target strains		
Bacillus cereus	ATCC 2	Gram-positive bacteria (JECFA list)
Bacillus circulans	ATCC 4516	
Staphylococcus aureus	ATCC 6538	
Streptococcus pyogenes	ATCC 12344	
Escherichia coli	ATCC 11229	Gram-negative bacteria (JECFA list)
Serratia marcenscens	ATCC 14041	
Saccharomyces cerevisiae	ATCC 36375	Yeast
Aspergillus niger	ATCC 16404	Mould
Producing strains		
Mortierella alpina	CBS 168.95	Arachidonic acid producing strains
	CBS 169.95	
Mucor miehei	Gist-brocades MCB 2A-B	Negative control
Penicillium chrysogenum	NRRL 1951	Positive control

Being a test for food samples, the JECFA method does not prescribe the cultivation method for the production organisms. Therefore, a range of 8 different media described for the production of secondary metabolites was used (Samson, 1995c), both in liquid and solid form. In addition, supernatant samples from two pilot scale *M. alpina* production fermentations, taken at various time points during the fermentation, as well as the crude AA-containing oil extracted from the biomass, were tested with the JECFA method.

The results are presented in the Table 4 and Table 5. The only liquid cultures to cause consistent and significant growth inhibition of the target organisms, were those of the positive control, *P. chrysogenum* (Table 4). *M. alpina* caused some growth inhibition of one target strain after cultivation on one growth medium. But since similar small effects were observed for the negative control, *M. miehei*, and since no consistent inhibition pattern was observed, this is not considered significant. We conclude that liquid cultures of *M. alpina* do not show capacity for antibiotic production. This includes the samples from the pilot plant fermentations, as well as the oil that was produced, which did not show growth inhibition towards any of the target organisms.

On the other hand, the *M. alpina* strains caused moderate growth inhibition of Gram-positive bacteria when grown on some of the solid media. The term ‘moderate’ is used to indicate that the inhibi-

Table 4
Growth inhibition by liquid culture supernatants

Producer	Target							
	B. cereus	*B. circul.*	*S. aureus*	*S. pyogen.*	*E. coli*	*S. marc.*	*S. cerevis.*	*A. niger*
YES-medium								
M. alpina 168			+					
M. alpina 169								
P. chrysogenum								
M. miehei					+			
Czapek YE								
M. alpina 168								
M. alpina 169								
P. chrysogenum		++	+++	+++				
M. miehei								
HD medium								
M. alpina 168								
M. alpina 169								
P. chrysogenum		+	++++	++++				
M. miehei		+						
R-medium								
M. alpina 168								
M. alpina 169								
P. chrysogenum		+	+++	+++				
M. miehei								

The degree of growth inhibition is expressed in a semi-quantitative fashion as follows: (+) zone of no-growth extending 0–4 mm from the paper disk or agar plug; (++) 4–6 mm; (+++) 6–15 mm; (++++) 15 mm and more. Where no response is indicated, growth inhibition was not observed. The composition of the production media (Yeast Extract Sucrose, Czapek Yeast Extract, Hanson-Dostalek and R-medium) is described in Samson et al. (1995).

tion was much less pronounced than the strongest response caused by the positive control, *P. chrysogenum*, but clearly stronger than any effect caused by the negative control, *M. miehei*. Growth inhibition towards Gram-negative bacteria, or towards the yeast and the mould, was not observed. This suggests that this may be a genuine antibiotic activity, caused by a secondary metabolite, although it cannot be excluded that a more general type of growth inhibition (for instance by organic acids or lipids) could be specific towards Gram-positive bacteria as well.

We conclude that *Mortierella alpina* shows a moderate antibiotic activity in solid state culture, but not in liquid culture. It is therefore advised that this last culture mode be used for production, until the identity of the antibiotic principle has been elucidated.

7. Conclusion

We conclude, on the basis of the literature reviewed and on the studies executed, that there is no scientific basis connecting *Mortierella alpina*, nor any other member of the genus, with the exception of *M. wolfii*, with disease or the production of toxins. Under certain conditions some antagonistic activity against Gram-positive bacteria or wheat seedlings may be observed, but such effects are quite common among fungi used for the production of food ingredients (Barbesgaard et al., 1992; Nevalainen et al., 1994), and there is nothing to suggest that these pose any threat to consumers, production personnel or the environment. In addition, toxicological tests of the final product (high-AA triglyceride oil) have not shown toxic effects.

Table 5
Growth inhibition by solid culture agar plugs

Producer	Target							
	B. cereus	*B. circul.*	*S. aureus*	*S. pyogen.*	*E. coli*	*S. marc.*	*S. cerevis.*	*A. niger*
YES-agar								
M. alpina 168	+	+	+	++				
M. alpina 169				+				
P. chrysogenum	+		+++		+	++	+	+
M. miehei								
Czapek YE agar								
M. alpina 168	+	+		++		+		
M. alpina 169		+		+				
P. chrysogenum	++	++	+++	++++	+	++	+	+
M. miehei								
HD agar								
M. alpina 168								
M. alpina 169								
P. chrysogenum		+	+++	++++				
M. miehei								
R-agar								
M. alpina 168	+			+				
M. alpina 169				+				
P. chrysogenum			+++	++++				
M. miehei								

Degree of growth inhibition expressed as in Table 4. The production media used in Table 3 were solidified by the addition of 2% (w/v) agar.

It is concluded that the AA production strains of *Mortierella alpina* should in general be considered safe for the production of food ingredients. Of course, such a general statement cannot suffice to determine the safety of a product for a specific application, but it is hoped that this may contribute to the safety assessment of products derived from this newly applied organism.

8. Note added in proof

After submission of this manuscript, a number of papers have been published on the effects of *Mortierella alpina* oils on animals (including a 90 days' subchronic toxicity study) and humans. Most of these studies have used high-AA oil with the trade name ARASCO® (Martek Biosciences), although some of the papers erroneously state that the oil was extracted from algae. One study has used oil enriched in eicosatrienoic acid from a new *M. alpina* strain. These new results correspond with the ones cited in the main text, in that there are clear physiological effects of PUFA supplementation, but no apparent toxic effects (see references below).

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