# For Debate: May the Use of the Polyene Macrolide Natamycin as a Food Additive Foster Emergence of Polyene-Resistance in *Candida* Species?

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## Abstract

Natamycin is approved almost worldwide as a food additive for surface treatment of cheese and sausages. Its use is considered to be safe as Natamycin is extremely sensitive to ultraviolet light and an acidic pH, so that products exposed to light in the retail industry and food stores are likely free from Natamycin. However, the use of an acid-, heat- and light stable Natamycin formulation in yoghurt has recently been authorized in the USA as well as in Australia and New Zealand. Furthermore, yoghurt is stored in sealed cups in refrigerated shelves, so it will not be exposed to light and thus not be inactivated during storage. Consequently, the resident flora will be exposed to Natamycin and it may exert a resistance selective pressure on faecal *Candida* spp. hypothetically selecting strains being resistant to Amphothericin B. In this review literature has been evaluated addressing the questions if Natamycin may foster emergence of polyene-resistance. This concern is supported by the facts that first, polyene-resistance could be elicited *in vitro* and *in vivo*. Second, as Azoles being used in agriculture and hospitals as well as polyenes share some common resistance mechanisms a polyene-resistance reservoir does exist in environmental and clinical fungal isolates. Third, Natamycin may amplify Amphothericin B resistance as fourth, resistance can in principal be spread amongst fungi by horizontal gene transfer. To preserve clinical efficacy of Amphothericin B for treatment of serious, life threatening infections, the use of Natamycin as a food-preservative should be limited to an absolute minimum.

**Keywords:** Polyene resistance; Food additive; Exposition of faecal flora; Selective pressure; Preservation of Amphothericin B

## Introduction

Preservation of food is of concern ever since mankind began to socialize and to migrate. Both, a growing population and thus a growing crisis of food supply, and the evolution of an industrial society living in large metropolitan areas demands that wheat yields continue to increase and fungal spoilage as well as losses of food to be reduced to a minimum. Thus, plant protectives and food additives for preservation of foodstuffs have been developed and accepted by the regulatory authorities. Diverse technologies of plant protection and food preservation have been developed such as use of chemical preservatives or use of antimicrobial agents [1]. Antifungal Azoles are used in agriculture and the lantibiotic Nisin being active against Gram-positive bacteria and the polyene macrolide (in the following "polyene") Natamycin being active against filamentous fungi but inactive against bacteria are used as food preservatives. However, resistance development is a serious risk associated with the use of antimicrobials as food additives and environmental fungicide use, respectively [2-6]. Although the overall level of resistance to antifungal agents is still low, studies have revealed that azole-resistance and surprisingly–Amphothericin B-resistance, too, could indeed be detected in environmental strains of *Aspergillus fumigatus* [6-11] as well as in environmental *Candida* spp. Strains [12-15]. These studies indicate that the extensive agricultural use of antifungals triggers resistance development in environmental fungi beyond the limits of structural classes of antimycotics thus probably creating a threat to human health.

The polyene Natamycin, too, is used in agriculture and may be used in horticulture. It is approved in the USA as a fungistat to prevent fungal contamination of growth media in enclosed mushroom production facilities and for indoor post-harvest treatment of several fruits Table 1 [16,17].

| Country | Matrix in/on which Natamycin is permitted | Maximum permitted level (mg/L, mg/kg, mg/ surface area) |
|---|---|---|
| EU | Surface of cheese +sausages | 1 mg/dm$^2$ |
| SA | Surface of cheese | 10 mg/kg |
| | Plastic coating of cheese | 500 mg/kg |
| | Sausages, manufactured fish products, canned food | 6 mg/kg |
| | Manufactured meat products | 500 mg/kg on casing or 6 mg/kg in contents |
| | Fresh fruit, fruit juices | 5 mg/kg |
| | yoghurt | 10 mg/kg |
| | Wine, alcoholic beverages | 30 mg/L |
| USA | Surface of cheese +sausages | 20 mg/kg |
| | Soft tortillas, salad dressing | 20 mg/kg |
| | yoghurt | 5-10 mg/kg |

|  | Liquid egg products | 15 mg/kg |
|---|---|---|
|  | Citrus, avocado, kiwi, stone-fruit, mango, pomegranate, pome fruit | 6.6-13.3 oz./100 gallons |
|  | pineapple | 0.18-1.44 oz./100 gallons |
|  | cherries | 3.3-13-3 oz./100 gallons |
|  | mushroom | 0.36-0.73 oz/1000 feet2 |
| Canada | Surface of cheese | 20 mg/kg |
|  | Grated/shredded cheese | 10 mg/kg |
| Mexico | tortillas | 20 mg/kg |
|  | bread | 14 mg/kg |
|  | beverages | 5 mg/l |
| Australia | Surface of cheese | 15 mg/kg |
| New Zealand | Surface of cheese | 12.5 mg/kg |
|  | beer | 5 mg/l |
| ARE | Permitted food additive |  |
| Kuwait | Permitted food additive |  |
| Russia | Surface of cheese | 5 mg/kg |
|  | Canned vegetables | 2.5 mg/kg |

**Table 1:** Selected examples for food legislation on the use of natamycin (modified according to 16, 17; EU=European Union; SA=South Africa; USA=United States of America; ARE: United Arab Emirates).

It is also used in the USA as an additive to food or drinking water of broiler chickens for the reduction of poultry condemnations [17,18]. Furthermore, patents have been granted for the use of Natamycin in treatment of bananas and potatoes [19,20] or tulip bulbs [21]. Active packaging systems coated with Natamycin have been described for food preservation [22,23]. Natamycin is approved almost worldwide as a food additive for the preservation of cheese and sausages. The use of Natamycin in agriculture and horticulture may result in a dietary exposure to Natamycin. However, exposure is considered to be insignificant as Natamycin is applied indoors only and directly to commodities [17]. The use of Natamycin for surface treatment of cheese and sausages is also considered to be safe as Natamycin is extremely sensitive to ultraviolet light [24], so that products exposed to light in the retail industry and food stores are likely free from Natamycin. Natamycin formulations used in agriculture or horticulture, and for surface treatment of cheese and sausages are aqueous suspensions of Natamycin crystals [25,26].

Recently, the use of Natamycin in yoghurt has been authorized in the USA [27] as well as in Australia and New Zealand [28]. Moreover, yoghurt products containing 5-10 mg Natamycin/kg are commercially available in South Africa, Canada and China. In addition, Natamycin may be added to wine, alcoholic beverages or fruit juices Table 1. However, Natamycin is not only degraded by UV light but also at an acidic pH as it prevails in yoghurt, fruit juices and wine. Therefore, a chemically and microbiologically stable Natamycin formulation had to be developed [29,30]. The new formulation protects Natamycin from degradation by heat, light, and high or low pH and offers the

advantage of a slow release [29,30]. In addition, yoghurt is stored in sealed cups in refrigerated shelves, so that Natamycin will not be exposed to light and thus not be inactivated during storage. Furthermore, the matrix yoghurt may shield Natamycin from degradation caused by an acidic pH. This assumption is supported by findings that turkish yoghurt purchased in food stores contained 0.1 to 4.89 mg Natamycin/kg yoghurt although turkish dairy products should not contain any preservatives [31,32]. Natamycin recovery from yoghurt samples spiked with pure Natamycin was 89% to 99.5% [32,33] and it was biologically active throughout the study period of 28 days [34] and 40 days [35]. Unfortunately, production and sampling dates were not specified in these publications, but data demonstrate that pure Natamycin retained its biological activity in stored turkish yoghurt products for more than four weeks. The characterization of the novel Natamycin formulation in the corresponding patents and the supportive evidence provided by the descriptive data from Turkey support the notion that the physicochemical properties as well as the biological and pharmacodynamic characteristics of the novel Natamycin slow release formulation to be used for the preservation of yoghurt, beverages, fruit juices, wine, etc. is not identical with those of the conventional formulation used for surface treatment of cheese and sausages. Furthermore, it should be considered that the use of a light- and acid stable Natamycin formulation as an additive to yoghurt, beverages, fruit juices, wine, etc. may expose the human resident fungal flora directly to the selective pressure of an anti-infective agent, while the use of a light- and acid unstable Natamycin formulation in agriculture or horticulture and as a food preservative may expose the environmental flora, if at all.

The theory that the consumption of Natamycin containing yoghurt may expose the resident faecal flora to Natamycin thus fostering emergence of resistance to polyenes in *Candida* spp. and jeopardizing the clinical efficacy of Amphotericin B [36] has been discussed controversially [37,38]. Evidence has been presented that Natamycin exerts a negligible *in vitro* resistance-selective potential in pathogenic yeasts and other probably opportunistic fungal pathogens [39]. Overall, development of Amphotericin B resistance seems to be slow under *in vitro* conditions and has seemingly not yet been a factor causing clinical problems; furthermore, reports on the recovery of resistant isolates from patients during Amphotericin B therapy are scarce [40], although a number of studies have described *in vitro* induction and *in vivo* selection of Amphotericin B resistance.

Reasons for concern were that the consumption of Natamycin containing yoghurt, beverages, fruit juices, wine, etc. may expose the human faecal *Candida* spp. flora to antimicrobially active drug concentrations [36]. Consumption of yoghurt containing the novel Natamycin formulation may result in an estimated 2-day daily intake of Natamycin from background and proposed uses for the total US population of 0.61 to 1.22 mg/day [41]. Assuming that the entire amount of Natamycin consumed with yoghurt is deposited in the faeces and is freely available and thus antifungally active, mean faecal Natamycin concentrations exceed its MICs for *Candida* spp. [36]. Exposition of the gastrointestinal flora to Natamycin will be greater following wine drinking as in South Africa the maximal permitted Natamycin concentration in wine and alcoholic beverages amounts to 30 mg/L [16]. Consequently, Natamycin may exert a resistance selective pressure on faecal *Candida* spp. under these particular circumstances. Furthermore, fungi harbouring polyene resistance originating from environmental sources or having been selected as a consequence of Amphotericin B- or Nystatin therapy may render other faecal fungal species polyene resistant by horizontal gene transfer

[36]. This hypothesis implies that first, polyene resistance can indeed be elicited *in vitro* and *in vivo*; second, that a polyene resistance reservoir does exist; third, that in case a polyene resistance reservoir does exist, Natamycin may exert a resistance selective potential thus amplifying Amphotericin B resistance, and fourth, that resistance can be spread. In the following literature is reviewed addressing these hypotheses.

## *In vitro* Development of Polyene-Resistance

Continuously increasing minimal inhibitory concentrations (MICs) of Nystatin and Amphotericin B, respectively, developed in 33.6% and 63.4% of the strains studied following at least 10, and up to 100 transfers of the strains on polyene containing medium. MICs of Amphotericin B for *Candida* spp. increased approximately 500 to 1000 fold after 100 transfers [42]. MICs of *C. albicans*, *C. stellatoides*, *C. parakrusei*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* increased after 50 subcultures in Amphotericin B containing medium 1.2, 4.5, 25.0, 57.0, 60.0, 200.0 fold, respectively [43]. Exposure of *C. albicans* to the polyene candidin revealed that its MICs increased 14 and 150 fold after 14 and 25 transfers, while the Amphotericin B MICs increased 10-fold [44]. The same group described that *C. albicans* could be rendered 150-fold candidin and/or 4 to 60 fold Amphotericin B resistant but not Nystatin-resistant after 20 to 30 transfers in either of these polyenes [45]. Exposure of 12 strains of *C. albicans* to Amphotericin B for a total of 52 transfers revealed that MICs of Amphotericin B for all of the strains increased, but in only three strains resistance developed [46]. Likewise, development of Amphotericin B resistance was recorded from the 10th transfer onwards for a total of 58 transfers; after about 10 transfers MICs were increased about three to fivefold only; induction of resistance proceeded rapidly once this moderate increase in MICs has been reached [47]. Increased Natamycin-MICs could be generated in *S. cerevisiae* and *S. bailii*. Natamycin MICs increased slowly up to the 20th transfer, with a steep increase thereafter [48]. Others failed to generate polyene-resistant strains of *Candida* spp. Short term exposure to Nystatin resulted in a five-fold increase in MICs for one strain only, whereas the remaining four were unaffected [49]. Streekstra et al. [39] described that MICs of Natamycin increased minimally in 13 out of 20 tested strains and 4 strains showed a >2-fold increase in MICs following 12 to 36 transfers in Natamycin containing medium; one of these strains also showed significantly increased MICs of Amphotericin B and Nystatin. Furthermore, MICs of Natamycin remained unchanged in fungi isolated from cheese warehouses following 25 transfers [50] and no change in the composition or the sensitivity of the contaminating fungal flora isolated from cheese- or sausage factories has been observed [50-52].

Obviously, discrepant data were obtained either following long-term exposure with more than 30 transfers or short-term exposure with about 25 transfers or less. Thus, the use of small, rather than large increments of polyene concentrations and a large number of transfers over a long period of time are necessary to raise polyene-resistance *in vitro*, since significant increases in MICs (and thus induced polyene-resistance) had not occurred until 20-30 transfers had been made [43-49]. Consequently, long term exposure to sub-inhibitory Natamycin concentrations may in theory represent a potential hazard.

Apart from phenotypic changes, i.e. increasing MICs, any exposure of microorganisms to antimicrobials exerts a stress which is sensed by fungi and to which the microorganism responds in order to survive in a hostile environment. Such sensing and cellular signalling mechanisms contribute as much to antifungal drug-resistance as alterations in the drug target [53,54]. *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus* and *Trichophyton rubrum* develop compensatory responses related to changes in the cell membrane caused by exposure to sub-inhibitory concentrations of Amphotericin B, Nystatin, Azoles and other drug classes [55-67]. Changes in gene expression triggered by exposure to sub-inhibitory drug concentrations affects genes involved in eg. drug-target, i.e. ergosterol biosynthesis including genes identified as contributing to resistance, but also genes involved in transport, osmotic tolerance, oxidative stress and other genes more. This pleiotropic drug-resistance network contributes to the acquisition of resistance to antifungals beyond structural limits and spanning the fungal kingdoms [53,54].

## Longitudinal Surveillance Studies Indicating the Existence of a Polyene Resistance Reservoir

Harmonized CLSI and EUCAST methods of susceptibility testing [68-75] defined the Amphotericin B susceptible- and resistant breakpoints according to EUCAST definition as <1 mg/l and >1 mg/l, respectively for *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* [76] whereas the susceptible- and resistant breakpoints for *A. fumigatus* and *A. niger* are <1 mg/l and >2 mg/L, respectively for other *Aspergillus* species evidence allowing breakpoint definitions is insufficient [77]. In the following only those studies are reviewed which have adopted these standards for the quantitation of minimal inhibitory concentrations (MICs). Data from routine susceptibility tests allowing a qualitative characterization of susceptible or resistant isolates only have not been considered.

As Natamycin is used nowadays for topical treatment of fungal infections only but no longer for systemic or oral treatment of fungal infections anymore, susceptible and resistant breakpoints have not been defined, so that by definition a quantitative measure for the characterization of a clinical isolate or a laboratory generated mutant as "Natamycin-resistant" is not existent. Furthermore, Natamycin has never been included into surveillance studies as it is used just for topical application, so that the question if a cross-resistance between Natamycin and Amphotericin B may exist amongst clinical isolates cannot be answered.

Although several longitudinal surveillance studies like the global SENTRY Antimicrobial Surveillance Program or ARTEMIS Global Antifungal Surveillance Program were and still are being conducted, information on the Amphotericin B susceptibility pattern is scarce as this polyene is not routinely included into these programs.

The SENTRY study revealed that in 2003 only 9.5% and 11.5% of *Aspergillus* spp. and *A. fumigatus* strains, respectively, isolated from infected, normally sterile body sites of hospitalized patients were inhibited at <1 mg/L of Amphotericin B. Concentrations inhibiting 50% (MIC 50) of the *C. albicans*, *C parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. neoformans* strains isolated from normally sterile body sites of hospitalized patients in North America, Europe, and Latin America amounted uniformly to 1 mg/L, so that based on breakpoint definitions 50% of the isolates have to be classified as non-susceptible [78]. Data generated three- and five years later in North- and Latin-America, Europe, and the Asia-Pacific region were not significantly different; in a few *Candida* species a decrease of MIC50 values by one titration step was noted and 71.4% of the *A. fumigatus* isolates were inhibited by <1 mg/L Amphotericin B, Table 2 [79,80]. Analoguous data were generated in the course of the ARTEMIS study

[81,82]. Fifty percent of the *C. glabrata* [81] and *C. krusei* [82] isolates studied were inhibited at 1 mg/L. A german/austrian multicenter study revealed that 11.3% of all *Candida* spp. isolates was resistant to Amphotericin B, 1.8% showing a complete cross-resistance to Azoles [83].

| Species | Range | MIC50 | MIC90 | %res. | Region | Year | Ref. |
|---|---|---|---|---|---|---|---|
| *Candida* spp. | 0.25-2 | 1 | 1 | | SENTRY global | 2003 | 78 |
| *C. albicans* | 0.25-2 | 1 | 1 | | | | |
| *C. glabrata* | 0.25-2 | 1 | 1 | | | | |
| *C. krusei* | 2-Jan | 1 | 1 | | | | |
| *Aspergillus spp.* | 4-Jan | 2 | 4 | | | | |
| *A. fumigatus* | 16-Jan | 2 | 4 | | | | |
| *Candida* spp. | 012-2 | 0.5 | 1 | | SENTRY global | 2006-2007 | 79 |
| *C. albicans* | 0.12-1 | 0.5 | 1 | | | | |
| *C. glabrata* | 0.12-1 | 1 | 1 | | | | |
| *C. krusei* | 0.12-2 | 1 | 1 | | | | |
| *Aspergillus spp.* | | | | | | | |
| *A. fumigatus* | 0.12-2 | 2 | 2 | | | | |
| *Candida* spp. | 0.06-2 | 0.5 | 1 | | SENTRY global | 2008 | 80 |
| *C. albicans* | 0.12-1 | 0.5 | 1 | | | | |
| *C. glabrata* | 0.25-1 | 1 | 1 | | | | |
| *C. krusei* | 0.25-1 | 1 | 1 | | | | |
| *Aspergillus spp.* | | | | | | | |
| *A. fumigatus* | 0.25-1 | 0.5 | 1 | | | | |
| *C. glabrata* | 0.06-16 | 1 | 2 | | ARTEMIS global | 2002 | 81 |
| *C. krusei* | 0.03-16 | 2 | 2 | | | 2001-5 | 82 |
| *Candida* spp. | | | | 11.3 | 1) | | 83 |
| *C. albicans* | 0.016-1 | 0.25 | 0.5 | | Denmark | | 84 |
| *C. glabrata* | 0.016-4 | 0.5 | 1 | 1.6 | | | |
| *C. krusei* | 0.016-4 | 1 | 1 | 4.7 | | | |
| *C. glabrata* | 0.25-2 | 1 | 2 | | Iran | | 85 |
| *C. albicans* | 0.25-2 | 0.5 | 1 | | Brasil | 2015 | 86 |
| *C. glabrata* | 2-Jan | | | | | | |
| *C. krusei* | 2-Jan | | | | | | |
| *Candida* spp. | | | | 7.8 | India | | 87 |
| *C. tropicalis* | | | | 17.6 | India | | 88 |
| *C. albicans* | | | | 3 | UK | | 90 |

**Table 2:** Amphotericin B susceptibility pattern for *Candida* species expressed as minimal inhibitory concentrations (MIC) inhibiting either 50% (MIC50) or 90% (MIC90) of the strains tested (% res. =% resistance, i.e. amphotericin MIC>1 mg/L; Ref=reference; 1)=Germany/Austria).

Danish fungemia isolates of seven different *Candida* species collected over a five year period from 2004 to 2009 tended to be by one to two dilution steps more susceptible [84] than those collected in North- or Latin America and Europe in the course of the SENTRY study. But nevertheless, 1.6% of the *C. glabrata* and 4.7% of the *C. krusei* as well as 16.3% of the non-Candida isolates had to be classified as Amphotericin B resistant, Table 2 [84]. These representative longitudinal nationwide studies or international surveillance programs revealed that a significant number of clinical isolates were inhibited by Amphotericin B concentrations higher than the susceptible breakpoint of 1 mg/L. A nationwide study in Iran demonstrated that the geometric mean MIC of Amphotericin B for C. glabrata was 1.1 mg/L with a resistance rate of 27.5% [85]. Furthermore, point prevalence studies in Brazil [86] and India [87,88] demonstrated that Amphotericin B resistance among *Candida* spp. isolated from hospitalized patients in tertiary care hospitals amounted to 12.7% [86] and 7.8% to 17.5% [87,88], respectively. Other studies indicated that Amphotericin B resistance remained rare [40,89,90]. *Candida* spp.-strains being simultaneously azole- and Amphotericin B-resistant due to loss-of-function mutations in ERG3 have been isolated, too [91-98].

In general, data quoted above demonstrate that a significant number of strains of various *Candida* species and *A. fumigatus* were inhibited by Amphotericin B concentrations which exceed the susceptible breakpoint and that a significant number of strains was isolated which were resistant to Amphotericin B as well as Azoles. Surveillance studies cannot be correlated with clinical outcome and therapeutic success or failure indicating that *in vitro* susceptibility testing alone is not sufficient to predict clinical efficacy of these antifungal agents [97]. However, this aspect is not relevant in the context of the use of a polyene as a food preservative hypothetically exerting a resistance-selective pressure on fungal populations harbouring polyene- and/or azole-resistance.

## Polyene Resistance Recovered from Patients Treated either with Amphotericin B or Natamycin as well as Patients not Having been on Antifungal Therapy

It has long been documented that polyene-resistance emerged under therapy of immunosuppressed patients with Amphotericin B [99-107]. Recently, *C. albicans* strains were isolated consecutively from biopsies and faeces of an immunocompromised patient which acquired stepwise resistances to Azoles, Echinocandins, and Amphotericin B [108]. The gastrointestinal tract and central venous catheters were identified as sources of *Candidal* sepsis in these cases of disseminated candidemia [109]; in general, the gastrointestinal tract is the reservoir for *Candida* spp. Infections [110,111].

Polyene-resistant strains could not only be isolated from immunocompromised patients but from almost healthy, immunocompetent individuals as well [36]. Oral treatment of gastrointestinal *Candida* spp. colonization with 400 mg Natamycin for ten days in 356 colonized patients resulted in a reduction in

susceptibility to Natamycin during exposure to this agent as compared to the susceptibilities of pre- and post-treatment isolates. Strains with Natamycin MICs of 1.25 mg/L could be isolated from 56% of these subjects prior to dosing, but from only 33% during exposures, and again 51% five days after cessation of therapy and 60% three months later [112]. Thus, the reduction of Natamycin-susceptibilities is a drug-exposure related pharmacodynamic effect. This phenomenon supports the notion that exposure to Natamycin bears a resistance-selective potential. Also, *Candida* spp. isolated from women with vaginal candidosis was less susceptible to Natamycin than isolates from healthy women [113]. Although the authors did not describe that the fresh clinical isolates were withdrawn from Natamycin treated patients, data demonstrate that strains with reduced susceptibilities could be isolated. Natamycin MICs of 15 mg/L and >50 mg/L were determined for two out of eight *C. albicans* strains freshly isolated from vaginal specimens (range 5 to >50 mg/L) as compared to a previously reported values of 3-6 mg/L [113].

Unfortunately, no systematic studies have been performed so far on the effect of polyenes on the gastrointestinal flora, so that the question if gastrointestinal *C. albicans* or other *Candida* spp. may harbour polyene resistance mechanisms can currently not be answered. But the studies quoted above clearly demonstrate that polyene-resistant strains of *Candida* spp. could be isolated from patients.

## Environmental Sources of Amphothericin B Resistant Fungi

It is well documented that azole-resistance has been selected in environmental fungi due to their agricultural use and evidence has been presented that azole-resistant strains could be isolated from environmental samples as well from azole-naïve- and azole treated patients [7,8]. These strains were not only azole-resistant, but some of them were resistant to Amphothericin B, too. In one study, samples were taken from outdoor air across the province of Madrid, from hospital air, and from hospitalized patients being on antifungal therapy or not. MIC50- and MIC90-values, geometric means and ranges of MICs of Amphothericin B were almost identical for all the strains studied irrespective of their origin. MIC90-values for *A. fumigatus* isolated from patients being on antifungal therapy or not taking any antimycotics amounted uniformly to 2 mg/L [114]. This finding indicates that patients infected with Amphothericin B-resistant *A. fumigatus* may likely have been colonized from the environment. In agreement with this study, another study revealed that in Spain and Austria environmental Amphothericin B resistant *A. fumigatus* strains could be isolated [8]. Hence, it is not surprising that almost all strains of *A. fumigatus*, *A. flavus* and *A. terreus* isolated from the air sac of falcons were Amphothericin B resistant [115,116]. As reviewed recently, resistance mechanisms were identical in environmental as well as clinical isolates, thus strongly suggesting that the mutation in the clinical isolate has been acquired from an environmental strain [98]. Also, many potentially human pathogenic fungal species have their natural habitat in the environment [117], and fungal spores are spread over long distances by circulating air flows [98], so that potential pathogens and antifungal resistances may originate from the environment.

These findings demonstrate that Amphothericin B resistance and resistance to both, polyenes and Azoles, in environmental fungi seems to be widespread.

## Cross Resistance, Multidrug Resistance, and Horizontal Gene Transfer

Data summarized above demonstrate that strains resistant to both, polyenes and Azoles could be isolated from environmental as well as clinical sources. Therefore, the question is, if Natamycin may exert a resistance-selective potential. One study has demonstrated that polyene-resistance induced by Amphothericin B or Nystatin lead to cross-resistance to Natamycin [42]. Likewise, a Nystatin-resistant isolate of *Candida stellatoidea* was also resistant to Amphothericin *A*, Amphothericin *B*, endomycin, filipin and Natamycin [45,118]. Molzahn and Woods [119] described that Natamycin selected for cross-resistance to Nystatin, but vice versa, Nystatin did not select for Natamycin-resistance in *S. cerevisiae*; these authors had not included Amphothericin B into the study. Phenotypically Natamycin- and Amphothericin B cross-resistant strains have been described [120]. Efficacy of both polyenes in a *Candida keratitis* model was directly correlated to their MICs, i.e. low MICs were associated with high reductions of viable counts and vice versa [120].

Not only polyenes, but chemically unrelated drug classes like Azoles select for polyene-resistance, too. Polyenes like Amphothericin B exert their fungicidal activity by binding to Ergosterol, followed by channel formation that further increases their activity. Natamycin interacts with Ergosterol without forming ion-channels, but it impairs membrane fusion *via* perturbation of Ergosterol-dependent priming reactions that precede membrane fusion [121-124]. Azoles disturbe fungal cell membrane sterol composition by inhibiting demethylation of Lanosterol and in the late step of Ergosterol biosynthesis also the desaturation of the sterol moiety, encoded by genes ERG11 and ERG 5 [125]. Several mechanisms have been documented to be involved in the resistance to Azoles as well as polyenes. They include active efflux, target enzyme alterations or its absence [126-129]. Amino acid alteration in the target enzyme ERG11 or the replacement of Ergosterol by other sterols in the membrane encoded by ERG3 are examples. As Ergosterol being essential for the mode of action of Azoles as well as polyenes is absent in ERG3 loss-of-function mutants, these strains escape the antifungal effect of both drug classes [91-98]. Other ERG-gene defects also confer multi-drug-resistance [98]. Alterations in sterol- and phospholipid composition as well as changes in membrane structure account for different polyene-cross-resistance patterns [130,131] and were found in clinical isolates resistant to Azoles as well as polyenes [98,132-137]. The situation is probably aggravated by the finding that a mutator phenotype caused by a mismatch repair defect is prevalent in *C. glabrata* [138]. This genetic mechanism promotes the acquisition of resistance to multiple antifungals. Strains carrying alterations in mismatch repair exhibit a higher propensity to breakthrough antifungal infections than non-mutator strains. Mutator strains were recovered at a rate as high as 55% from patients [128] thus giving rise to concern. Thus, Azoles as well as polyenes trigger resistance development in environmental and clinical fungal isolates as both drug classes share some common resistance mechanisms.

It could be argued that a colonization of a patient with a resistant strain acquired from the environment or hospital may probably not represent a hazard as many pathogenic yeasts are asexual and therefore not involved in intra- or interspecies exchanges of genes, i.e. horizontal gene transfer (HGT). Therefore, it has been concluded that resistance traits will not occur in fungi and an explosive expansion of resistance is unlikely to occur [139]. However, intra and interspecies gene transfers within fungi and even between bacteria and fungi have been described [140-149]. In principle, HGT could play an important role in the

spread of resistance mechanisms between food-borne and pathogenic yeasts [148] as well as amongst fungi residing in the gastrointestinal tract. The interspecies exchange of plasmids harbouring resistance genes could be demonstrated *in vitro* [148], and secretion systems conferring drug-resistance could be transferred from bacteria to fungi [150,151]. These examples demonstrate that in principle a horizontal transfer of resistance genes within and between fungal species is possible.

## Discussion

Clearly, the question "if the use of the polyene macrolide Natamycin as a food-additive may foster emergence of polyene-resistance development in *Candida* species" is a hypothetical question as the potential of Natamycin to select for polyene cross resistance in general or Amphotericin B resistance in particular has never been addressed systematically. This may be due to three reasons: first, susceptibility criteria allowing the classification of a strain as "Natamycin-susceptible" or "Natamycin-resistant" have never been defined; second, systematic studies on the propensity for resistance development in environmental and pathogenic strains have never been performed, and third, the European Food Safety Authority concluded that there was no concern for the induction of Natamycin-resistance [152] based on surveys in cheese warehouses and in dry sausage factories [50-52] as well as the difficult induction of Natamycin-resistant mutants in fungi [42]. However, aerogenic exposure of environmental fungi somewhere in distant vicinities of the production facility during the surface treatment of cheese or sausage to microbiologically active concentrations of a light unstable Natamycin formulation is very unlikely, so that this finding does not appear to be particular pertinent. Above all, different Natamycin formulations are in use either for surface treatment of cheese and sausages, or preservation of yoghurt, wine, beverages, etc. In principle, Natamycin is preferable to many other preservatives as it is free from odour and colour so that it causes no taste perversion and therefore does not adversely affect consumer acceptance. Because of its light- and acid instability, Natamycin will be degraded on the surfaces of cheese and sausages by the time of purchase by the consumer, so that the consumer will be exposed to negligibly low concentrations of Natamycin, if at all. Because of these characteristics and a favourable safety profile the Joint Food and Agriculture Organization of the United Nations and World Health Organization Expert Committee on Food Additives (JECFA) regarded the use of Natamycin for surface treatment of cheese and sausages as safe for human use [153]. However, a new light- and acid stable slow release formulation reducing inactivation of Natamycin for several weeks has been developed [29,30] for the preservation of yoghurt, wine, and beverages. Natamycin concentrations in food containing this new formulation range from 5-10 mg/kg yoghurt to 30 mg/L wine. Furthermore, Natamycin has been formulated as a cyclodextrin inclusion complex as it is barely soluble in beverages. Soluble concentrations of the conventional formulation are much lower than needed for a preservation of the product till the end of its shelf life, but this cyclodextrin inclusion formulation allows the production of beverages containing up to 500 mg/L of solubilized Natamycin (reviewed in 36). Thus, different Natamycin formulations are used for surface treatment of cheese and sausages on the one hand and preservation of yoghurt or wine on the other hand; the formulations differ from each other in stability- and solubility, and thus probably also in their pharmacological- and/or toxicological profiles. Also, the consumer is either not exposed to Natamycin because of its instability to light and acid, or will be exposed to Natamycin concentrations ranging from 3.33 mg/kg to 6.66 mg/kg faeces following consumption of 100 g of yoghurt containing either 5 ppm or 10 ppm Natamycin, 19.98 mg/kg of faeces following consumption of one litre of wine containing 30 mg/L, or maximally 3,333.0 mg/kg faeces following consumption of 1 L of beverage containing 500 mg of a Natamycin cyclodextrin-complex [36] provided the entire amount of Natamycin consumed with yoghurt or wine is deposited in the faeces and is freely available.

Faecal Natamycin concentrations are within the microbiologically active range. As summarized above, fungi developed polyene-resistance upon long term *in vitro* exposure to sub-inhibitory Natamycin concentrations, a finding which is supported by two observational clinical studies. Polyene-resistance seems to be ubiquitous in the environment as well as in hospitals, probably also due to the use of Azoles in agriculture selecting for polyene-resistance, too, so that even antimycotic naïve patients may be colonized with polyene-resistant fungal pathogens. Consumption of Natamycin containing food may thus exert a polyene-resistance selective pressure.

The use of Natamycin as a food preservative confronts us with a paradox. On the one hand use of antibiotics as growth promotors in food producing animals has been banned in 2006, and action plans have been put forward aiming at a mitigation of the risk of antibiotic resistance development, so that maximal residue levels in food animals and withdrawal times to reduce drug concentrations below levels safe for human consumption have been defined both in the US and the EU [154,155]. Maximal residue levels of most antibacterials in edible tissue range in the US from 0.1 to 0.01 ppm [156]. To define if drug levels are safe for human consumption, the probabilities have to be assessed that first, bacteria in the animal population will acquire resistance, second, that humans will ingest the resistant bacteria in food products, and third, that ingesting the bacteria will result in adverse health outcomes [157]. Consequently, this is an indirect and an unquantified hazard as not drug residues but resistance genes of microbial origin may be transferred from animals to humans *via* the food chain. However, addition of Natamycin to food may expose the human resident flora directly and quantifiably to a selective pressure. If Natamycin in itself may cause emergence of polyene-resistance in the gastrointestinal fungal flora and/or may act as an Amphotericin B resistance selector may be probable but is speculative. But still, the reservoir of Amphotericin B resistant fungal species should not be enlarged by an inappropriate use of antifungals in general and of polyenes in particular. Clinical efficacy of Amphotericin B should be preserved as it is used to treat serious, life threatening infections. Therefore, the use of any anti-infective agent as a food-preservative should be limited to an absolute minimum.

## Declaration of Interest

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