

Medium chain fatty acid ethyl esters – activation of antimicrobial effects by *Malassezia* enzymes

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Summary

Free medium and short chain fatty acids are known to have broad antimicrobial activity. However, their practical use in topical therapy is limited by their intensive smell and acidity. Surprisingly, a nearly identical antimicrobial effect was found with the ethyl ester derivatives of these fatty acids, but only against *Malassezia* (M.) yeast, not against *Candida* spp. Obviously, these esters are hydrolysed by M. enzymes, thus generating a selective activation of antimicrobial activity especially in areas well populated with these yeast ('targeting'). Octanoic acid ethyl ester (CAS 106-32-1) was found to be most suitable. In an agar dilution test, the minimal inhibitory concentrations against *M. globosa*, *M. pachydermatis* and *M. sympodialis*, respectively, ranged between ~5 and 10 mmol l⁻¹ after 10 days of incubation. The effect started immediately and was not delayed by other lipid sources applied simultaneously. Based on these data, fatty acid monoesters may represent a new therapeutic concept in M.-associated diseases.

Key words: *Malassezia* spp., hydrolytic activity, ethyl esters, antimicrobial effects, targeting, atopic dermatitis.

Introduction

Malassezia (M.) spp. belong to the resident skin flora of many warm-blooded animals. However, they are involved in skin diseases such as pityriasis versicolor, seborrhoeic eczema, atopic dermatitis, *Malassezia* folliculitis and in animal otitis externa.¹ With the exception of *M. pachydermatis*, all 14 *Malassezia* species known so far require an external lipid source for growth, i.e. they are obligatorily lipid dependent.² Lipid dependence is known to be based on a defect in the synthesis of myristic acid (C14 : 0), which serves as the precursor of long-chained fatty acids.^{3–5} At the molecular level, this metabolic deficiency can be explained by the absence of a gene encoding fatty acid synthase

in the genomes of *M. globosa* and *M. restricta*,⁶ which is compensated by an abundance of genes encoding hydrolases that can provide fatty acids. Using different fatty acid (mono-)esters, Mayser *et al.*⁷ found that M.-dependent hydrolysis was critically dependent on the alcohol moiety. The authors observed greater extracellular lipase activity and more pronounced production of free fatty acids in *M. sympodialis* than in *M. furfur*. Hydrolysis was best effected in ethyl esters.

Medium chain fatty acids like Capric acid C10 : 0, Caprylic acid C8 : 0 or Caproic acid C6 : 0 are known to have broad antimicrobial activity.⁸ The effects of the free fatty acids on different *Malassezia* spp. in comparison with *Candida* (C.) spp. were analysed in this study. As the practical use of these fatty acids in topical therapy is limited by their intensive smell and acidity, their ethyl ester derivatives were investigated for potential antimicrobial activity, too.

Material and methods

Reference strains and wild-type strains of the following *Malassezia* and *Candida* spp. were included in the investigation (Table 1).

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The wild-type strains were differentiated by their physiological and biochemical properties (Tween resp. carbohydrate auxanography, morphology on rice agar), as described elsewhere.⁹ Cultures of *Malassezia* spp. were maintained on modified (m) Dixon-agar,⁹ of the *Candida* spp. on 4% Sabouraud dextrose agar (SDA).

Table 2 shows a list of free fatty acids and their ethyl ester derivatives tested. All substances were obtained from Sigma-Aldrich, St. Louis, USA. Purity was $\geq 99\%$.

Agar dilution test

Cultures of *Malassezia* sp. were first grown aerobically on selective agar for pathogenic fungi (Merck, Darmstadt, FRG), overlaid with a thin layer of olive oil at 32 °C. After 8–10 days, cells were harvested and suspended in olive oil. By determining cell counts with a Neubauer chamber (dilution media xylene), suspensions were prepared to give a density of 10^4 , 10^5 , 10^6 , 5×10^6 CFU cm⁻² respectively. *Candida* spp. were grown on 4% SDA and treated in the same way with the difference being suspended in water. After autoclaving mdixon agar was allowed to cool down to ~60 °. Free fatty acids and their ethyl ester derivatives were added in geometric dilution over a range of 1.25–640 mmol l⁻¹. However, to avoid pH-dependent effects especially in the case of the pure fatty acids, each concentration was prepared individually after addition of the amount of test substance and adjusting to pH 5.5 with the addition of 2 m NaOH. After intensive stirring, the different concentrations were poured out in one well each of 24-well plates (1.75 cm²/well, Greiner bio-one, Frickenhausen, FRG). One well with mdixon agar without any additive served as control for growth. Growth was assessed daily for 20 days.

Table 1 Strains tested.

Species	Strains tested
<i>M. furfur</i>	CBS 7019, CBS 1878, 8 wild-type strains
<i>M. sympodialis</i>	CBS 7222, 7978, ATCC 42132, 8 wild-type strains
<i>M. pachydermatis</i>	CBS 1892, CBS 1879, 8 wild-type strains
<i>M. globosa</i>	CBS 7966, 9 wild-type strains
<i>C. krusei</i>	10 wild-type strains
<i>C. parapsilosis</i>	10 wild-type strains
<i>C. glabrata</i>	CBS 138, 9 wild-type strains
<i>C. guilliermondii</i>	10 wild-type strains
<i>C. albicans</i>	CBS 562; 9 wild-type strains

CBS, Centraalbureau voor Schimmelcultures, www.cbs.knaw.nl; ATCC, American type culture collection, www.atcc.org.

Minimal inhibitory concentration (Mic) was defined as the first concentration with no visible growth.

Coincubation of *C. albicans* and *M. sympodialis*

C. albicans (CBS 562, 10^4 CFU cm⁻²) and *M. sympodialis* (CBS 7222, 10^6 CFU cm⁻²) were also coincubated on mdixon agar with octanoic ethyl ester (concentration 1.2–150 mmol l⁻¹) for 10 days.

Thin-layer chromatography and densitometry

Thin-layer chromatography (TLC) was performed to determine the amount of free octanoic acid (FOA) generated from ethyl octanoate by *M. sympodialis* CBS 7222. Cultures were first grown aerobically on selective agar for pathogenic fungi (Merck) and overlaid with a thin layer of olive oil at 32 °C. After 8–10 days, cells were harvested and suspended in pure ethyl octanoate. By determining cell counts with a Neubauer chamber (dilution media xylene), suspensions were prepared to give a density of 5×10^5 cells μ l⁻¹. Selective agar for pathogenic fungi (Merck) was poured out on small glass petri dishes (3 cm in diameter) and overlaid with 100 μ l of the suspension. After incubation at 32 °C in the dark (0, 1, 3, 6 and 24 h), the suspension was taken up with 1 ml chloroform and transferred to gas-dense tubs. Ten microlitre of each sample taken up with CHCl₃ was applied to HPTLC 60-Plates (Merck) by means of a Camag Linnomat IV and fractionated by thin-layer chromatography (elution media hexane:diethylether:formic acid 80 : 20 : 2 v/v/v). After development, each plate was dipped in a solution of Primulin (Sigma P7522) (Stock 0.1% in methyl alcohol; final solution in Aceton 1 : 10 v/v) according to Wright.¹⁰ Results were

Table 2 Fatty acids and their ethyl derivatives tested.

Number of carbon atoms	Common name	Molecular formula	Chemical name/CAS
6 : 0	Caproic acid	C ₅ H ₁₁ COOH	Hexanoic acid/142-62-1
8 : 0	Caprylic acid	C ₇ H ₁₅ COOH	Octanoic acid/124-07-2
10 : 0	Capric acid	C ₉ H ₁₉ COOH	Decanoic acid/334-48-5
8 : 0	Ethyl caproate	C ₈ H ₁₆ O ₂	Hexanoic acid ethyl ester/123-66-0
10 : 0	Ethyl caprylate	C ₁₀ H ₂₀ O ₂	Octanoic acid ethyl ester/106-32-1
12 : 0	Ethyl caprate	C ₁₂ H ₂₄ O ₂	Decanoic acid ethyl ester/120-38-3

quantified densitometrically with a Camag TLC Scanner 2 using Camag software (Camag, Muttentz, CH, USA) and parallel processing of authentic octanoic acid and octanoic ethyl ester standards. Absolute values were obtained by comparison with the corresponding standard curves. GC analysis of the free fatty acid generated was omitted as former studies have shown that *Malassezia* does not transform free fatty acids in the external milieu.⁷

Results

Among the different inocula tested 10^6 CFU cm⁻² was most suitable and chosen for further testing. Smaller inocula resulted in a prolonged incubation time especially with the *Malassezia* spp., higher ones were difficult to assess. For comparison, 10 days of incubation were chosen for both the *Malassezia* spp. as well as for the *Candida* spp., although the latter were growing somewhat faster. Furthermore, with repeated testing not all of the 10 strains of each species were growing. In these cases, this is noted in square brackets in the Tables 3 and 4.

All of the *Malassezia* and *Candida* strains tested showed a growth reduction after exposition against the free medium chain fatty acids (Table 3).

Effectiveness can be graded as C 8 : 0 > C 6 : 0 >> C 10 : 0. Esp. with octanoic acid the *Malassezia* spp. *M. globosa*, *M. sympodialis* and *M. pachydermatis* seemed to be most sensitive; however, the results obtained with *C. albicans*, *C. guilliermondii* and *C. glabrata* were comparable. Less sensitive were the *M. furfur*, the *C. parapsilosis* and the *C. krusei* strains.

Table 4 shows the results obtained with the ethyl esters. Again with the exception of *M. furfur* the other *M. spp.* tested seemed to be most sensitive, but interstrain variability was higher. Totally different were the results obtained with the *Candida* spp. They showed remarkable lower sensitivity than with the free fatty acids.

For ethyl octanoate, the MICs against *M. globosa*, *M. sympodialis* and *M. pachydermatis* were ~2–3 times higher than with the FOA, but 32–106 times higher with the *Candida* spp. One exception was *C. krusei* with a relationship of 3.6 (Fig. 1).

Incubation of *C. albicans* and *M. sympodialis* on mdixon medium with Octanoic ethyl ester resulted in a MIC of 2.5 mmol l⁻¹ for *M. sympodialis* and of >160 mmol l⁻¹ for *C. albicans* respectively. Mixed suspension used as inoculum resulted in a MIC of 10 mmol l⁻¹. At this concentration, no growth of the *C. albicans* inoculum was seen, probably based on the release of FOA by *M. sympodialis*.

Table 3 Mic (mmol l⁻¹; mean and range) of different *Malassezia* and *Candida* spp. against different medium chain fatty acids after 10 days of incubation, inoculum 10^6 CFU cm⁻². If <10 strains were evaluated, the number is given in square brackets.

	C 6 : 0	C 8 : 0	C 10 : 0
<i>M. globosa</i>	3.1 (1.2–5)	1.6 (1.2–2.5)	16.5 (5–40)
<i>M. sympodialis</i>	6.25 (2.5–10)	3.75 (2.5–10)	23.5 (5–80)
<i>M. pachydermatis</i>	8.2 (2.5–10)	2	4.75 (2.5–10)
<i>M. furfur</i>	18 (10–20) [n = 6]	17.5 [5–20]	140 (40–160)
<i>C. albicans</i>	20 [n = 8]	5 [n = 9]	26.7 (20–40) [n = 9]
<i>C. parapsilosis</i>	40	20	145 (40–160)
<i>C. guilliermondii</i>	20 [n = 6]	5 [n = 9]	22.2 (20–40) [n = 9]
<i>C. krusei</i>	75 (40–80)	18.75 (10–20)	35 (20–160)
<i>C. glabrata</i>	40	6 (5–10)	46 (20–80)

Table 4 Mic (mmol l⁻¹; mean and range) of different *Malassezia* and *Candida* spp. against different medium chain fatty acids ethyl esters after 10 days of incubation, inoculum 10^6 CFU cm⁻². If <10 strains were evaluated, the number is given in square brackets.

	Hexanoic acid ethyl ester	Octanoic acid ethyl ester	Decanoic acid ethyl ester
<i>M. globosa</i>	12.9 (2.5–20) [n = 6]	5.1 (1.2–20)	50 (20–80)
<i>M. sympodialis</i>	21 (10–40)	10 (5–40)	51.25 (2.5–80)
<i>M. pachydermatis</i>	58.75 (5–80) [n = 8]	4.7 (2.5–10) [n = 9]	24 (10–40)
<i>M. furfur</i>	33.3 (20–40) [n = 6]	226 (20–320)	640
<i>C. albicans</i>	330 (80–640) [n = 8]	533 (160–640) [n = 9]	640 [n = 9]
<i>C. parapsilosis</i>	120 (80–320)	640	640
<i>C. guilliermondii</i>	106.7 (40–160) [n = 6]	426.7 (160–640) [n = 9]	640 [n = 9]
<i>C. krusei</i>	87.5 (20–320)	67.5 (20–320)	100 (20–640)
<i>C. glabrata</i>	424 (40–640)	432 (160–640)	640

Thin-layer chromatography and densitometry

Figure 2 shows the generation of FOA from octanoic ethyl ester by *M. sympodialis* CBS 7222 ($5 \times 10^5 \mu\text{l}^{-1}$; 100 μl ester). Hydrolysis starts immediately and half of the amount of the ester was hydrolysed at about 5 h.

Discussion

Antimicrobial effects of short and medium chain fatty acids against fungi have been known for decades.¹¹ In the present investigation, we could confirm antimicrobial activity against the *Malassezia* and *Candida* spp. tested for the medium chain fatty acids capric acid C10 : 0, caprylic acid C8 : 0 and caproic acid C6 : 0. Caprylic/octanoic acid was most effective under the conditions given. Most sensitive were the *Malassezia* spp. *M. globosa*, *M. sympodialis* and *M. pachydermatis* with a very low interstrain variability. However, the

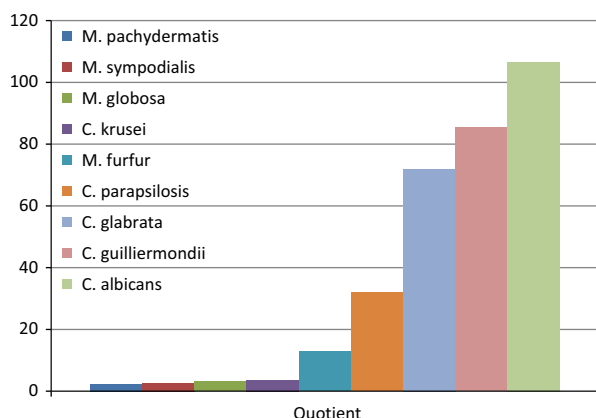


Figure 1 Relationship of the Mics obtained with ethyl octanoate and the corresponding free fatty acid for the different species.

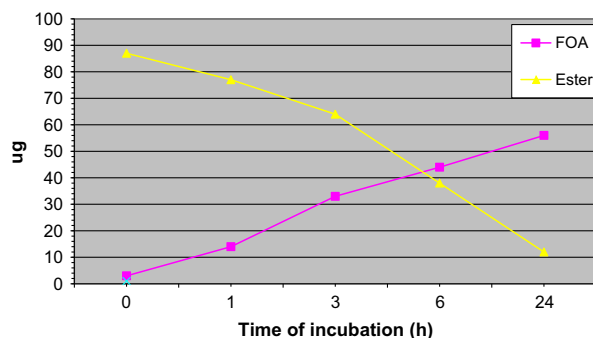


Figure 2 Time-dependent hydrolysis of ethyl octanoate by *M. sympodialis* CBS 7222 (5×10^5 CFU μl^{-1} ; total volume 100 μl).

results obtained with the *Candida* spp. *C. albicans*, *C. guilliermondii* and *C. glabrata* were comparable. In contrast, totally different were the data with the ethyl ester derivatives of these fatty acids. Ethyl esters were chosen as former studies have shown that hydrolysis was best effected in these esters.¹¹ Furthermore, they are readily available, stable and of low cost. Octanoic acid ethyl ester (CAS 106-32-1) was found to be most suitable. With the exception of *M. furfur*, the Mics of ethyl octanoate against the other *Malassezia* spp. tested were only 2–3 times higher than with the corresponding free fatty acid, but were still in a range of $<10 \text{ mmol l}^{-1}$. The reduced sensitivity and the high interstrain variability of *M. furfur* might be based on the reduced hydrolytic capacity of this species.⁷ Most remarkable was the dramatically reduced antimicrobial efficacy of the ethyl esters against *Candida* spp. Obviously, these yeast do not hydrolyse the ethyl esters, so that the corresponding fatty acid as the antimicrobial active principle is not released. This is well in line with the observation that most of these ethyl esters were generated in fermentation processes by *Candida* spp. and with their fruity odour serve as flavours in some foods. However, more sensitive were the strains of *C. krusei*. It is not known whether this result might be based on hydrolysis as there are no data available in the literature.

As shown densitometrically with *M. sympodialis* yeast-dependent hydrolysis of ethyl octanoate started immediately. There is no preferential utilisation of other lipid sources offered simultaneously like olive oil or Tween 40 as parts of the mdixon agar. In conclusion, the hydrolytic exoenzymes of *Malassezia* involved in these processes seemed to be rather unspecific.

This underlines that the antimicrobial effects of ethyl esters found in this investigation can be of therapeutical value. Topical application of these esters might be of benefit in *Malassezia*-associated diseases in which an increase in *Malassezia* numbers plays a significant role. Their antimicrobial potential is selectively activated by the target organism itself esp. in areas with highest populations, since the activation of these agents is exclusively mediated by yeast-specific enzymes. In contrast to azole antimycotics antimicrobial resistance seems not to be a problem for the free fatty acids. Ethyl esters are cheap, they are widely used as natural flavours and are non-toxic and non-allergenic. In contrast to the free fatty acids, topical therapy with the esters is not limited by their intensive unpleasant smell and acidity. However, in our test system, the inocula were 100 times higher than the populations reported by Leeming *et al.*¹² for healthy skin

(1.1×10^4 CFU cm⁻² on the chest; 8.9×10^3 on the back, 4.8×10^3 forehead, 10^4 on the cheek). But higher yeast numbers may be of relevance in malassezia-associated diseases. Among these seborrhoeic eczema/dandruff, malassezia-triggered atopic eczema, Malassezia folliculitis and animal Malassezia otitis are of specific interest.^{1,13–15} In these very common skin diseases, antimycotics are of therapeutic value but targeting with known antimycotics is not possible and azole resistance is an emerging problem.¹⁵ There is a known interaction of topicals and the malassezia microflora. Previous studies have shown that topical application of lipid-containing formulations, such as suntan cream or oil bath, can support growth and thereby exacerbating Malassezia-related skin diseases.^{16,17} Ethyl esters of medium chain fatty acids esp. ethyl octanoate can reverse these growth promoting effects by generating a selective activation of antimicrobial activity esp. in areas well populated with Malassezia yeast ('targeting'). They may be suitable for topical application in that way, as fatty acids esters already used in topicals as refatting agents and spreading promoters (e.g. isopropyl myristate, stearate) are replaced equimolar by ethyl octanoate. The concentrations thus obtained may be in the range in which effectiveness was shown in this study. However, this concept has to be supplemented by investigations *in vivo*. Furthermore, the resident Malassezia microflora can be utilised to influence the other microflora as shown in the preliminary coinubation experiment. For ethyl octanoate, the growth of *C. albicans* was dramatically reduced by coinubation with *M. sympodialis*. This might be due to a specific effect of medium chain fatty acid generated and/or simply to reduction of skin surface pH. For example, growth of *S. aureus* – in contrast to *S. epidermidis* – was dramatically reduced at low pH.¹⁸

The use of ethyl esters esp. ethyl octanoate in well-designed topical preparations might therefore represent a new therapeutic principle which uses the malassezia microflora to gain antimicrobial effects. These agents are designed such that they only act locally at the site with the highest Malassezia density, since the activation of these agents is exclusively mediated by yeast-specific enzymes.

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Conflicts of interest

The author states no conflict of interest. For the use of esters of medium-chained saturated acids for the prophylaxis and treatment of Malassezia-associated diseases, there is a patent application at the Justus Liebig University Giessen (Deutsche Patentanmeldung 10 2013 009 616.2).

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