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Effect of Australian tea tree oil on the viability of the wall-less bacterium *Mycoplasma pneumoniae*

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In vitro assays using a variety of essential oils revealed a particularly high antibacterial effect of Australian tea tree oil (TTO) on a great number of gram-negative and gram-positive bacteria of unrelated phylogenetic origin. In the present study, the susceptibility of cell wall-less bacteria such as the human pathogenic bacterium *Mycoplasma pneumoniae* to Australian tea tree oil was examined. The minimum inhibitory concentration (MIC) was determined to be 0.006% (v/v) TTO for the wild type and to 0.003% (v/v) TTO for mutants of *M. pneumoniae* which lost the ability to adhere to host cells (cytadherence-negative). The MIC and the MBC (minimum bactericidal concentration) for *M. pneumoniae* are 100 times lower than those for all other eubacteria tested. Electron microscopy with negatively stained cells as well as with ultrathin sections revealed a tendency to ovoid or round cells after oil treatment whereas the untreated cells of the wild type exhibit a flask-shaped morphology with a tip-like structure at one pole of the cell. The integrity of the mycoplasmal membrane seems not to be affected by TTO since no leakage of the *Mycoplasma* cell was observed after oil treatment. In the HET-CAM test TTO did not show any visible signs of irritation in concentrations less than 25%. Although the active component in TTO that has anti-mycoplasmal activity is not known, it seems very promising to use TTO tentatively for mouth washing and inhalation in case of *Mycoplasma-pneumoniae*-infection.

1. Introduction

The taxonomic class of the wall-less bacteria (*Mollicutes*) comprises six different genera. Two of these genera, namely, *Mycoplasma* and *Ureaplasma*, contain human pathogenic species. *Mycoplasma hominis*, *M. genitalium,* and *U. urealyticum* may cause genital infections whereas *M. fermentans* and *M. penetrans* are found systemically in AIDS patients [1]. *M. pneumoniae* colonizes the epithelium of the human respiratory tract and may cause tracheobronchitis or even atypical pneumonia, in particular in children between 5 and 7 years of age or in adults between the age of 30 and 35. In these age groups *M. pneumoniae* accounts for 20–30% of all cases of community acquired pneumonia. Infections caused by *M. pneumoniae* may be followed by several complications of the ear, the heart, the nervous system or the skin, which in turn might be associated with immunopathologic reactions [2].

Mycoplasmal infections can be usually cured by tetracyclines, macrolides, and fluoroquinolones. However, mycoplasmas can even survive long-term antimicrobial treatment, particularly in an immunocompromised host. Mycoplasmas are generally resistant to rifampicin, β-lactam antibiotics, glycopeptides, polymyxin, nalidixin acid, and trimethoprim. Resistance to these antimicrobial agents is linked to characteristic mycoplasmal features such as the lack of a cell wall or a particular structure of the RNA polymerase. Recently, there have been several reports on acquired resistance of *Mycoplasma* species. *U. urealyticum* and *M. hominis* isolates have become resistant to tetracycline, usually after acquisition of the *tetM* determinant [3]. Fluoroquinolone resistance was described recently for *M. hominis*, because of the substitution of a serine by a leucine in the DNA gyrase [4]. Macrolides are very effective against *M. pneumoniae* and *M. genitalium*; however, recently a macrolides-resistant *M. pneumoniae* strain exhibiting a punct-mutation in the 23S rRNA gene was isolated, resulting in a decrease of the binding affinity of erythromycin to the ribosome [5]. Acquired antibiotical resistance of mycoplasmas may lead to severe problems in curing mycoplasmal infections because of their innate resistance to a considerable number of anti-infectives.

Considering these problems there is an urgent need to search for new antimicrobial agents with bactericidal activities against these pathogens. *In vitro* assays revealed that Australian tea tree oil (TTO), used as a component of a diverse range of pharmaceutical and cosmetic products, is a potent antimicrobial agent affecting the viability of a broad spectrum of bacteria and yeast such as *Escherichia coli, Staphylococcus aureus, Aspergillus niger,* and *Candida albicans* [6–9]. These studies were extended to assess the efficacy of tea tree oil against wall-less bacteria. The wild type and two cytadherence-negative mutants of *M. pneumoniae* were chosen as the representative test organisms for examining the susceptibility of this class of bacteria to tea tree oil.

Additional, we investigated TTO for its potential to irritate the mucous membrane using the chorioallantoic membrane (CAM) of chicken eggs as the sensitive test system (HET-CAM test). The result of this investigation may be of interest in regard to the subsequent therapeutic application of tea tree oil (e.g., for inhalation, mouth washing) in children and adults.

2. Investigations and results

2.1. Chemical characterization of fresh and aged Australian tea tree oil

Since the chemical composition of Australian tea tree oil (TTO) affects the growth of *M. pneumoniae*, the essential oil was chemically characterized before using it in the bioassay. The results are listed in the Table. The major components were identified by comparing their mass spectral data with those of authentic terpene standards and the literature data as well as by coinjection with authentic substances.

Fresh TTO: The Australian tea tree oil is a complex mixture of approximately 100 compounds. The commercial essential oil was derived from *Melaleuca alternifolia* (Myrtaceae). It consisted mainly of terpinen-4-ol (37.6%), γ-terpinene (21.0%), and α-terpinene (11.2%). There were also small amounts of closely related monoterpenes, e.g., α-pinene, β-pinene, p-cymene, 1,8-cineol, terpinolene, and α-terpineol as well as the sesquiterpenes aromadendrene,

Table: Main compounds of Australian tea tree oil (ALVA, Wallenhorst): fresh distilled oil and aged oil (2 months old)

Australian tea tree oil Mono- and Sesquiterpenes	Concentration (%) Fresh distilled oil	Concentration (%) Aged oil (2 months old)
α-Pinene	2.4	2.8
β-Pinene	1.4	1,6
α-Terpinene	11.2	5.2
p-Cymene	2.0	11.5
1,8-Cineole	3.9	3.9
γ-Terpinene	21.0	13.7
Terpinolene	3.5	2.8
Terpinen-4-ol	37.6	35.0
α-terpineol	2.1	3.4
Aromadendrene	0.7	0.8
Viridiflorene	1.0	1.3
δ-Cadinene	1.1	1.2
Peroxide number	25 ppm	> 500 ppm

viridilorene, and δ-cadinene (Table). The commercial tea tree oil used in our investigation meets the Australian Standards (< 15% 1,8-cineole; > 30% tepinen-4-ol) [7, 8].

Aged TTO: Fresh TTO is chemically stable when stored in sealed, inert brown flasks and protected against prolonged high temperatures, the ingress of air, and particularly against light. On the other hand, TTO stored in transparent flasks and exposed to light showed a rapid deterioration (s. Tab. 1). In the oxidized oil, the p-cymen content increased dramatically from about 2% to 11.5%, whereas the content of α-terpinene, γ-terpinene, and terpinolene was reduced to smaller amounts. Simultaneously, the peroxide number of the oil increased from about 25 ppm (fresh distilled oil) to greater than 500 ppm in the oxidized oil [10, 11].

2.2. Effect of tea tree oil on Mycoplasma pneumoniae

After tea tree oil treatment the metabolically inactivated wild type-strain lost its ability to adhere to glass or plastic. The MIC for the wild type was 0.006% (v/v) tea tree oil and for the mutants 0.003% (v/v). No growth could be observed after transfer of the tea tree oil-treated cultures into fresh medium, indicating that the MIC is identical to the minimum bactericidal concentration (MBC) of 0.006% (v/v) for the wild type and 0.003% (v/v) tea tree oil for the mutants. The MIC and the MBC for *M. pneumoniae* were 100 times lower than those for all other tested eubacteria. The efficacy of aged oil as an antimycoplasmal agent decreased by a factor of 2 in comparison to fresh oil. Thus, the MIC as well as the MBC of the altered tea tree oil was determined to be 0.012% (v/v) for the wild type and 0.006% (v/v) for the mutants.

2.3. Ultrastructural data

While essential oils were extensively tested against a broad spectrum of bacteria, yeast, and fungi, the interaction between essential oils and bacteria which ultimately induces the antibacterial activity is not well understood. Essential oils are complex mixtures of highly lipophilic compounds. Therefore, the mechanism of action of essential oils has generally been described as being nonspecific and resulting in alterations of the cytoplasmic membrane [12].

In the case of *M. pneumoniae* we investigated both negatively stained cells and ultrathin sections of oil-treated and -untreated mycoplasmas by electron microscopy. After oil

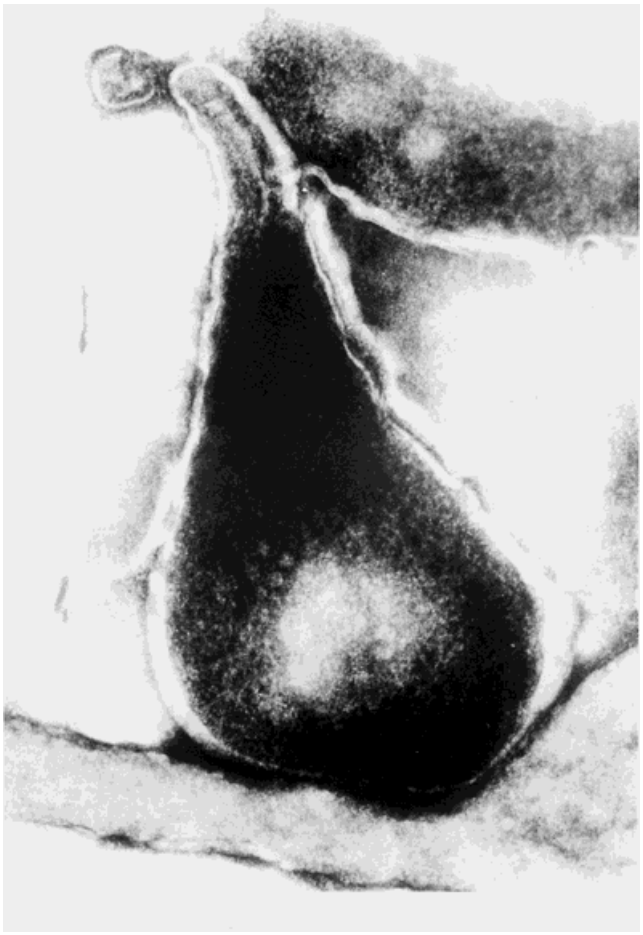

Fig. 1: *Mycoplasma pneumoniae*. Untreated cell with a flask-shaped morphology with a tip-like structure at one pole of the cell

treatment mycoplasma cells showed an ovoid or round morphology, whereas the untreated cells exhibit a variety of morphological features, including pear-shaped or flask-shaped bacteria with a tip-like structure at one end of the cell body (Fig. 1, 2). However, the overall structure of the bacterial cell membrane seemed not to be disturbed by exposure to tea tree oil.

2.4. Effect of tea tree oil on the mycoplasmal membrane

After tea tree oil treatment of *M. pneumoniae* no extracellular lactate dehydrogenase activity could be measured even after an incubation time of 60 min, indicating that

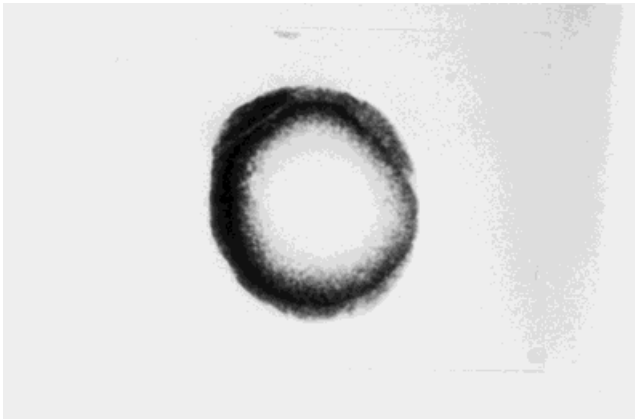

Fig. 2: Mycoplasma pneumoniae. Rounded cell shape after treatment with tea tree oil

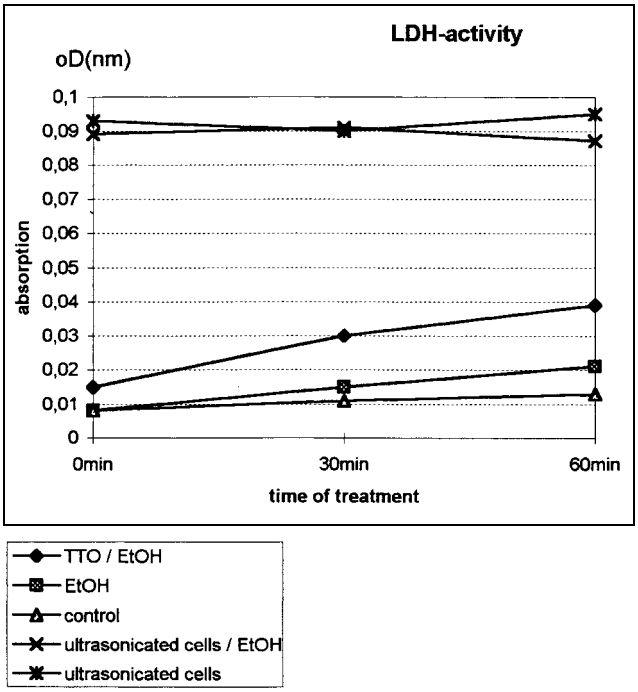

Fig. 3: Measurement of the extracellular lactate dehydrogenase activity at 340 nm. LDH = lactate dehydrogenase; TTO = tea tree oil; EtOH = ethanol.

tea tree oil did not cause leakage of the mycoplasmal membrane. In the supernatant of ultrasonicated mycoplasmas a tenfold increase in lactate dehydrogenase activity compared to supernatants of untreated or tea tree oil-treated mycoplasmas was observed, which proved the presence of lactate dehydrogenase in the *Mycoplasma* cell. Control experiments revealed that neither ethanol nor tea tree oil affected the lactate dehydrogenase activity (Fig. 3, 4).

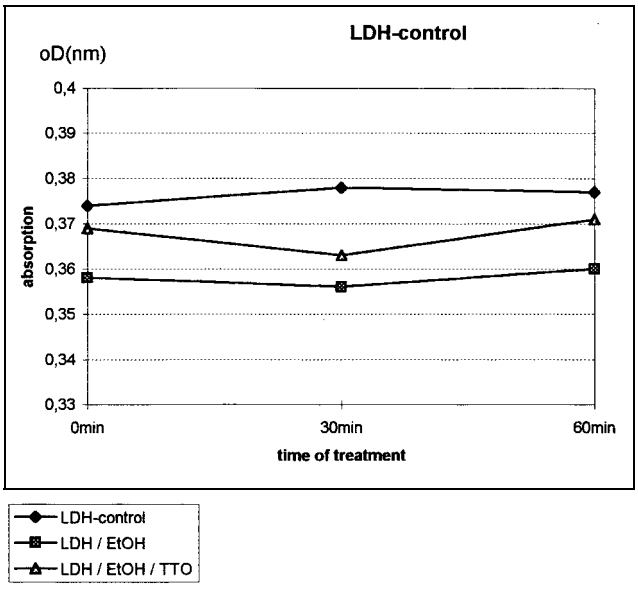

Fig. 4: Control measurement of the lactate dehydrogenase activity at 340 nm. LDH = lactate dehydrogenase; TTO = tea tree oil; EtOH = ethanol

2.5. Effect of tea tree oil on the mucous membranes

Australian tea tree oil was investigated for its potential to irritate mucous membranes. As an alternative to animal testing (e.g., Draize rabbit eye test), the HET-CAM test was employed. It is assumed that acute, irritating effects on the small vessels of the chorioallantoic membrane are similar to those induced by the same substances in the rabbit eye [13, 14]. Therefore, the effects of tea tree oil on the chorioallantoic membrane, such as hemorrhage, lysis, and coagulation, were evaluated to assess potentially irritating properties of this essential oil.

It is known from the literature [7, 8, 15] that tea tree oil possesses skin-irritant potential when applied as neat oil to both intact and abraded skin of albino rats (Draize index: 5). In contrast, when tea tree oil was diluted to 25% in paraffin oil and applied to the shaved skin of rabbits for 30 days it failed to produce any visible signs of irritation.

Our results with tea tree oil in the HET-CAM test support the finding that diluted tea tree oil is not or only moderately irritant to the skin and mucous membranes. Tea tree oil diluted to 20% in olive oil produced moderate irritation. Less than 20% of tea tree oil in olive oil as well as olive oil itself did not cause any adverse effects to CAM.

3. Discussion

An antimicrobial activity of Australian tea tree oil against gram-negative and gram-positive bacteria as well as against fungi has been demonstrated in several studies [6 to 8, 16]. Australian tea tree oil is a complex mixture of mono- and sesquiterpenes. The most effective antimicrobial components of the tea tree oil were shown to be terpinen-4-ol, and α-terpineol [9, 17–19]. *Mycoplasma pneumoniae,* a member of the cell wall-less bacteria proved to be about 100 times more sensitive to tea tree oil than all other gram-positive or gram-negative bacteria tested so far. The reason for this phenomenon is not yet well understood. The lipophilic terpenes of tea tree oil proved to have surface active properties such as reduction of surface tension. Moreover, these terpenes are potent membrane-penetrating reagents. Once inside the cell, they may interfere with the metabolism of the micro-organisms causing the bactericidal or fungicidal effects. The ovoid or round mycoplasma cells seen after oil treatment may be a result of breaking the surface tension of the cells or of an as yet unknown mechanism affecting the cytoskeleton. As a result of the altered cell structure, mycoplasmas lost their ability to adhere to glass or plastic. Although ultrastructural data exhibited no signs of cell membrane destruction it cannot be excluded that tea tree oil might interfere with specific targets in the cell membrane, for instance, with ion channels or carrier proteins. However, the data described in the present paper do not suggest any adverse effects of tea tree oil on the permeability of the mycoplasmal cell membrane, since no extracellular lactate dehydrogenase activity was detected after oil treatment. This finding could be confirmed by preliminary tests with the mycoplasmal membrane-associated enzyme prolyl iminopeptidase, which exhibited considerable activity even after oil treatment of intact mycoplasmal cells. To learn more about the mode of action of tea tree oil, in addition to the ultrastructural data, more biochemical data are also needed.

Interestingly, an increase in the antimycoplasmal efficacy of the tea tree oil towards nonadherently growing mutants of *M. pneumoniae* by a factor of 2 was observed. We suggest that this may be caused either by the more fragile nature of the mutants or by some degree of protection of the adherent wild-type strain by its contact with the culture flask. The antimycoplasmal efficacy of altered oil with a content of > 500 ppm peroxide decreased by a factor of 2 compared to fresh oil, indicating that the viability

of *M. pneumoniae* is not impaired by peroxide but by the intact components of fresh tea tree oil. For other eubacteria the aged oil showed a higher degree of antimicrobial activity, probably because of an oxidative killing of the cells. It is known from a considerable number of studies that *M. pneumoniae* is extremely resistant to the peroxides [20]. *M. pneumoniae* seems to have developed a hitherto unknown protective mechanism against oxidative agents since this pathogen produces high concentrations of hydrogen peroxide itself. The cause for hydrogen peroxide production is a truncated respiration chain in conjunction with the lack of catalase and superoxidismutase. The same innate mechanism may protect *M. pneumoniae* from the adverse effects of peroxides in the aged tea tree oil. However, this hypothesis needs further evaluation.

There are only few case reports of toxic effects of tea tree oil in humans [7–9]. If neat tee trea oil was used, side effects such as allergic contact dermatitis or local irritations were reported [7, 9]. Considering these facts, it is very interesting that diluted tea tree oil (< 20%) did not cause any irritation when applied to the shaved skin of rabbits [15] or CAM, respectively.

The results presented here demonstrate that Australian tea tree oil is a potential candidate for a new therapeutic agent among the limited number of efficacious anti-infectives available against wall-less bacteria. In regard to the subsequent therapeutic application of TTO in children and adults it is also of interest that diluted TTO (less than 20%) exhibited in the HET-CAM test none or only moderate irritations to mucous membrane. Furthermore, Hayes et al. [21] found that TTO exhibits a cytotoxic effect to HeLa cells at 0.27% (IC_{50} value). In contrast to HeLa cells, mycoplasmas responded under the prevailing conditions clearly more sensitively to tea tree oil (MIC: 0.006%). This finding is of great interest because the human cell line HeLa is phenotypically similar to human skin fibroblasts. Considering all these experimental facts, it seems very promising to use Australian tea tree oil tentatively for inhalation and mouth washing in case of Mycoplasma infection. Nevertheless, clinical studies are needed to evaluate in detail antimicrobial efficacy versus adverse effects in treating mycoplasmal infections in humans.

Since particularly the mycoplasmas causing genital tract infections increasingly develop resistance to tetracycline and fluoroquinolones, studies should be extended to determine the susceptibility of genital tract bacteria such as *M. hominis, M. genitalium,* and *U. urealyticum* to Australian tea tree oil.

4. Experimental

4.1. Australian tea tree oil

The Australian tea tree oil (TTO) tested was a commercial product supplied by ALVA, Wallenhorst, Germany.

4.2. Aged Australian tea tree oil

Twenty milliliters of TTO were poured into a transparent 1-liter flask, which was sealed and exposed, at room temperature, to the light and warmth of summer to autumn days on a window sill over a period of 2 months. Samples of the altered oil were then subjected to gc analysis of the degradation products, and the chromatograms were compared with those of unchanged (fresh) oil. The formation of peroxides in TTO was determined by a commercial kit (Perex Test, Merck, Darmstadt, Germany). The kit has a color scale for the determination of peroxides in aqueous and organic solution [11].

4.3. Terpene standards

Standards including 1,8-cineole, p-cymene, α-pinene, β-pinene, α-terpinene, γ-terpinene, terpinolene, terpinen-4-ol, α-terpineol were purchased from Roth, Karlsruhe, Germany, and aromadendrene and viridiflorene from Fluka, Buchs, Switzerland.

4.4. GC method

TTO was analyzed as 1% solution in n-hexane containing tridecane as the internal standard. GC was performed using a Carlo Erba GC 6000 chromatograph equipped with a Spectra Physics Integrator SP 4290. The GC column was a 15-m × 0.25-mm (i.d.) fused silica capillary column coated with OV 1 (phase thickness: 0.25 μm) and with helium as the carrier gas (flow rate: 2 ml/min.); split: 1 : 5. Temperature program: The initial column temperature was 40 °C for 4 min. Subsequently, the temperature rate was programmed from 40 °C to 300 °C in two steps, first 4 °C/min. up to 120 °C and then 10 °C/min. up to 300 °C. Injector temperature: 250 °C; detector temperature: 300 °C; injection volume: 1 μl.

4.5. GC-MS method

A gas chromatograph Carlo Erba HRGC 4160 was coupled via an open, split interface to a Finnigan MAT 4500 mass spectrometer (MS). GC column: 30-m × 0.25-mm (i.d.) fused silica capillary column coated with OV 1 (phase thickness: 0.25 μm). Split: 1 : 20. Temperature program: 46 °C for 4 min; 3 °C/min. up to 76 °C and then 4 °C/min. up to 136 °C and 6 °C/min. up to 300 °C. EI ionizing voltage: 70 eV.

4.6. Strains and culture conditions

Only two virulent isolates of *Mycoplasma pneumoniae*, FH and M129-B18 are commercially available. For the studies described in this article we used the isolate M129-B18 (ATCC 29342) and two previously described spontaneous hemadsorption-negative mutants (M5 and M6). M5 lacks two membrane proteins of 90 and 40 kDa, which are involved in effective cytadherence. This mutant resembles the avirulent, high-passage strain B176 [22–24]. M6 lost the HMW1 protein and exhibits a C-terminal truncation of the adhesin-related, 30 kDa protein by 5 kDa (loss of 8 out of 13 proline-rich repeat sequences) [25]. *M. pneumoniae* M129-B18 (ATCC 29342) and the spontaneous mutants M5 and M6 were grown for 24 h at 37 °C in 137 cm^2 Roux flasks containing 120 ml modified Hayflick medium [26] before adding various concentrations of tea tree oil.

4.7. Treatment of M. pneumoniae with tea tree oil

To log-phase cultures of the adherent growing strain *M. pneumoniae* M129 (wild type) and of the two described non-adhering mutants lacking membrane or cytoskeleton proteins TTO was added to give the following final concentrations: 5×10^{-2}%, 2.5×10^{-2}% to 19.5×10^{-5}% (dilution steps: 1 : 1). To keep the oil in solution the growth medium contained 1% (v/v) ethanol. Controls consisted of culture medium with 1% ethanol without TTO. After cultivation for another 12 h, the minimum inhibitory concentration (MIC) was determined by the color changing test, using phenol red in the culture medium as an indicator of the pH. Metabolically active mycoplasmas produce acid which can be detected by the change of the indicator phenol red from red (pH 7.5) to yellow (pH 6.8 or less). Once the mycoplasmas are inactivated, no acid is produced and therefore the red color of the culture medium persists. To evaluate bactericidal activity the TTO treated cultures were diluted in fresh media ten times and incubated for 48 h. Aging (= oxidative alteration) of TTO correlates with the peroxide concentration [10]. To evaluate the effect of oxidized TTO (peroxide number: > 500 ppm) on its antimicrobial efficacy the procedure described above was also carried out with altered oil. All experiments were performed two times.

4.8. Permeability testing

To assess the effect of TTO on the mycoplasmal membrane, cell permeability was tested before and after oil treatment. Extracellular activity of lactate dehydrogenase served as a marker for the bacterial membrane integrity. Lactate dehydrogenase activity was determined with a test-combination kit from Boehringer Mannheim according to the method described by the manufacturer. In the presence of lactate dehydrogenase lactate is oxidized to pyruvate and NAD^+ is reduced to NADH. The increase of NADH (measured at 340 nm) is equivalent to the enzyme activity. Lactate dehydrogenase activity in supernatants of oil-treated (for 1 h) and -untreated mycoplasmas was measured. As positive controls, the supernatants of ultrasonicated *M. pneumoniae* cells with and without ethanol (1%) were used, since ethanol served as a solvent for TTO in all test systems. For each test, a pellet of a 50-ml log phase culture of *M. pneumoniae* was resuspended in 1 ml PBS and treated with either ultrasonication or ethanol (1%), or TTO (0.006%) in 1% ethanol. One control was not treated at all. Furthermore, 100 μl of the supernatant of each sample was added to the standard test system provided by Boehringer Mannheim containing NAD^+, pyruvate and lactate (for more experimental details see enclosed test outlines). The effect of TTO or ethanol on the activity of lactate dehydrogenase was assessed by adding each reagent and a combination of the two to the test system containing lactate dehydrogenase, NAD^+, and lactate. All experiments were performed two times.

4.9. Transmission electron microscopy

Before and after treatment with TTO, cultures of the wild type were fixed with glutaraldehyde (2%) (Serva, Heidelberg, Germany) for 10 min. The untreated wild type was scraped off the flask and Formvar-coated copper grids (Plano, Wetzlar, Germany) were inoculated with a drop of fixed oil-treated and -untreated bacterial suspensions. Thereafter, the specimen were negatively stained with phosphotungstic acid pH 7.0. Transmission electron microscopy was carried out with a Philips CM10 electron microscope.

4.10. Hen's egg chorioallantoic membrane test (HET-CAM test)

The HET-CAM test is an organotropic test in which test material is applied to the sensitive chorioallantoic membrane (CAM) of chicken eggs on day 9 of embryonation [13, 14]. We used fresh, fertile eggs (7 eggs per concentration tested) from a white Leghorn race supplied by a commercial breeding farm (LSL Rhein/Main, Schaafheim, Germany). After incubating at 37 °C for 9 days, the eggs were opened at the side of the air chamber (= blunt end) by a small electric rotating saw-blade. Subsequently, the chorioallantoic membrane was revealed by opening up the interior egg membrane. Different concentrations of TTO (1%, 5%, 10%, 15%, 20%, 25%, 30%, 50%, 100%) were dissolved in olive oil and 0.2 ml of the solution were transferred to the chorioallantoic membrane. Additionally, we used 0.5% SDS as a positive control and olive oil and 0.9% NaCl solution as negative controls. All tests were performed two times. Our CAM protocol called for an observation period of 5 min. after application of test material. For assessing the reaction of tea tree oil at CAM the latter was examined for the severity of hemorrhage, lysis, and coagulation. Irritation was evaluated as *no irritation*: no reaction of TTO at CAM; *moderate irritation*: mild hemorrhage, dilatation of capillary and blood vessels; and *strong irritation*: strong hemorrhage, coagulation, and lysis.

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