The antimicrobial effect of Indian lemongrass (Cymbopogon citratus) and investigation of the potential therapeutic possibility of pitted keratolysis in case of skin infection

Doctoral (PhD) theses

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CHAPTER 1: General introduction and objectives

The topic of my thesis is the investigation of the antimicrobial effect of the essential oil of Indian lemon grass, within which we also focus on the effect against the causative agents of skin surface infection, pitted keratolysis. One of the most pressing public health problems today is the widespread existence and worldwide spread of resistance to traditional and therapeutic antibiotics. This fact encourages researchers to search for and use alternative antimicrobial substances that may be suitable for the treatment of infections caused by various human pathogens (Swamy et al., 2016). In this regard, the essential oils obtained from plants and their main chemical components are potential antibacterial agent candidates. Due to their antibacterial activity, essential oils can inhibit the growth of bacteria (bacteriostatic effect) or cause the bacterial cell to die (bactericidal effect) (Swamy et al., 2016). Previous studies confirm that Cymbopogon citratus essential oil and its main component, citral, have strong activity against a wide spectrum of fungal and bacterial species (Naik et al., 2010; Shi et al., 2017). Based on these previous literature data and a preliminary selection, we concentrated in our experiments on the antibacterial effect of the essential oil of Indian lemongrass, examining the possibility of its effectiveness against the infectious agents previously associated with pitted keratolysis. Pitted keratolysis is a common skin surface bacterial infection that affects the topmost layer of the skin, the stratum corneum, but as we know from early cases, in an advanced state it can also penetrate deeper layers, significantly affecting the quality of life of those affected (Singh and Naik, 2005; Leeyaphan and others, 2019; Bunyaratavej et al., 2018). Typical symptoms of this local infection include unpleasant foot odor, and the affected area becoming more and more sensitive, which later experiences a burning and itching sensation, and then even continuous pain (Law et al., 2019). Antibiotics are currently used for its treatment, of which the two most common agents are erythromycin and clindamycin. After successful therapy, it often happens that the infection recurs shortly after treatment (Kaptanoglu et al., 2012). In some cases, the reason for unsuccessful therapy is resistance to the above agents (Leung and Barankin, 2015) or rapid reinfection.

The sample to be examined for my work was provided by the case of a 43-year-old man, on both of whose soles there were pits (pits) typical of the infection and crater formations resulting from their fusion. In addition to the unpleasant odor present as another typical sign of infection, the patient also reported an irritating, burning sensation and itching. The symptoms appeared during the warm summer period. It can also be known that the patient has been wearing closed shoes for a long time. From the available literary data, our knowledge about the etiological agents of the infection is limited, but it was during this work that we had the opportunity to isolate a bacterial species from a domestic case, which has not been associated with PK until now, and on which our work was significantly focused. Since the localization of the infection can be ideal, an alternative antimicrobial, e.g. for the implementation of essential oil-based treatments, in the framework of this work, we aimed to go around this possibility, the didactic goals of which were to:

identify the bacterial species from the affected sole areas and determine the etiological agent(s),

● we examine the effectiveness of essential oils with antibacterial properties, in such a way that they are tested on a bacterial isolate cultured from the affected pits, likely to be a pathogenic agent (*Bacillus thuringiensis*), and on the bacterial species responsible for causing pitted keratolysis,

● we examine more thoroughly the antimicrobial effect of Indian lemongrass oil, which has one of the most effective antibacterial properties and also inhibits the spore formation of *B. thuringiensis*,

● identify the active components of Indian lemongrass against *B. thuringiensis*,

● determine the in vitro effectiveness of Indian lemongrass mixed in different ointment bases and concentrations on the aetiological agents of pitted keratolysis,

● in a skin penetration model, we determine the dissolution efficiency of individual components from different lubricant pads.

● with whole transcriptome analysis (WTA), we examine the gene expression pattern changes in *B. thuringiensis* under the influence of Indian lemongrass essential oil and compare them with the changes in the presence of erythromycin.

CHAPTER 2: Characterization of bacteria isolated from a case of pitted keratolysis using classic microbiological methods

1. Materials and methods

1.1. Bacteria used, their identification

During my work, we isolated bacteria from pitted keratolysis lesions of the soles of a 43-year-old healthy man. We ruled out the possibility of erythrasma by examining the infected sole with a UV lamp. Impressions and scrapings were taken and these samples were inoculated onto Luria Bertani (LB), brain heart infusion (BHI), blood, anaerobic blood (hemin and vitamin K1 content) and Sabouraud agar, then a part of it was aerobic and elevated (5%) CO2 for 24 hours was grown by incubating in its presence, while anaerobic blood agars were incubated under anaerobic conditions for 48 hours. We prepared the culture media ourselves and the bacterial growth on them was carried out at 37 °C in a thermostat. Anaerobic plates were evaluated after 14 days, while Sabouraud plates were evaluated after 7 days of incubation.

Then followed the identification of the bacteria, during which 10 colonies from colonies with the same morphology were taken with an inoculum, and then identified using MALDI-TOF MS (De Carolis et al., 2014). Since, in one case, MALDI TOF-based identification did not yield results, we therefore resorted to 16S rRNA sequence-based determination. For this purpose, universal prokaryotic 16S rRNA primers (Uni16S27_F: AGAGTTTGATCCTGGCTCAG, Uni16S1492_R: GGTTACCTTGTTACGACTT) were used (Tabei and Ueno, 2010).

The 1465 bp long product was amplified with the proofreading Pfu PCR enzyme kit (Thermo Scientific, F-531) and then ligated using the pJET1.2/blunt vector (Thermo Scientific, K1231). The inserted fragment is sequenced with primers after sequencing, the missing part was bridged using the Macr_16S_2 (5' GGCTAACTACGTGCCAGCAG) primer. All sequencing was performed with the ABI310 system according to the ABI Big Dye Sequencing Protocol using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific). CLC Sequence Viewer version 7.6 was used to align the obtained sequences, and then they were blasted on the NCBI website (www.ncbi.nlm.nih.gov).

The isolated strains were frozen in our strain collection.

1.2. Breeding conditions

Among the bacteria examined for daily experiments, *B. thuringiensis, S. simulans, S. haemolyticus, Macrococcus* sp. were inoculated onto Müller-Hinton (MH) medium. species, while *S. parasanguinis* and *S. mitis* species were incubated on blood agar in a CO2 thermostat. The incubations took place at 37 °C for 24 hours, after which the bacteria were grown in MH medium at 37 °C for 24 hours, except for *Streptococcus*. In the case of the two *Streptococcus* species, we prepared a suspension in PBS from the bacteria taken from the fresh blood agar on the day of the experimental work, as these species could not be grown in a liquid medium.

1.3. Enzymatic tests

In order to determine the pathological significance of the isolated bacteria, we examined their enzymatic activity. For this, we observed their ability to break down casein, gelatin, lipid and lecithin.

Extracellular protease production was tested on modified milk agar (2% w/v peptone, 0.6% w/v NaCl and 2% w/v agar) (Chu, 2007). At the time of preparation, the total calculated for the agar concentration half of the amount of liquid was added to the culture medium components in the form of distilled water, and after autoclaving, the previously heated (42 °C) 1.5% milk was added in the same amount as the water. The isolates were inoculated onto the solidified culture medium and their protease activity could be read after 1-2 days of incubation after the inoculation by observing the clearing zones that appeared around the positive isolates.

The lipase activity of the isolates was tested on Tween 80 agar. The autoclaved stock solution (10 g of peptone, 5 g of NaCl, 0.1 g of CaCl2 and 15 g of agar in 1 liter of distilled water) was cooled to ~65 °C, and then 5 ml of Tween 80 solution (Slifkin, 2000) was added. We inoculated the solidified culture medium

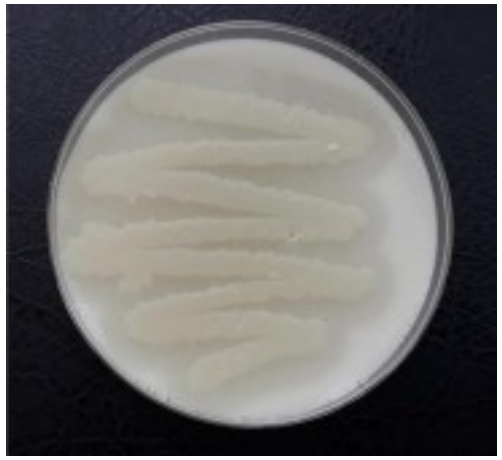
tested bacteria, and after 1-2 days of incubation, we evaluated them based on the amount of punctate precipitates formed in the agar around the positive colonies.

Lecithinase activity was tested on agar prepared using egg yolk. The washed, disinfected eggs with 70% alcohol were broken open, then the yolks were separated in a sterile container and diluted to 40% v/v with sterile distilled water. 10 ml of this was mixed with 90 ml of the previously prepared, autoclaved BHI agar cooled to ~55 °C. After inoculation, the solidified media were incubated for 3 days at 28 °C. The appearance of turbid zones indicating the formation of fatty acids indicates the positivity of the enzyme test (Jonit et al., 2016).

For gelatin hydrolysis, carbon-containing gelatin columns were used (Lányi, 1980). The sterile columns were added to the nutrient solution suspension of the bacteria and then incubated at rest for 3 days at 30 °C. The integrity of the gelatin cubes daily we checked. The production of gelatinase was indicated by the settling of carbon particles to the bottom of the tube (Kohn, 1953).

2. Results

2.1. Enzymatic tests



Detection of strong protease activity of B. thuringiensis on milk agar. The presence of a halo phenomenon along the serpentine-like spread of the bacteria can be clearly observed, which indicates the digested milk protein and thus the ability of the tested species to secrete a protein-degrading enzyme.

CHAPTER 3: Essential oil-based antibacterial tests

1. Materials and methods

1.1. Bacterial isolates and culture conditions

The bacterium *B. thuringiensis* was isolated from lesions on the sole of a 43-year-old man (Schneider and Schweitzer, 2021), while *Kytococcus sedentarius* (DSM 20547) and *Dermatophilus congolensis* (DSM 44180) were obtained from the German National Tribal Collection (DSMZ). (Leibniz Research Institute, Braunschweig, Germany). During my work, all bacteria were cultivated under aerobic conditions. Tryptone soy medium (TSA) (Oxoid, New York, NY, USA) was used for the growth of *B. thuringiensis* bacteria (37 °C), while blood agar was used for the culture of *D. congolensis* (37 °C) and *K. sedentarius* (30 °C) we used When determining the minimum inhibitory and bactericidal concentrations for all three species, we worked with tryptone soy broth (TSB) (Oxoid, New York, NY, USA).

1.2. Used essential oils

Twelve essential oils (rosemary (Rosmarinus officinalis), lemongrass (Cymbopogon citratus), clove (Eugenia caryophyllata), sage (Salvia sclarea), cinnamon (Cinnamomum zeylanicum), citronella (Cymbopogon nardus), eucalyptus (Eucalyptus globulus), fennel (Foeniculum vulgare)), spearmint (Mentha spicata), peppermint (Mentha piperita), lemongrass (Citrus limon) and thyme

(Thymus vulgaris)) were tested against the 3 bacterial species. For our experiments, we used the essential oils of AROMAX (Aromax Kft., Hungary), the purity of which was checked by gas chromatography before the tests.

1.3. Antibacterial test drop method

The antimicrobial effect of the above essential oils was performed on agar plates using the drop plate method. Before starting the experiment, the germ counts of the bacteria were synchronized based on their optical density. During this, they were pre-suspended in PBS and adjusted to 0.2 (~108 CFU/ml) at 600 nm. 100 µl of the *B. thuringiensis* suspension was spread on TSA, while for *K. sedentarius* and *D. congolensis* 100 µl were spread on blood agar. After drying, 5 µl of each essential oil was dropped onto the bacterial lawns on the surface of the culture media. The TSA blood agar containing *B. thuringiensis* and the blood agar containing *D. congolensis* were incubated at 37 °C, while the media containing *K. sedentarius* were incubated at 30 °C for 24 hours. The next day, the diameters of the inhibition zones were measured and expressed in mm.

We proceeded in the same way to determine the inhibition zones of citral and α-terpineol. The antibiotics erythromycin and clindamycin were used as controls in all cases.

1.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The minimum inhibitory concentration is the smallest concentration that still effectively inhibits the growth of bacteria. The minimum bactericidal concentration is the smallest concentration that kills 99.9% of pathogens (Moreira et al., 2005). The MIC and MBC values were determined using the so-called macrodilution test. The different isolates were grown and their optical density was synchronized (OD600=0.2) (CLSI, 1999). 5-5 ml of TSB medium using it, we created a dilution series: for these medium volumes, 0.5; 1; 2; 4; 8; 16; 32; 64 µl of essential oil was added, so the 0.1; 0.2; 0.4; 0.8; 1.6; 3.2; Concentrations of 6.4 and 12.8 mg/ml were obtained. 5 µl of the bacterial suspensions were measured into each tube. The control tubes either did not contain bacteria (essential oil purity test) or both bacteria and essential oil (medium control).

Incubation took place at 37 °C, with the exception of *K. sedentarius* (30 °C), then the inhibitory concentrations were determined visually the next day (*B. thuringiensis*) and after 3 days (*D. congolensis* and *K. sedentarius*). To determine the MBCs, after thorough vortexing, 10 µl amounts were dropped onto TSA and blood medium from the tubes in which there was no visible bacterial growth. The drops were flowed in parallel on the surface of the culture media, then they were incubated overnight and evaluated the next day.

The MIC and MBC values of citral and α-terpineol were also determined using the same method using a dilution series of 12.8 mg/ml to 0.1 mg/ml. Antibiotics erythromycin and clindamycin were used as controls.

1.5. Spore formation-inhibition test

An overnight culture of *B. thuringiensis* was used to investigate the sporulation inhibition of essential oils. A 1000x dilution was made from this, and then allowed to grow at 37 °C for 3 hours to promote

the transformation from spores that may be present into a vegetative form. The CFU was determined from the culture thus obtained, and then 5 µl of the suspension was added to the test tubes, into which 5 ml of TSB nutrient solution had previously been measured, and the Indian lemongrass essential oil was added concentrations: 0.5; 1; 2; 3; 4; 8; 16; 32; 64; 128 and 256 µl (final concentrations: 0.1; 0.2; 0.4; 0.6; 0.8; 1.6; 3.2; 6.4; 12.5; 25 and 50 mg/ml). After 24 h incubation at 37 °C, 1 ml of these were pipetted into flasks containing 25 ml of TSB from the tubes in which there was no visible growth. After 48 hours of incubation at 37 °C, 10 µl amounts were dropped onto TSA mediums in order to detect live bacteria. The absence of colonies indicated that sporulation did not occur in the original 5 ml volumes and thus the survival of the bacteria was prevented. We repeated our experiment 3 times.

2. Results

2.1. Antibacterial test drop method

Based on the inhibition zones (20–36 mm), thyme, cinnamon and Indian lemongrass proved to be the most effective essential oils for all three tested bacteria.

The other essential oils differed in their effectiveness for the three species. Lemon oil was only effective against the bacterium *D. congolensis*, while clary sage only showed sensitivity against *K. sedentarius* and *D. congolensis* species, and against eucalyptus only *B. thuringiensis* and *D. congolensis* showed sensitivity among the tested species. From the side of bacteria, the most sensitive bacteria was *D. congolensis*, as all tested essential oils showed some antibacterial effect against this species. 2.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The antibacterial effect of the essential oils was also tested in a liquid medium in order to determine the minimum inhibitory and bactericidal concentrations. In the macrodilution tests, all essential oils showed bactericidal effects against all three bacterial species at different concentrations.

2.3. Spore formation inhibition test

No bacterial growth was detectable after 24 h of incubation when the concentration of Indian lemongrass essential oil was above 0.2 mg/ml (MIC value) for B. thuringiensis. After 1 ml amounts were transferred from the optically clear tubes to the flasks containing 25 ml of nutrient liquid, after further incubation (24 hours, 37 °C) and after confirmatory instillation, we obtained that there were no living bacterial cells Indian lemongrass essential oil 12.8 mg/ when using concentrations above ml. In all other cases, there was no inhibitory effect, i.e. the nutrient solutions became turbid after 24 hours of incubation.

CHAPTER 4: Composition of the essential oil of Indian lemongrass, investigation of its antibacterial effect using methods based on separation

1. Materials and methods

1.2. Thin Layer Chromatography-Direct Bioautography ((Thin Layer Chromatography Direct Bioautography (TLC-DB))

The total composition and specific antibacterial components of the essential oil of Indian lemongrass were visualized on 2 pre-prepared (100 ◦C, 30 min) 5 × 10 cm 60 F254 TLC plates (Merck, Darmstadt, Germany) as previously described (Kovács et al., 2018), few with modification. Aliquots of 0.2 µl were dropped onto the horizontal thin line at the bottom of the plates; ethanol was used as a solvent control. Citrate (20 mg/ml; Sigma Technology Hungary, Budapest, Hungary) and α-terpineol (100 mg/ml; Sigma Technology Hungary) were used due to their known running parameters. Development of TLC plates in a double-walled chamber containing a mixture of toluene-ethyl acetate 97:3 (CAMAG, Muttenz, Switzerland).

In the case of *D. congolensis*, the plates were incubated for 1 hour and for the other two bacteria for 10 seconds in 50 ml bacterial suspension (TSB) (3 x 108 CFU/ml). The plates were then incubated in a steam chamber (for *B. thuringiensis* at 37 °C for 2 hours, for *D. congolensis* at 37 °C for 6 hours, for K. sedentarius at 30 °C for 6 hours). The next day, the plates were immersed in an aqueous solution of MTT (0.05 g/90 ml) for 10 seconds, and then incubated under appropriate conditions in a steam chamber until white spots appeared. The antibacterial activity of the separated components is indicated by the appearance of white spots against a bluish background (Kovács et al., 2018).

In order to visualize the separated components of the Indian lemon grass essential oil, the other plates (not treated with MTT) were immersed in ethanolic vanillin-sulfuric acid reagent and then heated at 90 °C for 5 minutes. The separated components were characterized according to the Rf values, based on two known standards (citral, α-terpineol) and the Kovats index. Definition of Rf value: the distance traveled by the given component divided by the distance traveled by the solvent front. At a known temperature for a given system, this is the characteristic of the component and helps to identify the components (Daintith, 2008). The Kovats retention index is a concept that transforms the retention time (time spent on the GC column of a given component) (Anjum et al., 2023) into a much more reliable and repeatable system (Idroes et al., 2019).

1.2. Demonstration of the antibacterial effect of citral and α-terpineol - instillation method

1.3. Detection of antibacterial components by bioautography

The 1.2. we also used the method described in chapter 2 to detect the antibacterial components in the case of the three tested bacterial species.

2. Results

2.1. Thin Layer Chromatography-Direct Bioautography ((Thin Layer Chromatography Direct Bioautography (TLC-DB))

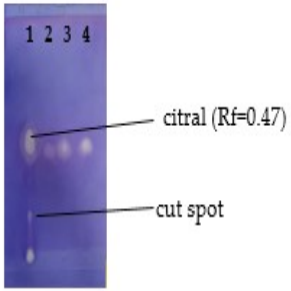
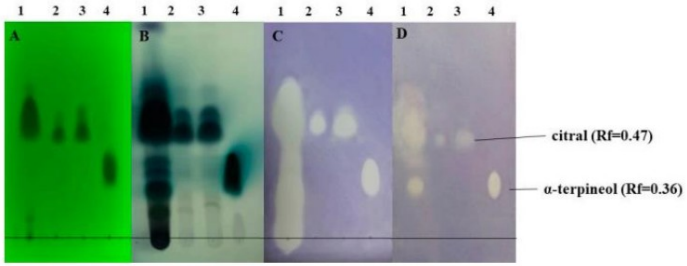


Figure 3. Compounds with antibacterial activities of lemongrass essential oil revealed on *B. thuringiensis* by TLC-DB. Order and quantity of the volatile test materials on the plate was the following: 1-lemongrass EO (0.2 mg); 2-citral (0.02 mg); 3-citral (0.04 mg); 4-citral (0.08 mg).

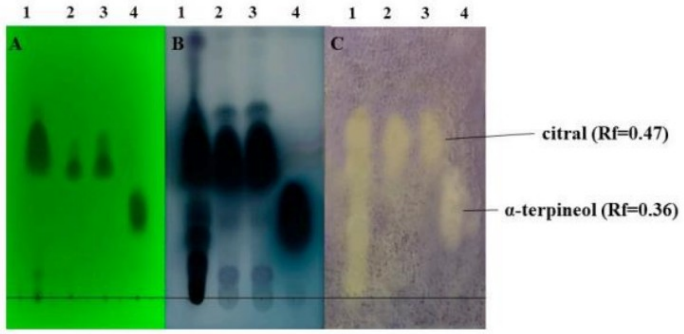
2.2. Demonstration of the antibacterial effect of citral and α-terpineol - instillation method

We observed a significant difference in the antibacterial activity of citral and α-terpineol when the instillation method was used. In the case of citral, a larger zone of inhibition was measured for all three bacterial species compared to α-terpineol. The difference was most pronounced in the case of D. congolensis and B. thuringiensis and was minimal in the case of the bacterium K. sedentarius.

2.3. Detection of antibacterial components by bioautography



Antibacterial components of Indian lemongrass essential oil after TLC-DB. (A) Plate under UV 254 nm illumination, (B) TLC plate viewed in visible light after vanillin-sulfuric acid treatment, (C) TLC-DB experiment: bioautograph of B. thuringiensis, (D) TLC-DB experiment: bioautograph of K. sedentarius (the light zones represent the antibacterial effect). Mobile phase: dichloromethane and toluene-ethyl acetate 93:7 v/v. The volumes of the components used were as follows: Indian lemongrass essential oil-6 µl, citral 4.5 and 6 µl, α-terpineol-1.5 µl. The concentrations of the stock solutions were as follows: Indian lemongrass essential oil-200 mg/ml, citral-20 mg/ml, α-terpineol-100 mg/ml. The application order and amount of the volatile test substances were as follows: 1-Indian lemon grass essential oil (1.2 mg); 2-citral (0.09 mg); 3- citral (0.12 mg); 4-α-terpineol (0.15 mg).



Antibacterial components of Indian lemongrass essential oil after TLC-DB. (A) plate under UV 254 nm, (B) plate viewed in visible light after vanillin-sulfuric acid treatment, (C) TLC-DB experiment: bioautograph of D. congolensis (bright zones represent the antibacterial effect). Mobile phase: dichloromethane and toluene-ethyl acetate 93:7 v/v. The volumes used were as follows: Indian lemongrass essential oil: 1 µl, citral: 4.5 and 6 µl, α-terpineol: 4.5 µl. The Indian lemon grass essential oil was applied without dilution, and in the case of citral and α-terpineol, the concentration of the stock solutions was 100 mg/ml. The application order and amount of the volatile test substances were as follows: 1-Indian lemon grass essential oil (undiluted); 2-citral (0.45 mg); 3-citral (0.6 mg); 4-α-terpineol (0.45 mg).

CHAPTER 5: In vitro testing of different ointments containing Indian lemongrass essential oil, dissolution tests

1. Materials and methods

1.1. Used lubrication pads

- Cremor aquosus
- Hydrogel "95%"
- Lipogel
- Vaseline

We mixed 1, 3 and 5% Indian lemongrass essential oil into these pharmacy base creams and then used them for in vitro testing.

1.2. Course of antibacterial tests

The initial logarithmic culture of *B. thuringiensis* was used for our experiment: 50 µl of the bacterial suspension was dropped onto the surface of LB agar, and then we waited until the drops dried. Then, using a sterile inoculum, we applied the essential oil-containing ointments and ointment controls (essential oil-free) to the surface of the bacterial instillations. After one hour of incubation, 3 x 3 mm cubes were cut from the agar containing the ointment using a sterile scalpel, and then the samples were placed in 300 µl of PBS. After thorough vortexing, the entire amount of 300 µl was poured onto the surface of the culture medium, and then spread with a sterile glass rod. Finally, the Petri dishes were incubated at 37 °C and read the next day the results. In case of growth, we counted the colonies and evaluated the obtained results. We also performed this in vitro experiment with another 10 Bacillus isolates.

1.3 Process of dissolution experiments

A flow-through model system was used to investigate the skin penetration of Indian lemongrass emulsified in different ointments. During this, the skin was supposed to be represented by a synthetic membrane, through which the components contained in the ointment and which could be dissolved were washed out of the ointment with the help of a circulation system, and samples were taken from this liquid after 30, 60, 90, 120 and 300 minutes. The samples were collected and the components found in them were subjected to qualitative and quantitative analysis by GC-MS analysis.

Traditional STIRWELLTM PAMPA sandwiches (Pion Inc.) were used for the dissolution experiments. The given tissue-specific membrane was formed on the filters of the upper acceptor plate immediately before the measurements. In the case of this skin PAMPA (Skin PAMPA), commercially available plates were used, which already had a lipid matrix characteristic of the stratum corneum of the skin. Before the measurements, the membrane of the Skin PAMPA was hydrated overnight with the hydration solution used for this purpose (Hydration Solution, Pion Inc.), and the sandwich was only used if it was properly hydrated. Hydration can be confirmed by visual inspection of the cells' filters. The membrane is suitable for measurement if its surface is light when turned towards the light, on the other hand, an insufficiently hydrated membrane has a dark shade. The permeability is considered in the PAMPA experiments in a traditional arrangement examined, i.e. the upper plate was the acceptor phase, while the donor phase was formed by the lower plate. Ointments with different compositions were dissolved in dimethyl sulfoxide (DMSO) using a concentration that was allowed by their solubility and UV spectrophotometric

properties (5-20 mM). 180 µl of Britton-Robinson buffer solution containing 1 v/v% DMSO stock solution with different pH was measured into the cells of the bottom plate of PAMPA sandwiches. In addition, the cells each contained a magnetic stirrer. 200 µl pH 7.4 Britton-Robinson buffer was pipetted into the cells of the upper acceptor plate. We covered the assembled system with wet filter paper to prevent evaporation of the solutions and a plastic lid that fits the PAMPA plates. The formed sandwich was placed on a magnetic stirrer (Gut-BoxTM, Pion Inc.) and the stirring intensity was adjusted so that the NKVR was approximately 40 µm thick. The total duration of the measurements was 5 hours.

1.4. SPME-GC-MS analysis

The essential oil and ointment samples were analyzed using the SPME-GC-MS method.

SPME-GC-MS: gas chromatography and mass spectrometry method after solid-phase microextraction sampling.

10 µl of essential oil or 1 ml of sample was tested in a 20 ml headspace (HS) bottle sealed with a silicone/PTFE septum. The static vapor field analysis of the sample was performed by solid-phase microextraction (sHS-SPME) using a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic sampler. After incubating the sample at 100°C for 5 minutes, the 65 µm film thickness StableFlex

A divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) was introduced into the vapor space of the sample, the extraction was performed for 20 minutes at 100°C, and then it was transferred to the injector of the gas chromatograph, where the desorption was 250° It happened on C, for 1 minute. In the case of the essential oil, the injection was performed in split mode (with a split ratio of 1:90), in the case of ointments and other liquid samples, in splitless mode. Finally, the fiber was cleaned and conditioned in high-purity nitrogen gas at 250°C for 15 minutes.

Analysis was performed on an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) on a Supelco SLB-5MS capillary column (30 m × 250 µm × 0.25 µm). After a 3-minute isothermal stage, the temperature of the column rose to 60-250 ºC at a rate of 8 ºC/min, and the final temperature was maintained for 1 minute. The carrier gas was high-purity helium 6.0, the flow rate was 1.0 ml/min (37 cm/s), in constant flow mode. The detection was performed with a quadrupole mass-selective detector in electron ionization mode (70 eV), full scan mode (41–500 amu, 3.2 scan/s). Data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). During the quantitative identification, the retention time and mass spectra of the components were compared with the data of standards and the NIST 2.0 library, and the percentage evaluation was carried out by area normalization.

2. Results

2.1. Antibacterial tests

The Hydrogel ointment containing the essential oil in concentrations of 1%, 3% and 5% resulted in complete purification of the *B. thuringiensis* bacteria cultured from our patient's foot. The Cremor ointment with 3% and 5% essential oil content also completely inhibited the growth of the bacterium, but at the same time, when

applied at a concentration of 1%, it resulted in only a small amount of inhibition. In the case of lipogel and vaseline ointments, there is no inhibition even at the highest essential oil concentration of 5%. This was observed in the bacterial species *B. thuringiensis* cultured from our patient's foot. After that, it can also be seen from the experiments carried out with additional *Bacillus* species that the essential oil was most effective in the Hydrogel lubricant, and the essential oil was not/less effective in the case of the hydrophobic lubricant.

2.2. Ointment dissolution tests

We performed a test of ointments with a concentration of 1% Indian lemongrass.

In sample 2 (Hydrogel), only the component called p-Cymen-8-ol was present. The neral component is found in larger quantities in the lipogel and vaselinum samples, with a measured amount of 18.6 m/m% in the vaselinum sample. Neral was present in all samples except the hydrogel sample. It can also be said about geranial that it occurs in significant quantities in samples 3 and 4 (lipogel 19.9 and vaselinum 40.1 m/m%) and is absent from the hydrogel sample. In the vaselinum sample, geraniol, in the hydrogel sample, geranyl acetate and γ-cadinene was present in the highest proportion. γ-cadinene was not detectable in the lipogel and vaselinum samples.

2.3. Skin penetration model results

Based on the data, it can be seen that most components of essential oil origin could be measured from the Hydrogel 95% + 1% samples.

The appearance of p-cymen-8-ol is possible due to the decomposition of limonene or citral (geranial + neral). The decomposition of limonene can produce limonene oxides and α-terpineol. The decomposition of linalool can produce α-terpineol, geraniol and nerol. The decomposition of geraniol can produce linalool, α-terpineol, nerol (He et al., 2018)

In each sample, a component appeared at 16.5 minutes, but it could not be identified.

CHAPTER 6: Gene expression tests

1. Materials and methods

1.1. RNA isolation

We started a logos phase from the overnight culture of *B. thuringiensis*. After reaching the appropriate germ count (OD600=0.2), a sample was taken at time 0 for RNA isolation, and the initial CFU value was determined. Next, 15 µl of Indian lemongrass was measured into a flask; in another, 300 µl of erythromycin (30 mg/ml) was added. In addition, there was another flask containing only bacteria (control). After seven and 15 minutes of incubation, 20-20 ml samples were taken from the flasks and the number of germs was determined at each time point, and RNA isolation was started. The samples taken at the specified time were centrifuged (4 minutes, 8000 rpm) and washed with 1 ml of RNAprotect® Bacteria Reagent solution (Qiagen). After thorough vortexing, each tube was allowed to incubate for at least 5 minutes. After centrifugation, the supernatant containing the RNAprotect solution was carefully aspirated with a pipette. Then, 200 µl of lysozyme solution (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) was added to the tubes, and after thorough vortexing, they were incubated for 30 minutes at room temperature. The contents of the tubes were again vortexed every 10 minutes. 20 µl of proteinase K solution (20 mg/ml) was added to each tube and incubated for 10 minutes at room temperature. After the end of the incubation time, 700 µl of RLT buffer (Qiagen) was pipetted into the tubes, to which 7 µl of β-mercaptoethanol was also added. After centrifugation, 500 µl of ice-cold 96% ethanol was added to 500 µl of supernatant, then the tubes were carefully turned upside down to mix their contents. 700 µl samples were transferred to Qiagen columns, followed by short grouting after step (1 min, 8000 xg), the samples were washed with 500 µl RPE (Qiagen) solution (2x). After the washing steps, the tubes were centrifuged at 8000 xg (15 sec). Finally, we did a pre-dissolving washing liquid discharge (fuge: 1 minute, 8000 xg), then dissolved our samples with 50 µl of DEPC-treated water (incubation at room temperature: 1 minute, then fuge: 1 minute, 8000 xg).

2. Results

2.1. Transcriptomic analysis result

Complete transcriptome studies showed that 2,109 genes out of 5,054 genes of *Bacillus thuringiensis* were affected by changes in the environmental factor caused by Indian lemongrass. Of these, the transcription of 986 genes was downregulated, while the expression of 1123 genes was increased. The obtained data can form the basis of many subsequent studies analyzing the antibacterial effect of Indian lemongrass more thoroughly, but in the context of this work, we limit our obtained preliminary results to only two main functions. It is clear from this that the increased expression of general stress genes is experienced in the 7th and 15th minutes. In the case of the 15th minute, the answer often rises drastically compared to the 7th minute (WP_000557321.1; WP_000448820.1; WP_000522903.1). In addition, the down-regulation of the genes affecting sporulation, compared to the control, is only manifested in 15 minutes. Their

involvement is not the same. In addition, the transcription of several transcription and translation factors was affected to a different degree and sign.

CHAPTER 7: Conclusions and theses

• The most pronounced effect was experienced in the case of Indian lemongrass, cinnamon and thyme essential oils

• Of the components, neral makes up 26.1% and geranial makes up 34.5% of the essential oil. These are the main components of essential oil.

• In addition to citral, α-terpineol can also contribute to the antibacterial effect of Indian lemongrass, although the percentage of this latter component is much lower than that of the main component itself.

• The antibacterial tests showed that the hydrogel base can produce a much more characteristic effect, while, on the other hand, IC mixed with hydrophobic ointment bases (lipogel and vaselinum) is not effective.

• To support this, we carried out the skin penetration experiment in the next step, which showed that the IC components were dissolved in a much higher proportion and in a shorter time from the Hydrogel ointment base than in the case of hydrophobic bases

• The importance of these results lies in the fact that in certain cases (e.g. skin surface infections) antibiotic-containing ointments can be replaced by essential oil-containing therapeutic agents, thereby reducing the amount of antibiotics entering the environment and, albeit to a small extent, contributing to the reduction of the evolutionary pressure that antibiotic- one of the driving forces of the spread of resistance.

• The stress situation affecting the transcription of 2109 genes in the genome of *Bacillus thuringiensis*, which consists of 5054 genes, shows that the environmental appearance of the essential oil of Indian lemongrass disrupts normal transcription. This situation is characterized by a drastic upregulation of the transcription of general stress genes, which suggests that the bacterium is trying to react with a general stress response, which also increases drastically over time.