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EVALUATION OF ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS, IDENTICAL NATURAL COMPOUNDS AND MEDICINAL PLANTS EXTRACTS ON BACTERIA OF VETERINARY INTEREST

VALUTAZIONE DELL'ATTIVITÀ ANTIMICROBICA DI OLI ESSENZIALI, COMPOSTI NATURALI IDENTICI E ESTRATTI DA PIANTE OFFICINALI SU BATTERI DI INTERESSE VETERINARIO

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ABSTRACT

According to WHO, antibiotic resistance is one of the biggest threats to global health, food security, and development today. Antimicrobial resistance has become a critical problem for infection treatment both in human and veterinary medicine, both in food-producing and affection animals. Finding alternatives to traditional antibiotics molecules is critical to fighting antimicrobial resistance identified by international authorities such as EFSA, WHO, and OIE.

This study aimed to test the antimicrobial activity against four bacterial strains (*Escherichia coli* - *EC*, *Salmonella* Typhimurium - ST, *Staphylococcus aureus* - SA, and Methicillin-Resistant *Staphylococcus aureus* - MRSA) of compounds suggested as an alternative to antibiotics as Essential Oils - EOs - (Cinnamon, Lavender, Tea tree, Mint, Oregano, Rosemary, Clove bud, Thyme), Nature Identical Compounds - NICs - (Carvacrol, Cinnamaldehyde, Menthol, Terpineol, Thymol) and Plants Extracts – PEs- (Marshmallow, Chamomile, Mallow). EOs and NICs were also tested in combination with Tween 20 and Tween 80 (only for EOs) as emulsifiers.

The results showed that for all the tested EOs average Minimal Inhibitory Concentration (MIC) value is lower than 4% and lower than 2% for the most. Overall, MIC was lower in the presence of Tween 20 for all the EOs and tested bacteria. The most susceptible bacterium to EOs was EC*;* contrariwise, the most resistant was ST. The lowest MIC average among all the tested EOs was found for Oregano oil, followed by Thyme oil, Tea tree oil, and Rosemary oil. The highest MIC average was found for Clove oil and Cinnamon oil, followed by Lavender oil and Mint oil. For what concerns PEs, no antimicrobial activity against the four tested strains was detected. Checkerboard assays between EOs and Tween 20 showed a prevalence of indifference (37,5%) and additivity (34,4%) among all tested EOs and bacteria, compared to antagonism (15,6%) and synergy (12,5%). Interesting to consider is that EOs MICs generally increased after about one year, showing a time-dependent decrease in antimicrobial activity.

Among all the tested NIC, the highest MIC was found for Menthol and Terpineol. The lowest MIC was found for Cinnamaldehyde against SA. For what concerns checkerboard assays between NICs and Tween 20, generally the combinations showed

a prevalence of indifference, no synergy, and additivity for Terpineol with Tween 20 against EC, ST, and SA and Menthol with Tween 20 for EC.

In conclusion, we can hypothesize that the administration of these essential oils, identical natural compounds, and their respective active ingredients may benefit gut microbiota, acting as inhibiting agents against the most common pathogenic bacteria of zootechnical animals. Their combination with Tween 20 could be helpful to lower the doses administered in husbandry.

INTRODUCTION

The critical importance of antimicrobial resistance

According to WHO, antibiotic resistance is one of the biggest threats to global health, food security, and development today [1]. Antimicrobial resistance has become a critical problem for infection treatment both in human and veterinary medicine. The lack of molecules active against pathogenic bacteria and fungi makes these microorganisms more and more dangerous for human and animal health. Furthermore, through cross resistances, entire classes of antimicrobial molecules are becoming less effective or ineffective at all. This has become a problem in treating infections in all species at all ages [2]. The damaging effects of antimicrobial resistance (AMR) are already manifesting worldwide. Antimicrobial-resistant diseases currently claim at least 50,000 lives each year across Europe and the US alone, with many hundreds of thousands more dying in other areas of the world [3].

Mechanisms of antibiotic resistance

Antimicrobial resistance can be due to the lack of antimicrobial target or the microorganisms' ability to inactivate the antimicrobial molecules [4]. As antimicrobials are classified by their means of action, and they also have classes of bacteria towards which they can exercise their effect. Therefore, some bacteria and fungi species are naturally resistant to one or various antimicrobial classes. In this case, all strains belonging to those species will be resistant [4].

One of the innate forms of antimicrobial resistance is the absence of the molecular target in the microorganism. Therefore, the antimicrobial molecule is not able to find the substrate to exert its action. For example, antimicrobial glycopeptides, such as vancomycin, are ineffective against Gram-negative bacteria because of their chemical nature. Their molecular weight and size are so elevated that it makes it impossible for the antimicrobial to enter the Gram-negative cellular wall and meet their target [5].

Otherwise, some species can acquire genes that give antimicrobial resistance. In this case, the strains that have acquired the resistance gene will be resistant to a specific antimicrobial or an antimicrobial family. Typically, gene resistance gained by the

acquisition of plasmids shows rapidly: the transmission of the plasmid responsible for the resistance phenomenon is related to the replication rate of the bacteria involved. Conversely, the selection of resistant strains takes longer because it entails an accumulation of gene mutations [6]. Resistance genes attribute resistance activity through different mechanisms: production of enzymes, efflux pumps, altered cell wall [4].

The problem in veterinary medicine

The use of antibiotics is a common animal husbandry practice both as treatment, metaphylaxis, and prophylaxis: measures [7]. Moreover, antimicrobials had held the role of growth promotor factors, as weight gain was observed among animals treated with subtherapeutic doses of antibiotics [8]. As already noted, the administration of antibiotics induces a selective pressure of antimicrobial-resistant organisms (AMRO); therefore, in 2006 European Union banned the administration of antibiotics as growth promoters in animals [9]. Following that moment, the use of antibiotics has been reserved for medical use only [9]. The prophylactic measure highly recommended by OIE is to apply for an immunization program through the administration of vaccines [10]. Since this path is not always feasible, and there are no vaccines that protect all infectious diseases, the use of prebiotics and/or probiotics is also encouraged to help the beneficial flora so that it counteracts the replication of pathogens [11].

Another essential aspect to be taken into consideration is that for zootechnical animals exist withdrawal periods. During this period, any food product coming from the treated animal cannot be admitted to consumption [10]. This is a critical issue because failure to observe the drug withdrawal period implies the consumption of products with residues by the consumer, which in this case means the administration of subtherapeutic doses of antibiotics. EFSA indeed has indicated how zoonotic AMROs show resistance to both human and food-producing animals' antibiotics [12]. To protect consumer safety and avoid antibiotic-resistance phenomenon, Maximum Residual Limits (MRLs) have been set, i.e., the maximum concentration of residues of a given drug allowed in animal-derived food. The doses and withdrawal times have been established to reach the final consumer with residues below the permitted MRLs [13].

Use of antimicrobial in livestock production

Katakweba et al. report that the antibiotics most used in livestock production and found on the farm are tetracycline, sulphonamides, and penicillin-streptomycin [14]. Landers et al. have pointed out that there is no uniformity in the national laws on the use of antibiotics in various animal species. Instead, is a differentiation between antibiotics for mainly human use and those for primarily veterinary use. They also observed that the use of antibiotics in food-producing animals is correlated to an increase in the appearance of antimicrobial resistance in humans [15]. Huijbers et al. have investigated the transmission of antimicrobial resistance to humans through a complex model that comprehends domestic animals, wildlife, humankind, soil, water, and air/dust. Bacteria can be passed through drinking water, animal meat, milk, other animal food products. Also, if withdrawal periods are not respected, a variable amount of antibiotics might be accidentally assumed by consumers [16,17].

Newly introduced systems such as Classyfarm in Italy, which tracks parameters concerning biosecurity, animal welfare, production, nutrition, consumption of antimicrobial drugs, injuries found at the slaughterhouse, are the future perspective to evaluate animals and husbandries. These platforms can highlight management and sanitary issues [18]. Maybe in the future, it will be possible through software and/or algorithms to strictly identify which animals will need to be administered antibiotics or alternative compounds.

European legislation on antibiotics in veterinary medicine

Reg. EU 2019/6 states that the clinical examination and consequent diagnosis must precede the prescription of antibiotics in animals [19]. In Reg. EU 2016/429 was decided to put the responsibility and supervision under the veterinarian, the only professional figure able to examine animals and establish the need for antibiotic treatment based on the animal's health status [20]. The European Medicines Agency (EMA) categorized antimicrobial molecules for use in veterinary medicine. These are divided into four categories which indicate the criteria for using the molecules that belong to them from most to less critical use. All antimicrobial molecules enlisted are to be used only when medically needed.

"Category A - Avoid" is reserved for human medicine, currently are not authorized in veterinary medicine in the European Union (EU). These antimicrobials may not be used

in food-producing animals, and they may be given to companion animals only under exceptional circumstances.

"Category B - Restrict" refers to quinolones, third and fourth generation cephalosporins, and polymyxins. Antibiotics in this category are critically important in human medicine, and their use in animals should be restricted to mitigate the risk to public health.

"Category C - Caution" covers antibiotics for which alternatives in human medicine generally exist in the EU, but only a few other options are available in specific veterinary indications. These antibiotics should only be used when there are no antimicrobial substances in Category D that would be clinically effective.

"Category D - Prudence," which includes antimicrobials to be used as first-line treatments whenever possible. In general, these antibiotics can be prudently used in animals. It means that unnecessary use and long treatment periods should be avoided, and group treatment should be restricted to situations where individual therapy is not feasible [21].

Multidrug-resistant organisms

It must be noted that just the use of antibiotics itself induces a selective evolutionary pressure [22]. Therefore, the use of antimicrobial susceptibility testing, especially before administration of critically important antimicrobials, is encouraged, and a reflection will be made upon the use of currently available tests and novel rapid diagnostic testing methods to improve rational prescribing [23]. Of course, the lack of new molecules active against microorganisms makes the battle harder and more complex every day. This phenomenon has flowed into the involuntary selection of multidrug-resistant organisms (MDROs) [24]. MDROs are defined as microorganisms, predominantly bacteria, resistant to one or more classes of antimicrobial agents [24]. Although the names of certain MDROs describe resistance to only one agent (e.g., Methicillin-Resistant *Staphylococcus aureus* - MRSA, Vancomycin-Resistant *Enterococcus* - VRE), these pathogens are frequently resistant to most available antimicrobial agents [24]. MDROs much more easily develop and spread in hospital environments such as intensive care units (ICUs), long-term care facilities, and pediatric, geriatric, oncological wards [25]. Once MDROs are introduced into a healthcare setting,

transmission and persistence of the resistant strain are determined by the availability of vulnerable patients, selective pressure exerted by antimicrobial use, increased potential for transmission from more significant numbers of colonized or infected patients ("colonization pressure"), and the impact of implementation and adherence to prevention efforts [26]. In these situations, they find immunocompromised subjects widely treated with many antibiotics, which make the perfect place for them to be put under pressure and selected. Often, the hospital personnel are a vehicle of transmission from one patient to another, rather than the carrier inside wards [3].

Natural alternatives to antibiotics

The scientific community's efforts have been directed to finding new compounds active against pathogen microorganisms to combat antibiotic resistance. The alternative strategies proposed were various. The most significant are reported below [27].

Bacteriocins

Bacteriocins are peptides produced by specific bacterial strains that exert antimicrobial activity. They have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain [28]. Bacteriocins, as well as antimicrobials, have a different mean of action. For example, colicin E2 – produced by *E. coli*– is an enzyme that exerts on DNA with endonuclease activity [29], cloacin DF13 – produced by *Enterobacter cloacae* – has RNAse activity [29], nisin - produced by *Lactobacillus lactis* subsp. *lactis* - causes a membrane depolarization [30]. Also, they may be produced *in situ* by probiotics [31]. In fact, various kinds of bacteriocins are found to be produced by most bacterial species [32].

Phage therapy

Another alternative to antimicrobial drug use has been found in phage therapy. This is based on the natural bacteriophage lytic activity: infection and killing of the bacterial cell [33]. Vantages of phage therapy are their broad activity against both Gram-positive and negative strains, and they are mostly free of side effects; they are highly specific. The disadvantages are that bacteria can still develop resistance. If they are immunogenic, they will be removed by the immune system, they are very specific, so there might be the need to make phage cocktails [34].

Fatty acids

Medium and short-chain fatty acids (SMCFA) have been proven to affect dysmicrobism in growing animals positively [35]. These have been proposed as an alternative to antimicrobial drugs as they showed *in vitro* bacteriostatic/bactericidal effects. These molecules have also been proved to diminish the finding of pathogens in treated animals' gut microbiota and consequently the onset of diarrhea. Furthermore, animals treated with medium and short-chain fatty acids had a lesser need for antibiotics [36, 37]. SMCFA indeed exert a positive effect on gut function as they improve cell turnover through beneficial effects such as an "emollient effect" [38,39].

Plants derivatives

Among the alternatives to antibiotics, and the object of this work, are plants derivatives. It has been suggested that plants interested in traditional and folk medicine might be a resource of active ingredients functional against microorganisms [40].

Furthermore, it has been suggested that cross associations of these compounds may exert a synergistic action, both for the intestinal environment and the organism [41].

Use of alternative to antibiotics in veterinary medicine

Ebani et al. and Rusenova et al. tested various essential oils (EOs) in bacteria and fungi strains of veterinary origin. They have suggested the use of EOs in veterinary medicine as antibacterial and antifungal compounds, as they have encountered inhibitory effects against microorganisms tested, among which are comprehended Gram-positive (*Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Mycobacterium* spp.…), Gram-negative (*E. coli*, *Salmonella* spp., *Pseudomonas, Campylobacter…*), and fungi (dermatophytes, *Malassezia pachydermatis, Aspergillus* spp., *Sporothrix* spp.) strains. Also, Ebani indicated that a mix of EOs might have applications as disinfectants [17,42].

Essential oils, natural identical compounds, and plant extracts as alternatives to antibiotics

Essential oils

Essential oils (EOs) are secondary metabolites of aromatic plants known in traditional medicine as versatile remedies [43]. EOs are hydrophobic compounds that can pass through the bacterial cell wall. Unlike antibiotics, essential oils' active principle is not made of just one molecule; Instead, it is a complex mixture of various compounds that together give its properties. It has been suggested that within an EO exists a relation of synergy between its ingredients [44].

Of the many components of EOs, principal active ingredients responsible for antimicrobial activity are terpenes, terpenoids, and aromatic compounds. These molecules establish EOs properties [45, 46]. Composition of EOs is deeply determined by botanical characteristics (species, part of plant object of the EO extraction…), but also the age of the plant, the environment (light, humidity, soil, …) in which it grew or was cultivated [47, 48]. This is the reason for the importance of the lot from which the EOs come [49]. The way the EO has extracted influence significantly its composition and, therefore, its antimicrobial activity [50].

Antimicrobial resistance towards EOs has already been detected by several research groups, especially in those bacterial species that have already shown a tendency toward antimicrobial resistance [44, 51]. In a study comprehensive of fifty-two different EOs, the most promising MIC found were Oregano, lemongrass, and bay, which exerted antimicrobial activity at a concentration of ≤2%. The same study underlines how important it is to consider pre-EO-extraction conditions of plants [45, 52]. Studies have shown Tea tree oil to exert promising antimicrobial and antifungal activity, particularly against *Candida albicans* [53].

Furthermore, it has been proven how EOs can positively influence rumen activity [54].

Natural Identical Compounds

Nature Identical Compounds (NICs) are chemically synthesized counterparts of the pure bioactive compounds of EO. They comprehend a variety of chemical classes [45]. The use of NICs allows combining pure compounds according to the target to achieve

appropriately. Thus, they represent a promising non-antibiotic tool [55, 41]. Many works have already shown NICs antimicrobial properties. Mechanisms of action are various, based on chemical and physical characteristics [56].

NICs have also shown antibiofilm, anti-inflammatory, and antioxidant properties [41].

Thymol, Carvacrol, and Cinnamaldehyde have shown an effective antimicrobial activity against Gram-negative *Vibrio anguillarum* at MIC concentrations of 1.88 mM, 1.88 mM, and 3.75 mM, respectively. The same study suggested that NIC could lower the minimal bactericidal concentration (MBC), acting as an adjuvant [56].

Cinnamaldehyde has been proven to exert anti-inflammatory, anticoccidial, and various biological activities [57].

The advantage of using NICs instead of EOs is having a standardized compound that is less variable in composition and properties. This becomes important also during the association process looking into synergy interactions [41].

Plant extracts

PEs can be obtained from various parts of the plant: flowers, roots, cortex, etcetera are all valid to create a PE. Also, for PEs, the extraction processes deeply characterize the PE properties. For plant extracts, the lot characteristics are as important as for EOs [5].

Various studies have taken folk medicine as a guide to test and prove the antimicrobial activity of PEs. Scientific literature mainly focused on foodborne diseases. Ríos et al. found that the most promising plants with antimicrobial properties were Mint, Thyme, Oregano, Cinnamon, salvia, and Clove [58].

Emulsifiers of EOs

Tween 20

Tween 20 is a nonionic emulsifier surfactant composed of polyoxyethylene sorbitan monolaurate [59]. Various studies have investigated the use of Tween 20 as a dispersive agent vehicle for EOs [60]. Hammer et al. used Tween 20 as an emulsifier in Mueller-Hinton agar media with a concentration of 0.5% (v/v) [53]. To enhance oil solubility in broth media, Tween 20 was added to the agar at 0.5% [45, 61].

A study suggested that Tween 20 helped NIC Carvacrol exert an antimicrobial action by forming a microemulsion that makes it more easily soluble in the culture broth [62].

Gomez-Lopez et al. found that Tween 20 activity depends on molecules used with it because if they are more soluble in Tween 20, they may lack in microorganism-molecule interaction. Therefore, it becomes fundamental to distinguish action with each compound that is put to the test. However, such influence has not been detected when Tween concentration was below 0.5% [59].

FDA has confirmed the safety of Tween 20 for oral administration [63]. Also, several studies have deepened the inquiry of Tween 20 as a carrier of many compounds *in vivo*, such as antitumoral chemotherapeutics [64,65] or spices [66].

A study by Castro et al. reported that the behavioral effect of Tween 20 negatively influenced mice activity, suggesting the onset of side effects [67].

Tween 80

Tween 80 is an emulsifier composed of polyethylene glycol sorbitan monooleate. It is found in scientific literature been used as a vehicle for EOs and other lipophile compounds. Indeed, Tween 80 can form microemulsions in which these compounds are transported into the organisms that are being inoculated [62].

Tween 80 has been used to evaluate *Tetradenia riparia*'s EO's antioxidant activity [68]. Jiang et al. used Tween 80 to enhance Rosemary oil solubility in sterile saline solution at a concentration of 0.5%(v/v) [45,69,70]. Hammer et al. used Tween 80 to magnify oil solubility at 0.001% (v/v) [53]. Instead, Ma et al. tested EOs with Tween 80 at various concentrations and found that high concentrations of Tween 80 exert an effect of reducing the EOs availability and consequently a decrease in the power of the antimicrobial action. The hydrophobic bond indeed reduced the possibility for the EO to interact with bacteria [62].

Tween 80 has also been used by Gazim et al. in oral suspension with ethanol and water (1:1:10) to help the administration of *Tetradenia riparia* EO. *In vitro* studies for *Tetradenia riparia* were conducted with a final concentration of 2% in each well [71].

Repercussions on *in vivo* animal behavior in mice of Tween 80 were studied by Castro et al. and, among all substances tested. It was the most positive, suggested it did not

play any role on side effects onset. Also, it did not affect the bioavailability of drugs administered alongside it [67].

Per os administration of Tween 80 is approved by FDA as a vehicle for other compounds [63]. Tween 80 has also been tested in several works *in vivo* as carriers for drugs or other substances requiring oral administration [72–74].

AIM OF THE WORK

In this work of thesis, it was decided to evaluate the antimicrobial activity against four bacterial strains of veterinary interest of alternative antibiotic compounds, specifically essential oils (EOs), nature-identical compounds (NICs), and plants extracts (PEs), alone and in combination with two different emulsifiers (Tween 20 and Tween 80).

In addition, the activity of these compounds was evaluated in two different moments, at one year from each other, to assess the possible change in efficacy over time of EOs and NICs, depending on the conservation of the compound as a potential additive in the feeds that are stored on the farm.

This work aimed to investigate new natural antimicrobial solutions that can be administered to livestock with feed, which can eventually replace traditional antimicrobials administered both in therapy and in prophylaxis and/or metaphylaxis.

MATERIALS AND METHODS

Chemicals

EOs tested in this work were the following:

- Clove buds (C*aryophyllus aromaticus*) [i]
- Cinnamon (*Cinnamomum zeylanicum*) [ii]
- Lavender (*Lavandula angustifolia*) [iii]
- Tea tree *(Melaleuca alternifolia*) [iv]
- Mint (*Mentha piperita*) [v]
- Oregano (*Origanum vulgare*) [vi]
- Rosemary (*Salvia rosmarinus*) [vii]
- Thyme (*Thymus vulgaris*) [viii]

Rosemary and Tea tree EOs were not diluted and used as a stock solution. Cinnamon, Clove bud, Lavender, and Mint EOs were diluted in DMSO to make a stock solution at the concentration of 6,250%. Oregano and Thyme EOs were diluted in DMSO to make a stock solution at the concentration of 0,2%.

NICs tested were:

- Carvacrol [xi]
- Cinnamaldehyde [xii]
- Menthol [xiii]
- Terpineol [xiv]
- Thymol [xv]

Carvacrol, Cinnamaldehyde, Menthol, Terpineol were diluted in DMSO to make a stock solution at the concentration of 2048 µg/ml. Thymol was diluted in DMSO to make a stock solution at the concentration of 4096 µg/ml.

Aqueous PEs tested were the following:

- Marshmallow (*Althaea officinalis*)
- Chamomile (*Chamomilla recutita)*
- Mallow (*Malva sylvestris*)

Chamomilla recutita's, Malva sylvestris', and *Althaea officinalis'* PEs were diluted in sterile demineralized water to make an aqueous stock solution at the concentration of 1:25 600, 1:25 600, and 1:12 800, respectively.

Bacterial strains

In this work, four reference bacterial strains were used to test each compound: *Escherichia coli* (*E. coli*) ATCC 25922, *Salmonella* Typhimurium (*S.* Typhimurium) ATCC 14028, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, and *Methicillin-Resistant Staphylococcus aureus* (MRSA) ATCC 43300.

Culture media

Compounds were tested with MIC assay using different culture media: Müeller-Hinton broth (MHB), MHB with 0,5% of Tween 20, and MHB with 0,5% of Tween 80. The only exception was for *Staphylococcus aureus*, which test concentration of Tween 20 was lowered at 0,25% due to a growth difficulty at 0,5% of Tween 20 of the growth control wells.

Evaluation of antimicrobial activity

The antimicrobial activity of these compounds was tested with the microdilution broth method for Minimal Inhibitory Concentration evaluation (MIC assay). In this way, it was possible to evaluate the action of the compounds against bacteria quantitatively and determine the Minimal Inhibitory Concentration (MIC). MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in µg/ml [26]. After establishing MIC value, it is possible to evaluate the microbicidal activity through the determination of Minimal Bactericidal Concentration (MBC). MBC is the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 hours under a standardized set of conditions [26]. The results were read by the unaided eye and then through spectrophotometry. In some cases, where the interpretation was difficult because of the turbidity of the EOs themselves, Alamar blue dye (Resazurin) was used [75].

Many experimental drugs and compounds do not dissolve readily in water or saline and, thus, are difficult to administer. Therefore, it is mandatory to use vehicles, which can be solvents, detergents, or vegetable oils [76]. These compounds, however, might have an antimicrobial activity of their own; therefore, it was decided to evaluate checkerboards containing both vehicles and compounds.

Inoculum preparation

All the experiments in this work were performed following the Clinical and Laboratory Standard Institute guidelines with some modifications [77]. Four or five bacterial colonies were withdrawn from solid fresh cultures of each strain and then inoculated in 6 ml of MHB. The bacterial suspension was incubated for 24 hours at 37 °C in aerobic atmosphere in static conditions. After incubation, the suspension was centrifuged at 2000 rpm for 20 minutes at 4 °C to obtain the pellet containing all bacterial cells. After eliminating the supernatant, the pellet was resuspended in phosphate buffer (PB) 10 mM pH 7. The suspension was adjusted through optical density (OD) reading using Biophotometer plus (Eppendorf, Hamburg, Germany) spectrophotometer (λ = 600 nm). The OD value correspondent to 10^8 CFU/ml suspension at 600 nm in a 1 cm light path cuvette is within the range of 0.08 - 0.13. The calibrated bacterial suspension was further diluted at 1:100 depending on the assay in sterile MHB or MHB and emulsifiers Tween 20 and Tween 80.

Fifty microliters of the bacterial suspension containing 10^6 CFU/ml were inoculated into each well to obtain a final concentration of 5 x 10^5 CFU/ml. Plates were incubated for 24 h at 37° C in aerobic atmosphere. All the microbiological assays were performed within 30 min after the inoculum standardization [78].

Minimal Inhibitory Concentration (MIC) assay for EOs

In 96-well microtiter plates, three experiments were performed with three replicates each of serial dilutions of EOs for each bacterial strain. The first well of each replicate was filled with 100 µl of EO stock solution. Each well from columns 2 to 10 was filled with 50 µl of MHB. Then the stock solution was serially diluted by transferring 50 µl from the previous well to the next, serially twofold diluting the compound. The final

range of EOs dilutions was 3,125-0,0061%. Finally, 50 µl of bacterial inoculum were added to each well, including growth controls.

The second to last (11th) well from each row was growth control (GC) and filled with 50 µl of MHB and 50 µl of bacterial inoculum. The last well from each row was sterility control (CS) and was filled with 100 µl of MHB.

Minimal Inhibitory Concentration (MIC) assay for NICs

The NIC was diluted to a stock concentration of 102,4 mg/ml in DMSO for Carvacrol, Terpineol, and Cinnamaldehyde and 204,8 mg/ml in DMSO for Thymol and Menthol, and subsequently each compound was further diluted to a concentration of 4096 µg/ml in MHB.

Two-fold dilutions 2048-2 µg/ml range of the stock solution were performed in a 96-well microtiter plate (Greiner, Milan, Italy): in each well, fifty microliters of the bacterial suspension containing 10^6 CFU/ml were added to obtain a bacterial concentration of 5 x 10^5 CFU/ml.

Growth and sterility controls were performed for each bacterial strain and each tested compound.

The first well of each replicate was filled with 100 µl of stock solution. Each well from columns 2 to 10 was filled with 50 µl of MHB. Then the stock solution was serially diluted by transferring 50 µl and after was added 50 µl of bacterial inoculum.

Minimal Inhibitory Concentration (MIC) assay for PEs

Plants extracts were diluted in an aqueous solution with sterile deionized water to a stock concentration of 12800 µg/ml for Marshmallow and 25600 µg/ml for Chamomile and Mallow.

Twofold dilutions 12800 (6400 for Marshmallow)-25 (12,5) µg/ml range of the stock solution were performed in a 96-well microtiter plate (Greiner, Milan, Italy): in each well, fifty microliters of the bacterial suspension containing 10^6 CFU/ml were added to obtain a bacterial concentration of 5 x 10^5 CFU/ml.

Growth and sterility controls were performed for each bacterial strain and each tested compound.

The first well of each replicate was filled with 100 µl of stock solution. Each well from columns 2 to 10 was filled with 50 µl of MHB. Then the stock solution was serially diluted by transferring 50 µl and after was added 50 µl of bacterial inoculum.

Checkerboard assay

To evaluate the combined activity between Tween 20 and EOs/NICs/PEs, checkerboard plates were set up following the protocol indicated by Meletiadis et al [79].

In 96-wells microtiter plates, serial dilutions of each EO from 3,1% to 0,012% were tested in combination with Tween 20 at a concentration ranging from 6,2% to 0,1% in MHB against the previously indicated bacteria.

For NICs and Tween 20, for Carvacrol, Thymol and Cinnamaldehyde were used serial dilutions from 256 µg/ml to 1 µg/ml in combination with the same dilution range of Tween 20 (6,2% to 0,1%), and 2048 µg/ml to 8 µg/ml was the dilution range used for Menthol and Terpineol.

Plates were inoculated with a bacterial suspension of 5×10^5 CFU/ml, adjusted spectrophotometrically as mentioned in the "Inoculum preparation" paragraph. Each plate was incubated overnight at 37 °C in anaerobic atmosphere.

After overnight incubation, to evaluate the antimicrobial activity, 10 µl of Resazurin (Alamar blue dye) were added to each well [75]. Immediately after the Resazurin inoculation, plates were further incubated for two hours at 37 °C in the dark. The antimicrobial activity is found when the Resazurin is not metabolized and the well does not turn pink.

Fractional Inhibitory Concentration index

To evaluate the antimicrobial effect of two compounds in association, the Fractional Inhibitory Concentration Index (FIC-index) was calculated for each association. FIC Index allows measuring the degree of synergy between antimicrobials [80].

The MICs of each compound tested (EO or NIC) individually and in combination with Tween 20 were registered, and the results were included in the following formula:

$$FIC = MIC_{a\ in\ combination}/MIC_{a\ single\ compound} + MIC_{b\ in\ combination}/MIC_{b\ single\ compound}$$

Based on the FIC index value, the antimicrobial activity of the associations could be synergistic, additive, indifferent, or antagonist. In particular, the compounds are synergic if the FIC is ≤ 0,5, additive if the FIC is between 0,5 and 1, indifferent if the FIC is between 1 and 4, and antagonist if the FIC index is > 4 [79].

RESULTS

For each tested compound is reported the average MIC value obtained and its standard deviation (SD).

Evaluation of MIC values

Essential oils

EOs analysis was conducted at the end of 2019 and the first part of 2020 with Tween 20, Tween 80, and without emulsifier. During the elapsed time between the two tests, EOs were stored in their original packaging at 4 °C, as indicated by the producer. In 2021 EOs' MICs were reevaluated. Obtained MIC values are resumed in the following tables.

All MIC values without emulsifier were below 4%, the highest result (3,47%) was obtained with Mint oil against *S.* Typhimurium (Table 1). For all tested oils, MIC values were generally lower in combination with Tween 20 in comparison with the two other conditions (without emulsifier, with Tween 80).

For what concerns the single EOs, lowest MIC average among all the tested strains was found for Oregano oil (0,08% ± 0,06%), followed by Thyme oil (0,19% ± 0,15%), Tea tree oil (0,29% ± 0,15%) and Rosemary oil (0,91% ± 0,48%). Highest MIC average were instead found for Clove oil and Cinnamon oil (respectively 4,13% ± 5,08% and 3,33% ± 4,54%) followed by Lavender oil (2,57% ± 1,01%) and Mint oil (1,75% ± 1,08%).

The bacterial strain generally less sensitive to the tested EOs was *S.* Typhimurium (1,88%), while the most sensitive was *E. coli* (1,17%). Considering single EOs, the followingly are reported as the most sensitive microorganisms:

- Cinnamon oil: MRSA (0,1%), followed by *S. aureus* (0,11%), both with Tween 20
- Clove buds oil: *S. aureus* (0,1% with Tween 20)
- Lavender oil: MRSA (0,1% with Tween 20)
- Mint oil: *S. aureus* (0,025% with Tween 20)
- Oregano oil: *E. coli* (0,012% without emulsifier), *S. aureus* (0,012% without emulsifier), *S.* Typhimurium (0,012% without emulsifier)
- Rosemary oil: MRSA (0,2% with Tween 20)

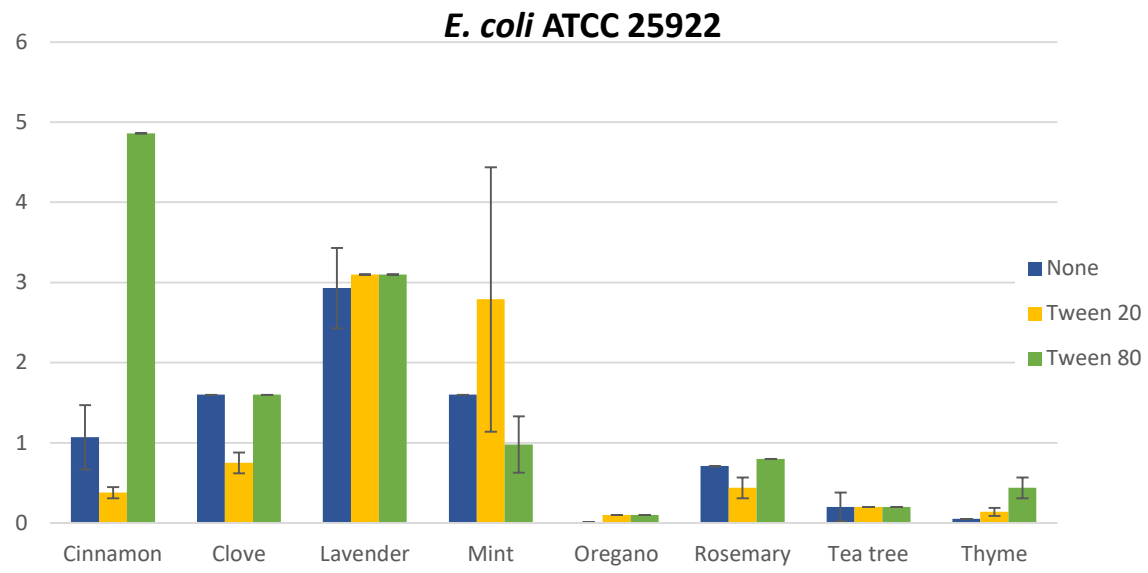
- Tea tree oil: *S. aureus* (0,1% with Tween 20)
- Thyme oil: *E. coli* (0,05% without emulsifier), MRSA (0,05% with Tween 20), *S. aureus* (0,05% with Tween 20).

E. coli ATCC 25922

EOs MIC values for *E. coli* ranged between 0,012% and 4,86%: lowest value was found for Oregano EO without emulsifier, highest was for Cinnamon EO with Tween 80. For *E. coli,* the MIC average of all EOs was 1,02% without an emulsifier, 0,98% with Tween 20, and 1,5% with Tween 80.

EOs that showed average MIC values of the three emulsifier conditions lower than 1% were the following, from lowest to highest: Oregano (0,07%), Tea tree (0,2%), Thyme (0,21%), Rosemary (0,65%) oils. Clove, Mint, and Cinnamon oil were still below 2,2%. Lavender oil MIC values were highest at 3,04%.

Figure 1: Y-axis: MIC (%) values of EOs alone, with Tween 20, and with Tween 80 against *E. coli* ± standard deviation. *X*-axis: different EOs tested.

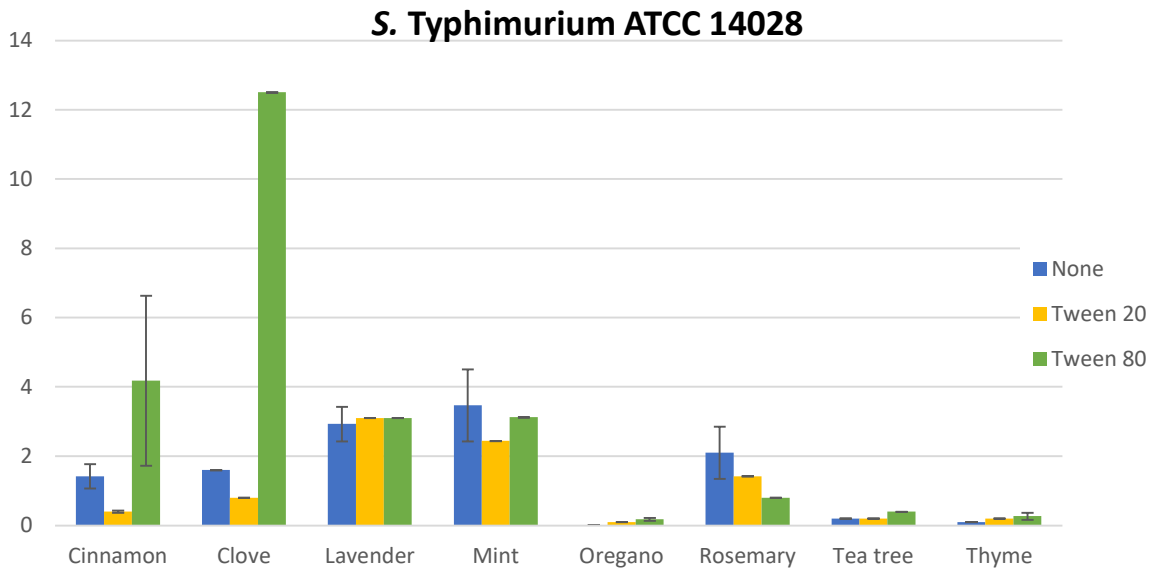


Salmonella Typhimurium ATCC 14028

EOs MIC values for *S.* Typhimurium ranged between was 0,012% and 12,5%: lowest value was found for Oregano EO without emulsifier, highest was for Clove oil with Tween 80. MIC average of all EOs against *S.* Typhimurium were 1,48% without an emulsifier, 1,08% with Tween 20, and 3,07% with Tween 80.

EOs that showed average MIC values of the three emulsifier conditions lower than 1% were the following, from lowest to highest: Oregano (0,10%), Thyme (0,19%), Tea tree (0,27%) oils. Rosemary, Cinnamon, Mint, and Lavender oils showed MICs between 1,4% and 3,05%. Clove showed the highest overall MIC for *S.* Typhimurium with a value of 4,97%.

Figure 2: Y-axis: MIC (%) values of EOs alone, with Tween 20 and with Tween 80 against *S.* Typhimurium ± standard deviation. *X*-axis: different EOs tested.



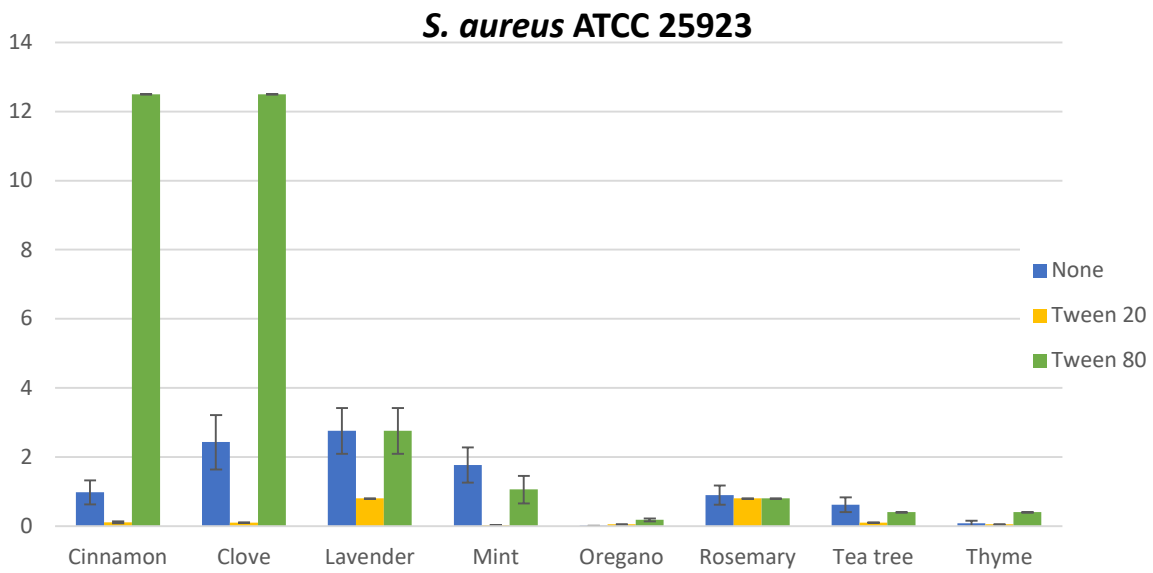
S. aureus ATCC 25923

EOs MIC values for *S. aureus* ranged between was 0,012% and 12,5%: lowest value was found for Oregano EO without emulsifier, highest was for Clove oil with Tween 80. For *S. aureus,* the MIC average of all EOs was 1,19% without an emulsifier, 0,25% with Tween 20, and 3,82% with Tween 80.

EOs that showed average MIC values of the three emulsifier conditions lower than 1% were the following, from lowest to highest: Oregano (0,08%), Thyme (0,18%), Tea tree

(0,37%), Rosemary (0,83%), Mint (0,95%). Lavender (2,11%), Cinnamon (4,53%), Clove (5,01%) oils instead showed much higher MIC values.

Figure 3: Y-axis: MIC (%) values of EOs alone, with Tween 20, and with Tween 80 against *S. aureus* ± standard deviation. *X*-axis: different EOs tested.



MRSA ATCC 43300

EOs MIC values for MRSA ranged between 0,025% and 12,5%: lowest value was found for Oregano EO without Tween 20, highest was for Clove oil with Tween 80. For MRSA, the MIC average of all EOs was 1,26% without an emulsifier, 0,25% with Tween 20, and 3,99% with Tween 80.

EOs that showed average MIC values of the three emulsifier conditions lower than 1% were the following, from lowest to highest: Oregano (0,09%), Thyme (0,18%), Tea tree (0,33%), Rosemary (0,75%). Mint (1,25%) and Lavender (2,1%) oils were still below 3%. Cinnamon (4,7%) and Clove (5,24%) were the highest MICs registered for MRSA.

Figure 4: Y-axis: MIC (%) values of EOs alone, with Tween 20 and with Tween 80 against MRSA ± standard deviation. *X*-axis: different EOs tested.

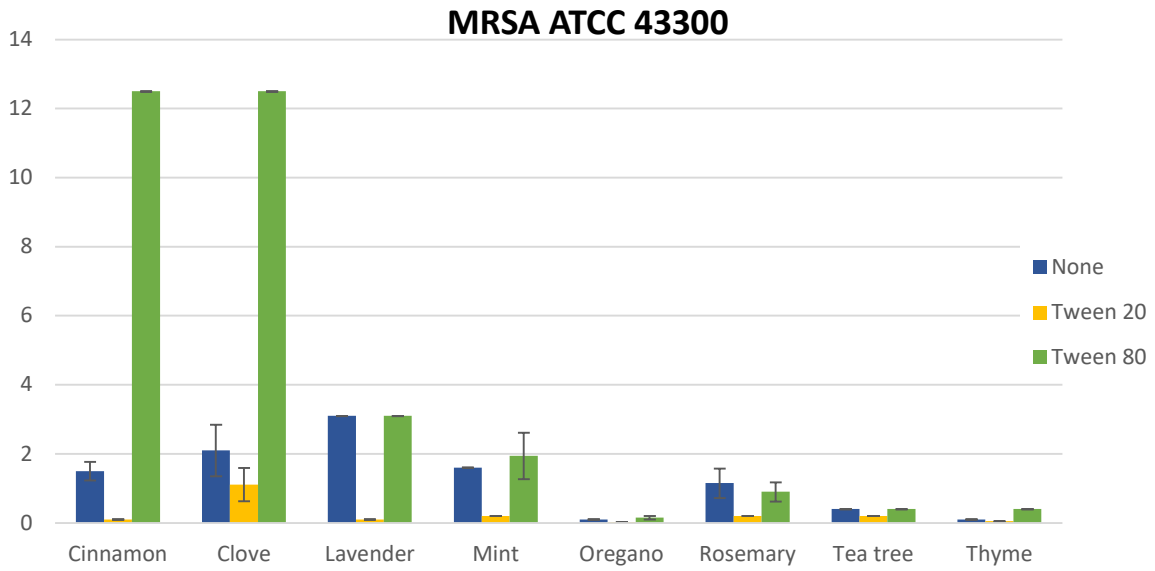


Table 1: MIC (%) of EO and relative SD against bacterial strains of interest evaluated in 2019-2020.

EO	Emulsifier	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*S. aureus* ATCC 25923	MRSA ATCC 43300
Tea tree oil	None	0,2 ± 0,0	0,2 ± 0,0	0,622 ± 0.21	0,4 ± 0,0
	Tween 20	0,2 ± 0,0	0,2 ± 0,0	0,1 ± 0,0	0,2 ± 0,0
	Tween 80	0,2 ± 0,0	0,4 ± 0,0	0,4 ± 0,0	0,4 ± 0,0
Rosemary oil	None	0,71 ±0,18	2,1 ± 0,75	0,9 ± 0,28	1,15 ± 0,43
	Tween 20	0,44 ± 0,13	1,42 ± 0,35	0,8 ± 0,0	0,2 ± 0,0
	Tween 80	0,8 ± 0,0	0,8 ± 0,0	0,8 ± 0,0	0,9 ± 0,28
Oregano oil	None	0,012 ± 0,0	0,012 ± 0,0	0,012 ± 0,0	0,1 ± 0,0
	Tween 20	0,1 ± 0,0	0,1 ± 0,0	0,05 ± 0,0	0,025 ± 0,0
	Tween 80	0,1 ± 0,0	0,18 ± 0,04	0,18 ± 0,04	0,15 ± 0,05
Thyme oil	None	0,05 ± 0,0	0,1 ± 0,0	0,08 ± 0,16	0,1 ± 0,0
	Tween 20	0,14 ± 0,05	0,2 ± 0,0	0,05 ± 0,0	0,05 ± 0,0
	Tween 80	0,44 ± 0,13	0,27 ± 0,1	0,4 ± 0,0	0,4 ± 0,0
Cinnamon oil	None	1,07 ± 0,4	1,42 ± 0,35	0,98 ± 0,35	1,5 ± 0,27
	Tween 20	0,38 ± 0,07	0,4 ± 0,0	0,11 ± 0,03	0,1 ± 0,0
	Tween 80	4,86 ± 0,0	4,18 ± 2,45	12,5 ± 0,0	12,5 ± 0,0
Mint oil	None	1,6 ± 0,0	3,47 ±1,04	1,77 ± 0,51	1,6 ± 0,0
	Tween 20	2,79 ± 1,65	2,44 ± 0,8	0,025 ± 0,0	0,2 ± 0,0
	Tween 80	0,98 ± 0,35	3,125 ± 0,0	1,06 ± 0,4	1,94 ± 0,67
Lavender oil	None	2,93 ± 0,5	2,93 ± 0,5	2,76 ± 0,66	3,1 ± 0,0
	Tween 20	3,1 ± 0,0	3,1 ± 0,0	0,8 ± 0,0	0,1 ± 0,0
	Tween 80	3,1 ± 0,0	3,1 ± 0,0	2,76 ± 0,66	3,1 ± 0,0
Clove oil	None	1,6 ± 0,0	1,6 ± 0,0	2,43 ± 0,79	2,1 ± 0,75
	Tween 20	0,75 ± 0,13	0,8 ± 0,0	0,1 ± 0,0	1,11± 0,48
	Tween 80	1,6 ± 0,0	12,5 ± 0,0	12,5 ± 0,0	12,5 ± 0,0

Natural Identical Compounds

NICs analysis was conducted at the end of 2019 and in the first part of 2020 without emulsifier.

All average MIC values among all strains were between 224 µg/ml (Cinnamaldehyde) and 2361 µg/ml (Menthol) (Table 2). The lowest MIC value among all NICs was found for Cinnamaldehyde against *S. aureus* (128 µg/ml). Conversely, the highest was found for Menthol against *S.* Typhimurium (4096 µg/ml).

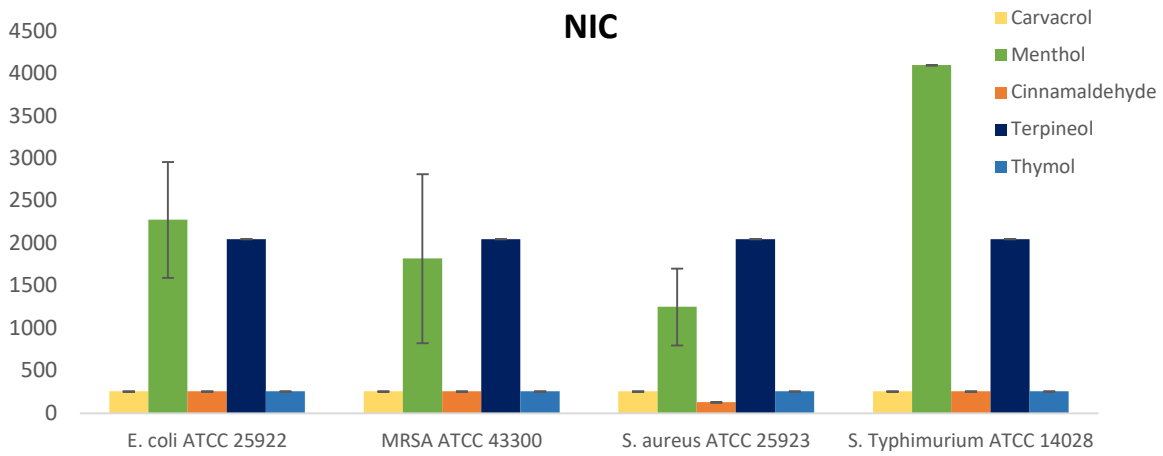
The bacterial strain generally less sensitive to the tested NICs was *S.* Typhimurium (1382,4 µg/ml), while the most sensitive was *S. aureus* (787,91 µg/ml).

From lowest to highest, MIC values were found for 1) Cinnamaldehyde, 2) Carvacrol and Thymol with the same values, 3) Terpineol, and finally 4) Menthol.

Table 2: MIC (µg/ml) of NIC against bacterial strains of interest evaluated in 2019-2020.

NICs	Emulsifier	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*S. aureus* ATCC 25923	MRSA ATCC 43300
Carvacrol	None	256 ± 0,0	256 ± 0,0	256 ± 0,0	256 ± 0,0
Cinnamaldehyde	None	256 ± 0,0	256 ± 0,0	128 ± 0,0	256 ± 0,0
Terpineol	None	2048 ± 0,0	2048 ± 0,0	2048 ± 0,0	2048 ± 0,0
Thymol	None	256 ± 0,0	256 ± 0,0	256 ± 0,0	256 ± 0,0
Menthol	None	2275,55±682,67	4096 ± 0,0	1251,55±451,54	1820,44±995,15

Figure 5: Y-axis: NICs' MIC (µg/ml) values against bacterial strains of interest ± standard deviation. *X*-axis: different bacterial strains and NICs tested.



Plant extracts

With plant extract overall, at the tested concentrations no MIC values were obtained, then no antibacterial activity of these compounds was detected (Table 3). In addition, plant extracts showed the difficulty of solubilization in an aqueous solvent. For this reason, no consideration can be taken regarding these compounds.

Table 3: MIC (µg/ml) of PEs against bacterial strains of interest evaluated in 2019-2020.

PEs	Emulsifier	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*S. aureus* ATCC 25923	MRSA ATCC 43300
Marshmallow	None	>6400	>6400	>6400	>6400
Chamomile	None	>12800	>12800	>12800	>12800
Mallow	None	>12800	>12800	>12800	>12800

Tween 20

MIC values were equal to or above 25% for all bacterial strains tested (Table 4). Only *E. coli* showed a MIC with SD different from zero and a higher MIC value (27,78%).

Table 5: MIC (%) of Tween 20 alone and relative SD against bacterial strains of interest.

BACTERIA	EMULSIFIER	VALUE (%)	SD
E. coli ATCC 25922	Tween 20	27,78	8,33
MRSA ATCC 43300	Tween 20	25	0
S. aureus ATCC 25923	Tween 20	25	0
S. Typhimurium ATCC 14028	Tween 20	25	0

Evaluation of Checkerboard MIC values

EOs and NICs with Tween 20 checkerboard assays analyses were conducted in the first half of 2021.

Essential oils and Tween 20

All average EOs in combination MIC values were within a range of 0,025% (Oregano on *E. coli* and *S*. Typhimurium, Thyme on MRSA) to 12,5% (Rosemary on *S*. Typhimurium).

All average Tween in combination MIC values were equal to 0,1%, except for Mint on *S*. Typhimurium (0,2%), Lavender on *S*. Typhimurium (0,4%), Rosemary on *E. coli* (0,8%) and Rosemary on MRSA and *S*. Typhimurium (25%).

The lowest MIC values obtained in combination (0,025% EO MIC in combination and 0,1% Tween 20 MIC in combination) were for Thyme on MRSA and Oregano on *E. coli* and *S*. Typhimurium. The highest MIC values obtained in combination were for Rosemary on *S*. Typhimurium: 12,5% EO MIC in combination and 25% Tween 20 MIC in combination.

Of all thirty-two interactions tested (100%), four (12,5%) were found to be synergic. Eleven interactions (34,37%) showed additivity. 37,5% of interactions (12/32) resulted in indifference. Five interactions (15,63%) showed antagonism.

All Clove buds oil interactions and 75% of Oregano oil interactions showed indifference. The resting 25% of Oregano oil interactions showed antagonism. 75% of Mint oil interactions wield additivity, 25% synergy. 100% of Cinnamon oil tests lead to antagonism.

For what concerns bacterial strains, on *S*. Typhimurium was observed synergy between Tween 20 and Mint/Lavender oils; on *E. coli* only, Rosemary showed synergy with Tween 20 and on MRSA only Thyme oil. No synergic associations were observed on *S. aureus*.

Additive associations were observed with Tween 20 and Tea tree, Thyme, Mint, and Lavender oils on *E. coli* and MRSA (except for Thyme). On *S. aureus,* additivity was observed with Tween 20 and Rosemary, Tea tree, and Mint oils.

Table 6: FIC index values, MIC of single EOs and in combination with Tween 20 in 2021, against the four tested bacterial strains.

EOs		*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*S. aureus* ATCC 25923	MRSA ATCC 43300
Tea tree oil	FIC	0,52 Additivity	0,52 Additivity	0,504 Additivity	0,504 Additivity
	MIC EO %	3,1 %	3,1%	6,2%	6,2%
	MIC EO combination %	1,6%	1,6%	3,1%	3,1%
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%
Rosemary oil	FIC	0,29 Sinergy	2,00 Indifference	0,504 Additivity	2,00 Indifference
	MIC EO %	6,2%	12,5%	6,2%	6,2%
	MIC EO combination %	1,6%	12,5%	3,1%	6,2%
	MIC Tween 20 combination %	0,8%	25%	0,1%	25%
Oregano oil	FIC	1,003 Indifference	1,003 Indifference	4,00 Antagonism	2,004 Indifference
	MIC EO %	0,025%	0,025%	0,025%	0,2%
	MIC EO in combination %	0,025%	0,025%	0,1%	0,2%
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%
Thyme oil	FIC	0,5 Additivity	3,88 Indifference	2,004 Indifference	0,06 Sinergy
	MIC EO %	1,6%	0,8%	0,4%	0,4%
	MIC EO combination %	0,8%	3,1%	0,8%	0,025%
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%
Cinnamon oil	FIC	8,00 Antagonism	15,5 Antagonism	4,00 Antagonism	8,00 Antagonism
	MIC EO %	0,2%	0,2%	0,2%	0,2%
	MIC EO combination %	1,6%	3,1%	0,8%	1,6%
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%
Mint oil	FIC	0,504 Additivity	0,14 Sinergy	0,504 Additivity	0,504 Additivity
	MIC EO %	0,4%	6,2%	1,6%	1,6%
	MIC EO combination %	0,2%	0,8%	0,8%	0,8%
	MIC Tween 20 combination %	0,1%	0,2%	0,1%	0,1%
Lavender oil	FIC	0,504 Additivity	0,27 Sinergy	1,00 Indifference	0,504 Additivity
	MIC EO %	6,2%	6,2%	3,1%	6,2%
	MIC EO combination %	3,1%	1,6%	3,1%	3,1%
	MIC Tween 20 combination %	0,1%	0,4%	0,1%	0,1%
Clove oil	FIC	2,004 Indifference	2,004 Indifference	2,004 Indifference	2,004 Indifference
	MIC EO %	0,1%	0,1%	0,1%	0,2%
	MIC EO combination %	0,2%	0,2%	0,2%	0,4%
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%

Figure 6: graphical representation of data collected in EO checkerboard assays in combination with Tween 20 for *E. coli*. *Y*-axis: MIC (%) value. *X*-axis: EOs tested and Tween 20.
The yellow line represents FIC index values for each association.

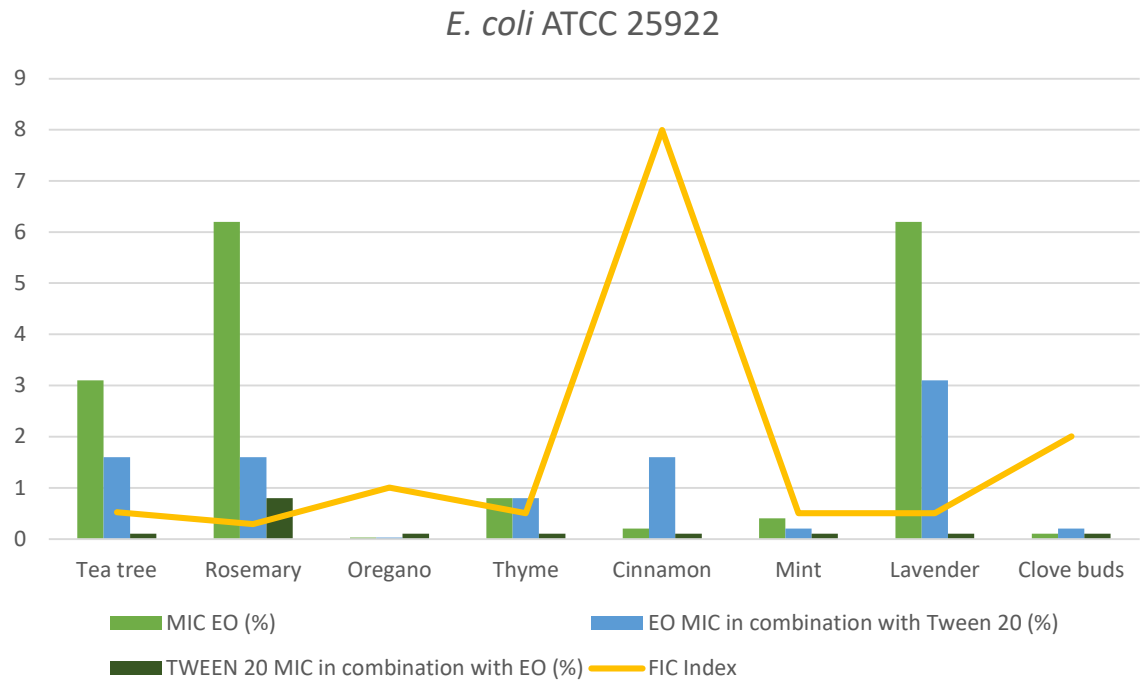


Figure 7: graphical representation of data collected in EO checkerboard assays in combination with Tween 20 for *S.* Typhimurium. *Y*-axis: MIC (%) value. *X*-axis: EOs tested and Tween 20.
The yellow line represents FIC index values for each association.

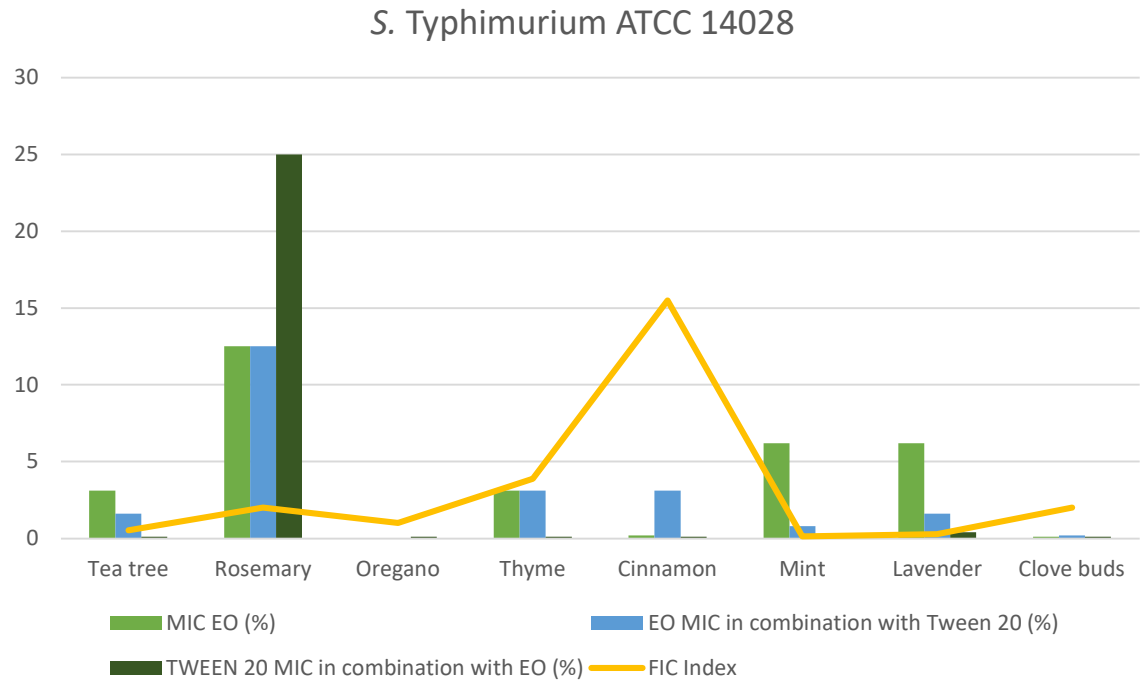


Figure 8: graphical representation of data collected in EO checkerboard assays with Tween 20 for *S. aureus*. *Y*-axis: MIC (%) value. *X*-axis: EOs tested and Tween 20.
The yellow line represents FIC index values for each association.

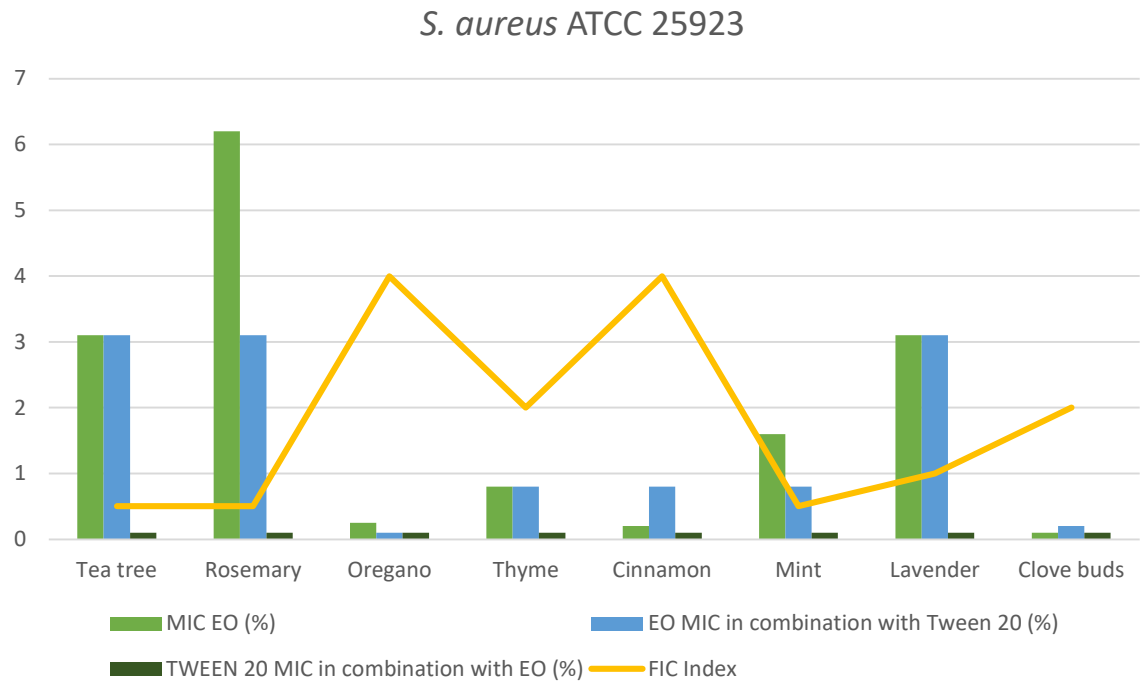
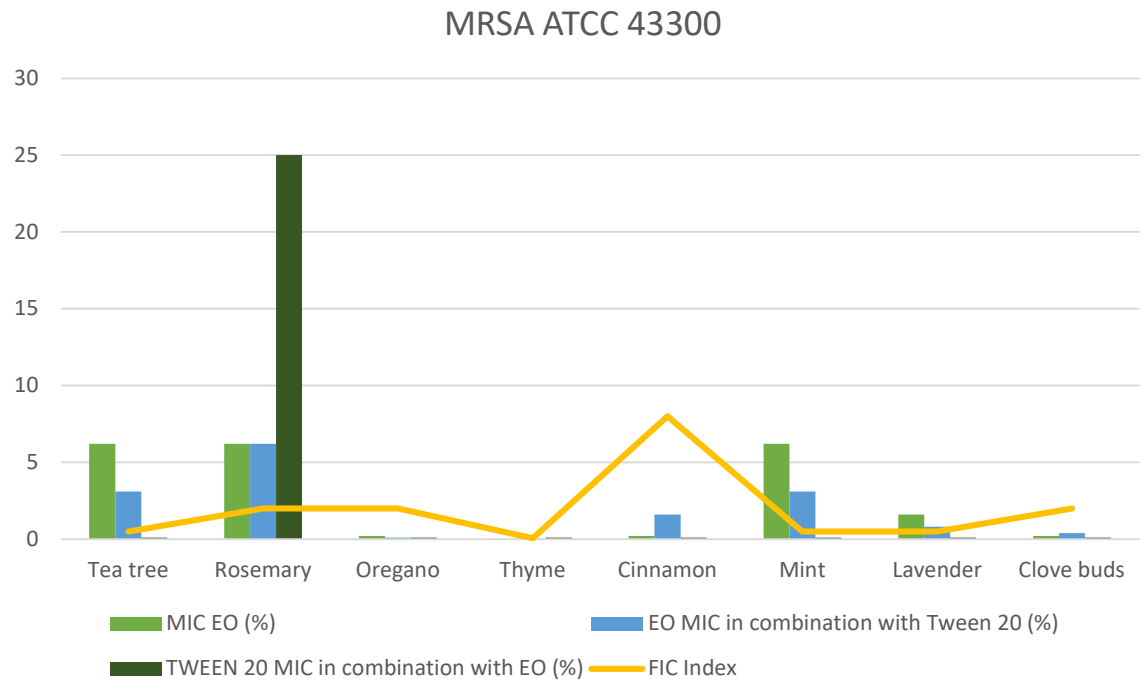


Figure 9: graphical representation of data collected in EO checkerboard assays combined with Tween 20 for MRSA. *Y*-axis: MIC (%) value. *X*-axis: EOs tested and Tween 20 .
The yellow line represents FIC index values for each association.



Variations over time in EOs MIC value

Altogether, MIC increased over time. Tea tree, Oregano, and Rosemary oils generally increased their MIC on bacterial strains by about 14, 7, and 6 times respectively. Thyme oil MIC, on average, increased among bacterial strains by 14 times, while on MRSA, MIC value in 2021 decreased. Lavender activity resulted in a doubling of MIC value.

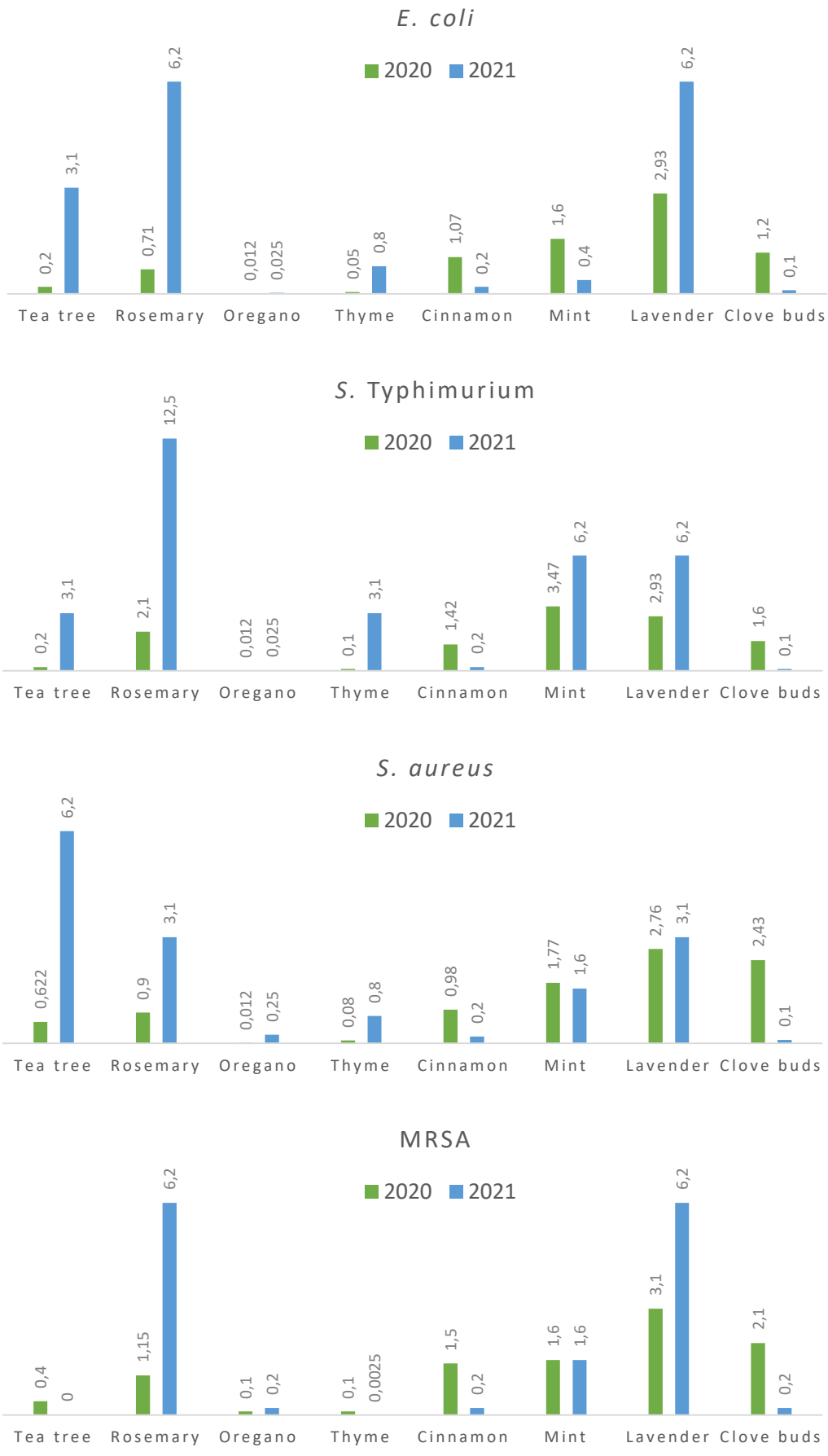
For Mint oil only, MIC value remained the same on MRSA (1,6%), while the rest of EOs MIC values changed.

Two oils MICs, on the opposite, were lower in 2021 than in 2020 on all bacterial strains tested: Cinnamon and Clove buds oil showed MIC values diminished by about a tenth.

Table 7: Comparison of all EO MIC values from 2019-2020 and 2021 tests.

EO	*E. coli* ATCC 25922		*S.* Typhimurium ATCC 14028		*S. aureus* ATCC 25923		MRSA ATCC 43300	
	2020	2021	2020	2021	2020	2021	2020	2021
Tea tree	0,2	3,1	0,2	3,1	0,622	6,2	0,4	6.2
Rosemary	0,71	6,2	2,1	12,5	0,9	3,1	1,15	6,2
Oregano	0,012	0,025	0,012	0,025	0,012	0,25	0,1	0,2
Thyme	0,05	0,8	0,1	3,1	0,08	0,8	0,1	0,0025
Cinnamon	1,07	0,2	1,42	0,2	0,98	0,2	1,5	0,2
Mint	1,6	0,4	3,47	6,2	1,77	1,6	1,6	1,6
Lavender	2,93	6,2	2,93	6,2	2,76	3,1	3,1	6,2
Clove buds	1,2	0,1	1,6	0,1	2,43	0,1	2,1	0,2

Figure 10: Comparison of EOs MIC values in 2020 and 2021 among bacterial strains of interest.



Natural Identical Compounds and Tween 20

All average NICs MIC values in combination with Tween 20 were within a range of 128 µg/ml (Cinnamaldehyde with *E. coli* and *S.* Typhimurium) to 4096 µg/ml (Menthol with *E. coli*, *S.* Typhimurium, *S. aureus*, and Terpineol with MRSA).

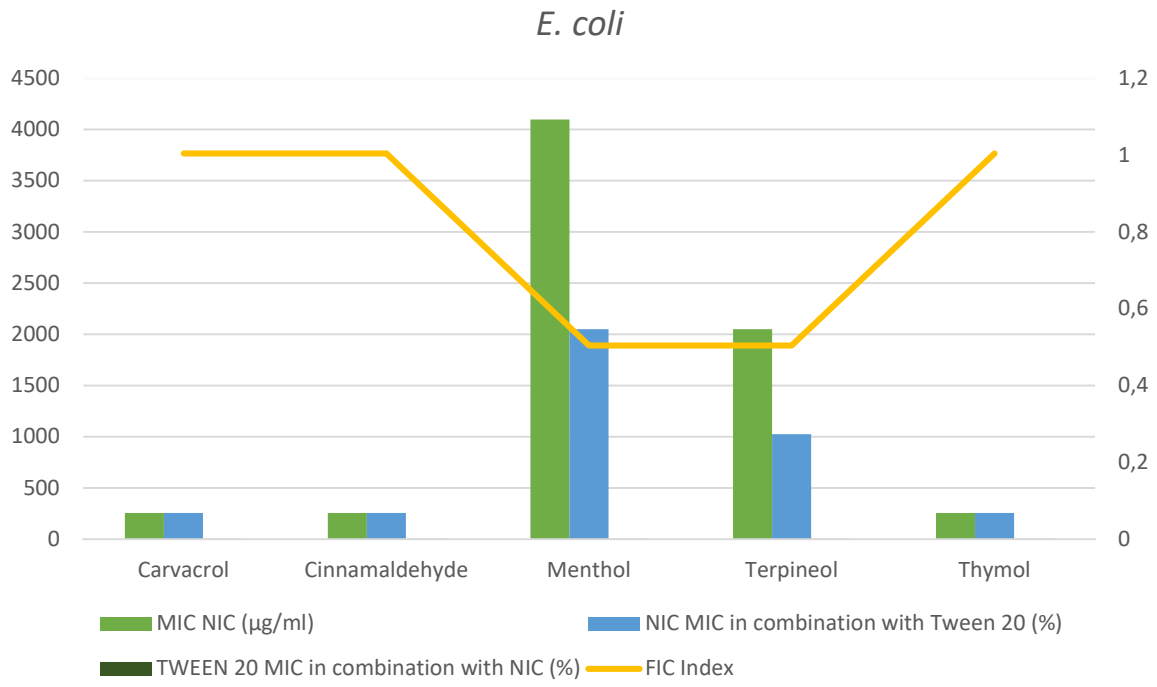
The lowest MIC values obtained in combination (128 µg/ml NIC MIC in combination and 0,1% Tween 20 MIC in combination) were for Cinnamaldehyde with *E. coli* and *S.* Typhimurium. The highest MIC values obtained in combination were for Menthol with *E. coli*, *S.* Typhimurium, *S. aureus*, and Terpineol with MRSA (NIC MIC 4096 µg/ml, Tween 20 MIC 25%).

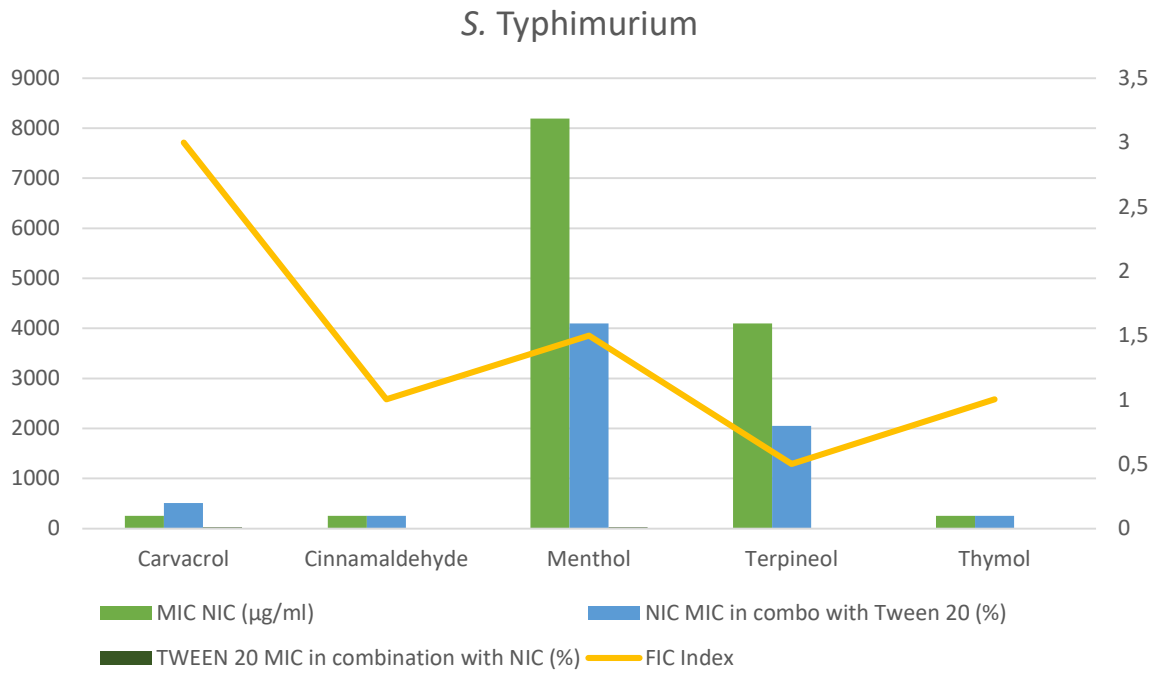
Of all interactions tested, sixteen (80%) were found to be indifferent. Four interactions (20%) showed additivity. Terpineol showed 75% additivity (with E. coli, MRSA, and S. aureus) and 25% indifference (with MRSA). Menthol showed 25% additivity (against S. Typhimurium) and 75% indifference (against *E. coli*, *S. aureus*, and MRSA). All the other tested NICs showed 100% indifference in combination.

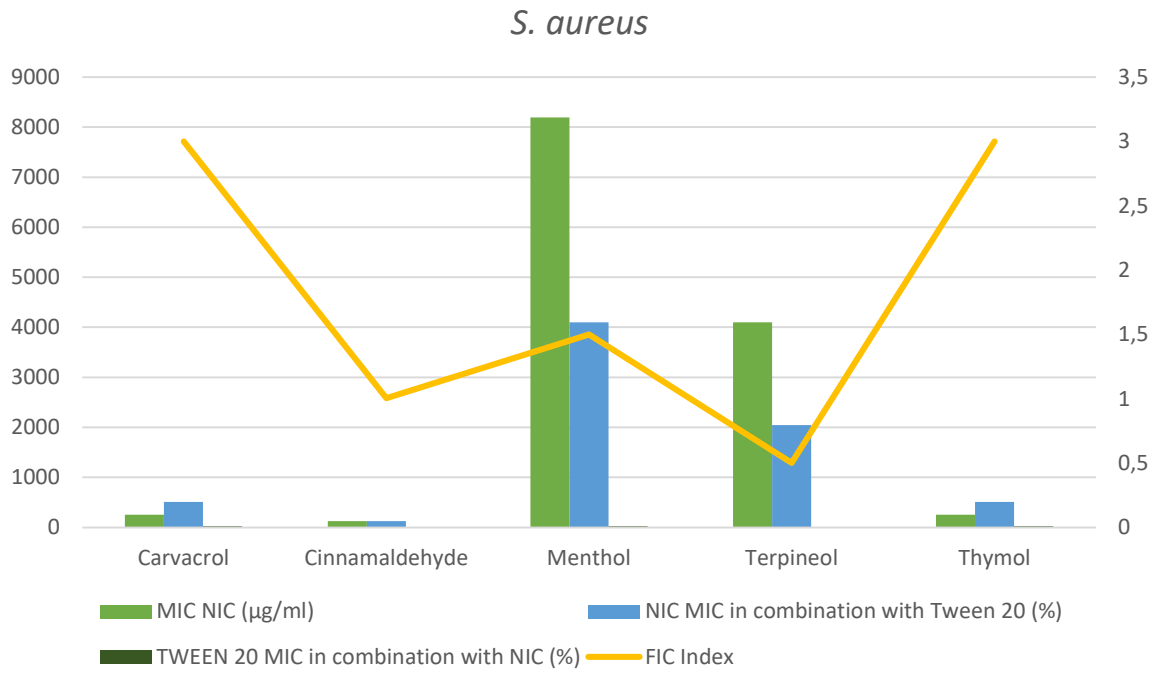
Table 8: FIC index values, MIC of single NICs and in combination with Tween 20 in 2021, against the four tested bacterial strains.

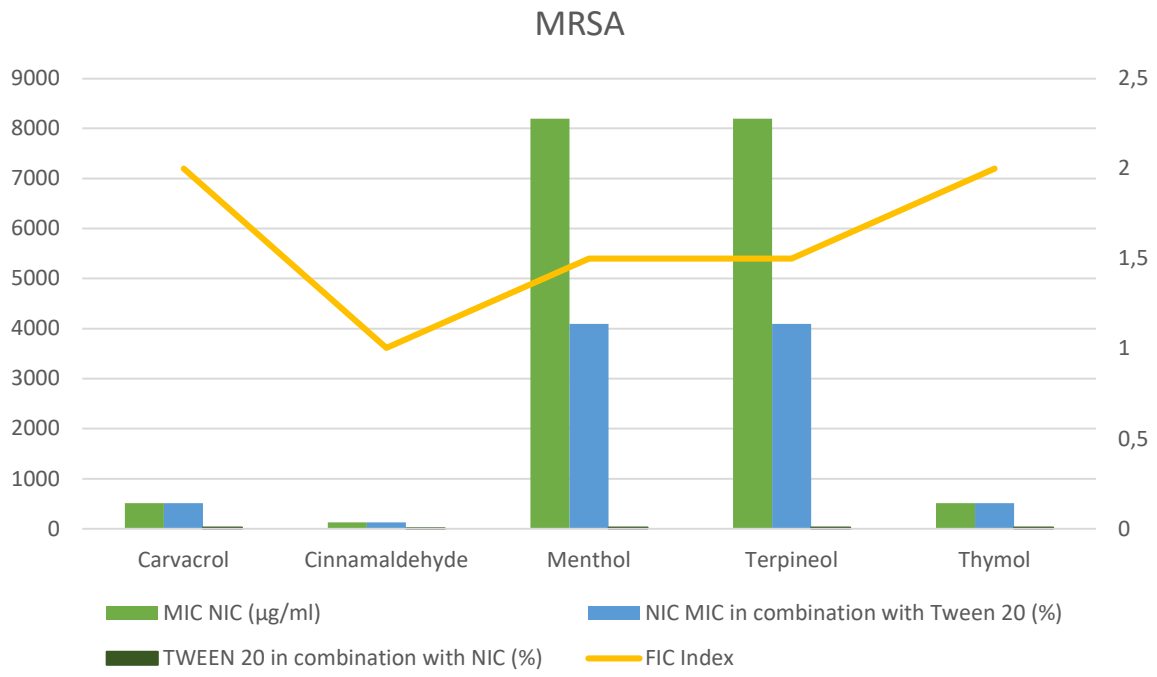
EOs		*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*S. aureus* ATCC 25923	MRSA ATCC 43300
Carvacrol	FIC	1,004 Indifference	3,00 Indifference	3,00 Indifference	2,00 Indifference
	MIC NIC µg/ml	256 µg/ml	256 µg/ml	256 µg/ml	512 µg/ml
	MIC NIC in combination µg/ml	256 µg/ml	512 µg/ml	512 µg/ml	512 µg/ml
	MIC Tween 20 combination %	0,1%	25%	25%	25%
Cinnamaldehyde	FIC	1,004 Indifference	1,004 Indifference	1,004 Indifference	1,004 Indifference
	MIC NIC µg/ml	256 µg/ml	256 µg/ml	128 µg/ml	128 µg/ml
	MIC NIC in combination µg/ml	256 µg/ml	256 µg/ml	128 µg/ml	128 µg/ml
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	0,1%
Terpineol	FIC	0,504 Additivity	0,504 Additivity	0,504 Additivity	1,5 Indifference
	MIC NIC µg/ml	2048 µg/ml	4096 µg/ml	4096 µg/ml	8192 µg/ml
	MIC NIC in combination µg/ml	1024 µg/ml	2048 µg/ml	2048 µg/ml	4096 µg/ml
	MIC Tween 20 combination %	0,1%	0,1%	0,1%	25%
Thymol	FIC	1,004 Indifference	1,004 Indifference	3,00 Indifference	2,00 Indifference
	MIC NIC µg/ml	256 µg/ml	256 µg/ml	256 µg/ml	512 µg/ml
	MIC NIC in combination µg/ml	256 µg/ml	256 µg/ml	512 µg/ml	512 µg/ml
	MIC Tween 20 combination %	0,1%	0,1%	25%	25%
Menthol	FIC	0,504 Additivity	1,5 Indifference	1,5 Indifference	1,5 Indifference
	MIC NIC µg/ml	4096 µg/ml	8192 µg/ml	8192 µg/ml	8192 µg/ml
	MIC NIC in combination µg/ml	2048 µg/ml	4096 µg/ml	4096 µg/ml	4096 µg/ml
	MIC Tween 20 combination %	0,1%	25%	25%	25%

Figure 11: graphical representation of data collected in NICs checkerboard assays in combination with Tween 20. From the top: *E. coli*, *S.* Typhimurium, *S. aureus*, MRSA.
Y-axis: MIC (%) value. *X*-axis: NICs tested and Tween 20.
It is graphically evident how the FIC index (yellow line) varies based on MIC proportion alone and in combination.






S. aureus
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
3,5
3
2,5
2
1,5
1
0,5
0
Carvacrol
Cinnamaldehyde
Menthol
Terpineol
Thymol
MIC NIC (µg/ml)
NIC MIC in combination with Tween 20 (%)
TWEEN 20 MIC in combination with NIC (%)
FIC Index


MRSA
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
2,5
2
1,5
1
0,5
0
Carvacrol
Cinnamaldehyde
Menthol
Terpineol
Thymol
MIC NIC (µg/ml)
NIC MIC in combination with Tween 20 (%)
TWEEN 20 in combination with NIC (%)
FIC Index

DISCUSSION

The methods used made it possible to evaluate the plants derivatives ability to inhibit bacterial metabolism and growth.

Optical density values measured as mentioned in the "Materials and Methods" chapter were not reported in the "Results" chapter because values obtained are unreliable. As a matter of fact, EOs emulsified with Tween 20, and Tween 80 have intrinsic turbidity that affects OD value reading. Also, the separation of the two phases when the emulsifier was not added does not allow an evaluation of this parameter.

For this reason, it was decided to substitute the OD reading with Resazurin assay, which also allows evaluating the bacterial metabolism (see "Materials and Methods" chapter).

Essential oils

EOs MIC results are encouraging, especially for oils such as Oregano and Thyme that inhibit all bacteria tested at a concentration lower than 0,2% without any help from emulsifiers; Tea tree has the same effect at a concentration lower than 0,63%. Furthermore, we consider very promisingly all MIC values under 1%.

A lower MIC value leads to lower production costs, linked to the price of the compounds due to a reduced quantity needed. Also, minor doses administered help avoiding the possible onset of side effects associated with a high administration (gut dysmicrobism, possible systemic absorption, toxic effects on kidneys and nervous system) [81].

This becomes interesting when considering the possibility of administration *in vivo*. This work has the aim to propose the oral administration of essential oils as prebiotics for zootechnical use. This would become highly advantageous for all organic and "BIO" productions.

The presence of EOs in animals diets would allow to administer fewer traditional antimicrobials and help to reduce the development of AMR in pathogenic microorganisms. The use of EOs supports the fight against pathogens while affecting only mildly physiological gut microbiota [82].

Natural Identical Compounds

Natural Identical Compounds also showed an interesting activity against the tested bacteria. Cinnamaldehyde, Thymol, and Carvacrol were the best among the tested NICs. Menthol and Terpineol were the less promising.

NICs are preferable to EOs as they are more standardizable and less subject to variations related to the plants characteristics [47,83]. This is very useful on an industrial level since the products on the market have a specific composition that does not need to be reevaluated every time a lot is changed.

Furthermore, being active ingredients, they can be quantified and administered at the necessary concentrations. They would probably be more expensive to produce but would be much more reliable on a medical level. The knowledge of correct posology allows avoiding overdose.

Plant extracts

Plants extracts showed the worst results among the tested compounds. Solubility problems or extraction issues of various kinds possibly can be responsible for the observed low activity. It could still be interesting to investigate the combination with multiple carriers (emulsifiers and other chemicals) to solve possible solubility issues.

Moreover, alternative extraction methods could be evaluated together with other plants life stages, to produce better results in terms of MIC values [47, 48].

Further tests could be performed to evaluate the antimicrobial activity of extracts derived from other plants species.

Emulsifiers

As expected, Tween 20 lowers MIC values. Probably this is due to the intrinsic antimicrobial properties of Tween 20 exerted on bacteria, as our results showed.

Some compounds, such as Oregano, Thyme, and Tea Tree, showed lower MIC values without Tween 20. However, it is essential to have found that the combination with other oils reduces the MIC of the Tween 20 and, therefore, any unwanted/toxic effects of the emulsifier or compounds. EFSA has already authorized the presence of Tween 20

in feed (food additive number E432) [84]. Results obtained with checkerboard assays, furthermore, showed that the association could reduce the EO quantity object of administration, in reason of the synergy/additivity of action with Tween 20.

Furthermore, Tween 80 exerted less activity in improving MIC values.

It is not clear why *S aureus* has showed growth issues in the presence of Tween 20. Further investigation must be carried forward.

Evaluation over time of EOs MICs

The comparison of MIC values of EOs in 2020 and 2021, after one year of storage at the temperature of +4° C, showed that for the most, EOs the MIC increased, except for Clove and Cinnamon oils, which decreased.

The increasing trend of MICs may be due to oxidation of oils components or other chemical alterations because EOs contain various organic molecules (terpenes, flavonoids, et cetera) [44]. Contrariwise, Clove and Cinnamon oil components might be minor subjects to these chemical alterations, and it is interesting to examine this characteristic further.

Limits of the study

The limits of the study match EOs properties limits.

We have evaluated the mentioned batches, characterized by specific and non-standard characteristics, on four reference bacterial strains.

No replicas of checkerboard assays were performed due to time constraints. It will be necessary to perform the replicates and verify the repeatability of the results obtained.

Further investigations

Future studies can consider EOs, NICs, and PEs activity against other microorganisms. It would be interesting also to investigate MIC values against yeasts and bacterial biofilm.

In the future, other Checkerboard assays can be evaluated, such as EOs with Tween 80 and/or other emulsifiers and NICs with Tween 20.

Future studies could evaluate the same activity in other plant species.

Future research could investigate the activity *in vivo*, particularly variations in microbiota composition following the administration of a feed enriched with essential oils, natural identical compounds, or plant extract and their associations.

CONCLUSIONS

Antimicrobial resistance has become a critical problem for infection treatment both in human and veterinary medicine. This work aimed to investigate new natural antimicrobial solutions to administer via feed to livestock, hopefully replacing traditional antimicrobial drugs.

In this work of thesis, the antimicrobial activity of alternative antibiotic compounds, specifically essential oils (Clove buds, Cinnamon, Lavender, Tea tree, Mint, Oregano, Rosemary, Thyme), nature-identical compounds (Carvacrol, Cinnamaldehyde, Menthol, Terpineol, Thymol), and plants extracts (Marshmallow, Chamomile, Mallow), alone and in combination with two different emulsifiers (Tween 20 and Tween 80) was evaluated against four reference bacterial strains of veterinary interest.

EOs MIC results are encouraging, especially for oils such as Oregano and Thyme that inhibit all the tested reference bacteria at a concentration lower than 0,2% without any help from emulsifiers; Tea tree has the same effect at a lower than concentration 0,63%. Furthermore, we consider as very good all MIC the values under 1%. Cinnamaldehyde, Thymol, and Carvacrol were the best among the tested NICs, while Menthol showed the highest MIC values. Plants extracts did not show evaluable results.

Most EOs and NICs had lower MIC values in combination with Tween 20. Results in combination with Tween 80 were often worse than with Tween 20 or without emulsifiers.

Treatment of zootechnical animals with EOs, NICs and their associations with Tween 20 will hopefully allow to reduce the use of antibiotics, and consequently, the selective pressure on pathogens of interest to become resistant. Moreover, the improvement of animals health status entails a reduction in production costs altogether.

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[i] OE0898 by Industrie chimiche Muller & Koster S.p.A
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[iii] OE0985 by Industrie chimiche Muller & Koster S.p.A
[vi] OE5370 by Industrie chimiche Muller & Koster S.p.A
[v] OE0228 by Industrie chimiche Muller & Koster S.p.A
[vi] OE0375 by Industrie chimiche Muller & Koster S.p.A
[vii] OE1318 by Industrie chimiche Muller & Koster S.p.A
[viii] OE0969 by Industrie chimiche Muller & Koster S.p.A
[ix] S0404041 by Frey + Lau GmbH
[x] S0400710 by Frey + Lau GmbH
[xi] S0401789 by Frey + Lau GmbH
[xii] S0400648 by Frey + Lau GmbH
[xiii] S0400715 by Frey + Lau GmbH

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Ad maiora semper,

Alicia