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# Comparison of In Vitro Methods for Assaying the Antibacterial Activity of a Mix of Natural Essential Oils Against Zoonotic Bacteria

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# Comparison of In Vitro Methods for Assaying the Antibacterial Activity of a Mix of Natural Essential Oils Against Zoonotic Bacteria

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**Abstract:** With the increasing occurrence of bacterial resistance, it is now essential to look for new alternatives to protect antibiotics curative utilization in One Health concept. Here we adapt and optimize a broth microdilution method and compare it against the broth macrodilution method for evaluating the antibacterial activity of a complex essential oils mix (EO mix) against 4 livestock pathogens Escherichia coli (E. coli), Bacillus cereus (B. cereus), *Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus.* Microdilution method performance (final volume well: 300 μL; inoculum: 106 CFU/mL), was evaluated following CLSI recommendations, by comparing each MIC of each 4 strains, with each MIC's obtained with macrodilution method (final volume tube: 2 mL; inoculum 106 CFU/mL). Microdilution was performed with automated plate reeder (Bioscreen C) and 3 bacterial growth parameters (OD max, lag phase, and growth rate) were calculated (Dmfit software). EO Mix MICs were determined for *E. coli*, *S. aureus* and *B. cereus*. Our results emphasize the importance of ensuring the accuracy of MIC results by performing 3 technical and 3 biological replicates, and combining OD max, lag phase, and growth rate to assess the impact of an EO mix at sub-MIC levels.

**Keywords:** Essential oils - plants extracts – antibacterial - microplate screening method

## 1. Introduction

Antibiotics have revolutionized modern medicine, enabling treatment for life-threatening infections. However, with the increasing occurrence of bacterial resistance against current antibiotics, it has now become essential to look for newer substances or even alternative methods to compensate for decrease in antibiotic power [1] . In fact, the European Union Summary Report on Antimicrobial Resistance reported worrying proportions of multidrug-resistant *Escherichia coli* isolated from broilers (37.7%), turkeys (47.6%), pigs (28.8%), and veal calves (18.8%) in 2020–2021[1]. The use of individual or mixed essential oils (EOs) or other natural plant extracts emerges as a sustainable alternative solution to limit the use of antibiotics.

EOs have natural antibacterial, antimicrobial, antiviral, and antifungal activities that help protect the host plant [2,3]. However, little is known about the potential use of EOs in livestock. Findings in the relevant literature concern the effects of medicinal plant species in livestock disease without going deeper into the isolated effects of their individual bioactive compounds. There is also only limited data on EOs [4]. However, it is important to study the efficacy of EOs on livestock pathogens, as EOs have been proposed for use as an alternative treatment to antibiotics in order slow the emergence of resistant bacteria [5,6]. Livestock animals are vulnerable to intestinal infections, especially during the weaning period, but the available antibiotic treatments are losing efficacy due to rising antibiotic resistance.

*Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Bacillus aureus* (*B. cereus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are commonly used as model organisms due to their diversity in cell morphologies and respiratory types, as well as their involvement in foodborne and/or zoonotic diseases. Several recent *in vitro* studies have demonstrated antibacterial action of EOs on many major foodborne pathogenic bacteria, e.g. *E. coli* [7], *S. aureus* [8], *Salmonella* [9], and *Listeria spp.* [10], that are also involved in diseases of livestock. *P. aeruginosa* is among the most abundant infectious microorganisms for animals and humans [4], causing respiratory infections such as pneumonia and enteric infections. Chickens are particularly vulnerable to intestinal infections caused by this zoonotic pathogen, and chicken carcasses and retail poultry products are a major vector of *P. aeruginosa* transmission to humans, especially after processing in abattoirs. Antibiotic resistance has made *P. aeruginosa* infection extremely difficult to treat [11]. *B. cereus* was identified as a sporulated model organism, as it is a known foodborne pathogen that produces enterotoxigenic toxins and has zoonotic potential due to its ubiquitous presence in soils [12]. *S. aureus* and *E. coli* cause diseases in monogastric animals from respiratory or intestinal infections to severe systemic infections [13] and have various implications in the processing and production of meat products [14]. Moreover, the formation of biofilms by *S. aureus* makes infections more challenging to treat [14].

Natural plant extracts are also widely used as nutritional additives to enhance growth parameters such as feed conversion, average daily gain, carcass quality, or milk yield [15], to decrease oxidative stress and inflammation, and to strengthen immunity [4]. In addition, all these mentioned effects work concomitantly with the prevention of enteropathies in farm animals [7]. The antibacterial and especially bacteriostatic action of phytogenic compounds have been demonstrated *in vitro* [16]. *In vivo*, their antimicrobial effect works by modifying the microflora ecosystem. Some EO compounds may selectively influence gut microflora. Cinnamaldehyde, for instance, was shown *in vitro* to be strongly inhibitory for coliform bacteria and *E. coli* [17].

Although many studies have explored the antibacterial effects of EOs, there is no standardized reference method, and no universally accepted indicators for repeatability and reproducibility. Tools for rapid and high-throughput screening of antibacterial natural plant extracts provide the means to quickly target livestock bacterial pathogens.

The CLSI [18,19] has standardized the agar dilution method to determine quantitative bactericidal activity of antibiotics. The CLSI has also issued recommended broth dilution methods for the determination of minimal inhibitory concentrations (MIC) [20] that use different principles to assess microbial growth or inhibition. MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test [20]. Conventional methods such as disc diffusion method and dilution tube method can prove time-consuming and require large quantities of test materials, along with other problems. Furthermore, plants extracts such as EOs are barely miscible in aqueous solution, since sedimentation quickly becomes a problem, and the interpretation of test results remains subjective and sometimes complicated by the colored plants extracts.

A number of other antibacterial assays only give an approximation of bacterial growth as they are based on visual observation of turbidity [8], which give subjective results that may not be reproducible between different laboratories. To overcome these drawbacks, different methods were compared and an assay with modified dilution and agitation parameters to yield a 'true' MIC value was proposed along with more information on target-strain growth parameters. By challenging the substance tests and bacterial targets with standardized parameters of the MIC method, our protocol can give reproducible and meaningful results. In this study, a detailed description of the modified method of MIC determination that is sensitive, rapid, robust, reliable and automatable wqs presented, and it can be successfully used to assess the antibacterial properties of natural plant extracts/EOs. The aims of this study were: (i) to evaluate the antibacterial activity of a complex mix of EOs (EO mix) as an MIC based on by macrodilution and microdilution methods for Gram-positive (*S. aureus*, *B. cereus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*), and (ii) to discuss and suggest

recommendations for the use of the most appropriate or complementary methods and indicators for MIC determination by the microdilution method.

## 2. Materials and Methods

### 2.1. Composition of the EO Mix

The EO mix was provided by Phytosynthese (Mozac, France). It is a blend of natural EOs with antimicrobial activity against Gram-negative pathogens. Its composition was analyzed in triplicate using aThermo Fisher Trace 1300 gas chromatography (GC) system (Thermo Fischer Scientific, Waltham, MA) coupled to a Thermo Fisher ISQ LT mass spectrometry (MS) system (Thermo Fischer Scientific, Waltham, MA). Carrier gas was helium used at a flowrate of 1.5 mL/min. Column temperature was initially 40°C then gradually ramped up at 4.7°C/min to 250°C. Diluted 0.5 μL samples were injected. The components of the EO mix were identified by comparing their mass spectra against the NIST 5 library of mass spectra (National Institut of Standards and Technology, Gaithersburg, MD)

### 2.2. Bacterial Strains

The bacteria were obtained from the of VetAgro Sup (France) research-school collection. Four strains of Gram-negative bacteria [*Escherichia coli* (CIP 59.8T), *Pseudomonas aeruginosa* (ATCC 27853)] and two strains of gram-positive bacteria [*Bacillus cereus* (wild strain) and *Staphylococcus aureus* (ATCC 25923)] were used. Cultures of the bacteria were maintained in aliquots of MHB broth with glycerol (concentration?) at -80°C throughout the study, and used as stock cultures.

### 2.3. MIC by the Dilution Tube Method

All bacterial strains were tested for MIC determination using a broth dilution method following NCCLS protocols. Briefly, triplicate tubes containing 5 mL of MHB medium were added with EO mix diluted as follows: progressive dilutions of EO mix were prepared 2:1 in Mueller–Hinton (MH) broth from 1⁄2 (v/v) to 1/16384. An overnight culture of strain grown in MHB broth at 37°C was added to target a final concentration of $10^6$ CFU/mL. Tubes were tightly capped, incubated at 37°C, and mixed by inversion. After 24h of incubation, the tubes were examined for visual inhibition of growth and a lack of color change of the MHB medium. MIC was defined as the lowest concentration of EO showing inhibition of visible growth (turbidity) and a lack of color change in the MHB medium compared with color of the controls.

### 2.4. MIC by the Automated Turbidimeter Measurements

The MIC of EO mix against *E. coli*, *B. cereus*, *P. aeruginosa* and *S. aureus* strains were determined using the broth microdilution method according to Kwieciński et al. (2009) [21]. All bacterial strains were incubated at 37°C in MH broth until the stationary growth phase was reached. A diluted bacterial suspension was added to a 100-well microtiter plate (Honeycomb microplate, Oy Growth Curves AB Ltd., Helsinki, Finland) at a final concentration of $10^6$ CFU/mL based on spectrophotometric absorbance measured at 600 nm (Shimadzu, 1280 UV-VIS, China). Serial twofold dilutions of EO mix were prepared and added to each well to obtain a final concentration range from 5.0 to 0.005 μL/mL. All wells contained from 1% to 5% DMSO (v/v) to enhance the solubility of the EO mix. In addition, there were solvent controls (test bacteria and MH broth containing from 1% to 5% DMSO), bacterial controls (test bacteria and MH broth), blank controls (MH broth containing from 1% to 5% DMSO and corresponding concentrations of EO mix), blank solvent controls (MH broth containing from 1% to 5% DMSO) and blank medium controls (MH broth). All plates were incubated at 37°C for 24 h, and growth was evaluated by an automated turbidimeter (Bioscreen C, Labsystems, Helsinki, Finland). Optical density (OD) measurement at the 420–580 nm wavelength band was

performed every 15 min after shaking for 20 s. MIC was defined as the lowest concentration of each antimicrobial agent for which there was no detectable growth for 24 h in liquid broth at 37°C.

Growth curves data were processed using the free DMFit curve-fitting software (v2.1; courtesy of the Institute of Food Research, Norwich, UK) and the function developed by Baranyi et al (1993) [22]. EO mix MIC/2 was the EO mix concentration where we observed bacterial growth recovery. Mathematical analysis enabled the determination of three growth parameters: ODmax data, which gives the maximum increases in OD during incubation, maximum growth rate (µmax), which occurs in the early exponential growth phase, and lag time (Lag T), which is time lapse before an OD increase as per Baranyi et al.'s (1993) function [22]. Extension of the lag time was calculated using the formula given in Hayouni et al. (2008)[23].

### 2.5. Statistical Processing

For each bacteria strain, the comparison of the 3 growth parameters between EO mix MIC/2 assays versus negative controls were analyzed in XLStat® premium software v2023.3.1.1416 (Addinsoft, France) using non-parametric Mann-Whitney and Kruskal-Wallis tests (0.05 significant level).

# 3. Results

### 3.1. Composition of the EO Mix

The EO mix was composed of 15 natural components. The main components were thymol (37.29%), carvacrol (28.4%), geranial (8.33%), neral (5.43%), p-cymene

### 3.2. Comparison of the Macrodilution vs Microdilution Methods for the Determination of EO Mix MIC

EO mix MIC values were determined according to macrodilution and microdilution method for 3 out of 4 bacteria strains (Table 1). No EO mix MIC was determined for P. aeruginosa.

**Table 1.** Comparison of the EO mix MIC (µL/mL) between microdilution and macrodilution methods for *E. coli*, *B. cereus*, *P. aeruginosa* and *S. aureus*.

| Bacterial Strain | Macrodilution Method EO Mix MIC (µL/mL) | Microdilution Method EO Mix MIC (µL/mL) |
|---|---|---|
| *B. cereus* | $6.08 \times 10^2 \pm 2.06 \times 10^2$a | $2.17 \times 10^2 \pm 6.53 \times 10^1$b |
| *P. aeruginosa* | N.D. | N.D. |
| *S. aureus* | $3.92 \times 10^2 \pm 2.41 \times 10^2$a | $3.69 \times 10^2 \pm 6.53 \times 10^1$a |
| *E. coli* | $1.39 \times 10^3 \pm 7.59 \times 10^2$a | $5.21 \times 10^2 \pm 1.95 \times 10^2$a |

N.D: not determined. Values are means of 9 replicates (3 biological replicates and 3 technical replicates in one biological replicate); values in a row with the same letter are not significantly different (p>0.05).

MIC values were not significantly different between the macrodilution and microdilution methods (p>0.05) except for the *B. cereus* strain (Table 1). MIC values were between $2.17 \times 10^2$ µg/mL and $1.39 \times 10^3$ µg/mL (Table 1). Bacterial strains showed different susceptilities to the EO mix: *S. aureus* was the most EO mix-susceptible strain (EO mix MIC between $3.69 \times 10^2$ µg/mL and $3.92 \times 10^2$ µL/mL) and *E. coli* had the highest EO mix MIC (EO mix MIC between $5.21 \times 10^2$ µg/mL and 1.39E+03 µg/mL).

### 3.3. Growth Parameters of the Microdilution Method

Bacterial strains growth parameters, obtained with microdilution method were presented in Table 2 :

**Table 2.** Growth parameters (µmax, lagT, and OD max) of *E. coli*, *B. cereus*, *P. aeruginosa* and *S. aureus* cultured with EO mix at progressive 2:1 dilution. Values with different letters between two growth conditions of the same

bacterial strain are significantly different (p<0.005). 'Negative control': bacterial strain in MHB; mt: Mean of three technical replicates ± SD; mb: Mean of 9 growth-condition replicates (3 biological replicates of 3 technical replicates) ± SD; N.D. not determined. .

| Bacterial Strains Growth Conditions (with or Without EO Mix) | | EO Mix MIC/2 (µL/mL) | | Lag Phase (h) | | Growth Rate (h-1) | | OD Max (uOD) | | R² | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mt* | mb** | mt | mb | mt | mb | mt | mb | mt | mb |
| *B. cereus* negative control | mt1 | | - | 0.093 ± 0.011 | 0.087a ± 0.026 | 3.728 ± 0.335 | 3.457a ± 0.897 | 0.737 ± 0.001 | 0.730 ± 0.039 | 81.72% | 92.50% |
| | mt2 | | | 0.110 ± 0.000 | | 4.269 ± 0.020 | | 0.684 ± 0.008 | | 99.50% | |
| | mt3 | | | 0.059 ± 0.022 | | 2.373 ± 0.309 | | 0.771 ± 0.008 | | 96.31% | |
| *B. cereus* with EO mix | mt1 | 108.39 ± 32.67 | | 0.672 ± 0.515 | 0.630b ± 0.407 | 0.052 ± 0.037 | 0.394b ± 0.557 | 0.112 ± 0.002 | 0.300b ± 0.140 | 91.59% | 87.90% |
| | mt2 | | | 0.250 ± 0.071 | | 1.110 ± 0.293 | | 0.388 ± 0.031 | | 77.75% | |
| | mt3 | | | 0.969 ± 0.000 | | 0.022 ± 0.006 | | 0.109 ± 0.001 | | 93.49% | |
| *E. coli* negative control | mt1 | | - | 0.079 ± 0.001 | 0.068a ± 0.011 | 5.358 ± 0.256 | 4.657a ± 0.628 | 0.862 ± 0.008 | 0.864a ± 0.023 | 98.04% | 97.50% |
| | mt2 | | | 0.063 ± 0.004 | | 4.420 ± 0.408 | | 0.873 ± 0.018 | | 96.46% | |
| | mt3 | | | 0.061 ± 0.014 | | 4.194 ± 0.467 | | 0.857 ± 0.044 | | 97.00% | |
| *E. coli* with EO mix | mt1 | 260.50 ± 97.50 | | 0.268 ± 0.248 | 0.546b ± 0.083 | 2.015 ± 3.306 | 0.660b ± 1.122 | 0.332 ± 0.270 | 0.179b ± 0.091 | 89.90% | 82.67% |
| | mt2 | | | 0.969 ± 0.000 | | (-)0.006 ± 0.035 | | 0.102 ± 0.001 | | 70.18% | |
| | mt3 | | | 0.402 ± 0.000 | | (-)0.029 ± 0.025 | | 0.103 ± 0.002 | | 87.93% | |
| *P. aeruginosa* negative control | mt1 | | - | 0.233 ± 0.016 | 0.029 ± 0.023 | 7.216 ± 0.557 | 7.513 ± 2.509 | 1.546 ± 0.008 | 1.618 ± 0.071 | 98.00% | 98.00% |
| | mt2 | | | 0.242 ± 0.036 | | 9.405 ± 4.265 | | 1.629 ± 0.068 | | 98.00% | |
| | mt3 | | | 0.213 ± 0.011 | | 5.918 ± 0.749 | | 1.679 ± 0.052 | | 98.00% | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *P. aeruginosa* with EO mix | mt1 | ind | ind | ind | ind | ind | ind | ind | ind | ind |
| | mt2 | | ind | | ind | | ind | | ind | |
| | mt3 | | ind | | ind | | ind | | ind | |
| *S. aureus* negative control | mt1 | - | 0.165 ± 0.053 | 0.150a ± 0.027 | 2.368 ± 0.606 | 2.146a ± 0.322 | 0.893 ± 0.127 | 0.940a ± 0.068 | 99.53% | 99.20% |
| | mt2 | | 0.139 ± 0.006 | | 2.035 ± 0.002 | | 0.955 ± 0.007 | | 99.02% | |
| | mt3 | | 0.144 ± 0.007 | | 2.035 ± 0.049 | | 0.971 ± 0.018 | | 99.06% | |
| *S. aureus* with EO mix | mt1 | 184.61 ± 32.67 | 0.184 ± 0.108 | 0.632b ± 0.405 | 1.507 ± 1.289 | 0.579b ± 0.975 | 0.500 ± 0.343 | 0.253b ± 0.257 | 99.22% | 57.44% |
| | mt2 | | 0.742 ± 0.393 | | 0.233 ± 0.403 | | 0.155 ± 0.087 | | 41.63% | |
| | mt3 | | 0.969 ± 0.000 | | (-)0.004 ± 0.006 | | 0.105 ± 0.002 | | 31.48% | |

The different letters indicate significant difference (p-value < 0.05) per strain between negative control (without EO mix) and with EO mix.

### 3.3.1. μmax

*B. cereus, E. coli* and *S. aureus* exposed to EO mix exhibited a significant decrease in growth rate (p<0.05) compared to their respective negative control (0.394 h-1 *vs* 3.457 h-1 for *B. cereus*, 0.660 h-1 vs 4.657 h-1 for *E. coli* and 0.579 h-1 *vs* 2.146 h-1 for *S. aureus*). For *P. aeruginosa*, no MIC could be determined and so no μmax could be calculated for sub-MIC. *P. aeruginosa* exhibited growth in all of the conditions tested. We were able to determine a μmax for *P. aeruginosa* in all of the conditions tested (see Appendix A Table 1). The μmax values of *P. aeruginosa* in technical replicates for each EO mix dilution were statistically compared against the controls (*P. aeruginosa* without EO mix): μmax values from dilution 1/10 to dilution 1/1280 were significantly different to μmax values of controls (p<0.005).

According to the means of the biological replicates (Table 2), there was no significant difference between μmax values of the 3 bacterial strains tested. However, *E. coli* tended to have higher μmax values than S. *aureus* and *B. cereus*.

### 3.3.2. lagT

*B. cereus, E. coli* and *S. aureus* exposed to EO mix exhibited a significantly higher lag time (p<0.05) compared to their respective negative controls (0.630 h *vs* 0.087 h for *B. cereus,* 0.546 h vs 0.068 h for *E. coli*, and 0.632 h *vs* 0.150 h for *S. aureus*) (Table 3). For *P. aeruginosa*, no MIC could be determined, and so no lagT could be calculated at sub-MIC.   There was no significant difference in *P. aeruginosa* lag phase between EO mix assays and controls without EO mix (see Appendix A Table 1).

According to the means of the biological replicates (Table 2), the lag phase was longer for *S. aureus* than *B. cereus* and *E. coli*, but the between-strain differences were not statistically significant.

### 3.3.3. ODmax

After 24 h of the experiment, OD max values for *B. cereus, E. coli* and *S. aureus* exposed to EO mix were significantly lower (p<0.05) than their respective negative control OD max values (Table 3). OD max values for *B. cereus* and *E. coli* decreased more than two-fold (0.300 vs 0.730 for *B. cereus* and

0.179 vs 0.864 for *E. coli*; Table 2). For *P. aeruginosa*, no MIC was determined and so no OD max could be calculated at sub-MIC. *P. aeruginosa* exhibited growth in all of the conditions tested. *P. aeruginosa* OD max values for 1/10, 1/80, 1/320, 1/640 and 1/1280 dilutions of EO mix were significantly different to control OD max ($p < 0.005$; see Appendix A Table 1)

According to the means of biological replicates, OD max was higher for *B. cereus* than *S. aureus* and *E. coli*. OD max values were significantly different between *E. coli* and *B. cereus* ($p < 0.005$) but not between *E. coli* and *S. aureus* or between *B. cereus* and *S. aureus*.

### 3.4. Technical and Statistical Comparison of the Two MIC Methods

#### 3.4.1. Microdilution Method

Repeatability was -described based on the SD of technical replicates calculated for each growth parameter. The SD values for technical replicates ranged from 0.000 to 4.265 (Table 2).

For the lag phase of negative controls, SD values for technical replicates ranged from 0.000 to 0.053.

For the growth rate of negative controls, SD values for technical replicates ranged from 0.002 to 4.265. For the OD max of negative controls, SD values for technical replicates ranged from 0.001 to 0.127.

The DMFit model showed a low SD for technical replicates of all negative controls except the *P. aeruginosa* strain.

For the lag phase of strains with EO mix, SD values for technical replicates ranged from 0000 to 0515.

For the growth rate of strains with EO mix, SD values for technical replicates ranged from 0.006 to 3.306. For the OD max of strains with EO mix, SD values for technical replicates ranged from 0.001 to 0.343.

The $R^2$ values for growth-model technical replicates ranged from 31.48% to 99.50%. $R^2$ values for growth-model technical replicates of positive controls ranged from 81.72% to 99.53%. $R^2$ values for growth-model technical replicates of strains with EO mix ranged from 31.38% to 99.22%.

Reproducibility was evaluated based on the SD of biological replicates calculated for each growth parameter. The SD values for biological replicates ranged 0.011 to 2.509 (Table 2).

For the lag phase of negative controls, SD values for biological replicates ranged from 0.011 to 0.027. For the growth rate of negative controls, SD values for biological replicates ranged from 0.322 to 2.509. For the OD max of negative controls, SD values for biological replicates ranged from 0.023 to 0.071.

For the lag phase of strains with EO mix, SD values for biological replicates ranged from 0.083 to 0.407. For the growth rate of strains with EO mix, SD values for biological replicates ranged from 0.557 to 1.122. For the OD max of strains with EO mix, SD values for biological replicates ranged from 0.091 to 0.257.

The $R^2$ values for growth-model biological replicates ranged from 57.44% to 99.20%. $R^2$ values for growth-model biological replicates of positive controls ranged from 92.50% to 99.20%. $R^2$ values for growth-model biological replicates of strains with EO mix ranged from 57.44% to 87.90%.

#### 3.4.2. Macrodilution Method

The reading of MIC results was determined by visible turbidity and by a spectrophotometer set to 600 nm. The high turbidity due to the EO mix affected the OD measurement. The MHB medium + EO mix of each dilution was used as blank to determine MIC, but it was higher than 1 unit for dilutions between 1/10 and 1/80, and so the turbidity due to bacterial growth could not be not well determined by spectrophotometry readings at a single wavelength for the dilutions between 1/10 and 1/80. However, for the following dilutions tested (i.e. from 1/160 to 1/ 10240), the OD of MHB medium + EO mix with MHB as blank was lower than 1 unit, making it possible to determine the turbidity due to bacterial growth based on spectrophotometry readings.

3.4.3. Comparison of the Macrodilution Method vs Microdilution Method

MIC values between the macrodilution method and the microdilution method were only significantly different for *B. cereus*.

### 3.5. Evaluation of the Effect of DMSO on Bacterial Growth

We investigated several effects of DMSO concentration on bacterial growth using 24h OD measurements (Figure 1). DMSO at 5% concentration impacted the growth of all bacterial strains (see Figures 1A, 1B, 1C and 1D). The growth rates of *E. coli*, *S. aureus* and *P aeruginosa* were less impacted by DMSO at 3% (Figures 1A, 1B, 1C). The growth of *B. cereus* was not impacted compared to *B. cereus* control at 3% DMSO concentration (Figure 1D). With DMSO 1%, the growth curves of *E. coli* and *S. aureus* were not different from their respective controls (Figure 1A and 1B). For *P. aeruginosa* with DMSO 1%, the growth curve exhibited the same OD max values as the control curve (up to 1.5 OD unit) (Figure 1C) but exponential-phase growth rate was greater in the control condition (Figure 1C). DMSO impacted all bacterial growth rates in a concentration-dependent manner.
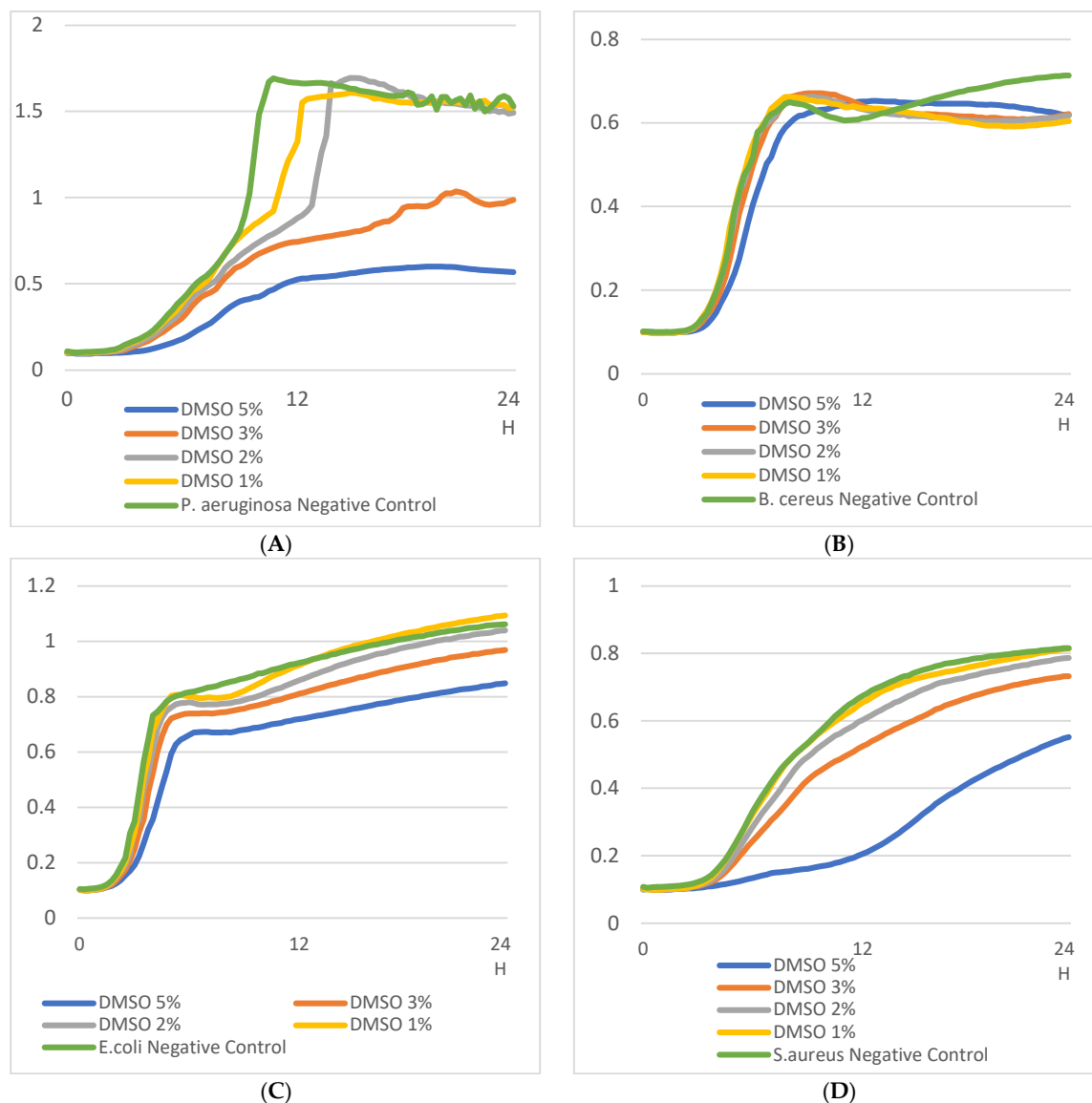


**Figure 1.** 24-h growth curves (OD) of (A) E. coli, (B) S. aureus, (C) P. aeruginosa, and (D) B. cereus at different DMSO concentrations (5%, 3%, 2%, 1%).

## 4. Discussion

The EO mix MIC values found in this study had SDs from 65.3 to 795 for MIC values ranging from 217 μg/mL and 1390 μg/mL (Table 1). MIC determination by the eye could be difficult with the macrodilution method, especially with a colored antibacterial solution such as EO mix. This method would likely lead to different results between operators and biological replicates. Coloration and incomplete dissolution of the EO mix could also lead to increasing SDs with OD reads. Both dilution methods showed limits for MIC determination with colored antibacterial substances.

Nevertheless, the automated microdilution method allowed to calculate additional bacterial growth parameters such as growth rate, lag phase, and maximal OD value. DMFit was suitably able to determine the MIC and to give further information on strain growth in the presence of EO mix. The high $R^2$ from positive controls confirmed the robustness of the DMFit model in these cases. Maximal OD value and lag phase parameters were efficient when there is a significant growth ($\mu$max>0).

DMSO was used in this study to solubilize the EO mix in MHB. DMSO impacted the growth of all model bacteria studied in a concentration-dependent manner (from 1% to 5%; Figure 1). The bacterial MICs found were from 217 to 1390 μL/mL for *E. coli*, *B. cereus* and *S. aureus* (Table 1). In these experimental conditions, DMSO proportions were between 0.001% and 0.008%: the effect of DMSO on bacterial growth did not affect the MIC results.

MIC values were only significantly different between the macrodilution method and the microdilution method for *B. cereus* (Table 1). The difference could be explained by several technical and/or biological reasons. Several technical and/or biological factors could explain this difference. *B. cereus* is a spore-forming, anaerobic, facultative, rod-shaped bacterium. Type of metabolism may impact the growth and thus the MIC of *B. cereus* depending on whether it is grown on the surface of an agar medium or in a tube in a broth medium. In previous experiments, we showed that *B. cereus* strains did not use the same metabolism for growth and are able to produce high concentrations of ethanol by metabolizing excess carbon (data not shown, personal source). *B. cereus* could potentially react differently to the EO mixture when it is grown in a microplate well than when it is grown in the microtube, as the microtube has a greater anaerobic zone.

The turbidity due to bacterial growth was not as visible as the color due to the EO mix, especially for dilutions between 1/10 and 1/80. Same problem occurred with spectrophotometer at a single wavelength; i.e. the color of the EO mix disturbed the OD measurement in dilutions from 1/10 to 1/80 (data not shown) [24,25]

The Bioscreen C reader measured OD in the 420–580 nm wavelength band and was able to detect bacterial growth more easily despite the high color of the EO mix. It thus enabled us to monitor *P. aeruginosa* growth in all dilutions tested and to determine all of the corresponding growth parameters.

The literature has identified other technical parameters that can affect MIC results (reference?). The adherence of EO to the polypropylene pipette tips could also affect the results (reference?). The emulsion of EO was critical and the agar microdilution, sonication and/or the use of emulsification agents such as DMSO, Tween 80, or Tween 20 were proposed to improve it. Hood et al (2003) [26] established an optimized broth dilution method, using 0.02% Tween 80 to emulsify the oils, and showed that it is as the most accurate method for testing the antimicrobial activity of hydrophobic and viscous EOs. The insolubility of EOs causes variability and instability of the OD measurement and the mixture [27]. With the microdilution method, we observed a decrease in OD during the first 2 hours that probably reflected partial sedimentation of the EO mix, but this phenomenon has no impact on MIC results. Zdoga. et al. (2001) [28] first observed this phenomenon when they mixed natural plant extracts with sterile water to make up a 2.5% DMSO/water/extract solution and found that the extracts precipitated from the solution. To overcome this problem, 100 mg of solid extract were prepared from aliquots on a separate polypropylene 96-well plate. The extracts were then dissolved in pure DMSO at an appropriate concentration and added to 95 μL sterile water on a sterile 96-well bioassay plate. We compared the MIC results after runs with continuous shaking *vs*

discontinuous shaking and found no difference between the MIC results (data not shown). This was no agreement with Vanegas et al (2021) [29] who observed that constant agitation guaranteed continuous contact of oil with bacteria and improved the MIC results. This divergence between findings may be due to one of two reasons. First, we used DMSO whereas Vanegas et al (2021) [29] used Tween 80 that could be more efficient in terms of producing an emulsion. Second, the constant agitation may not have been high enough to limit sedimentation and thus did not modify our MIC results. Donadu et al (2021) [30] and Li et al (2015) [31] used Tween 80 in broth and Tween 20 in agar with concentrations of 0.5% (v/v) and observed good dispersion of the oils in liquid medium. Tan et al. (2015) [25] reported that the addition of Tween 80 may be a limitation to using solvents, and that the concentration of Tween 80 used could tend to be low, ranging from "a few drops" to 0.02% and up to 5% for less-polar polyphenols like flavonoids. Oils may require up to 10% Tween 80. Ghosh et al (2013) [32] applied sonication to the emulsion for 30 min and observed a reduction in droplet size and an incremental increase in stability of the emulsion. However, Chen et al. (2023) [27] found that the agar dilution method had better repeatability than microbroth dilution but some operations caused some deviation of repeatability for agar dilution. They concluded that the microbroth dilution method was suitable for determining MICs for EOs. Tan et al. (2015) [25] added that the agar dilution assay could be an alternative to broth microdilution for testing less-polar natural extracts.

Chen et al. (2023) [27] showed that different EOs have different solvent requirements for tests against different bacteria, are so the limit of the solvents should be evaluated before use. Here we tested graduated concentrations of DMSO on the target strains and found that, in our experimental conditions, between 0.001% and 0.008% DMSO had no impact on the growth of the bacterial strains, and that DMSO could be used to dissolve the EO mix.

EOs are hydrophobic and have high viscosity. These properties may reduce dilutability or cause unequal distribution of the oil through the medium, which would lead to separation of the oil–water phases [33] and the formation of micelles [34]. The antimicrobial activity of EOs could be related to their ability to form micelles, which is a microbially-inactive form [35]. By opposition, Man et al (2019) [36] showed that some formulas of colloid or micelle suspensions of EOs such as oregano, thyme or lemon oil, can have some antimicrobial activity. This suggests that size of the micelles is a critical factor for the antimicrobial action of EOs. Man, et al (2019) [36] proposed a protocol for preparing EOs in solution without emulsion solvents and only with water mixed overnight and sonicated.

The volatility of the EOs tested is a factor that could affect the MIC results. Indeed, EOs are very complex mixtures of volatile components, and so long incubation times may result in evaporation or decomposition of some of the components during the testing period, which makes it important to set up specific experimental conditions to avoid these phenomena [37]. It may also be useful quantify the active EO residues at the end of the incubation period, which could serve to evaluate the proportion of EO potentially complexed with the culture medium.

The culture medium is also a factor that could significantly affect the MIC results. In this study, we used MH broth because it is the medium most commonly used for broth and agar dilutions for the standardized (internationally accepted) method for the MIC determination of natural substances. However, Hulankova (2022)[38]compared BHI, TSB and MHB and concluded that the MHB is far from ideal for determining the antimicrobial properties of EOs. EOs MIC values were significantly lower in MHB for all the pathogens. This was probably due to an interaction of EOs components with starch and to reduced ability of the bacteria to repair cell damage in a nutrient-deficient medium. TSB seems to be the most suitable candidate reference medium for any future standard broth dilution method for Eos antimicrobial testing.

The literature has clearly identified the most important factors causing variance in MIC between studies, which included incubation conditions, culture media, and the use of emulsifiers or solvents . Balouiri et al. (2016) [39] also mentioned other factors, such as inoculum size and the end-points determined.

Several authors have shown that bacterial inoculum size affects MIC determination. Koutsoumanis. et al. (2005) [40] and Lambert et al. (2000) [35] showed that the level of inhibitor factor

decreases with size of the inoculum. This suggests that MICs may depend on the concentration of the bacterial target encountered in the real situations [41]. Based on our results, the weak $R^2$ and high SD between the technical replicates and biological replicates for each bacterial strains MIC/2 (Table 2) could be explained by variability due to all of the factors listed above, but inoculum size could also play a major role. The heterogeneity of bacterial physiological state in the inoculum could also have an impact on the growth/no-growth boundary [42]. Tan et al. 2015 [25] added that regardless of the inoculum size used, the bacterial suspension should be used within 30 min after it has been adjusted, to avoid significant changes in cell numbers.

Of course, the growth/no-growth boundary is examined for the determined MIC, it is also possible to calculate the non-inhibitory concentration (NIC). Lambert et al. (2000) [35] defined NIC as the smallest concentration of inhibitory substance that observably slows normal growth. NIC is only observable with the automated method and it is an important indicator for evaluating the stage and timing of the bacteriostatic effect. In Supplementary data, we showed that the EO mix had an impact on the growth rate (μmax) of *P. aeruginosa* but not on the duration of its lag phase. Indeed, the lag phase of *P. aeruginosa* with the EO mix (at all concentrations tested) was not significantly different from the lag phase of *P. aeruginosa* controls (i.e. without EO mix). In addition, the growth of ps. aeruginosa in the presence of EO at dilutions from 1/10 to 1/320 was similar to that of the control without EO. Its growth rate decreased from 1/320th dilution of the EO mixture.

Microdilution-method MIC determination for EOs and plant extracts in general is a complex process. Many authors have worked with the CLSI recommendations and developed modified methods, with most of the effort focused on improving technical parameters. Other authors have focused on calculating parameters based on the OD, such as growth rate, compared to controls [41]. Vanegas et al. (2021) [29] reported that there are currently various *in vitro* approaches for evaluating the antimicrobial activity (MIC) of natural compounds, but despite the number of studies published, results fail to converge due to the fact that studies have used different methods that are not internationally standardized. Here we did not propose a new method per se, but rather a methodology for standardizing an antimicrobial screening method in each laboratory that could contribute to ultimately develop an international consensus method.

This study made it possible to determine the MIC of EOs via a reproducible and convenient high-throughput, time- and cost-effective method, as well as and the measurement uncertainty of MIC by microdilution method compared to macrodilution method. Moreover, we provided tools for characterizing the action of EO on bacterial growth, especially at sub-MIC EO concentrations. The combination of lag-phase duration, μmax and OD max values effectively detected growth or no-growth and identified which growth parameter(s) were affected by the EOs. Future experiments could focus on validation of the microdilution method on an anaerobic bacterial model. We also intend to explore other screening methods to prevent color issues of the EO mix and plant extracts.

The EO mix showed activity against 3 of the 4 model bacteria strains tested. Its composition was a blend of thymol, carvacrol, citral,p-cymene. Thymol and carvacrol, two phenolic isomers, have strong antibacterial activity against several bacterial strains [3,43,44]. At sub-MIC, carvacrol impacted both *E. coli* and *B. cereus* growth-curve kinetics that translated into lower OD max values [45]. These results are consistent with our study. In contrast, for calculated growth parameters, such as bacteriostatic effect, Pedreira et al (2024) [7] found a higher susceptibility to carvacrol for *E. coli* than *B. cereus*. Thymol and carvacrol have mainly been studied for their action against *P. aeruginosa* by inhibiting biofilm adherence and formation [46,47]. Maggini et al (2017) [48] found MICs values over than 0.5% (v:v) for *P. aeruginosa* exposed to oregano oil, with an oregano-oil composition of carvacrol >70% and thymol 21.5%. Given the composition of the EO mix studied here (see 3.1.), oregano MIC found by Maggini et al (2017) [48] was much higher than for the strongest EO mix concentration that we could have tested in our study, which explains why we were unable to find a EO mix MIC against *P. aeruginosa*. Citral also has well known antibacterial effects [49–51]. Citral inhibits bacterial growth in a species-dependent manner: *B. cereus* was shown to be more susceptible to lemongrass containing more than 70% citral compared to *E. coli* or *S. aureus* [52,53]. Furthermore, Gram-positive bacteria

were found to be more susceptible to lemongrass than Gram-negative bacteria [54]. These results are consistent with our study, as the Gram-positive bacteria (*B. cereus* and *S. aureus*) showed higher susceptibility to the EO mix than the Gram-negative *E. coli* (Table 1). Our study is also consistent with the conclusions of Murbach et al (2014) [55]. Nevertheless, we were unable to determine MIC values for *P. aeruginosa*. *P. aeruginosa* is known to demonstrate greater tolerance to volatile compounds than other Gram-negative bacteria such as *E. coli* [56]. Note too that among several volatile compounds tested by Cox & Marckham (2007) [56], *P. aeruginosa* was susceptible to trans-cinnamaldehyde, which was absent from the EO mix tested here. The absence of exposure to trans-cinnamaldehyde could explain why *P. aeruginosa* showed no growth and gave no MIC here. In addition, Naik et al. (2010) [54] found no susceptibility of *P. aeruginosa* to citral-rich lemongrass. Interestingly, Liu et al (2021) [57] showed that thymol inhibits *P. aeruginosa* growth by permeabilization of the outer and inner membrane at an MIC of 0.25 mg/mL. The fact that we failed to find any MIC for *P. aeruginosa* in this experiment may be due to the fact that the EO mix only contained 37.29% of thymol.

However, exposure to the EO mix from 0.005 μL/mL to 5 μL/mL affected *P. aeruginosa* growth parameters in a heterogeneous way. Growth parameters showed lag phases of *P. aeruginosa* from 0.15±0.04 h to 0.42±0.09 h (Appendix A, Table 1) and OD max values that varied from 0.99±0.11 to 1.25±0.10. However, the lag-phase values and OD max values were not always significantly different (p<0.05) from control values, and differences were not proportional to EO mix dilutions. This suggested a heterogeneous adaptation of *P. aeruginosa* to different. Nevertheless, all concentrations of the EO mix led to a decrease in growth rate (from 4.01±0.60 h-1 to 1.03±0.20 h-1) compared to control growth rate (7.51±2.51 h-1). This proved that the EO mix was able to reduce *P. aeruginosa* growth at all concentrations even if its antibacterial effect was not enough to completely inhibit bacterial growth. Consequently, we were unable to determine a MIC for *P. aeruginosa*. *P. aeruginosa* is well known to be able to adapt and survive to various environmental stressors via a broad range of resistance mechanisms, often present in the same clinical isolate [58]. *P. aeruginosa* can mobilize molecular mechanisms, efflux pumps, transcription regulation, and quorum sensing in various ways to counteract the effects of antibacterial drugs. These capabilities confer *P. aeruginosa* various potential strategies for surviving against EOs, even when they are blended with various other molecules.

The modes of action of various EOs against various bacterial strains are well documented, but little is known about the complex effects of EO mixes on bacterial cells. In previous work using flow cytometry, we demonstrated the mode of action of another EO blend (similar to the EO mix her but containing cinnamaldehyde) against Gram-negative *E. coli* [16]. The association of about 9% thymol and 8% carvacrol in the mix demonstrated bacteriostatic activity against *E. coli* by membrane permeabilization and pH perturbations. Thymol and carvacrol are known to inactivate bacterial enzymes and proteins and they also have a global effect on DNA and protein synthesis at sub-MIC levels [59]. This mode of action could also explain the decrease in bacterial growth rate found with sub-MIC EO mix in this study (Table 2), as DNA and protein synthesis are key for cell growth. Furthermore, the sustained loss of ions or metabolites due to exposure to an EO can compromise microbial metabolism and lead to cell death [60]. This same mechanism may have led to the reduced growth rate and OD max values found here. Thymol and citral have been reported to target bacterial membranes and disrupt bacterial homeostasis in a Gram-negative strain [61,62]. In particular, exposure to thymol led to cell membrane depolarization, decreased intracellular ATP concentrations and lower pHi. Cell membrane depolarization was also studied in our previous work exposing *E. coli* to the complex EO mix [16] and confirmed thymol action on cell membrane depolarization. Several terpenes such as thymol, carvacrol, and p-cymene targeted membrane integrity in another way against Gram-negative strain: the effect reported is an increase in the level of reactive oxygen species (ROS) [63]. ROS are known to cause lipid peroxidation in cell membranes, which affects membrane permeabilization. This mechanism could also explain the decrease in OD max with exposure to the EO mix compared to control (Table 2), as OD max is directly correlated with bacterial concentration.

This study focused on the antibacterial activity of the EO mix rather than individual EO components. This choice was motivated by the fact that it would be too hard to unravel the complex

synergistic, antagonist and/or additive effects of interactions between individual EO components [64], even with different essential oils [65]. There is increasing evidence of synergism between major and minor components within one EO but also between the major components themselves [66]. EO blends are expected to have synergies that will exert stronger antibacterial effects than individual EOs or their individual components [59,67]. In particular, thymol, carvacrol and eugenol at different combinations exhibit synergism or moderate synergism depending on their respective ratios [68,69]. In general, phenolic monoterpenes increase the bioactivities of other components in mixtures [70]. In particular, thymol and carvacrol exhibit antibacterial synergism or additive antibacterial effects against Gram-negative strains including *E. coli*, *S. aureus*, and *P. aeruginosa* [35,71]. A blend of carvacrol and p-cymene, which was one of the components of the EO mix studied here, exhibited synergistic effects against *B. cereus* [72], as did oregano oil associated with thymus oil [73]. Our study is consistent with this literature, as we found MIC values for these same bacterial strains with our EO mix composed of these related individual oils. This study points to the utility of exploring the activity of complex EO blends against several pathogenic bacterial strains.

## 5. Conclusions

This study statistically compared two *in vitro* methods, i.e. the macrodilution method and the microdilution method, for determining the MIC of a specific EO mix against 4 model pathogenic strains. Our experiments contribute to refining rules and tools for MIC interpretation, as well as providing further information on the effect of EOs on bacterial growth. This study represents one of the first attempts to specify the statistical parameters of a microdilution method to determine bacterial growth parameters (OD max, lag phase, and growth rate) and below the MIC of an EO mix. Our experiments clarified key technical specifications for determining the MIC of an EO mix. Our study emphasizes the importance of ensuring the accuracy of MIC results by:

- Performing three technical and three biological replicates using an automated system for accuracy
- Combining three growth parameters (OD max, lag phase, and growth rate) to assess the impact of an EO mix, particularly at sub-MIC levels.

We validated the *in vitro* antibacterial action on *E. coli*, *B. cereus*, *S. aureus* and *P. aeruginosa* of a specific EO mix that did not contain cinnamon oil, which is an oil known to have strong antimicrobial action. With our method, this EO mix showed bacterial inhibition on 3 out of 4 strains (based on MIC values) and exhibited significant action against growth of all 4 pathogens growth (based on growth parameters). In sub-MIC EO-mix conditions, *E. coli*, *B. cereus* and *S. aureus* showed increased lag phases and *E. coli*, *B. cereus*, *S. aureus* and *P. aeruginosa* showed decreased μmax. Analysis of bacterial growth parameters with our screening method helped to quantify the antibacterial effect of the EO mix. This work thus contributes to efforts to study the antibacterial effects of EOs via a robust and repeatable *in vitro* method. Repeatable methods that include combined growth parameters for screening pathogenic bacteria can help to discover new natural molecules and better formulate products of interest in phytogenics science. This effort could be crucial to help address the major global health challenges caused by antimicrobial resistance.

**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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