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Anna Jánosity

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Evaluation of the efflux mediated resistance-modulating effect of carvacrol in *Escherichia coli* using predictive microbiology methods

Anna Jánosity

Budapest

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Doctoral School

Name: Doctoral School of Food Science

Field: Food Science

Head: Livia Simon Sarkadi, DSc

Department of Nutrition

Institute of Food Science and Technology

Hungarian University of Agriculture and Life Sciences

Supervisors:

Gabriella Kiskó, PhD

Department of Food Microbiology, Hygiene and Safety, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences

József Baranyi, PhD

Institute of Nutrition, University of Debrecen

The applicant met the requirement of the Ph.D. regulations of the Hungarian University of Agriculture and Life Sciences and the thesis is accepted for the defense process.

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Head of the Doctoral School

....

Supervisor Supervisor

TABLE OF CONTENTS

1 INTRODUCTION

The aim of predictive microbiology is to describe the responses of microbes to the cellular environment by mathematical models. Furthermore, with the use of this knowledge the objective is to ensure the microbiological safety and quality of foods (McMeekin et al. 1993). However, recently predictive modelling ideas has been suitable to describe and optimize antibacterial therapy as well (Jánosity et al. 2021).

The spread of multidrug-resistant (MDR) microorganisms have reached an alarming threshold in the last decade, including pathogenic bacteria transmitted through the food chain. Currently, there is a competition between the development of bacterial resistance and the development of new antimicrobial agents. Nowadays, antibiotic resistance has become a global threat for both human and animal health and the misuse and overuse of antibiotics have resulted in the rapid developing bacterial resistance. The death cases per year caused by MDR pathogens might reach the 10 million for 2050 (Sharma et al. 2019; Sun et al. 2019).

Bacteria are able to develop antibiotic resistance by several ways, but one of the most important is the active efflux pump mechanism. Efflux pumps can be found in all bacterial species and responsible for removing toxic substances from the bacterial cells, including antibiotics (Teelucksingh et al. 2020). Accordingly, bacterial efflux pumps play an important role in antibiotic resistance, besides in bacterial pathogenicity and biofilm formation as well. Therefore, it is necessary to quantify the activity of efflux pumps. There are multiple methods for measuring efflux (Blair and Piddock 2016; Spengler et al. 2017) and to provide information about efflux pump activity, such as susceptibility measurements, direct and indirect fluorescence assays and the recently reported flow cytometric or mass spectrometric methods.

The use of efflux pump inhibitors (EPIs) can be a promising way to restore and enhance the efficacy of antibiotics. The search for small molecules that could block the expelling mechanism is an active and rapidly growing research area. EPIs can be derived from synthetic or either natural source. The main drawback of synthetic efflux inhibitors might be their toxic property which prevents their clinical application. Essential oils and their active components have been used for diverse purposes since ancient times such as antibacterial, antifungal, antiviral, anticancer agents. These natural antimicrobials have also shown excellent results against MDR bacteria. Changing the membrane permeability of bacteria is one of the main actions of essential oils and their components (Chouhan et al. 2017; Elshafie and Camele 2017).

Consequently, the essential oils are possible target for developing new efflux pump inhibitor molecules.

Carvacrol is a phenolic monoterpene produced by numerous aromatic plants such as thyme, oregano, wild bergamot. Carvacrol is a food additive generally recognized as safe (GRAS) and it is used in baked goods, frozen dairy foods, chewing gum, soft sweets, sauces and beverages. Several previously published articles have reported its antimicrobial activity and the potential efflux inhibitor effect of carvacrol (Khan et al. 2017; Magi et al. 2015; Sharifi-Rad et al. 2018). Based on this fact, optimization of the use of carvacrol as an efflux inhibitor and its combination with different antibiotics is a promising way to restore and enhance the activity of antibiotics.

2 OBJECTIVES

The aims of my PhD thesis are to form a comprehensive picture about (i) resistance weakening effect of carvacrol (used as an efflux pump inhibitor) (ii) its optimum inhibitor concentration according to the growth history of the organisms, moreover (iii) carvacrol's efficacy in combination with different antibiotics. To achieve these objectives, I have used predictive modelling methods and set the following tasks:

- Design and conduct research that gives practical implementation of the recommendation of Buss da Silva et al. 2019. Develop a highly reliable and accurate method to reach single cell concentration and measure their kinetics.
- Use the species of Enterobacteriaceae family and prove why *E. coli* strains are appropriate as model microorganisms even at single cell level experiments.
- Define resistance weakening activity of carvacrol by:
    - ❖ Describing the effect of carvacrol on the lag time extension of *E. coli* strains and find which concentration of it causes significant lag time extension.
    - ❖ Studying the efflux inhibitory effect of carvacrol via ethidium bromide (EtBr) accumulation, to determine the optimum concentration of carvacrol where the fluorescent dye accumulation is the highest which infer indirectly to efflux retention.
    - ❖ Meanwhile describing its concentration dependent membrane degradation effect.
    - ❖ Providing modelling methods for both types of fluorescent assays (indirect EtBr accumulation and membrane integrity assays).
    - ❖ Investigating whether the efflux mechanism of *E. coli* depends on the physiological state of the organism.
    - ❖ Studying whether the optimum inhibitor concentration of carvacrol varies among *E. coli* cultures obtained from different physiological states.
    - ❖ Defining the efficacy of carvacrol (from the range of optimal efflux inhibitor concentrations) in a combination with different antibiotics (applied in subinhibitory concentrations) to enhance the activity of antibiotics.

3 MATERIALS AND METHODS

3.1 Microorganisms

Two strains of *Escherichia coli* were used to study the *bacterial efflux mechanism* and *membrane integrity* changes: ŽM 370 (ATCC 11229), a clinically isolated pathogenic strain from the collection of the University of Ljubljana and *E. coli* ŽM 513 (VF 3584), a foodborne isolate from steak tartare derived from the Veterinary Faculty of the University of Ljubljana. Beside the above-mentioned *E. coli* strains, the bacterial culture of *E. coli* ATCC 25922 was also tested in *antimicrobial susceptibility measurements* and in *lag phase duration experiments*. This reference strain was obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary. For the *individual cell kinetics' measurements*, *E. coli* VF 3584, a foodborne isolate, and *E. coli* ATCC 25922, a reference strain were used. In addition to the two *E. coli* strains the following microorganisms were used: *Salmonella enterica* subsp. *enterica* ATCC 14028 and ATCC 13311 strains obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary and *Shigella sonnei* HNCMB 20044 clinical isolate which was from the (National Center for Epidemiology, Budapest 20044/99702).

3.2 Chemicals and antimicrobial agents

Carvacrol (5-Isopropyl-2-methyolphenol, Merck KGaA Darmstadt, Germany) was used as a natural compound and its effect was estimated on (1) EtBr accumulation, (2) bacterial membrane integrity, (3) lag time extension. Moreover, its (4) concentration dependent activity on the cells' antibiotics uptake rate was demonstrated with five antibiotics: (i) gentamicin sulphate and (ii) cefotaxime sodium salt (Sigma-Aldrich, Chemie, Steinheim, Germany); (iii) vancomycin hydrochloride (MedChem Express, Monmouth Junction, New Jersey, USA) and (iv) erythromycin and (v) ciprofloxacin hydrochloride (Cayman Chemical Company, Ann Arbor, Michigan, USA).

The stock solutions of gentamicin sulphate, cefotaxime sodium salt, vancomycin hydrochloride and ciprofloxacin hydrochloride were prepared in sterile water and the stock of erythromycin in DMSO (Sigma-Aldrich Chemie, Steinheim, Germany). The stock solutions of carvacrol were prepared in absolute ethanol (EtOH). The applied efflux substrate was EtBr (Sigma-Aldrich Chemie, Steinheim, Germany). NMP and PAβN were the tested as synthetic efflux inhibitors and obtained from CHESS GmbH (Mannheim, Germany). NMP stock solution was prepared in EtOH and stock solution of PaβN in sterile water. LIVE/DEAD BacLight Bacterial Viability

Kit (L-7012; Molecular Probes, Eugene, Oregon, USA) measured the changes of membrane permeability. To wash the cultures and resuspend for fluorescence probes, Phosphate Buffered Saline (PBS) tablets were used from Oxoid (Basingstoke, Hampshire, UK).

3.3 Measurements and methods

Broth microdilution method was used **to determine the minimum inhibitory concentration** (MIC) of carvacrol and the antibiotics following the instructions of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing 2021). Reading MIC endpoints were done after 24 h incubation at 37 °C (Cuenca-Estrella et al. 2002) and MIC values were expressed in mg/mL.

To **investigate the lag time distribution of single cells**, an indirect method was used. By measuring the single-cell-generated subpopulations' detection time, the time that was needed for the growing bacteria to reach a given concentration, the detection level ($5*10^7$- 10^8 cells/mL). The aim was to (i) provide a practical implementation of the recommendation of Buss da Silva et al. (2019) and to establish how the distribution of single-cell-generated detection times depended on (ii) the initial cell number and (iii) the five tested strains. Before the turbidity experiments, serial dilutions in Mueller-Hinton Broth were applied to stationary phase cells. The aim was to obtain, *ca.* 1-3 cells/well initial number via dilutions as the lowest cell density on a microtiter plate. A new dilution method was developed to reach the desired cell number in a well. The final volume was 200 μL *per* well. The initial concentration of the inoculum was set at OD_{600}=0.1 (Analytik Jena Specord 200 Plus Spectrophotometer).

The **slopes of the OD growth curves were used to determine the physiological states** of the microorganisms. The OD curves were measured at 37 °C, with the initial inoculum level of OD_{600}=0.1 (≈10^8 CFU/mL) in Tryptic Soy Broth. The turbidity of the wells was measured at 600 nm, using a microplate reader (Microplate reader, Safire 2, Tecan, Switzerland). Experiments were performed in triplicates using 96-well flat-bottom microtiter plates, with the final volume of 100 μL *per* well.

The **kinetics of EtBr accumulation and membrane degradation** were measured by using a VarioskanLUX multimode microplate reader (Thermo Scientific, Waltham, Massachusetts, USA) with the measurements performed according to Kovač et al. (2015) with some modifications. Before fluorescent assays, the overnight cultures were diluted in fresh Tryptic Soy Broth at OD_{600}=0.1, the same initial concentration as set in the determination of growth phases experiments. Three growth phases were tested: fast-, slow-, and non-growing phases.

To achieve them, freshly diluted cell cultures were incubated at 37 °C for 0.5 h, for 4 h and for 12 – 16 h, respectively. The incubation times were decided according to the OD growth curves. Following the incubation, cell cultures from different growth phases were washed and resuspended in PBS, then diluted to OD_{600} = 0.2 which is equivalent to *ca.* 10^9 CFU/mL. Thus, the final test cultures used in fluorescent probes had the same concentrations. After the dilution, carvacrol was added in different concentrations to the PBS washed cultures. This treatment was quantified by the ratio between the carvacrol concentration and its MIC value. The 17 test-concentrations were chosen from sub-inhibitory range, between 0.1 and 0.5 MIC, which corresponds to 30 mg/L and 150 mg/L of carvacrol. The interval was divided equidistantly with steps of 7.5 mg/L. Similarly, NMP efflux modulation effect was tested in 100, 200 and 300 mg/L concentrations and efflux inhibition activity of PaβN was tested only in 22 mg/L concentration as it was suggested by Kurinčič et al. (2012). Finally, fluorescent dyes (EtBr or LIVE/DEAD BacLight Bacterial Viability kit) were added to both the treated and the non-treated cultures. These tests were carried out in three dependent and two independent replicates using flat-bottom black microtiter plates. The intracellular EtBr accumulation was measured without carvacrol treatment as a reference assay and when the tested 17 sub-inhibitory carvacrol concentrations were applied. Relative fluorescence units (RFUs, produced at λ_{ex}=500 nm and λ_{em}=608 nm) described the cell responses. The LIVE/DEAD BacLight Bacterial Viability kit was used to detect the changes in the membrane integrity caused by carvacrol. The intracellular propidium iodide penetration measures the cytoplasmic membrane damage, which was quantified by relative fluorescent units (RFU values), measured in 60-s intervals over 1 h at λ_{ex} =481 nm and λ_{em}=510 nm. As a negative control, the membrane integrity of heat-treated cultures (at 80 °C for 15 min) was also measured.

Carvacrol effect on the detection time extension was investigated **via turbidity measurements**. The applied sub-inhibitory concentrations ranged from 45 to 120 mg/L (0.15 – 0.4 MIC) with steps of 7.5 mg/L. Turbidity readings were carried out at 620 nm (Microplate reader, Sunrise, Tecan, Switzerland) measured in Mueller-Hinton Broth at 37 °C for 18 hours. Readings were made in every 30 minutes, with 30 seconds of shaking time prior them. According to the study of George et al. (2015), turbidity-based readings were used to record T_{det} values of bacteria. The initial inoculum concentration was set to $5*10^5$ CFU/mL.

Antimicrobial Susceptibility Tests were carried out to describe the efficacy of combined treatments. The broth microdilution method was used for measuring the combined activity of antibiotics and carvacrol. The dispensing device was epMotion 5075 Automated Pipetting

System (Eppendorf AG, Hamburg, Germany). Following the preparation, microtiter plates were incubated at 37 °C for 24 hours and then read by microplate reader (Sunrise, Tecan, Switzerland) at 620 nm, two times with 30 seconds of shaking time prior them. Plastic, flat-bottom 96-well microdilution trays were used, with a specific layout, developed for these measurements. Each set of experiments (five antibiotics were tested against three *E. coli* strains) were carried out in dependent duplicates and independent triplicates, in total 45 plates were evaluated.

3.4 Data analysis

Primary models of EtBr accumulation and membrane integrity changes of microorganisms were analyzed at each concentration of carvacrol, described by the temporal variation of F_S (fluorescent signal) values, in the unit of RFU. Signals were measured and fitted by primary models: (A) saturation model of EtBr accumulation or (B) a dissipation model of membrane integrity:

$$Fs(t) = Fs_0 + (Fs_{max} - Fs_0) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad \text{(A)}$$

$$Fs(t) = Fs_0 - (Fs_0 - Fs_{min}) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad \text{(B)}$$

Here, $Fs(t)$ is the Fs value at the time t elapsed from an initial time t_0; Fs_0 is its value at the initial time; Fs_{max} is its theoretical (asymptotic) maximum; Fs_{min} is its theoretical minimum; and r is the exponential rate at which the $Fs(t)$ function converges to Fs_{max} or Fs_{min}, depending on the type of fluorescent assay, described by the primary models, finally, ε is the random measurement error.

In the **secondary models**, the ratio between the highest (Fs_{max}) and lowest (Fs_0) fitted Fs values were chosen to quantify the efficacy of carvacrol as EPI in the secondary models. This is the factor by which the Fs values increased from Fs_0 to $Fs_1 = Fs(1)$. The variation of the natural logarithm of this parameter, as a function of the carvacrol concentration, *x*, was modelled for each strain and growth phase, by an asymmetric, convex, bi-linear (triangle-) function notated as B_s:

$$y_{EP}(x) = ln\frac{Fs_1}{Fs_0} = B_s(x) = y_{opt} \cdot \begin{cases} \frac{(x - x_{min})}{(x_{opt} - x_{min})} & (x_{min} \le x \le x_{opt}) \\ \frac{(x_{max} - x)}{(x_{max} - x_{opt})} & (x_{opt} \le x \le x_{max}) \end{cases}$$

The $x_{min,}\ x_{opt,}\ x_{max}$ parameters are the minimum, optimum, and maximum concentrations defining the bi-linear function. In the first range, the $\frac{Fs_1}{Fs_0}$ was greater than 1 (i.e., the Fs values increased during the experiment). The scaling constant y_{opt} is the value of this ratio at the optimum carvacrol concentration. The s in the index of the B_s notation indicates that we expect the bi-linear function to depend on the physiological state of the culture. Outside the $[x_{min,} x_{max}]$ interval, it is assumed that the Fs values do not grow during the observation time, i.e., the B_s function is zero there. F-test was used to determine (i) whether the independent models can be merged and (ii) the optimum inhibitory concentrations of carvacrol.

$$F = \frac{RSS_1 - RSS_2}{n(p_2) - n(p_1)} \Big/ \frac{RSS_2}{N - np_2}$$

By **analysing the effect of carvacrol treatments on the lag time duration** of *E. coli*, the aim was to describe the effect of carvacrol on the bacterial lag time duration with the starting inoculum concertation of $5*10^5$ CFU/mL. In the primary model, the measured OD values (at 620 nm) were plotted as a function of time (h). Prior data analysis, the OD growth curves were standardized. The OD values were shifted to a common starting point to OD=0.1 to eliminate the initial noise. Detection time (T_{det}) values were calculated via linear interpolation at a fixed detection level (OD=0.15). In the secondary models, the recorded T_{det} values were represented as a function of carvacrol treatments. To describe the relation between the secondary model's variables, the power model was used:

$$T_{det} = \beta_0 * (121 - x)^{\beta_1} + \varepsilon \quad ,$$

where x represents the applied carvacrol treatment, ε is the error term of the model, β_0 and β_1 are the model parameters. Four independent replicates were generated *per* strain, with the same measurement settings. F test (Motulsky and Christopoulos 2004) decided whether the four datasets can be merged to an aggregated data set.

$$Ratio\ of\ relative\ differences\ of\ SS\ values\ = \frac{\left(ABS(SS_{merged} - SS_{sep})\right)}{SS_{sep}}$$

$$df_{relative\ differences} = \frac{df_{merged} - df_{sep}}{dfsep}$$

$$F = \frac{Ratio\ of\ the\ relative\ differences\ of\ SS\ values}{df_{relative\ differences}}$$

The diagnostic of non-linear regression models was done in R (package: moments - https://CRAN.R-project.org/package=moments).

Lag time extension (LE) as a response to stress factors is an important feature of bacteria since extended lag phase can bring survival advantages. Lag time extension as a function of carvacrol was defined according to Li et al. (2016).

$$LE = \frac{\lambda_C}{\lambda_0}$$

where λ_C is the lag time of a microorganism at the given concentration (C) of carvacrol, and λ_0 is the lag time at zero carvacrol treatment, measured as a positive control.

Evaluating the efficacy of combined treatments, the endpoint of OD values was recorded twice with a microplate reader (OD values of each microtiter plate was measured one after another). The average of the two dependent measurements was taken and normalized OD values based on the positive (OD_{max} – average of positive control values) and negative (OD_{min} – average of negative control values) controls. In that way the bacterial growth (%) could be expressed as:

$$Bacterial\ growth\ (\%_{\mathrm{OD}}) = \frac{OD_x - OD_{min}}{OD_{max} - OD_{min}}\ .$$

IBM SPSS Statistics, version 27 was used to carry out a multivariate statistical analysis. MANOVA investigated the differences between the combined treatments, where dependent variables were bacterial OD growth ($\%_{\mathrm{OD}}$) values when vancomycin, gentamicin, erythromycin, cefotaxime, and ciprofloxacin antibiotics were used together with factor variables carvacrol concentration (0, 75, 90, 105 mg/L) and antibiotics MIC rate (0.5; 0.25; 0.125; 0.0625).

4 RESULTS AND DISCUSSION

4.1 MIC of antimicrobials

The antibacterial activity of carvacrol was measured using the broth microdilution method. Its MIC value was estimated as 300 mg/L against *E. coli* ATCC 11229 and *E. coli* VF 3584 strains, the reference strain showed lower MIC 270 mg/L. The measured values concur with the data in literature (Dos Santos Barbosa et al. 2021). The determination of MIC of antibiotics was performed similarly by broth microdilution, the results are within the range reported in the literature (European Committee on Antimicrobial Susceptibility Testing 2022).

4.2 Distribution of bacterial single cell lag time under optimum growth conditions

Stochastic mathematical models are needed to describe and interpret the studying the growth kinetics of pathogens at low initial cell numbers, thus the variability of single cell behavior. Turbidity based readings and the recorded T_{det} values (at OD=0.15) were used to study the lag time distribution scatter of microorganisms. A specific dilution method was developed (as a practical implementation of Buss da Silva et al. 2019), to reach 1-3 cells *per* well of a microtiter plate which was proven to be optimal for experiments studying the single cell kinetics.

The initial concentration of the inoculum was set at OD_{600}= 0.1, from which four consecutive decimal dilutions were made, then 11-13 binary dilutions as needed to reach the desired cell concentration (*ca.* 1.6 cells/well), which was proven to be optimal for experiments studying single cells kinetics (Buss da Silva et al. 2019). The dilution rate was strain-dependent; therefore, a range is given for the number of binary dilutions.

The estimations for the initial cell/well numbers were obtained via (i) combination of plate counts and dilutions (c^*) and (ii) by means of the proportion of empty wells (ρ^*). No significant difference (F=0.78, p = 0.39, F_{crit}=4.20) was found between the two estimates of total number of cells (ρ^* and c^* values) obtained by the two methods. Thus, the faster and cost-effective estimate, ρ^* can be used to assess the initial number of cells in low inoculum level.

The benefit of measuring the lag time duration of single cells is that it can be easily compared to population level data and also revealing important scenarios regarding food safety issues. Strains of *E. coli, Shigella sonnei and Salmonella enterica* subs. *enterica*; the members of Enterobacteriaceae family were used at low inoculum level targeting a specific low concentration, 1.6 cells/well. Investigation established that the lag time duration of microbes was affected by the inoculum size. At the initial cell number of below *ca*. 20 cells *per* well,

high variabilities were observed in the recorded detection time (T_{det}) values, where *E. coli* strains showed the lowest scatter compared to the other investigated bacteria. The initial concentration could be set very accurately to 1–3 cells. Settings and results were repeatable and the applied binary dilution factor was well represented by the initial number of cells. Regarding food safety aspects, the presence of pathogenic and indicator bacteria is important in foods. Considering the individual behavior of each cell, the results may have significance in estimating the risk and calculating safe storage times for foods.

4.3 Carvacrol dose-dependent effect on the lag time duration

The effect of carvacrol in subinhibitory concentrations were quantified on the lag time duration of *E. coli* via turbidity-based readings. The recorded T_{det} values were proportional to the lag times. It became apparent that the increasing carvacrol concentration prolonged the lag period of bacteria which can be considered as a self-protection response to the antimicrobial treatment. Power function was used to describe the relationship between T_{det} and carvacrol treatments. The power functions, generated by independent replicates, could not be merged and the explanation efficacy of functions was different in terms of strains, but never lower than 70%. The effect of carvacrol was also expressed using lag time extension (LE) values. The increase in lag time duration was expressed in %. It can be concluded that 65–112 mg/L carvacrol concentrations caused 20–40% increase in the lag time duration. However, there were differences between the strains; the clinical isolate seemed to be the most resistant one. As carvacrol stock solution was prepared in EtOH, therefore its lag time extension effect was tested similarly to carvacrol treatments in 4, 6 or 8 µL volumes. A slight increase in the lag time duration was caused by EtOH, especially in the case of *E. coli* ATCC 25922. However, none of the tested EtOH concentrations induced significantly the lag time extension for the *E. coli* strains, p=0.08 (*E. coli* ATCC 25922)*;* p=0.22 (*E. coli* VF 3584); p=0.38 (*E. coli* ATCC 11229).

4.4 Fluorescent assays to describe effect of carvacrol on the efflux mechanism and membrane integrity of *E. coli*

Prior to the fluorescent assays, OD growth curves were used to define the fast-, slow- and non-growing phases of *E. coli* cultures. Based on the slopes of curves, the different physiological states were identified after 0.5, 4, and 14 h incubation. The cells, tested via fluorescent assays, were collected after these incubation times.

Ethidium bromide (EtBr) accumulation was measured and used to evaluate the effect of carvacrol on the efflux pump mechanism of *E. coli.* A combined experimental and numerical

procedure was used to study the efflux inhibitor efficacy of carvacrol and to optimize its concentration. Saturation functions were fitted to the temporal F_S (relative fluorescent signal) values obtained via indirect EtBr accumulation measurements at the selected carvacrol treatments, whereas dissipation functions were used to describe the membrane degradation effect of carvacrol. The saturation and dissipation functions were used in primary modelling.

In the secondary models, primary model parameters were used as a function of carvacrol treatment. Regarding the membrane integrity measurements, the effect of carvacrol can be described by a monotone decreasing function suggesting that the higher the carvacrol concentration, the lower the bacterial membrane integrity. The effect of carvacrol on EtBr accumulation (using the primary model parameter y_{EP} values) could be described by a bi-linear model, the breakpoint of the model estimated the optimum carvacrol concentration which caused the highest EtBr accumulation, thus the highest efflux inhibition. F-test proved (i) bi-linear fit represents an improvement over the linear fit and (ii) the existence of an optimum treatment. It became clear that the optimum EPI concentration of carvacrol depends on the growth phase of the culture. The relationship between physiological state and efflux activity and its inhibition was described in tertiary modelling. The bacterial efflux mechanism was found to be weaker in the fast- and slow-growing phases than in the non-growing phase. In the first two stages, lower carvacrol concentration – around 50 mg/L – was enough to reach the greatest efflux inhibition. However, in the non-growing, stationary phase culture the efflux was promoted. Thus, the optimum inhibitor value of carvacrol was around 75-95 mg/L.

The efflux mechanism of bacteria was described in the different physiological states without carvacrol treatment as well. Bacteria from fast- and slow-growing phases could not completely extrude EtBr out of the cells, so the efflux mechanism seemed to be weaker compared to the non-growing phase. In the non-growing phase, bacteria have a better-developed resistance mechanism. It might be due to the adaptation to secondary metabolites, formed during the growth of bacteria. The evaluation method was validated using NMP, a synthetic inhibitor. However, carvacrol, the natural agent, showed a higher efflux inhibition effect than NMP or PaβN in the applied concentrations. In summary, the concentration of carvacrol, as an efflux pump inhibitor was optimized according to the history of the organisms.

4.5 Antibiotic susceptibility tests using carvacrol in combination with five antibiotics

Based on the previous results, carvacrol showed a promising potential to mitigate bacterial resistance between its 50 and 95 mg/L concentrations. Therefore, the ability of carvacrol to

increase the bacterial sensitivity to antibiotics were tested at 75, 90 and 105 mg/L concentrations. Five antibiotics in sub-MIC levels (0.5, 0.25, 0.125, 0.0625 MIC) were used. The antibiotics differed in their modes of action and in their molecular sizes. Multivariate statistical methods were used to evaluate the effect of multiple factors simultaneously. The multivariate overall test of MANOVA (using Wilks' lambda (λ) test values) showed that the effect of antibiotics at all factors' levels (0.5, 0.25, 0.125, 0.0625 MIC) was significant ($\lambda<0.195$, $p<0.001$), which means that there are differences between the groups of antibiotics more precisely between their influence on the bacterial growth, even in the combined treatments. *E. coli* strains' influence was also found significant ($\lambda<0.558$, $p<0.42$) as well as the two factors interaction ($\lambda<0.183$, $p=0.016$). Factors interaction was only significant at 0.5 and 0.25 sub-MIC antibiotics level.

The types of antibiotics had significant effect on the bacterial growth at all factor levels and combinations ($F(4;10)>3.75$, $p<0.014$). However, evaluating the between-subject effects, some treatment combinations did not induce significantly different strains' responses. The least difference between the antibiotics could be made in their lowest concentration, at 0.0625 MIC. The strains' effect was less significant, only one case was found where the strains responded differently: when 0.125 MIC antibiotics was combined with 75 mg/L carvacrol ($F(2;38)=3.319$, $p=0.05$).

In the combined treatments, there were no dominant distinctions between the three strains' responses. However, examining the responses of bacteria, there were differences in terms of the five antibiotics combined with carvacrol. When the antibiotics were combined with 90 or 105 mg/L of carvacrol, the treatments significantly reduced the bacterial growth compared to the singular antibiotic treatment. The 75 mg/L carvacrol seemed to be less effective, applied in the combination with sub-MICs of antibiotics.

Conclusively, carvacrol increased the susceptibility of *E. coli* strains to all the tested antibiotics. The best treatment combinations were reached with cefotaxime, gentamicin and erythromycin. These antibiotics targeted the bacterial protein and cell wall synthesis. Thereby it is not surprising that carvacrol worked best with these drugs since the molecules have the same target. The least effective combined treatment was the mixture of carvacrol and ciprofloxacin. Note that even here the inhibition rate of ciprofloxacin still increased with an average of 50% in the combination with 105 mg/L carvacrol.

5 CONCLUSIONS AND RECOMMENDATIONS

Antibiotics saved millions of lives; however bacterial resistance has been accomplished to nearly all antibiotics. Due to the rapid bacterial resistance, there is a need for strategies to manage this emergent crisis. Small molecule inhibitors, that inhibit the drug efflux from bacterial cells, represent a new solution for combating antibiotic resistance. Beside the antimicrobial activity of natural volatile compounds, some of these have efflux inhibitor activity as well, such as carvacrol. Nevertheless, it was shown that carvacrol can act in synergy with some antibiotics, i.e., erythromycin, penicillin, ampicillin (Magi et al. 2015; Langeveld et al. 2014; Ventola 2015).

Results obtained in the current study suggest the same, carvacrol can act as a promising efflux inhibitor. It can be stated that carvacrol increases the bacterial sensitivity to various antibiotics. However, because of its antimicrobial activity, carvacrol can also induce a defensive cell response, which could evoke additional bacterial resistance in the long term. Therefore, to characterize its optimum concentration, applied in the combined treatment with antibiotics, is vital.

Predictive microbiology models were suitable to evaluate the EtBr accumulation. Data obtained via this indirect method served to describe the efflux modulation activity of carvacrol. Bi-linear model was used to define the concentrations dependent effect of carvacrol. The breakpoint of the fitted models referred to the optimum value of carvacrol which could be used as an EPI. Applying our evaluation method, 5≤ treatment concentrations should be used to determine the exact concentration of an inhibitor. In the stationary phase cultures, it was found that the efflux mechanism is enhanced, assuming that the cells adapt to various stress factors, and or due to the accumulation of secondary metabolites in the broth. Both scenarios could promote the bacterial efflux.

As the result of membrane integrity measurements, it was shown that carvacrol with its increasing concentration, has a monotone negative impact on the membrane of bacterial cells. The tested organism, *E. coli* possess flagella with a peritrichous arrangement and long fimbriae, which are correlated with pathogenicity. Moreover, the bacteria have specific fimbriae – pili on their surface for adhesion to target cells and colonization of various specific host epithelia. Since the carvacrol affects the bacterial cell membrane, additionally the morphological changes of *E. coli* – caused by carvacrol in the region of interest – could be assessed using transmission

electron microscopy (TEM) or scanning electron microscope (SEM) images to define effect of carvacrol on the filamentous structures.

In the combined treatments, carvacrol worked best with cefotaxime, gentamicin and with erythromycin, fold changes in MICs were also observed. These antibiotics targeted the bacterial membrane and protein synthesis and their molecular weights were lower than 800 g/mol. Therefore, to prove this statement that the differences in the inhibition activity are due to the mechanism and molecular size of antibiotics, more types of antibiotics could be involved in future studies. Similarly, synergistic action was shown by Obaidat et al. (2011) between carvacrol and tetracycline against Gram-negative bacteria. The action was explained by the enhanced permeability of bacterial cell wall, thus tetracycline influx through the bacterial cell wall was more successful. Similarly, synergy of carvacrol and erythromycin was shown by Magi et al. (2015), against clinical, erythromycin-resistant Group A Streptococci. These results indicate the evidence of a synergism, thus the need for further investigations to combine carvacrol with antibiotics. Understanding the underlying mechanism of synergy may lead to the development of safe drug combinations. There are few studies concerning the metabolism, bioavailability of carvacrol and targeted tissues. However, more studies are required including animal models and human studies (Sharifi-Rad et al. 2018).

On the other hand, Kiskó and Roller (2005) investigated that carvacrol and another essential oil component, p-cymene have synergistic interaction. Based on this fact, probably it would worth testing the resistance-weakening effect of these two natural components in the same way as it was shown with carvacrol.

In my doctoral thesis, the auspicious potential of carvacrol, as an efflux inhibitor is well characterized. Carvacrol was able to increase the bacterial sensitivity to antibiotics, thus it seems to be an attractive alternative to the conventional synthetic EPI agents. A better understanding and quantification of bacterial susceptibility using combined treatments will be a key in future research to combat the fast-spreading antibiotic resistance.

6 NEW SCIENTIFIC RESULTS

In my dissertation, the new scientific results can be summarized in four major points, with six sub-points.

1. Measuring the growth kinetics of bacteria at single cell level via turbidity-based readings

a. For studying the growth kinetics of single cells, a specific dilution method was developed. To estimate the single cell kinetics, the optimal initial concentration is proven to be *ca.* 1.6 cells/well by Buss da Silva et al. 2019. To reach the recommended 1-3 cells *per* well of a microtiter plate, the initial concentration of the inoculum was set to OD_{600}= 0.1, from which four consecutive decimal dilutions were made, then 11-13 binary dilutions, noting that the dilution rate was strain-dependent. The newly developed dilution method was highly accurate and repeatable. Moreover, it was established that the estimate of the average number of initial cells using the Poisson parameter (denoted by ρ^*) does not differ significantly from the results of the traditional plate count method. Applying this method, there is no need for traditional technique to estimate the concentrations which can be cost and time effective.

b. Turbidity-based readings recorded the T_{det} values (at OD=0.15) of single cell cultures which values are proportional to the lag time of bacteria. It was found that *E. coli* can be used as model microorganism even at single cell level due to the unexpected great difference between the lag time distributions of individual cells. All the tested microorganisms were the member of Enterobacteriaceae family. However, below *ca.* 20 cells *per* well concentration, the variance of T_{det} values of *Salmonella enterica* strains was generally higher than that of *E. coli* strains. Moreover, *Shigella sonnei* had two times greater lag time distribution interval.

2. Fluorescent assays to describe the changes in bacterial efflux activity and membrane integrity changes using carvacrol, a natural antimicrobial

a. Efflux modulation activity of carvacrol was investigated via EtBr accumulation measurements. As a novelty, predictive modelling methods were used which led to an appropriate numerical evaluation. EtBr accumulation and membrane integrity changes of microorganisms were analyzed at each concentration of carvacrol, described by the temporal variation of F_S (fluorescent signal) values, in the unit of RFU. Signals were measured and fitted by primary models: (A) saturation model of EtBr accumulation or (B) a dissipation model of membrane integrity:

$$Fs(t) = Fs_0 + (Fs_{max} - Fs_0) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad \text{(A)}$$

$$Fs(t) = Fs_0 - (Fs_0 - Fs_{min}) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad \text{(B)}$$

Here, $Fs(t)$ is the Fs value at the time t elapsed from an initial time t_0; Fs_0 is its value at the initial time; Fs_{max} is its theoretical (asymptotic) maximum; Fs_{min} is its theoretical minimum; and r is the exponential rate at which the $Fs(t)$ function converges to Fs_{max} or Fs_{min}, depending on the type of fluorescent assay, described by the primary models, finally, ε is the random measurement error.

In the secondary models, it was shown for the first time that a bi-linear function can describe the primary model parameters of EtBr accumulation as a function of carvacrol treatment. The ratio between the highest (Fs_{max}) and lowest (Fs_0) fitted Fs values was chosen to quantify the efficacy of carvacrol as EPI in the secondary models. This is the factor by which the Fs values increased from Fs_0 to $Fs_1 = Fs(1)$. The variation of the natural logarithm of this parameter, as a function of the carvacrol concentration, *x*, was modelled for each strain and growth phase, by an asymmetric, convex, bi-linear (triangle-):

$$y_{EP}(x) = ln\frac{Fs_1}{Fs_0} = B_s(x) = y_{opt} \cdot \begin{cases} \frac{(x - x_{min})}{(x_{opt} - x_{min})} & (x_{min} \le x \le x_{opt}) \\ \frac{(x_{max} - x)}{(x_{max} - x_{opt})} & (x_{opt} \le x \le x_{max}) \end{cases} .$$

The $x_{min}, x_{opt}, x_{max}$ parameters are the minimum, optimum, and maximum concentrations defining the bi-linear function. The scaling constant y_{opt} is the value of this ratio at the optimum carvacrol concentration. The s in the index of the B_s notation indicates that we expect the bi-linear function to depend on the physiological state of the culture. The convex manner established the optimum concentration of carvacrol as an EPI. This modelling approach was validated using NMP, a synthetic efflux inhibitor, as a positive control. Meanwhile with the same modelling approach, the monotone effect of carvacrol on the cell membrane integrity was described: the higher the carvacrol concentration the more significant is the membrane damage.

b. It was established for the first time that the optimum inhibitor concentration of an efflux inhibitor (carvacrol in this doctoral thesis) depends on the physiological state of bacteria. Three physiological states were tested: fast-, slow-, and non-growing phases. The optimum efflux pump inhibitor (EPI) concentration of carvacrol was found to be between 44-56 mg/L for fast-, slow-growing phases and 76-94 mg/L against *E. coli* strains in the non-growing phase. Similarly, it was established that *E. coli* is more resistant in the non-growing phase without treatment as well, as the bacteria have a well-developed efflux mechanism.

3. Measuring the lag time prolongation in *E. coli* as a function of carvacrol treatment

a. It was presented for the first time, via optical density measurements, how carvacrol prolongates the lag time of *E. coli* strains. It was also presented that the lag time duration as a function of carvacrol treatment, can be described by a power function. T_{det} values (measured at detection level of OD=0.15). In the secondary models, the recorded T_{det} values were represented as a function of carvacrol treatments. To describe the relation between the secondary model's variables, power model was used:

$$T_{det} = \beta_0 * (121 - x)^{\beta_1} + \varepsilon \quad ,$$

where x represents the applied carvacrol treatment, ε is the error term of the model, β_0 and β_1 are the model parameters. Evaluating the results, 20% increase in lag time duration was found as a response to 64-70 mg/L carvacrol treatment, except in the case of a clinical isolate (*E. coli* ATCC 11229), where a higher, 92 mg/L carvacrol induced the same response.

4. Antimicrobial susceptibility tests to describe the growth inhibition efficacy of carvacrol in a combination with different antibiotics

a. In the last part, it was shown that subinhibitory concentrations of carvacrol increase the susceptibility of *E. coli* to ciprofloxacin, cefotaxime, erythromycin, gentamicin and vancomycin. Antibiotics in the combination of 90 or 105 mg/L of carvacrol treatments significantly reduced the bacterial growth compared to the singular antibiotic treatment. Moreover, it was shown that carvacrol worked best with cefotaxime, gentamicin and erythromycin, and even halving in the value of MICs was also observed. Thereby, the natural agent promoted the most the antibacterial activity of those antibiotics of which (i) molecular weight was lower than 800 g/mol and (ii) targeted the bacterial protein and cell wall synthesis.

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