Hungarian University of Agriculture and Life Sciences

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

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Abbreviations

ANOVA – ANalysis of VAriance

ATP – Adenosine Triphosphate

DA – Discriminant Analysis

ECDC – European Centre for Disease Prevention and Control

EFSA – European Food Safety Authority

EP – Efflux Pump

EPI – Efflux Pump Inhibitor

EtBr – Ethidium Bromide

EtOH – Absolute Ethanol

EUCAST – European Committee on Antimicrobial Susceptibility Testing

Fs – Fluorescent signal

GRAS – Generally Recognized As Safe

LPS – LipoPolySaccharides

MANOVA – Multivariate ANalysis of VAriance

MDR – MultiDrug-Resistant

MIC – Minimum Inhibitory Concentration

OD – Optical Density

PBS – Phosphate Buffered Saline

RFU – Relative Fluorescence Unit

Sub-MIC – Sublethal Minimum Inhibitory Concentration

WHO – World Health Organization

QS – Quorum sensing

1 INTRODUCTION

The spread of multidrug-resistant (MDR) microorganisms have reached an alarming proportion 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. With the discovery of first antibiotics thereafter, appeared the antibiotic resistance as well. Nowadays, antibiotic resistance has become a global threat for both human and animal health, the misuse and overuse of antibiotics has resulted in the rapid developing of bacterial resistance. On the other hand, the future ability of antibiotics to control bacterial growth is uncertain and the death cases per year caused by MDR pathogens might reach the 10 million for 2050. Fighting against antibiotic resistance, the possible solutions could be the judicious use of antibiotics and the enhancement of bacterial susceptibility to antibiotics e.g., via efflux inhibitors (Sharma et al. 2019; Sun et al. 2019).

Bacterial resistance mechanism can be characterized as self-protection. When resistance is formed against an antibiotic, it is unable to reach the microbial target in adequate concentration. 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 are responsible for removing toxic substances from the bacterial cells, including antibiotics. Efflux pumps are membrane-embedded proteins, located in the outer membrane, within the periplasm and in the cytoplasmic membrane of Gram-negative bacteria (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 methods can be susceptibility measurements, direct and indirect fluorescence assays and the recently reported flow cytometric or mass spectrometric methods. EtBr is a well-studied efflux substrate; the dye is a DNA intercalating agent dye, so it gives differential fluorescence when located intra- or extracellular. Active efflux of drugs by transmembrane proteins promotes bacterial survival and may even contribute to the development of permanent resistance to them. Moreover, Gram-negative bacteria conform lower sensitivity to antimicrobial

agents, due to the complex outer membrane which restricts the diffusion of various compounds. The aforementioned facts are the abundant evidences that there is a need for new strategies to combat the growing threat of antibiotic resistance (Walsh and Wencewicz 2016).

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 and/or modulate the membrane permeability is an active and rapidly growing research area. EPIs could work as potential therapeutic agents (Pagès and Amaral 2009). Such inhibitors, in a combined use, could reduce the required dosage of chemically diverse agents but most importantly the required dosage of antibiotics. 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. Hunting for alternative antimicrobials derived from plants seems to be a solution (Mahamoud et al. 2007; Marchetti et al. 2012). The development of new EPI molecules is a leading strategy to mitigate the mechanism of bacterial efflux pumps.

Essential oils and their active components have been used for diverse purposes since ancient times, such as antibacterial, antifungal, antiviral and 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.

Escherichia coli is a Gram-negative bacterium, widely studied and used as a model microorganism. While most of the *E. coli* strains do not cause diseases, some serotypes can cause serious food-borne infections. *E. coli* is also commonly found in minimally processed ready-to-eat foods (Abass et al. 2020; Al Safadi et al. 2012).

Carvacrol is a phenolic monoterpene produced by numerous aromatic plants. Carvacrol, at high concentrations, is able to interfere with the cell membrane, by releasing the lipopolysaccharides (LPS) and making pores on the lipid bilayer of cytoplasmic membranes, bringing loss of integrity and increasing of its permeability. 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, the optimization of using carvacrol as an efflux inhibitor and combining it 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 members of Enterobacteriaceae family and prove why *E. coli* strains are appropriate 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 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 LITERATURE

3.1 Predictive microbiology

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. The basic strategy of food safety is to keep the product under well-controlled conditions. The concept of predictive microbiology can be used for the whole production chain: from primary processing, to distribution, storage and operations via monitoring temperature, water activity, pH values *etc.* of the product. Having such data makes it feasible to predict the growth kinetics of certain microorganisms in foods. While predictive microbiology methods can be used to economical benefits, its positive influence on health sustainability is even more important (McMeekin et al. 1993).

3.1.1 Evolution of predictive microbiology

The first predictive microbiology methods were introduced in the 30's but practical applications began in the 80's, only when an increase in the incidence of food poisoning outbreaks was observed and the use of desktop computation began started. Thus, the new research area was the result of collaborations between food microbiologists, mathematicians and statisticians (McMeekin et al. 2002). Thus, the evolution of predictive microbiology into a more exact science is well demonstrated (Baranyi and Roberts 2004). As of now, predictive microbiology has become a useful and practical concept, used by academia, food industry and regulatory authorities.

Predictive microbiology models can be used for many purposes. The application of predictive models to microbiology problems has been mainly focusing on the growth, inactivation and survival of microorganisms (Dominguez and Schaffner 2007). However, recently predictive modelling ideas has been suitable to describe and optimize antibacterial therapy as well (Jánosity et al. 2021). In this study, the objective was to estimate the optimum efflux inhibitor dose of carvacrol. The tested carvacrol concentrations were chosen below its 0.5 Minimum Inhibitory Concentration (MIC) value because (i) data in literature suggested this region (Kovač et al. 2015) and (ii) sub-MIC carvacrol concentrations were tested to find its concentration which has no influence on bacterial growth. In subsequence, the subinhibitory concentration range (between 0.1 and 0.5 MIC) of carvacrol was called the region of interest.

3.1.2 Mathematical models

Mathematical models are a set of assumptions, some of which are described by variables, parameters and error terms via (possibly differential) equations. In the absence of a standard nomenclature, which can change widely and may even be confusing, one is shown in Fig. 1, for a simple one-dimensional linear case. The independent variable (X) explains the magnitude of the measured signal quantifying the dependent variable (Y). This can be quantities like the growth rate, or probability of survival, etc. of bacteria. The dependence of Y on X is described by an equation with the parameters β_0 and β_1, whose values can be estimated by statistical/numerical methods applied to the raw data (experimental observations). A usual objective is to minimize the expected error (Ɛ) term, which is a stochastic variable between prediction and real outcome. To find the best fit to a given data, the expected (root-mean-square average) deviation between model and the observations should be minimized by choosing the model parameters appropriately. This minimum expected deviation is the most frequently used indicator on the goodness of fit for the model.

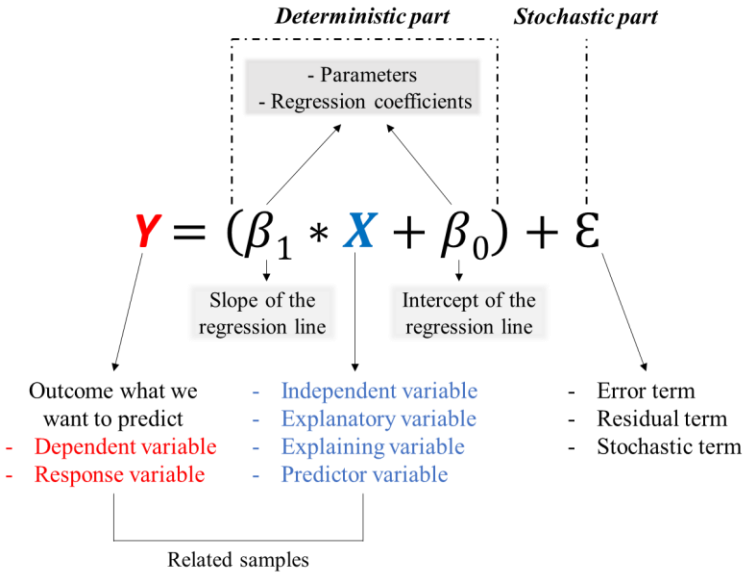

Fig. 1. A nomenclature for regressing a linear model

When developing and fitting even more complex models in a recursive way, starting from the zero-order model (the average value of the raw data) a vital point is this: Is it worth introducing more parameters (more complex model) for the sake of a better fit? Will the fit be significantly better, making it worth the possible sacrifice of parsimony? Moreover, the stochastic error also gives information about the model performance.

If we want to define what a mathematical model is, the phrase “art of omitting the unnecessary” was used by Baranyi (2005). The basis of modelling is about simplifying the experimental data and reducing its complexity. This is the first step in data management which can already be done in preliminary experiments (McMeekin et al. 1993).

3.1.3 On what bases do we classify mathematical models?

Classification of mathematical models can be based on questions like: (i) does the model take the causality into account (mechanistic or empirical) (ii) does it quantify the uncertainty of the variables (stochastic or deterministic), (iii) is the model dynamic (in which case the change is modelled not the state)? If yes, it is commonly described by differential equations.

3.1.3.1 Causality assessment: empirical versus mechanistic models

In the case of an empirical model, we simply describe a set of data in a concise way by highlighting the important and neglecting the unnecessary part of the dataset and possibly give information about the strength of the relationship between the variables. We only look at the relationship between the variables without knowing the direction of causality, so it is a descriptive model. On the other hand, a mechanistic model is based on an underlying process. It also considers preliminary knowledge, phenomenon and direction of causality behind the data. Significant differences can be observed between these two types of models. Generally, mechanistic models are preferred to empirical ones (McMeekin et al. 1993), as the former one is more suitable to predict microbial responses.

3.1.3.2 Quantification of uncertainty: deterministic versus stochastic models

Deterministic models deal with the outcome of a process while neglecting the individual scatter of datapoints. On the other hand, in stochastic models, the variables have probability distributions and estimating the errors of the model parameters is a major objective. The major difference between these is that the stochastic model cannot be derived from the deterministic one, but the latter can be derived from the former, by means of the expected values of the variables (Kutalik et al. 2005).

An example can be the following case: The lag time duration of individual cells is random, the lag time describes the adjustment period of one cell, therefore it is a stochastic process. However, the population lag is a deterministic concept, based on the dynamics of the whole bacterial population (Baranyi and Pin 2001).

The field of food microbiology has been typically featured by experiments performed at population level when the collective behavior of many cells is measured. However, at lower cell concentrations, the randomness of the individual cells' responses has a much greater influence on the collective actions of the cells than it does at higher concentrations. It is therefore of foremost importance to use stochastic modelling approaches to study bacterial growth in food matrices where pathogens usually occur at low concentrations (Koutsoumanis 2008).

3.1.4 Steps of modelling, primary, secondary and tertiary model

Mathematical models allow the prediction of bacterial actions in foods over time as a function of various factors, such as treatments or specific storage conditions. The term 'primary model' has been introduced for temporal responses, to measure the behavior of bacteria over time (e.g., the change in bacterial population density over time). This is a dynamic response to the cellular environment which is assumed to be constant during the observation interval (Ross and McMeekin 2003).

In the primary models, the response variable is usually the concentration (N) of a specific bacterium. It is usually expressed by its log$N$ value (Zwietering et al. 1991). To describe the effects of environmental (including stress-) factors on a primary model parameter (like on the maximum specific growth rate - μ_{max}), the term secondary model is commonly used (McKellar and Lu 2003). The environmental factors are typically continuous physical and biochemical variables, such as temperature, pH, water activity and factors like added food preservatives as well as the concentration of additives or antimicrobial agents. It is commonly assumed that the "μ_{max} vs. environmental factors" multi-dimensional surface is convex, inferring that there exists a unique, relatively small region of environmental factors where the maximum specific growth rate reaches its highest possible value. The further away are the environmental factors from their optimal combination, the lower is the corresponding μ_{max} value. It was shown that carvacrol, a natural phenolic compound, as the studied environmental factor in the secondary model (Jánosity et al. 2021) also has an optimum value when used as an efflux inhibitor molecule.

The parameters of the secondary model can vary not only across species, but strains as well, as shown by Baranyi et al. (2017). The authors pointed out that the term "tertiary model" is appropriate and logical for describing the dependence of secondary model

parameters on category variables such as strains / species or substrate / food types, or the physiological state of the culture.

3.1.5 Importance of replicates to estimate model parameters accurately

A model helps to evaluate the process and can predict the possible outcomes of it. However, if we perform measurements and repeat them, we could easily get different points for the measurements with the very same settings. That means that the best fitted line will slightly be different from the previous one. We can produce different predictions, since fitting the repeated experiments do not generate the same parameters (Jánosity et al. 2022). Comparing such independent or biological replicates is vital to build a robust and accurate model.

3.2 Major pathogenic bacteria

Most bacterial species are harmless and are often beneficial, but others can cause diseases. A pathogen also can be called as infectious agent since it infects its host. In 2019, there were more than 1.5 million global deaths associated with drug-resistant bacteria of which the leading pathogens were *E. coli* and *Staphylococcus aureus* both causing more than 600 000 deaths (Murray et al. 2022).

3.2.1 Foodborne pathogens

Foodborne diseases are caused by foodborne pathogens, which can infect healthy people and take advantage of immunocompromised conditions. Children under the age of five, pregnant women and elderly belong to the high-risk group. Billions of people are under risk and many death cases are the evident consequence of consuming unsafe food containing harmful bacteria, parasites or even chemical substances (Friel and Ford 2015). To avoid this, the aim of food safety specialists is to provide safe food and drink thus and reducing the number of hospitalizations.

Bacterial pathogens, that already gained resistance against a specific drug, are frequently resistant to multiple antimicrobial agents. These microorganisms are called MDR pathogens. Only 1% of the known bacteria are responsible for causing bacterial infections. However, the consequences can be serious. Some bacterial species such as *Bacillus cereus* and *E. coli* can produce chemicals and toxins as secondary metabolites. Therefore, the investigation of bacterial mechanisms conferring pathogenesis is a central theme of many studies (Kumar et al. 2013).

The latest multi-country outbreak in the EU was caused by a monophasic variant of *Salmonella enterica* Typhimurium in the spring of 2022. Ferrero recalled numerous Kinder products worldwide. In total, 156 infected cases were recorded through 10 EU countries and in the UK according to the European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA). Children under the age of 10 were the most affected ones, some of them have even been hospitalized. Although, the outbreak strain was a MDR bacteria, resistant to six types of antibiotics, there have not been any fatalities. The possible contamination point could be detected in the processing step of buttermilk (European Centre for Disease Prevention and Control, European Food Safety Authority 2022).

3.2.2 Antibiotic-resistant pathogens as the greatest threat

Recently, World Health Organization (WHO) published the antibiotic-resistant 'priority pathogens' that currently burden the greatest threat to human health and require adequate treatment (World Health Organization2017). In terms of antibiotic-resistant bacteria three priority groups were formed (Fig. 2).

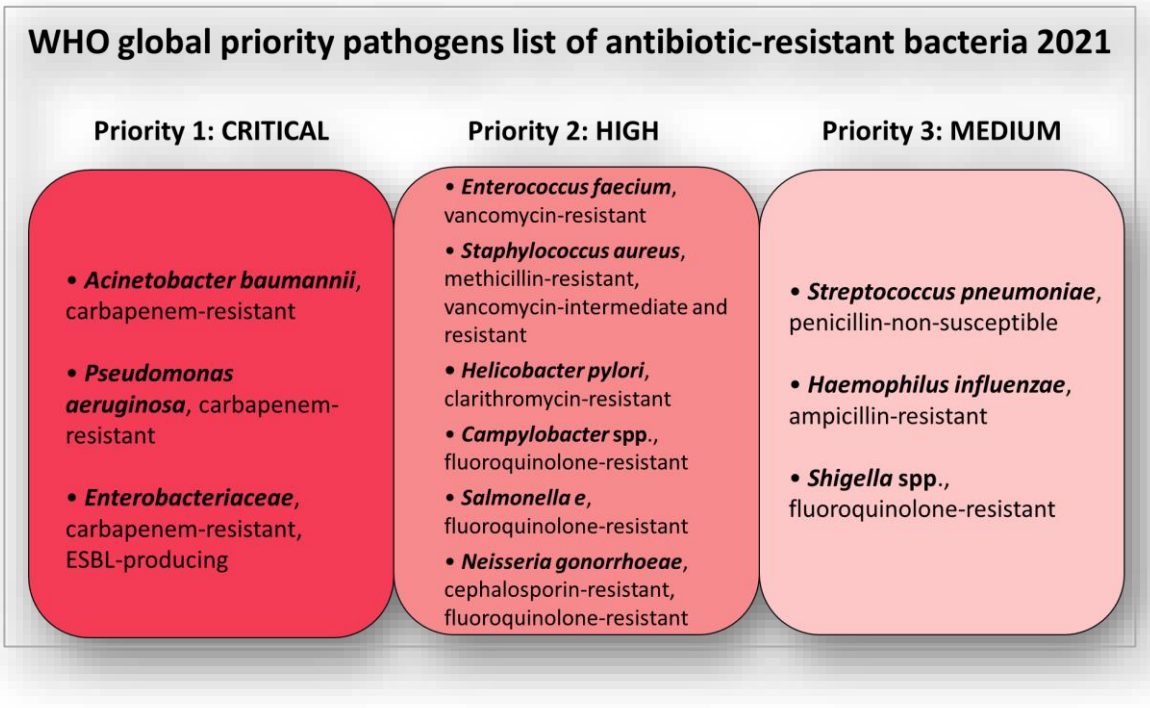

Fig. 2. Priority groups of antibiotic-resistant bacteria (World Health Organization 2017)

Not surprisingly, the members of priority group 1 contain only Gram-negative bacteria and some members belong to Enterobacteriaceae family like the extended-spectrum beta-lactamase (ESBL) producing bacteria.

3.2.3 Actions to fight against antimicrobial resistance

In 2015, World Health Organization issued a Global Action Plan and the United States, the National Action Plan as well to combat the antimicrobial resistance (World Health Organization 2015; Centers for Disease Control and Prevention 2015). The objectives were to improve the public awareness and knowledge of antimicrobial resistance via effective communication and education, to reduce the possible infections by preventive actions, to optimize the antimicrobial use and to increase the investment in pharmaceutical interventions.

National Action Plan highlights the needs and built-in activities to enhance domestic and international capacity to prevent the outbreaks of antibiotic-resistant infections. Activities included specific plans for the improvements as well as the expectations or outcomes which were anticipated for 2020. It is assumed that the aggressive actions would help to handle and slow down the serious drug-resistant threats which knock on our doors.

In 2017, two years later after issuing the National Action Plan, the European Commission also released an action plan against the resistant pathogens, with similar objectives (European Commission 2017). As concluded, in preventing the spread of resistant infections the most essential part is the judicious use of antibiotics which would also help to extend the antibiotics' useful lifetime.

3.2.4 *Escherichia coli* as a model microorganism

E. coli, which nowadays stands out as a model microorganism, was first isolated by an Austrian pediatrician, Theodor Escherich in 1885 (Escherich 1885). *E. coli* can be found naturally in the intestinal tracts of humans. Most forms of the bacteria are harmless, moreover commensals of the intestinal tract serving useful functions. However, some serotypes of *E. coli* are meaner than the others: food infections caused by the pathogenic *E. coli* (like Shiga toxin producing enterohemorrhagic *E. coli* (EHEC) may lead to watery or bloody diarrhea, fever, nausea and vomiting. In 2011, a novel *E. coli* outbreak was recorded in Germany due to *E. coli* O104:H4 and resulted 3816 cases with the highest number (845) of hemolytic uremic syndrome (HUS) and 54 deaths (Al Safadi et al. 2012). The strains of *E. coli* are non-spore forming bacilli (rod shape), Gram-negative bacteria. All the major bacterial efflux transporter superfamilies can be found in *E. coli* (Guelfo et al. 2010; Nishino and Yamaguchi,

2001; Teelucksingh et al. 2020; Zhou et al. 1998). The bacteria are approximately 0.5 μm in diameter and 1.0–3.0 μm in length. Within the periplasm, there is a single layer of peptidoglycan, and it has a typical subunit structure where the N-acetylmuramic acid is linked to a peptide consisting of L-alanine, D-glutamic acid, meso-diaminopimelic acid and finally D-alanine. Cells of *E. coli* are commonly fimbriated and motile in liquid media by means of peritrichous flagella.

The organism is a facultative anaerobe and ferments simple sugars such as glucose to form lactic, acetic and formic acids. The optimal condition for growth of *E. coli* is the temperature of 37-42 °C, with a wide range (7.5 to 48 °C) (Strocchi et al 2006). Most strains of *E. coli* are capable of growing within a pH range of approximately 5.5–8.0 with the optimal growth occurring at the neutral value, but some diarrheagenic *E. coli* strains can tolerate even pH 2.0. Thus, it is well demonstrated that the bacteria can adapt to a wide range of environmental conditions (Welch 2006).

3.3 Membrane permeability

Several studies suggested that both intrinsic and acquired resistance mechanisms are enhanced for Gram-negative bacteria, owing to their unique cell wall structure. Gram-negative organisms possess an outer membrane surrounding their cell wall, which can restrict diffusion of hydrophobic compounds through its lipopolysaccharide covering (Burt 2004; Dik et al. 2018).

In most cases, the cell wall of Gram-positive bacteria consists of peptidoglycan, to which other molecules, such as teichoic acid and proteins are linked. This cell wall structure allows hydrophobic molecules to easily penetrate the cells which can be the reason that Gram-positive bacteria are more susceptible to antibacterial treatments than Gram-negatives. The complex cell wall structure of Gram-negative bacteria contains a peptidoglycan layer (2–3 nm thick) which gives 20% of dry weight of cells. The peptidoglycan layer is much thinner than in case of Gram-positive bacteria (it is marked in red in Fig. 3).

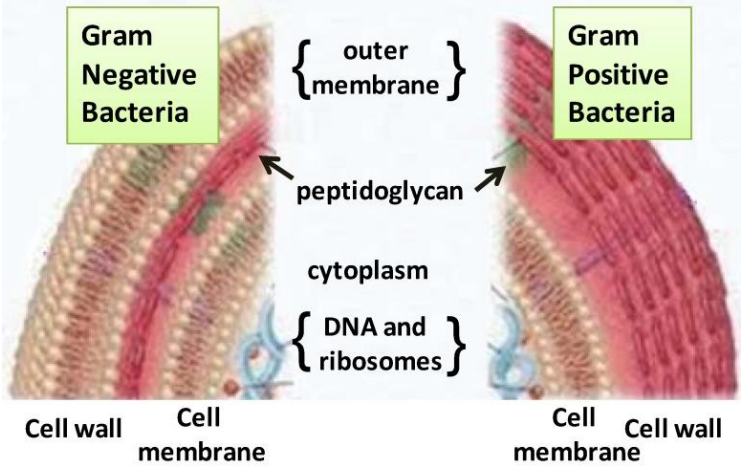

Fig. 3. Schematic diagram about the cell wall differences in Gram-negative and Gram-positive bacteria (Nazzaro et al. 2013)

Outside of the thin peptidoglycan layer lies the outer membrane, the presence of it in Gram-negative bacteria is the cause of the main difference of membrane permeability. The outer membrane is almost impermeable to hydrophobic compounds. It consists of a double phospholipid layer which is linked to the inner membrane by LPS. LPS consist of lipid A (core polysaccharide) and O-side chain. Through porin proteins and transmembrane channels only the small hydrophilic molecules can cross. Thus, Gram-negative bacteria can be more resistant to hydrophobic large molecular sized antibiotics (Nazzaro et al. 2013).

3.4 Physiological state of bacteria

In a closed system or environment (i.e., in a batch culture) with a given level of nutrients, the bacterial population grows until a certain point that the nutrients are depleted, or toxic by-products start to inhibit their own growth. This phenomenon marks the end of the exponential growth. By plotting the logarithm of cell density as a function of time, a typical bacterial growth curve will be obtained (Fig. 4). The sigmoid growth curve will be followed by a monotonously decreasing phase. Based on this curve, the physiological state of the bacterial culture can be divided into four categories: lag, exponential, stationary and death phase. Between the major phases, the transition periods can be characterized by “curvatures”, expressing how sudden the change is.

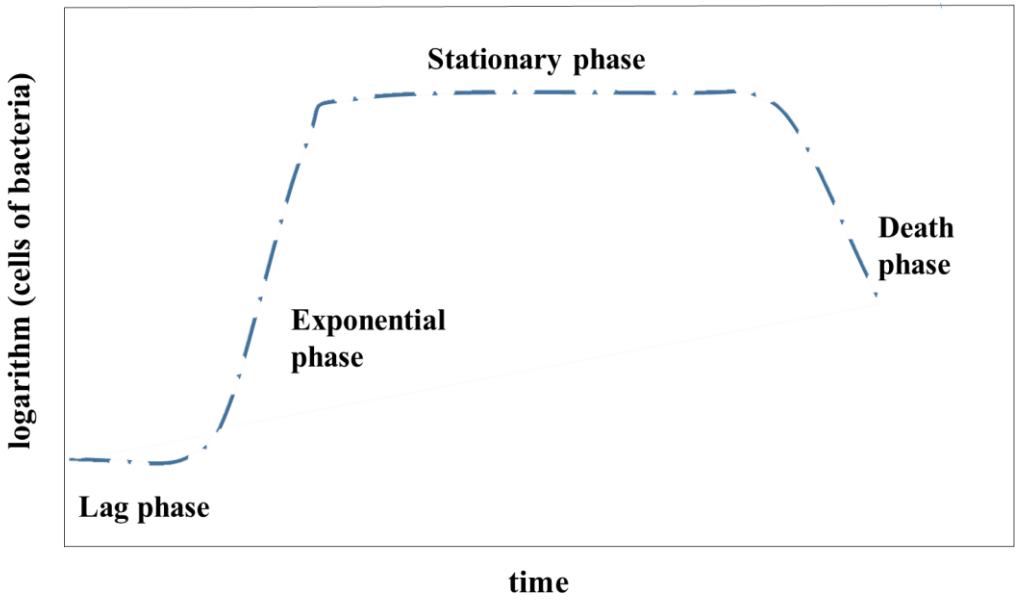

Fig. 4. Plotting the logarithm of cell numbers as a function of time

3.4.1 Lag phase of bacterial growth

Each phase is unique, but in terms of food safety issues, the lag and exponential phases are the most studied ones. Important primary model parameters of a sigmoid curve can be the maximum specific growth rate (μ_{max}), the lag parameter (λ) and the maximum population density (x_{max}). These are the main parameters of the Baranyi-model (Baranyi and Roberts, 1994) too, except that a q_0 extra initial value replaces the lag, which is an initial value, just like the initial cell concentration. It quantifies the initial physiological state of the cells (Eq. 1):

$$\frac{dx}{dt} = \frac{q_0 e^{n\mu_{max}t}}{1+q_0 e^{n\mu_{max}t}} \mu_{max} x \left(1 - \left(\frac{x}{x_{max}}\right)^m\right) \quad (1)$$

$x(0)=x_0$.

The n and m parameters characterize the curvatures (degree of non-linearity) before and after the exponential growth phase. Their default value is 1, respectively, which corresponds to the assumption that the lag (acclimation) period is controlled by a bottle-neck gene-product, like an enzyme needed for the new environment, and it affects the growth rate according to the Michaelis-Menten kinetics. The convergence to the stationary phase happens according to the logistic growth model. In what follows, the curvature parameters are assumed to be n=m=1 (default values), as suggested by Baranyi (1997).

The Baranyi-model is a dynamic one, applicable to a time-dependent environment, too. The lag parameter is a consequence of the initial physiological state via its various reparameterization and the respective interpretations:

- $\alpha_0=q_0/(1+q_0)$ is a proportion. If this proportion of the initial cells grow at the μ_{max} specific rate immediately after inoculation, without lag, while the other cells don't grow, then the same exponential phase would be produced as the original population has.
- Therefore, α can be called the "adjustment function" and the value of - ln (α) is equal to h_0. h_0 is frequently considered to be a quantification of "work to be done" during the lag time. h_0 is a dimensionless parameter from which the lag time λ (h) can be calculated according to Eq. 2.

$$\lambda = \frac{h_0}{\mu_{max}} \tag{2}$$

The initial physiological state can therefore be quantified by $\alpha_0 = \mu(0) / \mu_{max}$, where $\mu(t) = dx(t)/dt / x(t)$, is demonstrated in Fig. 5.

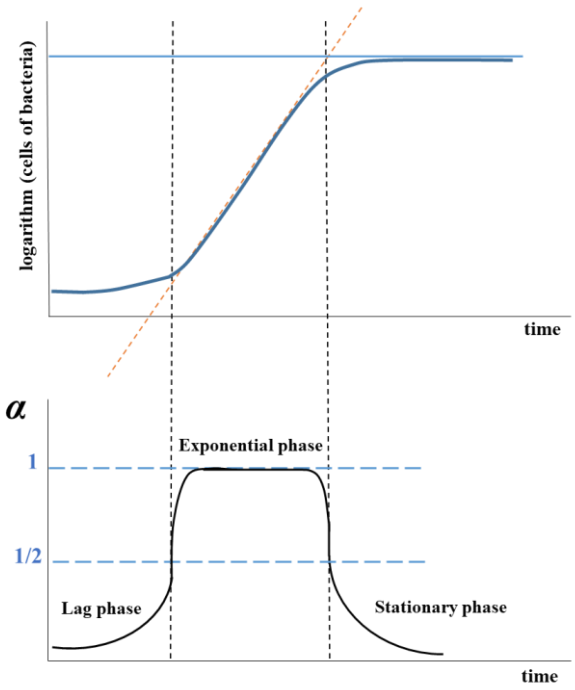

Fig. 5. The value of α, is a ratio of the actual to the maximum specific growth rate. It can be used to quantify the actual physiological state of the culture

The maximum specific growth rate is intrinsic to the bacterial population, characterizing the environment, which is assumed to be constant during growth. From that, the population doubling time (t_{dbl}) can be calculated according to Eq. 3.

$$t_{dbl} = \frac{\ln(2)}{\mu_{max}} \tag{3}$$

There is high correlation between the lag phase and maximum specific growth rate of the culture, where the former is inversely proportional to the latter. Both parameters depend on the actual environment although the lag depends on the cells' previous history as well. As an example, we set the very same inoculum concentrations for two bacterial cultures, but if we use different broth media for the inoculation and experiments, we are going to record longer lag phase for the same culture, since the cells must adjust to the new broth (Baranyi and Roberts 1994). A frequently used assumption is that the "work to be done" during the lag phase, $h_0=\mu*\lambda$, is constant for the "happy growth" region of temperature, i.e., where no significant stress is involved, just the process is slower or faster. This assumption is in fact a simple secondary model.

3.4.2 Tasks for bacterial cells in the different growth phases

Lag phase is the initial phase of bacterial growth. During the lag phase, the cells adapt to their new environment before entering exponential phase. Both phases represent vulnerability; the first one because of the adaptation period, the second one because of the focus on cell division. In a batch culture, the exponential growth typically ends due to the depletion of nutrient sources and the accumulation of metabolic products in the environment which triggers stress responses. The study of Navarro Llorens et al. (2010), stated that stress cross-protection is an often-occurring phenomenon. For example, nutrient-depleted stationary-phase cells fight against osmotic or temperature stress better than exponentially growing cells do. Furthermore, the entrance to stationary phase is a well-regulated process, where the involved general sigma factor *RpoS* (σs) regulates not only the entrance into stationary phase, but it is the master regulator of general stress response in *E. coli* too (Hengge 2008). In previous studies it was also found that bacterial susceptibility to antibiotics is growth rate dependent, so bacterial tolerance to drugs can be increased in non-growing and slow-growing phases (Greulich et al. 2015; Smirnova and Oktyabrsky 2018).

Therefore, similar observations were expected that bacterial efflux resistance is affected by the physiological state of the cells. This is the reason why the final step of the study is called “tertiary modelling”, where the physiological state of the culture will be an explanatory variable and optimum carvacrol concentration, while the parameter of secondary model, will be the response variable. To explore the adaptation efficacy of bacteria could lead to new approaches to prevent the clinical failure of antibiotics by slowing down the spread of antibiotic resistance.

3.4.3 Turbidity-based readings to record the detection time of bacteria

Optical density is a widely accepted method to measure bacterial kinetics. George et al. (2015) showed that the lag time of a bacterial population can be estimated indirectly via optical density measurements, since the distribution of detection times is considered as a proportional reflection of the distribution of lag times. The detection time of turbidity experiments is the time needed to reach a fixed turbidity level. To estimate the lag time of bacteria by this method, it is required that the detection level should be low enough that the culture reaches it in the exponential phase (e.g., set it to OD_{600}=0.15). This method can be used to describe the single cells lag time scatter as well, by plotting the detection times versus the logarithm of the inoculum size.

3.5 Stress responses of bacteria

Bacterial resistance is simply a self-defense mechanism. We can distinguish between intrinsic and acquired resistance of microorganisms. Intrinsic resistance is the primary resistance of microorganisms while acquired resistance is the general insensitivity of bacteria to drugs. However, bacteria have developed acquired resistance as a survival tool to protect themselves against the adverse effects of the environment (Blanco et al. 2016). Acquired resistance is typically a bacterial stress response, in which microorganisms acquire a capability to resist a particular antimicrobial agent, to which it was susceptible previously. Lag time prolongation could be mentioned as a typical “adjustment” response of bacteria (Walsh and Wencewicz 2016).

3.5.1 Lag time prolongation

It is widely accepted that bacteria under certain environmental stress factors (applied in sub-MIC levels) can prolong their lag phase duration. Stress factors can prolong the bacterial lag, as it can be seen in Fig. 6 (George et al. 2015). Sodium-chloride was

added to the broth and the authors found that the increasing concentration of NaCl positively correlates with the duration of lag phase.

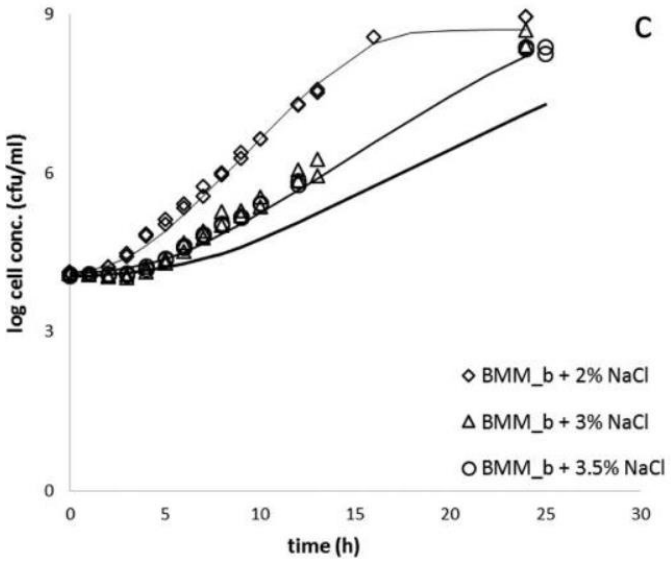

Fig. 6. In case of *Salmonella enterica* Typhimurium SL1344, the lag phase duration of the growing subpopulation increased with NaCl concentration (George et al. 2015)

In the lag phase of microbial growth, microorganisms adjust to various stress factors such as to a new culture medium or to an antimicrobial treatment before starting exponential growth. Thus, this phase is characterized by cellular activity with rare divisions. As a response to environmental factors, like antimicrobials, bacteria can prolong their lag period. This phase is considered to be an adjustment period. During this time, bacteria transform their transcriptome and proteome to produce cellular components required to divide (Hamill et al. 2020; Li et al. 2016).

The delay in growth, induced by a sub-MIC treatment, can bring survival advantages to bacteria to fight antibiotics. It was found that changes in the lag-time can be considered as “tolerance” and it influences the response to antibiotic stresses and may facilitate the subsequent evolution of antibiotic resistance. Quantifying the lag time may help us understand the ability of microbes surviving under wide range of conditions and also to define the highest concentration of an antimicrobial which does not cause significant change in the response (Fridman et al. 2014).

3.5.2 Effect of temperature on the lag time duration

In the study of Kandhai et al. (2006), the authors illustrated the temperature’s effect on the lag phase (λ) and specific growth rate (μ_{max}) of *Enterobacter sakazakii*. Temperature had great influence on both parameters. With increasing temperature, the

square root of μ_{max} strongly increased until a certain point then it sharply decreased. The Ratkowsky model (Fig. 7 - dashed line) was used to describe the relationship between the square roots of specific growth rates and temperature.

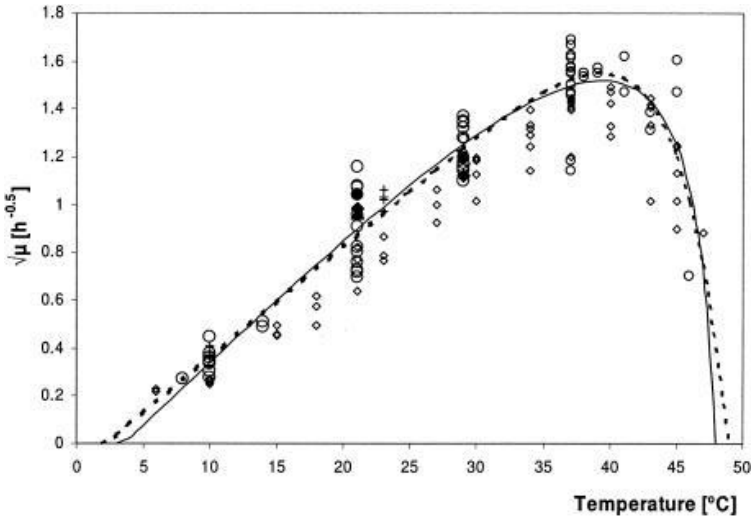

Fig. 7. Relationship between the square root of μ_{max} and temperature (Kandhai et al. 2006)

The lag time decreased with the temperature increasing. To demonstrate this effect, the logarithm of lag time values was plotted against temperature (Fig. 8) and fitted with a hyperbolic model (dashed line) and the reciprocal Ratkowsky model (solid line).

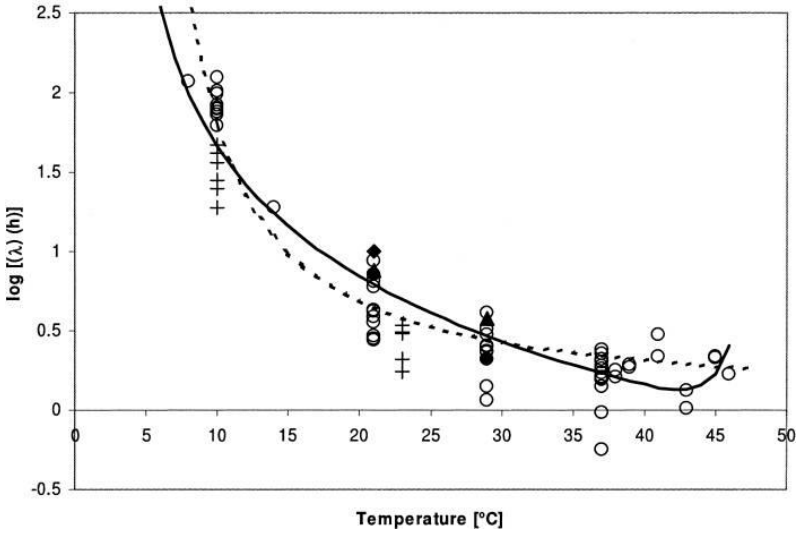

Fig. 8. Relationship between the log lag time values and temperature (Kandhai et al. 2006)

3.5.3 Quorum sensing, biofilm formation

Quorum sensing (QS) is a communication mechanism of bacteria also called as cell-cell communications. It regulates gene expression in response to environmental changes (Spengler et al. 2017). During the process, bacteria synthesize, recognize and

respond to extracellular signaling molecules. One of the responses can be the biofilm formation. Bacterial biofilm formation is also a form of self-protection. It can be the response for various environmental stress factors like nutrition depleted media or antimicrobials. This form of growth induces several resistance mechanisms at the same time such as reduced growth rate meanwhile increased expression of efflux transporter and other virulence factors like toxin production (Alav et al. 2018; Al Safadi et al. 2012).

3.5.4 Cell signaling systems and their influence on the expression of efflux pumps

MDR in Gram-negative bacteria may be caused by the overexpression of resistance–nodulation–cell division (RND) type genes. Most of the efflux genes are weakly expressed under normal conditions (except the AcrAB-TolC drug efflux system) (Sulavik et al. 2001).

The induced over-expression of drug efflux pumps and their regulation can be accomplished through the signal transduction system of bacteria, also known as the cell signaling system or the "crosstalk system" (Tiwari et al. 2017). It is basically the response of bacteria to the external factors and about the transmission of molecular signals. Signals are originated outside of the cells and transmitted to their interior via surface receptors, that is why it is called as surface response. The transmission process into the cells must be effective to ensure their appropriate adjustment. The two-component signal transduction system (TCS) consist of a sensor (histidine kinase) which receives the signal and its cognate response regulator, the receptor protein. Signals interact with receptor proteins connecting to the outside and inside of the cell. The regulator mostly acts as a transcriptional factor to control gene expression. *E. coli* has a total of 30 sensors and 34 response regulators. Nishino et al. (2009) found that 17 out of the total 34 response regulators are involved in the drug resistance of *E. coli*. Results suggested that these three crosstalk systems, namely EvgSA, BaeSR and CpxAR can induce the efflux pump expression of *E. coli*. The EvgSA cell signaling system increases multidrug resistance by the expression of the MdtEF and EmrKY pump; BaeSR system induces the expression of the two RND pumps (MdtABC and AcrD), and the CpxAR system responds to membrane disruption and controls the expression of MdtABC and AcrD.

A bacterium is able to become resistant by spontaneous mutation, adaptation, transduction, conjugation and transformation. Via transduction, resistance genes can

be transferred by bacteriophages. Through the conjugation, two bacteria connect via their cell membranes and then the microbes can exchange their genetic materials. Horizontal gene transfer (transformation) means that bacteria take up pieces of DNA directly from their environment (including antibiotic resistance genes coded on plasmids). Transferring bacterial MDR plasmids contributes to the development of new “superbugs” (Sun et al. 2019).

3.6 Classifications of antibiotics: mode of action, source of origin and molecular size

Antibiotics are drugs with antibacterial activity, usually with low molecular weight (<1000 Da). We can classify them based on their mode of action, molecular weight, targeted bacteria and source of their origin. The synthetic antibiotics have three classes: (1) sulfonamides (sulfa drugs) which were introduced in 1930s; (2) fluoroquinolones introduced in 1960s and (3) oxazolidinones introduced in 1990s. Sulfa drugs, which block a key enzyme in the process of folic syntheses, are still used 90 years later. The members of the second class, like ciprofloxacin, are broad-spectrum antibiotics blocking the DNA biosynthesis. The members of third class inhibit the bacterial protein synthesis. Natural antibiotics are produced by microbes, bacteria and fungi with the primary aim to inhibit other microbes in their environment (Walsh and Wencewicz 2016).

According to their mode of action, antibiotics can prevent the bacterial growth in seven ways: inhibit the synthesis of (i) cell wall, (ii) cell membrane, (iii) proteins, (iv) DNA gyrase, (v) RNA, (vi) RNA polymerase and (vii) Folic Acid (Fig. 9).

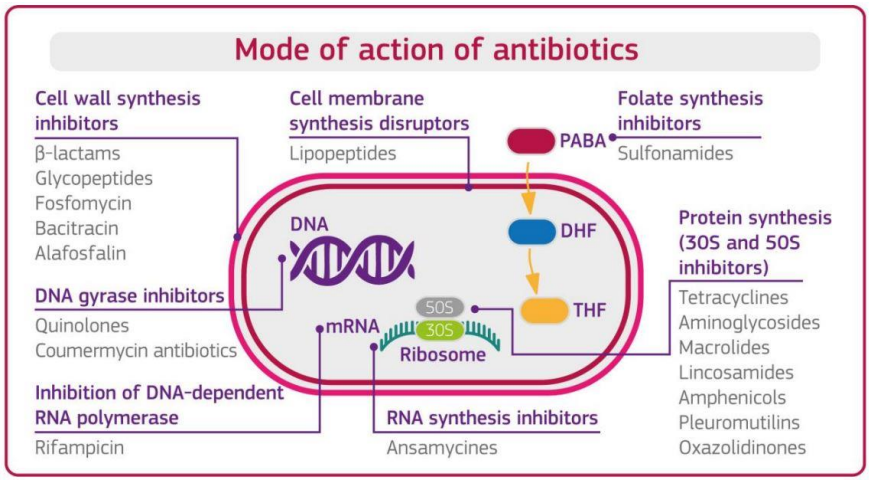

Fig. 9. Mode of action of antibiotics (Sanseverino et al. 2018)

Fig. 10 represents the timeline of antibiotic discovery. A 40-year gap can be seen in the discovery between 1960 and 2000. Either it is explained by the confidence that there were enough antibiotics or with the difficulty of finding new molecules. According to estimations, a typical R&D path for a new antibiotic molecule in large pharmaceutical companies can take up to 13 years including the drug discovery, clinical trials and the new drug application. The cost of it can even reach $1 Billion (Walsh and Wencewicz 2016).

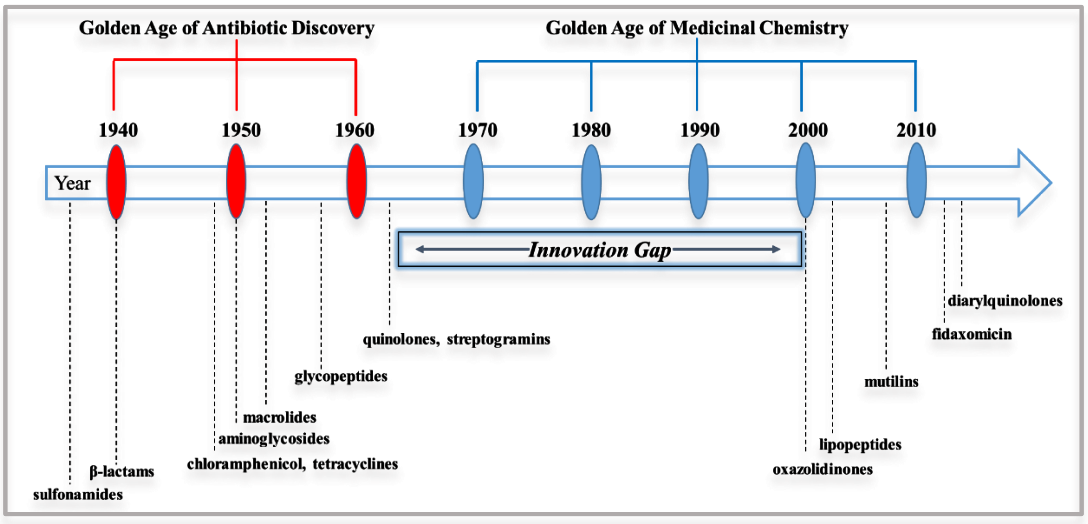

Fig. 10. Timeline of antibiotics' discovery (Walsh and Wencewicz 2016)

3.6.1 Antibiotic resistance and its consequences

In 2019, almost 5 million death cases could be connected to antimicrobial resistance. 1 million out of 5 million deaths are associated with: *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* ("ESKAP" pathogens) and with *Streptococcus pneumoniae* (Murray et al. 2022). Currently, the death cases per year caused by MDR pathogens can reach the 10 million in 2050 (Sun et al. 2019).

The Centers for Disease Control and Prevention (CDC) published its report on antibiotic resistance related threats highlighting that more than 2.8 million antibiotic-resistant infections occur in the U.S. yearly, resulting more than 35000 deaths. Moreover, the report characterized four groups: bacteria and fungi involving urgent threats, serious threats, concerning threats and the last group is the watch list pathogens (Centers for Disease Control and Prevention 2019).

3.6.2 Bacterial pathways to develop antibiotic resistance

The bacterial resistance (Fig. 11) can occur via (i) alteration of drugs' target sites, (ii) decreasing membrane permeability, (iii) active efflux of drugs, (iv) external factors and (v) inactivating or modifying the antibiotics (Kumar et al. 2013).

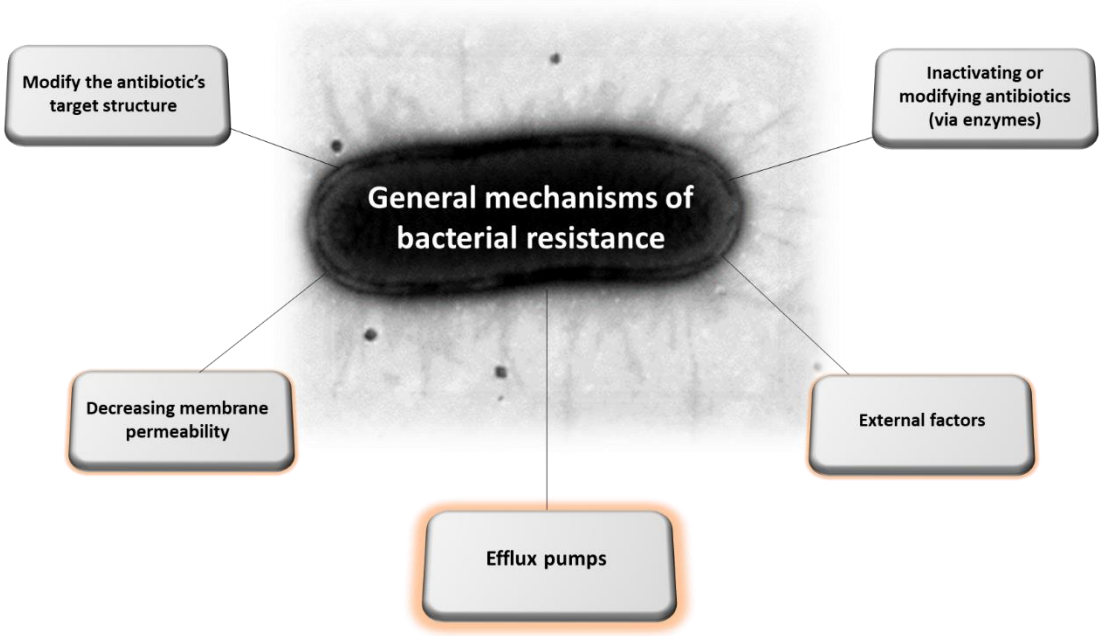

Fig. 11. Possible antibiotic resistance mechanisms utilized by bacteria

3.6.3 Is antibiotic resistance inevitable?

The antibiotic resistance has appeared almost simultaneously with the discovery of the first antibiotic. Thus, the resistance is an ancient and natural phenomenon. The occurrence of antibiotic-resistant bacteria can be connected with the overuse of antibiotics, inappropriate prescribing and extensive agricultural use, these actions contributed to the emergence and spread of MDR bacteria, which are resistant to various clinically relevant drugs (Alav et al. 2018; Walsh and Wencewicz 2016).

Resistant bacteria can spread via various routes. There are no boundaries that bacteria cannot across. The major routes, where MDR bacteria can spread, are: (i) person to person, since we all have our unique bacterial footprint; (ii) from animals to humans and vice versa; (iii) via the food industry; (iv) via drinking and irrigation water supplies (v) within health care facilities and (vi) through the international travelers and trading

systems (Pérez-Rodríguez and Mercanoglu Taban 2019). However, as of now resistant bacteria are truly everywhere, to prove this statement four examples follow.

1. Antibiotic resistance genes were detected in marine microbial communities close to the wastewater stream and in sediments in Florida (Griffin et al. 2020).
2. In Spain between 2008 and 2009 fecal samples were collected from wild raptors and an unexpectedly high proportion of multidrug-resistant *Salmonella* and *Campylobacter* isolates were obtained. However, the wild birds were not treated with antimicrobial agents before sampling (Molina-Lopez et al. 2011).
3. 416 mice were collected in New York City. Quinolone resistance gene were present 7% of the samples while 21% of the samples contained fluoroquinolones and β-lactam drugs resistance genes. Most importantly, 1% of mice were also positive for the methicillin resistance gene (Williams et al. 2018).
4. As the last example Veldman et al. (2013) collected 414 cloacal swabs from 55 different bird species, especially among aquatic-associated species (waterfowl, gulls and waders) in The Netherlands. Cefotaxime-resistant *E. coli* isolates were identified in 65 birds (15.7%) from 21 different species.

3.6.4 Influence of the molecular size of antibiotics on their uptake efficiency

The outer membrane of Gram-negative bacteria functions as an impermeable barrier. Antibiotics above 600 Da molecular weight cannot accumulate into the cells via passive diffusion. However, many antibiotics act inside the bacterial cells (Yamamoto et al. 2022). As an example, vancomycin is a large-scaffold antibiotic with a molecular weight 1450 Da (Walsh and Wencewicz 2016). The vancomycin-resistant Gram-negative bacteria are formed due to the inability of vancomycin to penetrate the outer membrane. Therefore, the enhancement of antibiotics uptake rate of bacteria is a key in the combat of antimicrobial resistance.

Large-scaffold antibiotics are not applicable to treat Gram-negative infections in health care because they cannot cross the outer membrane efficiently, but Muheim et al. (2017) reported a small molecule that made the outer membrane of *E. coli* more permeable. The authors monitored the growth of *E. coli* in 96-well microtiter plates in the presence of a sub-lethal concentration of vancomycin in combination with the small molecule. The aim was to increase the permeability of the outer membrane (with

the help of small molecule) until a certain point when vancomycin can cross the membrane, get access to the periplasm and inhibit the cell growth.

The aim was the same in this thesis, using carvacrol to make *E. coli* more susceptible to antibiotics. Moreover, to find whether the increased uptake rate was influenced by the mode of action or the molecular weight of the antibiotic. In this thesis ciprofloxacin (Baskin et al. 2002; Hangas et al. 2018; Linde and Lehn 2003), cefotaxime (LeFrock et al. 1982; Møller et al. 2016), erythromycin (Bortolin et al. 2017; Francis et al. 2013), gentamicin (Muheim et al. 2017; Siibak et al. 2009) and vancomycin (Antonoplis et al. 2019; Muheim et al. 2017; Wang et al. 2018) were used. The molecular weight, mode of action and MIC value of compounds against *E. coli* are summarized in Table 1.

Table 1. Antibiotics and their properties used in the thesis

Antibiotics	Molecular weight (g/mol)	Mode of action	MIC value against *E. coli* (mg/L)
vancomycin	**1449.2**	~ inhibition of the cell wall biosynthesis by targeting the d-Ala-d-Ala terminus of peptidoglycan	128-512
erythromycin	**733.9**	~ inhibition of protein synthesis by binding to the 50 S ribosomal	256
gentamicin	**477.6**	~ inhibition of bacterial protein synthesis by binding to 30S ribosomes	2-4
cefotaxime	**455.5**	~ inhibition of bacterial cell wall biosynthesis	0.032
ciprofloxacin	**331.3**	~inhibition of DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase	0.004-0.007

3.7 Efflux pumps and multidrug resistance

The feasibility that bacteria can obtain resistance by extruding antibiotics by plasmid-encoded proteins was first described in 1980 (Blanco et al. 2016). Bacterial efflux pumps can be specific for one substrate or for a range of structurally dissimilar compounds (such as antibiotics of multiple classes) (Webber and Piddock 2003).

Efflux is a general mechanism through which bacteria are able to extrude the potentially toxic compounds out of the cells (Spengler et al. 2017). Efflux systems are important determinants of both intrinsic and acquired bacterial resistances to antimicrobial agents. Efflux pumps are membrane proteins with broad specificity. Such pumps prevent drug accumulation inside the cells by transferring at least a portion of the drugs out from the cells. This mechanism is responsible for low, ineffective concentration of antimicrobial compounds inside the cells. Bacteria with active efflux system can increase their resistance level by acquiring simultaneously additional resistance factors. Most of the genes that encode these MDR pumps are normal constituents of bacterial chromosomes (Lomovskaya et al. 2000, Walsh and Wencewicz 2016).

The common feature of MDR efflux activities is their ability to recognize many structurally unrelated substrates. After identifying the drugs, pumps remove them from the cells. However, there are some efflux inhibitor molecules. As an example, to demonstrate the mechanism, the xenobiotic removal could be mentioned: the inhibitors directly bind to the hydrophobic regions of MDR proteins resulting inhibited transport process (Mire et al. 2003).

Regarding bacterial efflux transporters (Fig. 12), seven major efflux pump superfamilies can be distinguished (Hassan et al. 2015; Hassan et al. 2018). One of them are the drug transporters of the major facilitator superfamily (MFS), that able to carry solutes across the membrane while the energy of translocation is driven by chemiosmotic gradient of cations. The ATP-binding cassette (ABC) superfamily includes the members that use ATP hydrolysis to transfer small molecules as well as macromolecules from the cells. The small multidrug resistance (SMR) superfamily represent resistance to numerous antibiotics and to quaternary ammonium compounds. The superfamilies of RND and the multidrug and toxic compound extrusion (MATE) use the energy of cation (H^+) gradients to transport the antibiotics out of the bacterial cells. The RND tripartite drug transporters can only be found in Gram-negative bacteria. These pumps consist of a cytoplasmic membrane pump, a periplasmic adaptor protein and an outer membrane protein channel. As an example, the three-component AcrAB-TolC pump in *E. coli* could be mentioned, where (i) TolC, the outer membrane protein, (ii) AcrA, the periplasmic membrane fusion protein and (iii) AcrB, the inner membrane transporter are distinguished (Alav et al. 2018; Walsh and Wencewicz 2016). Recently, two additional transport families have been described.

The proteobacterial antimicrobial compound efflux (PACE) family of transport proteins are likely to be powered as MATE (by H^+ gradients). PACE efflux family mainly transports biocides and PACE family proteins are encoded by widespread Gram-negative pathogens such as *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Salmonella* and *Burkholderia* species (Hassan et al. 2015; Hassan et al. 2018). The p-aminobenzoyl-glutamate transporter (AbgT) family was shown to be a possible, novel family of antimetabolite efflux pumps, conferring bacterial resistance to sulfonamides (Delmar and Yu 2016).

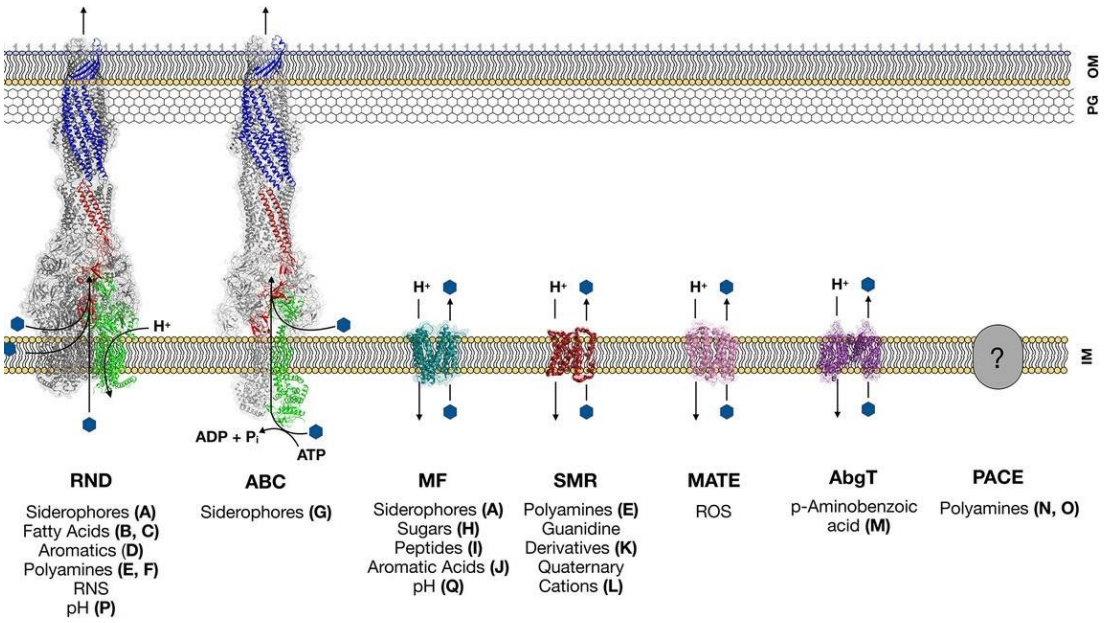

Fig. 12. The major families of bacterial drug efflux proteins and their energy-coupling mechanisms (Teelucksingh et al. 2020)

3.7.1 Measuring the efflux activity of bacteria

Various methodologies are described in the literature how to measure the activity of bacterial efflux pumps e.g., antibiotic susceptibility measurements (such as MIC determination). Comparison of MICs give us a hint about the efflux activity since isolates with greater expression of an efflux pump will be less susceptible to various antimicrobials than isolates with lower efflux pump expression. On the other hand, comparison of MICs obtained in the presence and absence of inhibitors can also be an effective approach as well.

Using fluorescent assays, the relative concentration of an efflux substrate via direct or indirect measurement methods can be defined. The former measures the efflux directly, as how much of a substrate is extruded from the bacterial cell, while the latter describes the efflux activity indirectly as how much of a substrate molecule can

accumulate inside the cells. Both types of method use dyes that give differential fluorescence intra- and extracellularly. To measure the direct efflux, commonly used substrates are EtBr (well-validated substrate of many efflux pumps), nile red, 1,2′-dinaphthylamine and doxorubicin. EtBr is a well-validated substrate of many efflux pumps, however nile red and 1,2′-dinaphthylamine cannot be used for certain type of bacteria whereas doxorubicin is precious. To determine the indirect efflux, the relative intracellular concentration of DNA intercalating dyes can be measured such as Hoechst H33342 or EtBr. Moreover, commonly used substrates also include fluoroquinolones e.g., ciprofloxacin and norfloxacin.

The direct measurement of bacterial efflux is a highly sensitive method and gives kinetic information about the export of substrate. However, measuring the intracellular accumulation of an efflux substrate is a less sensitive method compared to the direct one. It provides only indirect evidence of a change in efflux and affected by the rate of influx and the outer membrane permeability of the tested bacteria.

Recently, new methods have been described to assess efflux activity of bacteria. As such, flow cytometry can be used to measure the accumulation of a fluorescent dye in single cells. Thus, the difference in efflux activity between population and single cell level can be measured. However, quantitative mass spectrometry-based methods tend to be the best approach to measure the drug accumulation because it can be used to measure the exact concentration of the accumulated efflux substrate (Blair and Piddock 2016; Spengler et al. 2017).

3.7.2 Efflux pump inhibitors

Efflux pumps can be described as the gold shield of bacteria. Bacteria are able to protect themselves from antibiotics with the use of “EP shield”. Thus, the new direction is to use efflux pump inhibitors. Several families of efflux pump inhibitors (EPIs) have been described which are able to strongly decrease the intracellular concentration of antimicrobial drugs. Inhibitors of efflux transporters have the affinity for substrates of MDR transporters. This affinity may facilitate the drug’s entry into the cells and this large inhibitor–drug complex would effectively ‘cloak’ the drug and does not allow the efflux mechanism. The drugs in such complex can be hidden from MDR transporters. Inhibitors can bind directly to one or more binding sites on the protein, therefore blocking the transport of drugs. Inhibitors can also cause the

depletion of pump energy by the inhibition of binding ATP and modifying protein conformation (Mire et al. 2003).

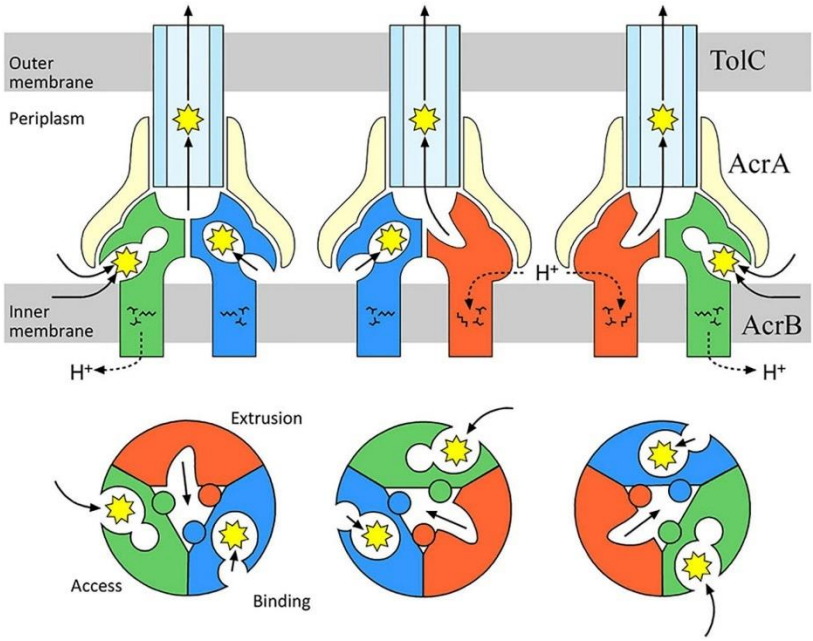

Fig. 13. Mechanism of AcrAB-TolC multidrug efflux system. Drugs are transported in sequence: from access (green) to the binding (blue), and then to the extrusion (red) monomer (Nishino et al. 2021)

The inhibition of efflux mechanism in Gram-negative bacteria can be achieved through various routes. Inhibition can be achieved by inhibiting the contact gene in the tripartite AcrAB-TolC pump, and its mechanism can be seen in Fig. 13. AcrA is responsible for connecting and coordinating the work of AcrB and TolC. To control the efflux the chemical structure of antibiotics could be changed, thereby hindering the molecule from the transporters. Blocking the outer membrane pores (TolC), which are responsible for the extrusion of antibacterial compounds, can be also an option. With the use of small molecules, it is possible to (a) control the expression of efflux genes, (b) collapse the energy-source mechanisms of membrane transporters and (c) inhibit the affinity site with the use of small molecules (Pagès and Amaral 2009).

There is a common methodology for testing the efficacy of these efflux inhibitors. It is based on the comparison of the intracellular concentration of the added antibiotic to the bacterial cell culture before and after adding the inhibitors. Using in vitro experiments fluorescent dyes can work as a proxy to illustrate the antibiotic accumulation. The molecule is considered to be a successful inhibitor if the antibiotic or dye can achieve higher intracellular concentration with the help of it. The drawback of the synthetic inhibitors is their toxic properties, which prevent their clinical

application (Askoura et al. 2011). Hence, treating bacterial infections in human or veterinary medicine using efflux pump inhibitors is not allowed. The development of new EPI molecules can be the future of combating bacterial efflux pumps and restore the activity of antibiotics. Moreover, controlling the efflux pumps could reduce the rate of spread of MDR bacteria (Mahamoud et al. 2007). Restoring the activity of antibiotics would allow to reduce the required dosage. Antibiotic treatment is one the most valuable treatments, however the side or adverse effects of it can significantly increase as the drug's concentration elevates (Mohsen et al. 2020). EP inhibitors together with antibiotics can decrease the bacterial resistance and reduce the MIC of the antibiotics by blocking the bacteria's expelling mechanisms (Bhardwaj and Mohanty 2012).

A successful EP inhibitor should be safe; non-toxic but most importantly, the molecule *per se* must not have antibacterial activity, as it should not prolongate significantly the lag phase or should not seriously affect the membrane integrity of bacteria. Why is it so important that the inhibitor molecule itself should not have antibacterial activity? Stress, above a certain level, but below the MIC, induce the bacterial resistance mechanism. In the study of Nové et al. 2020, the importance of stress level was well demonstrated. Their tested inhibitor molecule has increased the EtBr accumulation, however when the inhibitor treatment was combined with acidic pH, *E. coli* had to overcome the stress which resulted the upregulation of many genes involved in efflux mechanism.

Amaral et al. (2011) showed that the bacterial efflux activity was increased in acidic environment (pH 5) compared to above neutral pH (pH 8). This means that bacteria that overexpress one or more efflux pumps will be less susceptible to antimicrobials in acidic environment than at high pH. As a straightforward consequence, efflux inhibitors activity (such as CCCP efflux inhibition activity) is proved to be less effective at low pH. Besides, efflux activity can also be increased by adding substances such as 0.4% glucose and ions which provide metabolic energy to the cells. However, these substances can support increasing the efflux of cells at higher pH. The authors observed lower EtBr accumulation in the presence of sugar (0.4% glucose) at pH 8, than in the absence of sugar at the same pH level. As a consequence, it can also be stated that at low pH (pH 5) the bacterial efflux mechanism is less dependent on energy-providing compounds (glucose, ethanol or pyruvate). At acidic pH, the proton motive force provides sufficient energy to operate the pump.

3.7.3 Chemical EPI agents

Probably the most well-known laboratory EPIs are carbonyl cyanide-m-chlorophenylhydrazone (CCCP), Phenylalanine-arginine β-naphthylamide (PaβN) and 1-(1Naphthylmethyl)piperazine (NMP). All of them are broad spectrum inhibitors, showing a potential activity against Gram-negative bacteria and could reduce both the activity of AcrAB efflux system in Enterobacteriaceae family, and the erythromycin transport from *Campylobacter jejuni (*Kumar et al. 2013). PaβN is considered as a competitive natured inhibitor. It means that while the pump extrudes PaβN out of the cells, the antibiotic remains in the cells. In this way, the antibiotic compound can reach the concentration required for its activity on the target (Pagès and Amaral 2009). NMP was tested in several bacterial species such as in *Acinetobacter baumannii*, in different species of the Enterobacteriaceae family and in *E. coli*. NMP is considered to be the most potent compound against *E. coli*. However, NMP like all the listed compounds has serious chronic health effects, thus potentially hazardous to humans (Mahamoud et al. 2007, Marchetti et al. 2012).

3.7.4 Natural agents as efflux inhibitors

Numerous natural extracts have been shown to modulate bacterial drug resistance by inhibiting efflux pumps. However, identifying natural inhibitors and the exploration of EPIs from natural resources is still not a well-described area. The chemical diversity of the plants provides an attractive option to search for novel EPIs. According to previous studies, plants are capable of producing compounds which mitigate the MDR. These natural antimicrobials may serve as possible sources of plant-derived efflux inhibitors (Miladi et al. 2016). Many plant-derived active constituents have been reported so far with efflux pump inhibitory activity, including farnesol and geraniol, thymol and carvacrol, (-)-α-pinene, piperine, theobromine, p-coumaric acid and its derivatives, and resveratrol. These compounds showed activity against efflux pump systems in both Gram-negative and Gram-positive bacteria (Seukep et al. 2020).

In general, natural antimicrobials are more active against Gram-positive bacteria than against Gram-negative ones. Several natural agents can block the NorA MDR pump of *Staphylococcus aureus* such as: (i) 5'-methoxyhydnocarpin (5'-MHC), a natural EPI isolated from *Berberis* species, (ii) capsaicin from *Capsicum annuum* L. (*Solanaceae*), (iii) olympicin A isolated from *Hypericum olympicum* L. cf. uniflorum, (iv) reserpine isolated from *Rauwolfia vomitoria*, (v) carnosic acid from *Rosmarinus officinalis*, and

also (vi) thymol and (vii) carvacrol found in essential oils of thyme and oregano (Dos Santos Barbosa et al. 2021; Spengler et al. 2017).

The efflux inhibitor activity of (-)-α-pinene was well demonstrated in the study of Kovač et al. (2015). The component in sub-MIC concentrations (0.0625 and 0.125 MIC values) increased the susceptibility of *C. jejuni* to ciprofloxacin, erythromycin and triclosan as well. These concentrations, expressed as the proportion of the MIC values, gave us a hint about the region of interest, where carvacrol should have its optimum inhibitor concertation.

3.8 Natural antimicrobial compounds - essential oils and their components

Plant extracts and their secondary metabolites are common sources of antimicrobial agents. These substances are volatile components (essential oils), including coumarins and psoralens, acetylenes, flavonoid and non-flavonoid polyphenols and terpenes. Monoterpenes, like carvacrol (Table 2), (−)-menthol, γ-terpinene, (+)-α-pinene, (−)-β-pinene and p-cymene are the most important constituents of essential oils (Marchese et al. 2017).

Essential oils are aromatic oily liquids from flowers, buds, seeds, leaves, twigs, bark, various herbs, wood, fruits and roots. Steam distillation is the most commonly used method to extract the essential oils and their constituents. By an estimation, 3000 oils are known, of which about 300 are commercially important and some of them have antibacterial, antiparasitic and antiviral properties. The antibacterial activity of essential oils provides their suitable application (Bauer et al. 2001).

Table 2. Carvacrol is one of the active constituents of essential oils (Burt 2004)

Common name of essential oil	**Latin name of plant source**	**Major constituents and their compositions**
Oregano	*Origanum vulgare*	**carvacrol** (Trace-80%), thymol (Trace-64%), ɤ-terpinene (2-52%), p-cymene (Trace-52%)
Thyme	*Thymus vulgaris*	thymol (10-64%), **carvacrol** (2-11%) ɤ-Terpinene (2.31%), p-Cymene (10-51%)

To control the growth of pathogens in food products, sensorially unacceptable high doses would be needed from the essential oils' components. Essential oils' efficacy is reduced in food matrixes; thus, those concentrations mean two to four times higher than in vitro experiments applied (Belda-Galbis et al. 2014).

3.8.1 Antibacterial activity of essential oils

The natural antimicrobial compounds' activity can be similar to synthetic compounds, but the application of natural products is safer (Pisoschi et al. 2018). The antibacterial activity of essential oils was demonstrated against several foodborne pathogens, like *Listeria monocytogenes*, *Salmonella* Typhimurium, *E. coli* O157:H7, *Shigella dysenteria*, *B. cereus*, *S. aureus* etc. The results suggested that the Gram-negative organisms are slightly less susceptible to the treatments compared to Gram-positive bacteria. The essential oils' mode of action involves several targets. The hydrophobic attribution enables them to disrupt the bacterial cell membrane and mitochondria while causing the leakage of cell contents at high concentrations (2x MIC value) (Zhang et al. 2017, Chouhan et al. 2017). Some physical conditions are able to enhance the natural components' activity, like low pH, low temperature and low oxygen levels. Synergistic or merely additive effect of components derived form an essential oil has been observed (e.g.: using carvacrol and its precursor p-cymene) in fruit juices against pathogenic *E. coli* (Kiskó and Roller 2005). Some components are registered as flavoring; however, essential oils may cause undesirable organoleptic properties (Burt 2004). In the food industry, essential oils were tested as a preservative in eggplant salad, tzatziki, packed fresh meat, minced meat, vacuum-packed ham, baked goods, non-alcoholic beverages, mozzarella and in soft cheese (Bagamboula et al. 2004; Ultee et al. 1999).

3.8.2 Mechanism of action of essential oils and their constituents

The efficiency of natural compounds was found to depend on their chemical composition (Nazzaro et al. 2013). It has been shown that carvacrol (like thymol and eugenol) disrupts the cell membrane, via inhibiting the activity of ATPases. Aside from inhibiting those activities, carvacrol releases intracellular adenosine triphosphate (ATP) concentration and other cell components. The antimicrobial efficiency of carvacrol against foodborne pathogens is influenced by the position of hydroxyl (-OH) group on the phenolic ring and by the delocalized electrons. These proton exchangers are confining the hydrogen bonding capacity and decreasing the pH gradient across the cytoplasmic membrane (Burt, 2004; Pisoschi et al. 2018). The targeted

mechanisms and locations of essential oils in the bacterial cell are indicated in Fig. 14 based on the study of Burt (2004).

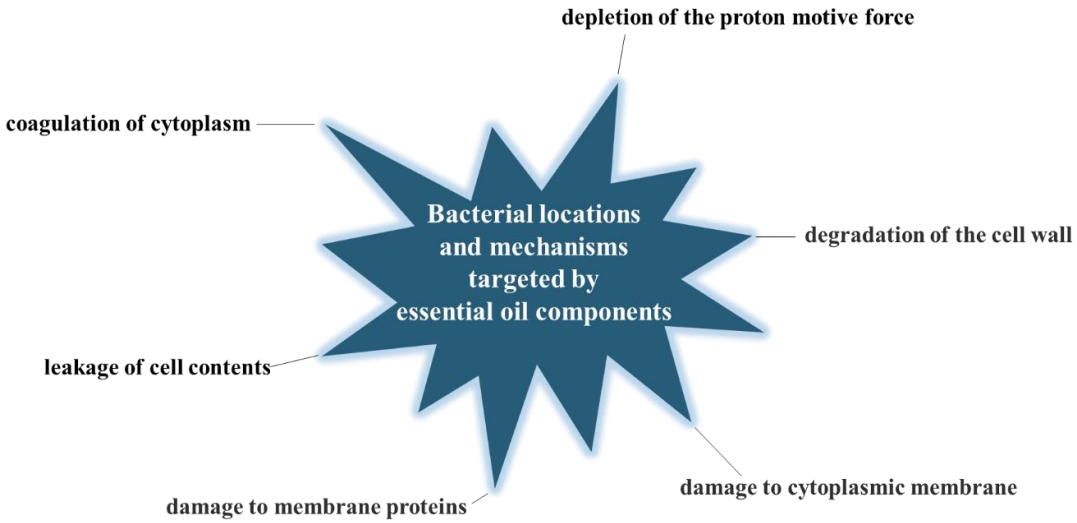

Fig. 14. The mode of antibacterial action of essential oils and their constituents

It can be concluded that essential oil components interact with the lipid bilayer of cytoplasmic membranes and make them more permeable which corresponds to the increased uptake and accumulation of drugs inside the bacterial cells.

3.8.3 Carvacrol, a natural antimicrobial

Carvacrol, a hydrophobic phenolic compound (Fig. 15), is one of the major compounds in essential oils of oregano, thyme, wild bergamot and pepperwort (Sharifi-Rad et al. 2018). Carvacrol is an oxygenated (hydroxylated) terpene, synthesized from p-cymene and α-terpinene. Carvacrol has been shown antimicrobial, antifungal, antiseptic and antioxidant activity. Furthermore, carvacrol effectively inhibits the formation of biofilms by up to 74–88% for Gram-negative microorganism and up to 86–100% for Gram-positive microorganism on technical surfaces including materials as stainless steel, polypropylene, polyethylene and PVC. Additionally, it is able to inactivate mature biofilms as well (Walczak et al. 2021). The antibacterial mode of action of carvacrol mainly occurs by membrane damage. Carvacrol is able to disintegrate the outer membrane of Gram-negative bacteria. Membrane damage results an increscent membrane permeability to protons and potassium ions leading to the depletion of intracellular ATP pool and affecting the proton-motive force (Kiskó and Roller 2005). Phenolic monoterpenoids, like carvacrol, at higher concentrations can denature proteins and as a result of it, releases lipopolysaccharides (LPS) and increases the

permeability of the cytoplasmic membrane to ATP subsequently leading to cell death (Burt 2004; Khan et al. 2017). Carvacrol is characterized as a growth inhibitor for different pathogens and also shown inhibitory activity against vegetative bacterial cells and toxin production in case of *B. cereus* (Ultee et al. 2002).

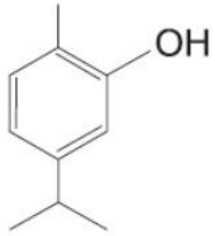

Fig. 15. Chemical structure of carvacrol, the major component of oregano essential oil (Burt 2004).

In the study of Ultee et al. (1999), the effect of carvacrol was evaluated on the intracellular ATP pool, the membrane potential, the pH gradient across the cytoplasmic membrane and the potassium gradient on a common food-borne pathogen, *B. cereus*.

3.8.4 Carvacrol as an efflux inhibitor

The antimicrobial activity of carvacrol is well demonstrated. Essential oils from edible plants are GRAS components and the use of carvacrol in food has been approved by European Parliament and Council (Khan et al. 2017). In sub-inhibitory concentrations where the molecule itself has no antibacterial activity, carvacrol can be used as an efflux inhibitor. In the "region of interest", carvacrol lacks toxicity, which is the major disadvantage of chemical inhibitors that limits their use to laboratories only.

The efflux inhibitor activity of carvacrol is demonstrated clearly by indirect method showing that the molecule can increase the accumulation of fluorescent dyes. However, its applied inhibitor concentration differs significantly in the literature. In the study of Miladi et al. (2016), carvacrol was used in 190 mg/L and even higher concentrations where 50% efflux inhibition was reported in *Salmonella* Enteritidis. Since the inhibitor itself should not have antibacterial activity, in our study the optimum inhibitor concentration of carvacrol in subinhibitory concentrations between 0.1 and 0.5 MIC values was investigated (Jánosity et al. 2021).

Synergistic action was shown by Obaidat et al. (2011) between carvacrol and tetracycline against Gram-negative bacteria, which action was explained by the

enhanced permeability of bacterial cell wall, thus tetracycline influx through the bacterial cell wall was more successful.

Carvacrol is active against resistant strains as well. MIC value of carvacrol was recorded as 256 mg/L against a wild type of *S. aureus* strain and shown the same MIC against a NorA-overexpressing strain (Dos Santos Barbosa et al. 2021). Authors found that carvacrol in subinhibitory concentration (0.125 MIC) had the same effect as a standard CCCP synthetic inhibitor. Carvacrol significantly reduced the MIC of norfloxacin against both strains in the combined treatment (MIC decrease from 64 μg/mL to 32 μg/mL and from 128 μg/mL to 64 μg/mL were observed). Therefore, the potential of carvacrol to increase the effectiveness of antibiotics is well characterized.

The combination of multiple drugs, like combining antibiotics, was already effective in clinical treatments and used many times to treat infections. Therefore, future treatments performed through combinations of antibiotics and natural antimicrobials may serve as the best strategy to inhibit resistant pathogens. Small molecules, like carvacrol, in a combination with antibiotics can target multiple mechanisms and effectively inhibit the bacterial resistance mechanism, including efflux pumps. Thereby increasing the efficacy of antibiotics.

4 MATERIAL AND METHODS

4.1 Microorganisms and chemicals

4.1.2 Cultivation of cultures

Bacterial cultures were derived from frozen stocks, from -80 °C storage. Preparation of frozen stocks can be seen in Fig. 16.

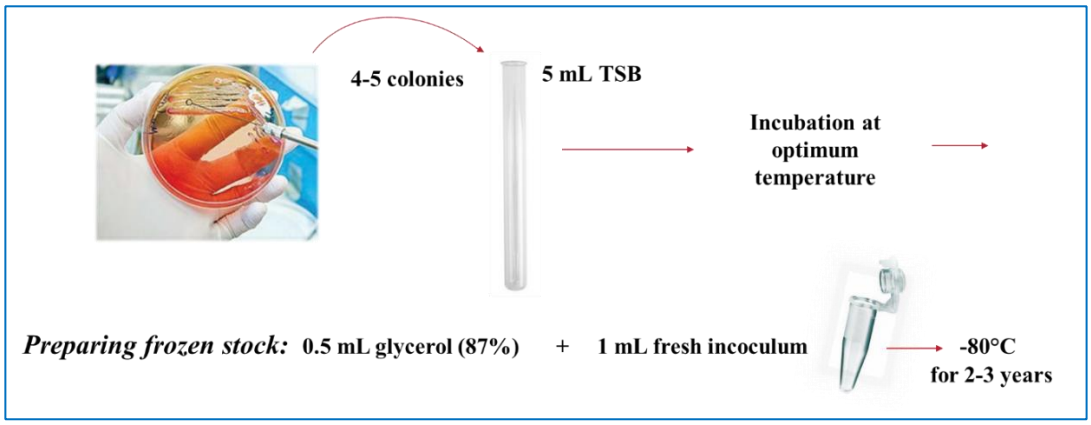

Fig. 16. Bacterial glycerol stock culture maintenance and storage

Form frozen stocks, the cultures were cultivated twice on Tryptic Soy Agar (TSA) plates (Merck KGaA, Darmstadt, Germany; Basingstoke, Hampshire, UK), denoted them as *F1* and *F2* plates, with 24 hours of incubation at 37 °C. The *F1* plates were used for one month and the *F2* plates, which were prepared from the *F1* plates, were used for a maximum of 1.5 weeks. For the experiments, the overnight cultures were prepared from the *F2* plates, as 4-5 bacterial colonies were inoculated either in 5 mL Mueller-Hinton Broth (Oxoid, Basingstoke, Hampshire, UK) or in Tryptic Soy Broth (TSB) (Basingstoke, Hampshire, UK) and were grown at their optimum temperature. TSB was only used for the fluorescence assays, where antimicrobial agent was not added to the broth during the experiments. Overnight cultures were always prepared in the same broth, that would be used in the experiments.

4.1.3 Organisms, culturing and growth conditions

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 two *E. coli* strains, the bacterial culture of *E. coli* ATCC 25922, a reference strain, was also tested in *antimicrobial susceptibility measurements* and in *lag phase duration experiments*. The reference strain was obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary.

For the *individual cell kinetics' measurements* (Fig. 17), *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 derived from the (National Center for Epidemiology, Budapest 20044/99702).

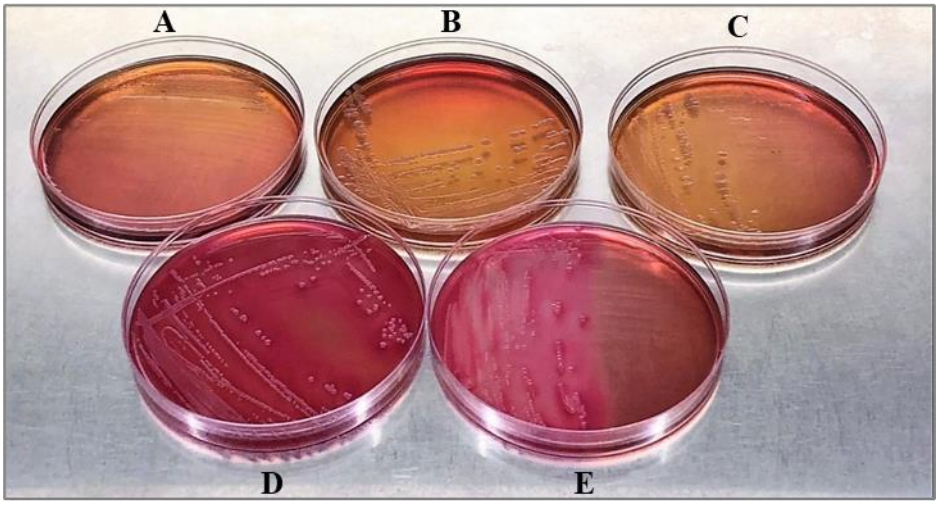

Fig. 17. *Salmonella enterica* subsp. *enterica* ATCC 13311 (A), *Salmonella enterica* subsp. *enterica* ATCC 14028 (B), *Shigella sonnei* HNCMB 20044 (C) (colorless, non-lactose fermenting colonies), *E. coli* VF 3584 (D) and *E. coli* ATCC 25922 (E) (pink, lactose fermenting colonies) at MacConkey Agar after 24 h incubation at 37 °C

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

4.2 Methods

Optical density readings are one of the most commonly methods used in microbiology laboratories for measuring the bacterial growth. In general, optical density measurements detect light scattering rather than light absorption. Turbidimetric measurements were carried out at 590, 600 and 620 nm. Thus, all the samples were measured near 600 nm wavelength, slight variation came from the different instruments.

4.2.1 Broth microdilution method to determine the MIC of antimicrobial compounds

The MIC of carvacrol and the antibiotics were determined by broth microdilution method following the instructions of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing 2021). The initial concentration of bacterial cultures was set to 5×10^5 CFU/mL (10 μL from the overnight culture were diluted in 10 mL fresh broth, proved by spread plate technique using non-selective agar plates). The reading of MIC endpoints was done after 24 h incubation at 37 °C (Cuenca-Estrella et al. 2002). Evaluations were carried out from bacterial pellets in U-bottom plates (manually) and via OD measurements (automated plate reader). The MIC values were determined by

the upper boundary of those carvacrol concentrations where the wells of microtiter plate did not become turbid (compared to the negative control – sterile broth). These MIC values were expressed in mg/mL.

4.2.2 Inoculum preparation and dilution prior to the single cell measurements via turbidity-based readings

Kinetic studies allow food processors to know whether a product is safe or not. Most of the microbiological methods have high detection limit: in case of turbidimetric measurements, it is *ca.* 10^7 cells/mL whereas in case of microscopic methods *ca.* 10^6 cells/mL of concentration. 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 needed for the growing bacteria to reach a given concentration was measured, the detection level ($\approx 5*10^7$- 10^8 cells/mL). The scatter of the detection times can give information about the variability of individual cell's lag times. The variability of single cell behavior at the early stage of bacterial growth has much greater influence on the overall growth than at higher concentrations. The average numbers of initial cells can be estimated by the proportion of those wells, which do not become turbid; or by the spread plate technique (Buss da Silva et al. 2019).

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. I have developed a dilution method 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 newly worked out dilution method is detailed in section 5.2, which approach lead to reaching the targeted *ca.* 1-3 cells/well initial concentrations prior the turbidity measurements.

The optical density readings were produced right after the appropriate dilutions by means of the automated Sunrise (Tecan Group Ltd., Switzerland) plate reader. The time (called detection time, T_{det}) needed for the cultures to reach OD_{det}=0.15 (detection level) at 590 nm was recorded, when the number of cells in a well was N_{det} (detection level number of cells). The effect of individual cells on the variation of lag time was

investigated under optimum growth conditions at 37 °C for 24 hours. Readings were made in every 30 minutes, with 20 seconds of shaking time prior them. The initial cell number per well was estimated by the proportion of those wells, which did not become turbid during the observation time. Before each set of experiments, the concentrations of the binary diluted samples were also estimated by traditional plate counting. The distribution of T_{det} was considered as a shifted version of the distribution of lag times in the wells, as the maximum specific growth rate was assumed to be the same for all wells. The N_{det} of each strain were estimated by the traditional spread plate method as George et al. (2015).

4.2.3 Fluorescent assays

4.2.3.1 OD growth curves to determine the physiological states of bacteria

The slopes of the OD growth curves were used to determine the physiological state of microorganisms. To determine slope values in the fast-growing phase, linear line was fitted to OD values recorded at 0; 0.5; 1; 1.5 hours; in the case of the slow-growing phase, linear line was fitted to OD values recorded at 3.5; 4; 4.5; 5 hours; and in the case of the non-growing phase linear line was fitted to OD values recorded at 13.5; 14; 14.5; 15 hours. The OD curves were measured at 37 °C, with the initial inoculum level of OD_{600}=0.1 (≈10^8 CFU/mL) in TSB. 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.

4.2.3.2 Culture preparation prior the fluorescent assays

The kinetics of EtBr accumulation and membrane degradation were measured by using a VarioskanLUX multimode microplate reader (Thermo Scientific, Waltham, Massachusetts, USA), where the measurements were performed according to Kovač et al. (2015) with some modifications. Culture preparations and treatments were the same before both types of assays.

Before fluorescent assays, the overnight cultures were diluted in fresh TSB 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, the efflux modulation effect of NMP 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.

4.2.3.3 EtBr accumulation assays

EtBr was used as a fluorescent substrate to evaluate the efflux pump (EP) mechanism and the modulating effect of carvacrol on this defense mechanism. The basis of this measurement is that the EtBr passes across the cytoplasmic membrane and accumulates inside the cell, where it fluoresces only when it is binding to DNA and excited by ultraviolet light. The EtBr penetrates the cytoplasmic membrane by passive diffusion and accumulates within the cell (Martins et al. 2013; Neyfakh et al. 1991). As a protective response, the EP proteins are activated and EtBr (at least partially) can be transported out of the cells. The process simulates the way how cells fight against antimicrobial agents (Blair and Piddock 2016).

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 readings were made in 45 s intervals over 1 h observation time. Solution of EtBr was prepared and used according to the manufacturer's instructions therefore the final volume was 100 μL in each well with the final concentration of 0.5 mg/L EtBr.

The aim was to characterize EtBr accumulation in slow-, fast and non-growing phases and to compare the activity of efflux mechanism in the growth phases and also study the inhibitor efficacy of carvacrol.

4.2.3.4 Membrane integrity assays

The LIVE/DEAD BacLight Bacterial Viability kit, as a mixture of the green-fluorescent dye SYTO 9 and propidium iodide, was used to detect the changes in the membrane integrity caused by carvacrol. The basis of this technique is that while green dye can pass through both damaged and undamaged cell membrane, red dye can only cross damaged membranes. The intracellular propidium iodide penetration measures the cytoplasmic membrane damage, which was quantified by relative fluorescent units (RFU values), measured in 60s 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.

4.2.4 OD growth curves to describe the effect of carvacrol on the lag time extension

OD growth curves were used to determine the highest concentration of carvacrol that does not cause significant lag time extension. Thus, the defined concentration could be used as an efflux inhibitor, assuming a not significant antibacterial activity.

The effect of carvacrol on 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. Each experiment included growth-control and uninoculated control wells. Since the carvacrol's stock solution was prepared in EtOH, as vehicle control 4, 6 or 8 µL EtOH were added to the bacterial suspensions, representing the volumes of 60, 90 and 120 mg/L in carvacrol's stock solution. Experiments were performed in 96-well flat-bottom plates, with the final volume of 100 µL *per* well. These tests were carried out in dependent duplicates and independent quadruplicates.

4.2.5 Antimicrobial Susceptibility Testing

Broth microdilution method was used for measuring the combined activity of antibiotics and carvacrol. The dispensing device (Fig. 18) was epMotion 5075 Automated Pipetting System (Eppendorf AG, Hamburg, Germany). At the same time

three plates could be prepared and the program, to execute the dispensing part, lasted for 2.5 hours.

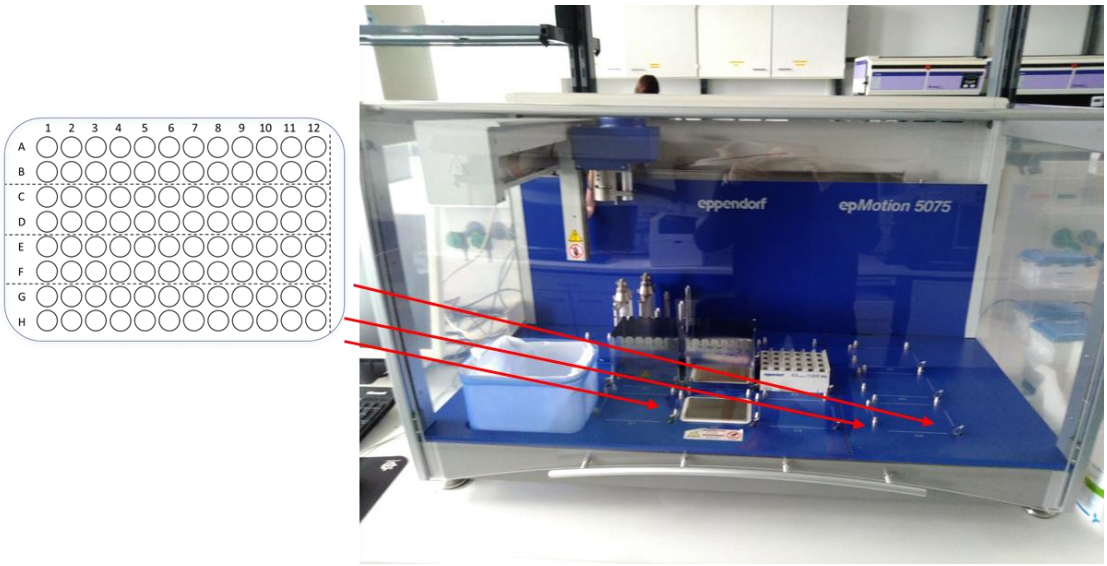

Fig. 18. epMotion 5075 Automated Pipetting System

Plastic, flat-bottom 96-well microdilution trays were used, layout of those is reported in section 5.5. Regarding the layout, the aim was to have the minimum number of commands to prepare the plates as fast as possible, furthermore high number of replicates were required from the positive and negative control wells which were used to normalize the final OD values. 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. Each set of experiments (5 antibiotics were tested against 3 *E. coli* strains) were carried out in dependent duplicates and independent triplicates, in total 45 plates were evaluated.

4.3 Data analysis

4.3.1 Numerical and statistical methods used to evaluate the fluorescent assays

4.3.1.1 Primary models

The changes of EtBr accumulation and membrane integrity 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 (Eq. 4) or (B) a dissipation model of membrane integrity (Eq. 5):

$$Fs(t) = Fs_0 + (Fs_{max} - Fs_0) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad (4)$$

$$Fs(t) = Fs_0 - (Fs_0 - Fs_{min}) \cdot (1 - e^{r \cdot t}) + \varepsilon \quad (5)$$

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.

For non-linear regression, the optimization algorithm of Levenberg-Marquardt was programmed in Microsoft Visual Basic for Application to minimize the Residual Sum of Squares (RSS) according to the Least Squares method, as shown by Press et al. (1990).

4.3.1.2 Secondary models

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) function notated as B_s (Eq. 6):

$$y_{EP}(x) = ln\frac{Fs_1}{Fs_0} = B_s(x) = y_{opt} \cdot \begin{cases} \frac{(x - x_{min})}{(x_{opt} - x_{min})} & (x_{min} \le x \le x_{opt}) \\ \frac{(x_{max} - x)}{(x_{max} - x_{opt})} & (x_{opt} \le x \le x_{max}) \end{cases} \quad (6)$$

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-test (Eq. 7) was used to decide whether a simplification of a model can be used, by reducing the number of its parameters. M_1 and M_2 were the models, where M_1 was nested in M_2. Let p_1 and p_2 be the respective parameter vectors. Let $n(p_i)$ denote the dimension of p_i (i = 1, 2; $n(p_1) < n(p_2)$). Fitting N data points by M_1 and M_2, we get RSS_1 and RSS_2 residual sums of squares ($RSS_1 > RSS_2$, since M_2 has more parameters). Using the Fisher-information concept, the difference between RSS_1 and RSS_2 can be defined as the loss of information when a simpler model (M_1) is used to describe the dataset. According to the classical F-test, the closer the regression models to linear, the more it is valid that the ratio:

$$F = \frac{RSS_1 - RSS_2}{n(p_2) - n(p_1)} \Big/ \frac{RSS_2}{N - np_2} \tag{7}$$

follows the F-distribution, with $n(p_2) - n(p_1)$ and $N - n(p_2)$ degree of freedom. Therefore, if this ratio is above the appropriate critical value F_{crit}, then the M_2 represents a significant improvement over M_1 (i.e., we cannot use the simpler M_1 instead of M_2, without losing significant amount of information). F_{crit} constant was chosen to correspond to the 5% significance level.

The above procedure was also used to decide whether two compatible datasets produce significantly different parameter estimates, in our case, whether the two independent replicates produce significantly different maximum points (the highest EtBr accumulation reached at a certain level of carvacrol treatment). Let $p(D_i)$ be the sets of parameter estimates produced by the two datasets (D_i, i = 1, 2). Can we say that the difference between two independent estimates, namely, $p^{(1)}$ of $p(D_1)$ and $p^{(2)}$ of $p(D_2)$, is not significant? In this case, the higher resolution model (M_2) is the one when all terms of $p(D_1)$ can be different from the respective estimates of $p(D_2)$. Therefore, the number of parameters to be estimated, when using M_2, is $n(p(D_1)) + n(p(D_2))$. In case of the lower resolution model (M_1), the two respective estimates can be considered the same: $p^{(1)} = p^{(2)}$.

4.3.2 Analyzing the effect of carvacrol treatments on the lag time duration of *E. coli*

By analyzing the OD growth curves, 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 (Eq. 8) was used,

$$T_{det} = \beta_0 * (121 - x)^{\beta_1} + \varepsilon \quad (8)$$

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 (Eq. 9,10,11 - Motulsky and Christopoulos 2004) decided whether the four datasets can be merged to an aggregated data set.

$$Ratio\ of\ relative\ differences\ of\ SS\ values = \frac{\left(ABS(SS_{merged} - SS_{sep})\right)}{SS_{sep}} \quad (9)$$

$$df_{relative\ differences} = \frac{df_{merged} - df_{sep}}{df sep} \quad (10)$$

$$F = \frac{Ratio\ of\ the\ relative\ differences\ of\ SS\ values}{df_{relative\ differences}} \quad (11)$$

The diagnostic of non-linear regression models was done in R (package: moments - https://CRAN.R-project.org/package=moments) with the next steps including: testing the significance of model parameter's estimations and the goodness of fits via t test, describing the model accuracy via F test (ANOVA) and calculating the root mean square error (RMSE) value of models.

One-factor analysis of variance (ANOVA) was used to determine statistically significant differences between the means of T_{det} values, measured at different concentrations of carvacrol. IBM SPSS Statistics (version 27) was used to carry out the analysis, employing the previously determined predicted models' values. When significant results were obtained with the application of ANOVA ($p<0.05$), Games-

Howell post hoc test (multiple comparisons) was used to find the highest concentration of carvacrol that causes significant lag time extension.

The sample sizes were n=48 for all cases, except for *E. coli* VF 3584 (n=36). Checking the assumptions, no serious outlier was detected with Mahalanobis distance based on Chi-square test score (with the lowest p=0.02). Shapiro-Wilk test examined whether the data were normally distributed. The results were significant at three carvacrol concentrations only. At (1) 97.5 mg/L (p=0.041), (2) 90 mg/L carvacrol treatments (p=0.049) with *E. coli* ATCC 25922, and at (3) 112.5 mg/L carvacrol treatments (p<0.001) with *E. coli* VF 3584. Levene's test measured the equality of error variances. The residuals had normal distribution (p>0.084), provided that the results at the highest carvacrol concertation were omitted.

Lag time extension

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 (Eq. 12) was defined according to Li et al. (2016).

$$LE = \frac{\lambda_C}{\lambda_0} \tag{12}$$

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.

LE values were calculated separately for the four independent replicates *per* strain. For each strain, a median value from the four replicas was used and finally determined the carvacrol concentrations that resulted lag time duration increase between 20 – 40%.

4.3.3 Methods used to analyze the efficacy of combined treatments

Following 24 hours of incubation, 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 OD values based on the positive (OD_{max} – average of positive control values) and negative (OD_{min} – average of negative control values) controls were normalized. In that way the bacterial growth (%) could be expressed (Eq. 13) as

$$Bacterial\ growth\ (\%_{\mathrm{OD}}) = \frac{OD_x - OD_{min}}{OD_{max} - OD_{min}}\ . \tag{13}$$

IBM SPSS Statistics, version 27 was used to carry out Multivariate Analysis of Variance (MANOVA), a multivariate statistical analysis. MANOVA investigated the differences between the combined treatments, where dependent variables were bacterial OD growth ($\%_{OD}$) 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).

Checking the assumptions of MANOVA: When the overall MANOVA was applied using both factors (strains and the groups of antibiotics), Levene's tests showed that the equality of error variances were significant ($p<0.05$) in the following cases of treatment combinations: (i) when 0.5 or 0.25 MIC antibiotics were combined with the 75 or 90 mg/L carvacrol treatments; when 0.125 MIC antibiotics were combined with the 0, 75 or 90 mg/L carvacrol concentrations; and when 0.0625 MIC antibiotics were combined with 0, 75 or 90 mg/L carvacrol treatments.

Analyzing the factors separately, Levene's test showed that the basic assumption of equality of error variances was also compromised in some other cases. Therefore, Games-Howell post hoc test was chosen for further investigations.

If MANOVA indicates significant results, discriminant analysis (DA) can be used as a follow-up analysis to estimate the differences between the tested groups. DA is a clustering method if a preliminary grouping is at hand, which allows us to classify samples based on a prior hypothesis to identify the variables with the highest discriminant power. This analysis defines the distance of a sample from the center of a pre-defined cluster. It creates a new set of axes, allocates the members of the same group as close together as possible and moves the groups as far apart from each other as possible (Héberger 2008).

Checking the assumptions of DA, the multivariate normality was not compromised based on skewness and kurtosis values. The groups' sizes were n=45. In DA the variables changed compared to MANOVA. In DA, the independent variables were the bacterial growth (%) in the presence of different antibiotics plus carvacrol treatments. The dependent variables were (i) the strains of *E. coli* (*E. coli* ATCC 11229, *E. coli* ATCC 25922, *E. coli* VF 3584) – model *1*; and (ii) the groups of antibiotics (cefotaxime, ciprofloxacin, erythromycin, gentamicin, vancomycin) – model *2*.

For data representation and visualization, median value from the three independent replicates was used to control the impact of variances. Balloon plots were prepared: using RStudio and R version 4.1.2 (2021-11-01), package: ggpubr (https://cran.r-project.org/web/packages/ggpubr/index.html).

5 RESULTS AND DISCUSSION

5.1 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 (*E. coli* ATCC 25922) showed lower MIC 270 mg/L. The measured values concur with the literature data (Dos Santos Barbosa et al. 2021).

The determination of MIC of antibiotics was performed similarly by broth microdilution, the results are summarized in Table 3. Within the three independent, two dependent replicates were also done.

Table 3. Antibiotics' MIC values against *E. coli* strains determined by broth microdilution method

	MIC values of antibiotics [mg/L]					
	Independent replicates	**vancomycin**	**erythromycin**	**gentamicin**	**cefotaxime**	**ciprofloxacin**
***E. coli* ATCC 25922**	*a*	165	32.813	1	0.123	0.032
	b	165	65.625	1	0.123	0.016
	c	82.5	65.625	2	0.123	0.016
***E. coli* ATCC 11229**	*a*	165	131.250	2	0.015	0.016
	b	82.5	131.250	2	0.031	0.016
	c	165	131.250	2	0.031	0.016
***E. coli* VF 3584**	*a*	165	65.625	1	0.123	0.032
	b	165	65.625	2	0.123	0.032
	c	165	65.625	1	0.061	0.032

E. coli ATCC 11229 seemed to be more resistant to gentamicin and erythromycin than the other two strains, however, it was also more sensitive to the antibacterial activity of cefotaxime. In case of ciprofloxacin treatment, the food isolate, *E. coli* VF 3584 showed a higher resistance.

The results are within the range reported in the literature (European Committee on Antimicrobial Susceptibility Testing 2022). Although, comparing these results to the

values highlighted in Table 1, lower MIC for gentamicin, erythromycin and vancomycin but higher in the case of ciprofloxacin was measured.

The results of broth microdilution method were recorded by plate reader (via OD values) as well as manually. Manually the pellets in the bottom of the wells indicated bacterial growth, when U bottom plates were used (Fig. 19) as suggested in the EUCAST protocol (European Committee on Antimicrobial Susceptibility Testing 2021).

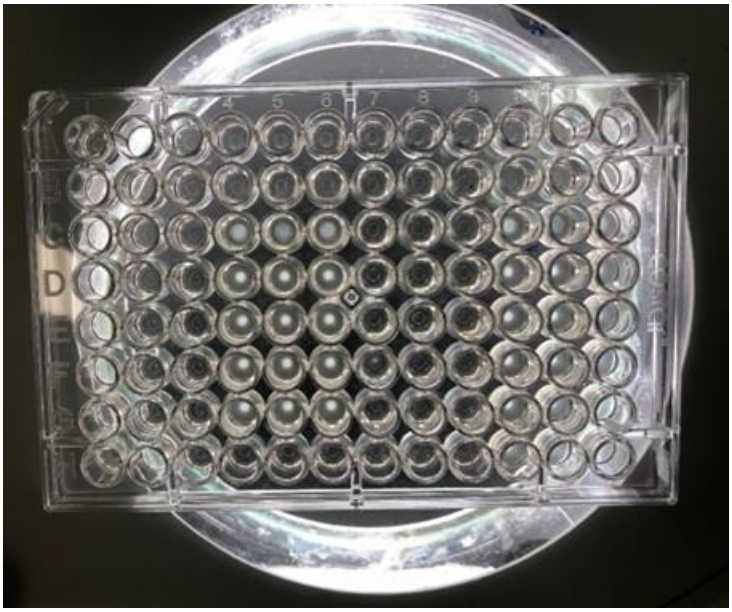

Fig. 19. U bottom microtiter plates used to record the MIC of antimicrobials manually. The pellet, at the bottom of the wells indicates the bacterial growth

5.2 Analyzing the growth kinetics of bacteria at low inoculum level

Studying the growth kinetics of pathogens at low initial cell numbers, stochastic mathematical models are needed to describe and interpret 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 single cell kinetics.

As for the layout, the dilution series started at *ca.* 10^3 cells/well, then nine more consecutive binary dilutions were inoculated into the wells of a microtiter plate (Fig.

20). The design followed the idea of Baranyi and Pin (1999), expecting that the variance of the detection times decreases with increasing inoculum level.

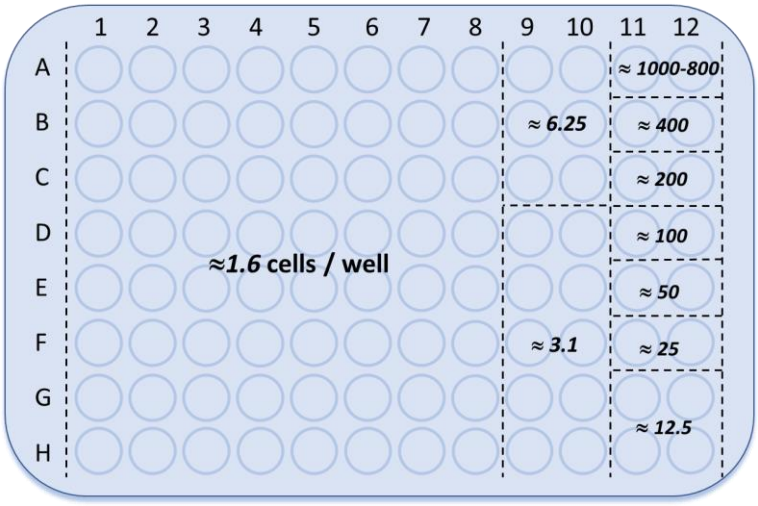

Fig. 20. Layout of microtiter plates, prepared for turbidity measurements, where the lowest initial cell concentration is around 1.6 cells/well

By traditional spread plate technique, the exact cell number of the five tested microbes at OD=0.15 detection level was estimated (Table 4). More accurately, if the concentrations are known, a better estimation for the variance of the lag time of the individual cell can be made.

Table 4. Cell numbers per well, measured at the detection level (OD_{590}=0.15) by spread-plate technique, concentrations are average values of three independent replicates

Mueller-Hinton broth	**OD_{590}=0.15** **CFU/well**	***N_{det}* (OD_{590}=0.15)** **LOG(CFU/well)**
E. coli VF 3584	$7.54*10^7$	7.88
E. coli ATCC 25922	$6.96*10^7$	7.84
Salmonella enterica subsp. *enterica* ATCC 14028	$7.42*10^7$	7.87
Salmonella enterica subsp. *enterica* ATCC 13311	$8.98*10^7$	7.95
Shigella sonnei HNCMB 20044	$5.2*10^7$	7.72

Note that, in the paper of George et al. (2015), the authors measured lower concentrations (N_{det}=$10^{7.2}$ cells at OD=0.15). The difference can be explained by the initial absorbance of the different applied broths.

Buss da Silva et al. (2019) pointed out that to assess the lag time distribution of individual cells by turbidity measurements, *ca.* 20% of the wells of a microtiter plate should not became turbid, which would allow to estimate the average number of cells in a well, (Poisson parameter denoted by ρ^* in what follows), at optimal precision. It was shown that the parameter can be calculated as:

$$\rho^* = \ln\left((w-2)/W_0\right) \tag{14}$$

which determines efficiently the expected number of initial cells in a well, where *w* is the total number of wells and W_0 is the number of those wells that show no growth (Eq. 14).

According to the layout shown in Fig. 20, at the lowest tested concentration out of the 64 wells, 12–13 should stay empty to study the single cell kinetics at the optimal cell concentration level. Such result is shown in Fig. 21.

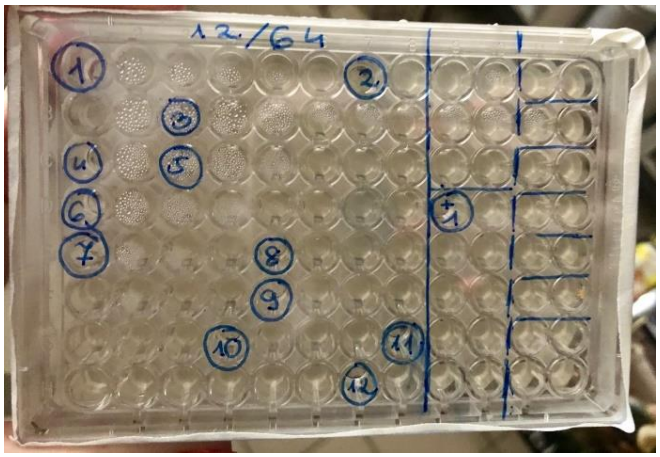

Figure 21. Result of a turbidity measurement. Individual cells of *E. coli* VF 3584 were incubated in 64 wells of the plate and out of those, 12 wells did not become turbid

The aim was to obtain *ca.* 1–3 initial cells *per* well at the lowest concentration, as suggested by Buss da Silva et al. (2019). The total number of cells, inoculated in the 96 wells of a microtiter plate, was estimated by plate counts (Fig. 22) and compared to the ρ^* parameter obtained via turbidity experiments.

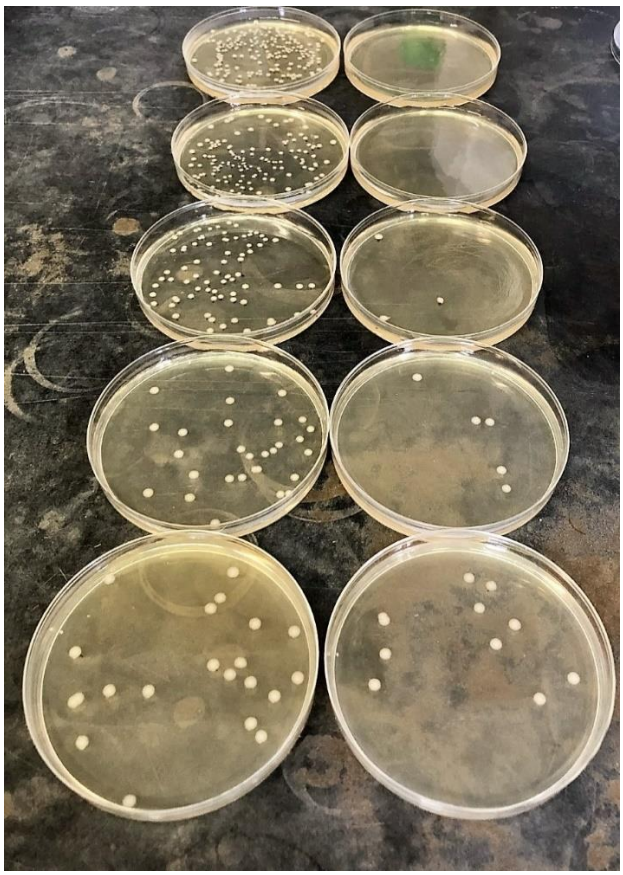

Fig. 22. Estimating the cell number of 10 serial binary dilutions (before the turbidity measurements) by spread plate technique using *E. coli* VF 3584

Table 5 contains 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^*). Different rows represent independent measurements.

To reach the desired 1-3 cells *per* well I have developed the following specific dilution method. As a starting point, the cell concentration was set to $OD_{600} = 0.1$ (considered as dilution 0). Then 4 decimal dilutions, followed by 11 ± 2 binary dilutions were applied to reach the required 1-3 cells *per* well as the lowest concentration. As mentioned above, the number of binary dilutions were strain dependent, the required numbers can be found in the second column. The third column contains the total dilution rate applied in the experiment compared to the starting point.

The fourth column, ρ^*, shows the inoculum level calculated by means of the proportion of empty wells. Finally, the last column, c^*, shows the estimated lowest cell number *per* well obtained via the traditional method. Before each set of experiment, c^* (CFU/200 µL) values were estimated in two replicates by traditional technique and their average is indicated in Table 5.

Table 5. Estimations for the values of initial cell numbers *per* well and the required dilution rate

Bacterial strains	**Required number of binary dilutions[a]**	**Log dilution rate[b]**	**Expected initial cell numbers *per* well, ρ^*, from the proportion of empty wells**	**Expected initial cell numbers *per* well, c^*, from plate counts and the dilution factor**
E. coli VF 3584	10	7.01	4.13	1.99
	11	7.31	1.90	1.71
	11	7.31	1.64	0.91
E. coli ATCC 25922	9	6.71	3.43	4.19
	10	7.01	1.29	2.23
	11	7.31	1.05	0.63
Salmonella enterica subsp. *enterica* ATCC 14028	10	7.01	3.06	2.35
	11	7.31	1.61	1.87
	12	7.61	0.66	0.65
Salmonella enterica subsp. *enterica* ATCC 13311	10	7.01	2.05	1.81
	11	7.31	1.29	1.67
	12	7.61	0.43	0.69
Shigella sonnei HNCMB 20044	10	7.01	4.13	2.51
	11	7.31	3.03	1.12
	12	7.61	0.73	0.76

[a] *Initial concentration of cultures was set to OD=0.1 then 4 decimal dilutions were done and then the certain number of decimal dilutions, required to reach ca. 1–3 cells per well.*
[b] *Numbers are the log values of the applied total dilution factors, from a cell concentration measured at OD=0.1 considered as the 0 dilution.*

In the case of *E. coli* VF 3584, after log(concentration)=7.31 decrease from the starting point, ρ^*=1.90 and 1.64 average cell numbers *per* well were obtained, which means that targeting a specific low concentration is well replicable. This is also supported by the fact that in the case of *E. coli* ATCC 25922, at twice as high concentration log(concentration)= 6.71 decrease, ρ^*=3.43 was measured, so double as many cells, as after log(concentration)= 7.01 decrease, ρ^*=1.29. Thus, the accuracy of the applied method is well demonstrated. Moreover, 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.

The lag time of the population can be estimated from the T_{det} detection times measured by these turbidity experiments. T_{det} is defined as the time when the population reaches a fixed turbidity level. Detection times from different inoculum levels are shown in Fig. 23, one independent replicate with each strain.

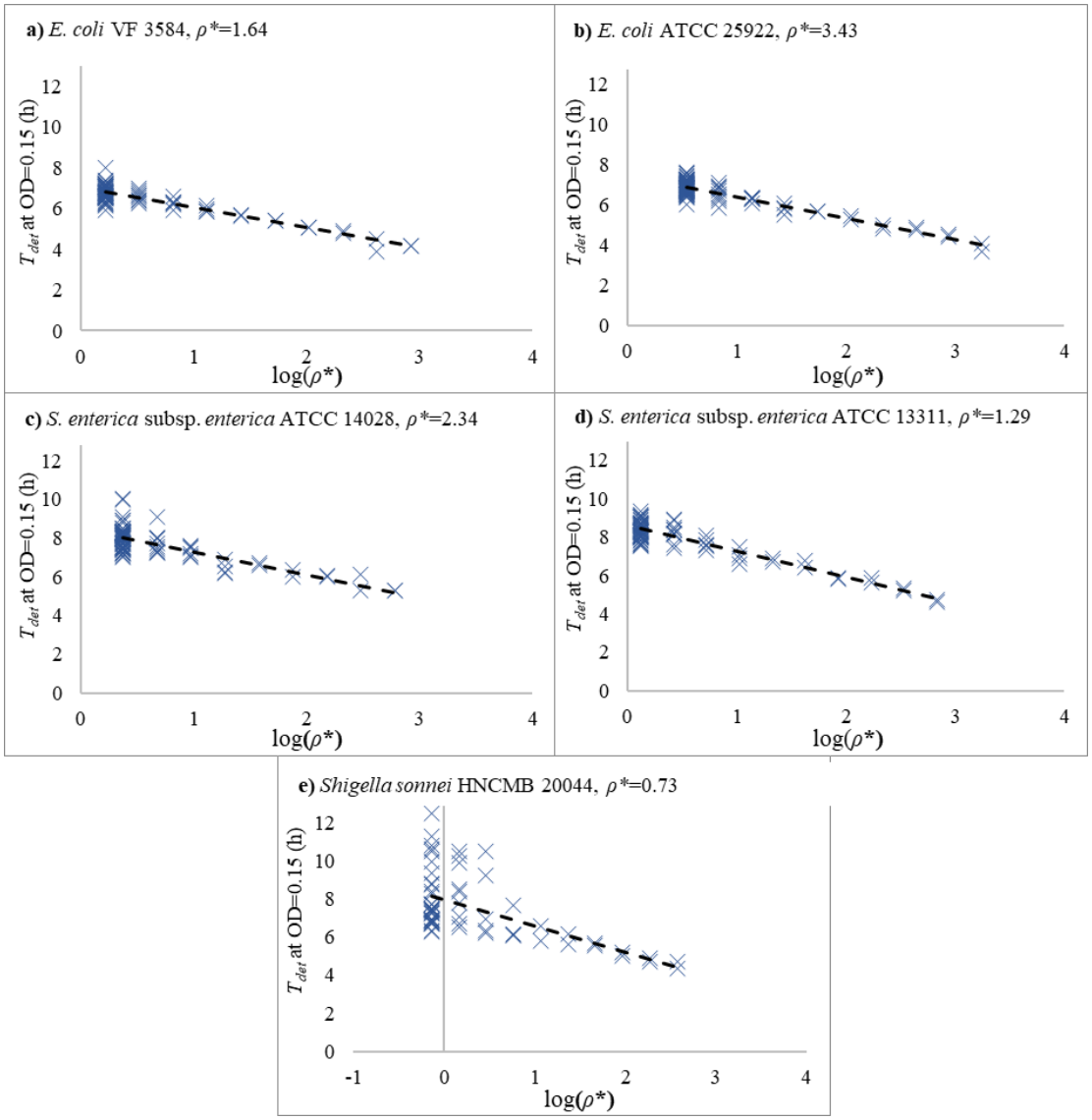

Fig. 23. Single cell kinetics of *E. coli* strains (a, b), *S. enterica* subs. *enterica* strains (c, d) and *Shigella sonnei* (e)

The detection times were plotted versus the inoculum size on logarithmic scale. At lower inoculum levels, the scatter of the detection times is quite big, but then it decreases as the initial cell number increases. Finally, at high initial cell numbers, the detection time becomes a linear function of the inoculum with a small variance. The reason behind this pattern is that the smaller the size of the inoculum, the bigger the

effect of the variability of the single cell lag times on the population growth. Since all the tested microorganisms are members of Enterobacteriaceae family, great difference between their lag time distributions at single cell level was not expected. However, below *ca.* 20 cells *per* well concentration, the variance of T_{det} values of *Salmonella enterica* subsp. *enterica* strains was generally higher than that of *E. coli* strains. Moreover, *Shigella sonnei* had two times greater lag time distribution interval at low concentrations than in the former cases. This could have been due to the difference in the specific growth rates of microbes.

Based on these results, *E. coli* can be used as model microorganism in antimicrobial studies because under optimum growth conditions it showed the lowest variance in the lag time distribution. This characteristic makes *E. coli* well modellable and feasible to study the effect of antimicrobial treatments.

In this part, the practical implementation of the recommendation of Buss da Silva et al. (2019) was provided. I could set up an efficient method for measuring the lag time variability of individual cells and describe it among strains. The lag times of microbes were affected by inoculum size and this was also reflected by the detection time. Below *ca.* 20 cells *per* well concentration, high variabilities were observed, partly because the initial cell number in a well is random, partly because of the variability of the single cell lag time. By means of the Poisson assumption, the parameter of this distribution (ρ^*) could be estimated and proved that its values do not differ significantly from the respective estimates obtained by the traditional dilution and spread-plate method.

5.3 OD growth curves

OD growth curves were used to study the effect of carvacrol on the lag time extension at population level, with a starting concentration of $5*10^5$ CFU/mL.

5.3.1 Primary models of OD growth curves

The primary models describe the observed OD values over the time. The detection times were recorded as the times needed to reach OD=0.15 value. Carvacrol added to the cultures' broth between 40–120 mg/L, while its MIC was between 270 and 300 mg/L. As a positive control, the OD growth curves of the non-treated cultures were measured as well. Two dependent replicates were recorded with each measurement settings (Fig. 24).

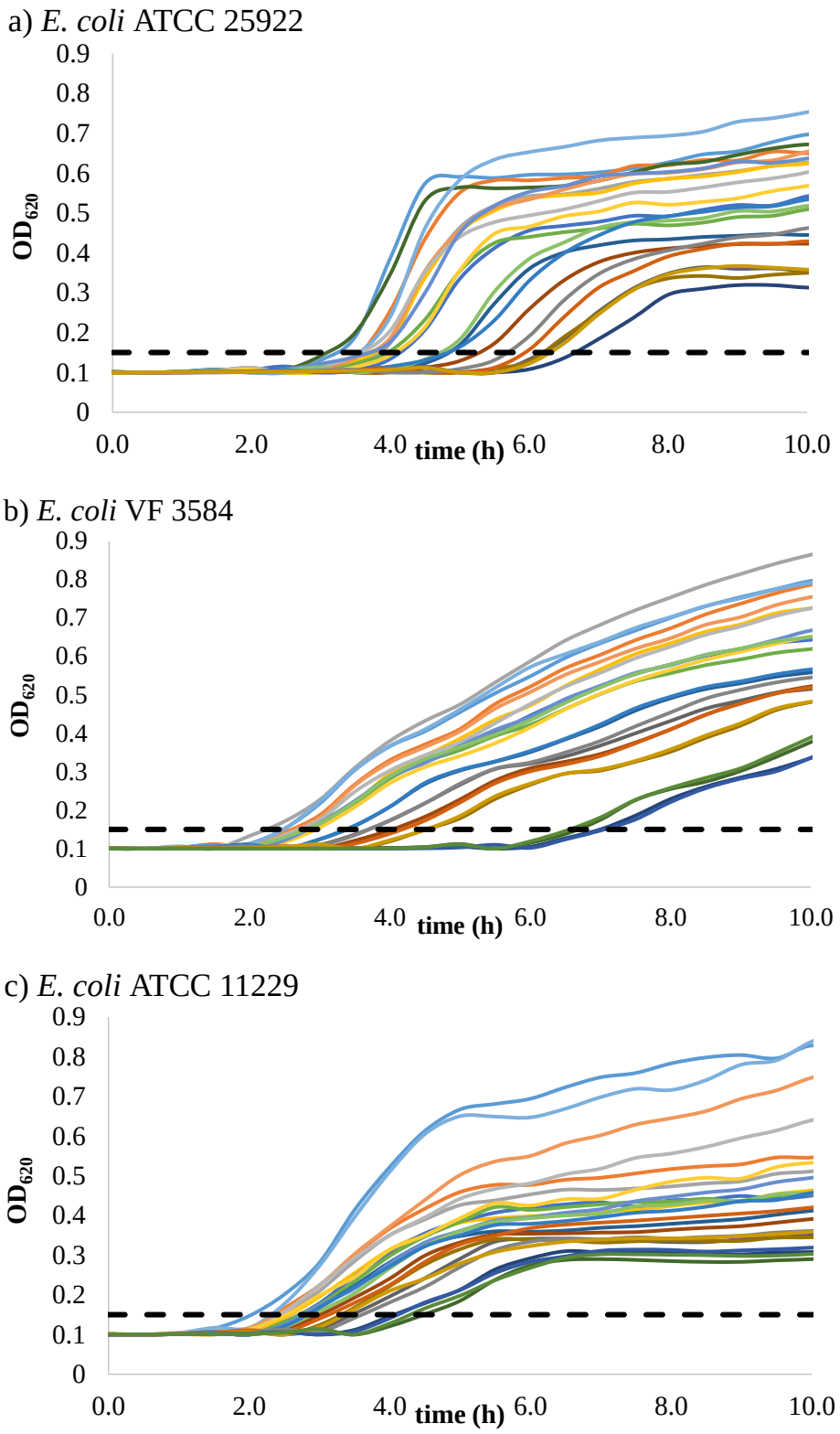

Fig. 24. OD growth curves of *E. coli* ATCC 25922 (a), *E. coli* VF 3584 (b) and *E. coli* ATCC 11229 (c) with a starting concentration of $5*10^5$ CFU/mL. OD values were measured at 620 nm and influenced by 17 sub-inhibitory carvacrol concentrations (between 45–120 mg/L). One independent replicate and two dependent replicates can be seen on each graph. Black dashed lines represent the detection level, at OD=0.15

5.3.2 Secondary models of OD growth curves

Assuming that the detection time of a microbe is proportional to their lag time, it was found that as carvacrol concentration increased, so did the lag phase duration of *E. coli***.** In the secondary models, the recorded T_{det} values (the chosen primary model parameters) were plotted against carvacrol concentrations. Four independent replicates were prepared with each strain, represented in Fig. 25.

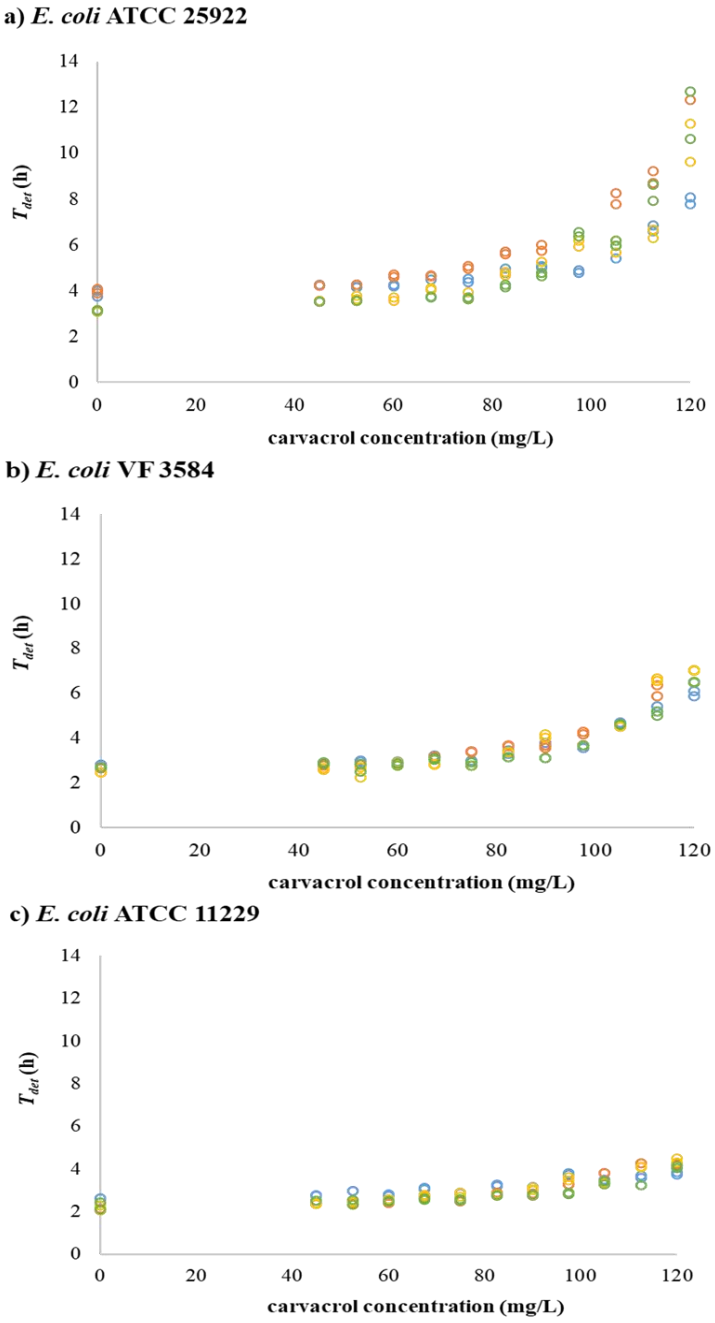

Fig. 25. T_{det} values versus carvacrol concentrations. Colors indicates the independent observed values of *E. coli* ATCC 25922 (a), *E. coli* VF 3584 (b) and *E. coli* ATCC 11229

It is clearly visible that around 70 and 110 mg/L carvacrol concentrations, as a response to the stress, each *E. coli* strain starts to increase their lag phase compared to the non-treated cultures.

E. coli ATCC 25922 was the most sensitive to carvacrol treatment. At 120 mg/L concentration the strain represented as high as 12.5 h T_{det} values with the highest variation, followed by *E. coli* VF 3584 which responded to 6.5 h T_{det} values and the clinical isolate, *E. coli* ATCC 11229 was the least sensitive to carvacrol.

As above mentioned, carvacrol stock solution was prepared in EtOH, therefore its lag time extension effect was tested similarly to carvacrol treatments. Vehicle control in 4, 6 or 8 µL volume was added to the bacterial suspension (final volume was 100 µL *per* well). The applied EtOH volumes represented the 60, 90 and 120 mg/L carvacrol treatment, added from its stock solution. On Fig. 26, the secondary models of EtOH effect are represented. Boxplot diagrams suggested a slight increase in the lag time duration 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 of *E. coli* strains, p=0.08 (*E. coli* ATCC 25922)*;* p=0.22 (*E. coli* VF 3584); p=0.38 (*E. coli* ATCC 11229).

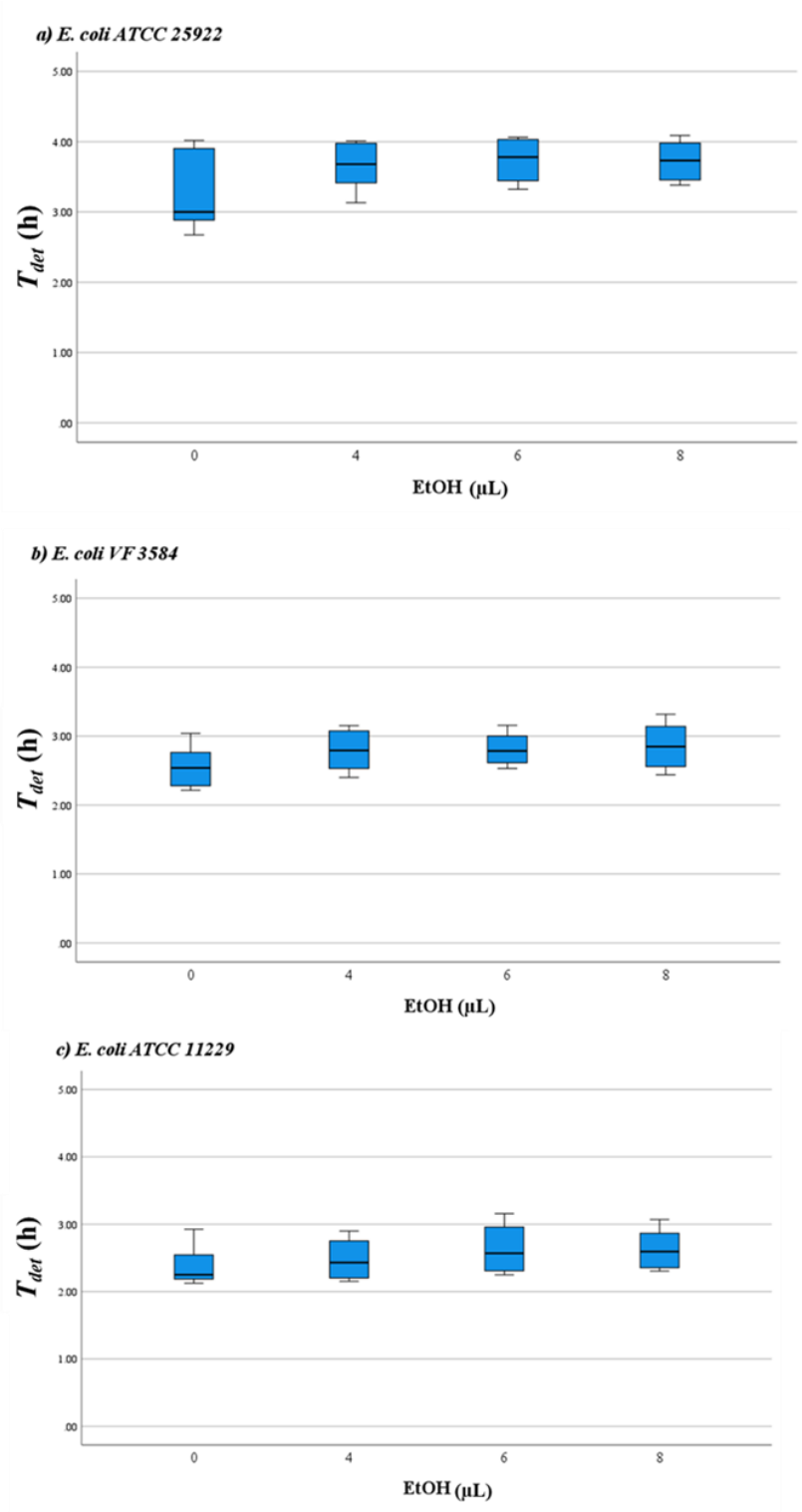

Fig. 26. Boxplot diagrams represent the measured T_{det} values (recorded at OD=0.15) as a function of EtOH treatment. Letters distinguish between the strains: a) *E. coli* ATCC 25922, b) *E. coli* VF 3584, c) *E. coli* ATCC 11229

Excluding that the EtOH significantly influences the lag time, the aim was to define the highest concentration of carvacrol which does not cause significant lag time extension, since high concentrations of carvacrol would induce the bacterial resistance in the long term. Presumably, the concentrations which cause only slight lag time extension, could be used as a safe efflux inhibitor. To describe the relationship between T_{det} and carvacrol treatment, the power function seemed to be suitable for fitting purposes (Fig. 27).

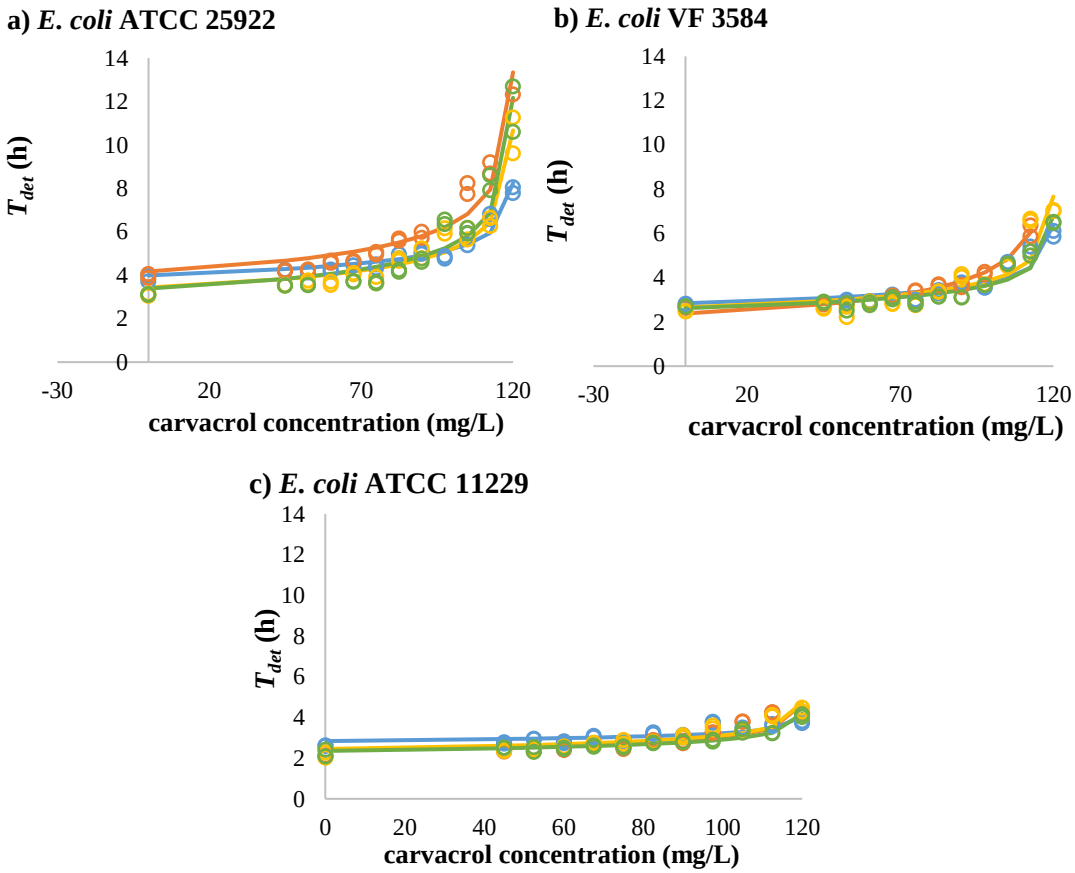

Fig. 27. Power function decribes the relationship between T_{det} and carvacrol treatments. Dots are the observed experimental values and the lines represent our fitted models.

The efficacy of explanation functions was different in terms of strains, but always above than R^2=0.70 . Out of the four replicates the lowest R^2 value, which infers to the worst fit, *per* strain was: R^2=0.93 (*E. coli* ATCC 25922); R^2=0.75 (*E. coli* ATCC 11229) and R^2=0.90 (*E. coli* VF 3584).

The four independent replicates with each strain measured the same response with same measurements settings, although, F tests showed that the functions generated by independent replicates cannot be merged (F>5.7, p<0.001). The model parameters can be seen in Table 6.

Table 6. Parameters of power models to describe the dose-dependent lag time prolongation effect of carvacrol

	Replicate 1	*Replicate 2*	*Replicate 3*	*Replicate 4*	
***E. coli* ATCC 25922**	8.228	13.341	10.667	12.168	$\mathbf{b_0}$
	0.151	0.242	0.237	0.267	$\mathbf{b_1}$
***E. coli* ATCC 11229**	4.027	4.656	4.721	4.178	$\mathbf{b_0}$
	0.073	0.138	0.137	0.120	$\mathbf{b_1}$
***E. coli* VF 3584**	6.374	12.788	7.664	6.791	$\mathbf{b_0}$
	0.168	0.351	0.221	0.199	$\mathbf{b_1}$

ANOVA highlighted that there are significant differences between the T_{det} values ($F>24.3$, $df_1=11$, $df_2=1$ $p<0.001$). Since the prolongation of lag time is dose dependent, multiple comparisons suggested (using Games-Howell post hoc tests) that 105 mg/L carvacrol treatment (almost) significantly prolongated the lag time of *E. coli* ATCC 11229 ($p=0.053$). In the case of *E. coli* ATCC 25922, the critical concentration was 97.5 mg/L carvacrol ($p=0.051$) while the effect of 75 mg/L carvacrol was significant on lag time extension in the case of *E. coli* VF 3584 ($p=0.04$).

However, these carvacrol values are the results of statistical computations and highly depend on the variances of the data that are quite different among the strains. T_{det} prolongation values were calculated as (Eq. 15):

$$T_{det}\ \text{prolongation} = T_{det\,0} - T_{det\ \text{at significant carvacrol treatment}} \quad (15)$$

where $T_{det\ at\ significant\ \text{carvacrol treatment}}$ is the T_{det} measured at those carvacrol concentrations which were evaluated as significant before. In the first case (*E. coli* ATCC 25922), ≈1.5 h was obtained as significant prolongation; in the second case (*E. coli* ATCC 11229), ≈0.7 h and in the last case (*E. coli* VF 3584) ≈0.5 h. Referring to that, it should be determined what is significant for the microorganisms.

The LE values were calculated at each carvacrol concentration, then taking the median of the four independent replicates, the lag time duration increase was expressed in %. Those concentrations of carvacrol that increased the lag time duration of bacteria with 20 to 40% are listed in Table 7.

Table 7. The Dose dependent effect of carvacrol on the bacterial lag time duration. The increase in lag time duration is expressed in %

	carvacrol concentration (mg/L)		
increase in lag time duration	*E. coli* ATCC 25922	*E. coli* ATCC 11229	*E. coli* VF 3584
20%	64.4	91.6	70.1
25%	73.2	99.4	79.0
30%	80.4	105.2	86.1
35%	86.2	108.7	91.8
40%	91.1	112.1	96.5

These LE values described more precisely the response of bacteria because the same pattern was observed as the OD growth curves suggested. Thus, the carvacrol tolerance over the strain changed as:

E. coli ATCC 11229 > *E. coli* VF 3584> *E. coli* ATCC 25922.

The lag phase extension was employed by strains to overcome carvacrol antimicrobial activity. The extension of lag phase is an important route to tolerate antimicrobial stress and to protect bacteria from subsequent adverse environmental factors. Therefore, it is important to study the effect of inhibitors on lag time duration.

5.4 Fluorescent assays

Two types of fluorescent assays were carried out, EtBr accumulation and fluorescent probes using the LIVE/DEAD BacLight Bacterial Viability kit. The former investigated the efflux mechanism, while the latter investigated the membrane integrity of both *E. coli* ATCC 11229 and *E. coli* VF 3584. Readings for the curves of EtBr accumulation were made in every 45 seconds, whereas the membrane degradation in 60 seconds over 1 h intervals. The effect of carvacrol was investigated in 17 subinhibitory concentrations between 30 – 150 mg/L concentration (with the step of 7.5 mg/L). In each set of experiments, two independent repeats and three technical replicates were generated.

5.4.1 Prior fluorescent assays, determining the growth phases of *E. coli* strains using the OD growth curves

OD curves were used to determine the fast-, slow- and non-growing phases of cultures with a starting concentration of OD=0.1 (Fig. 28).

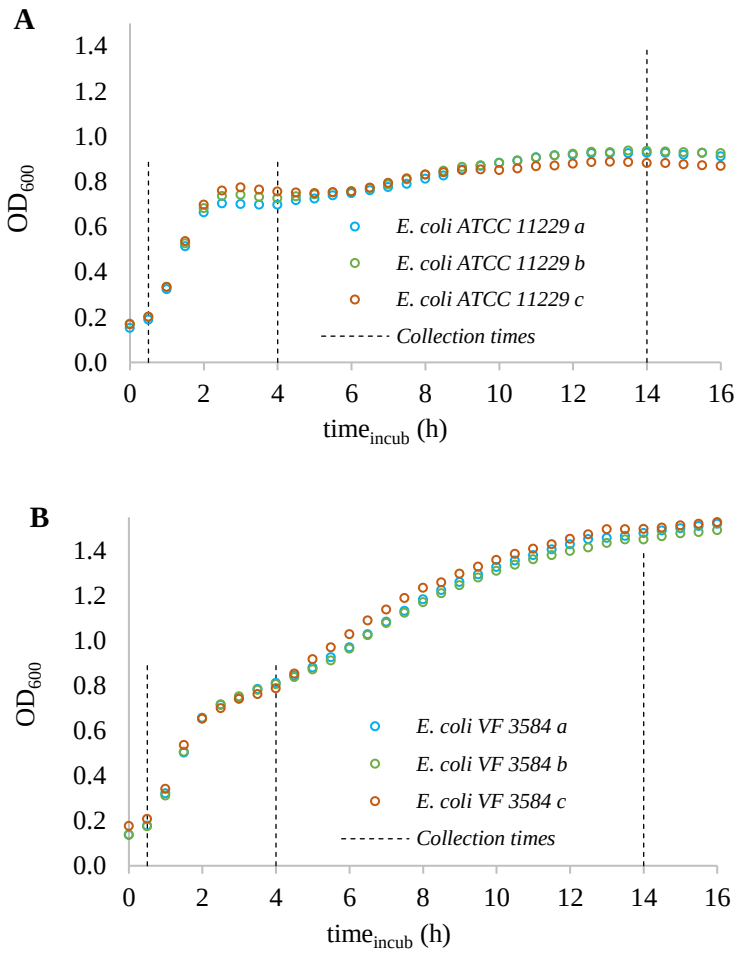

Fig. 28. OD_{600} growth curves of bacteria in triplicates. (A) *E. coli* ATCC 11229; (B) *E. coli* VF 3584. The starting concentration (shifted to 0.1 for all curves) was $\approx 10^8$ CFU/ml

The slopes of the curves defined the fast-, slow-, and non-growing phases of cultures. The different growth phases were identified after 0.5, 4, and 14 h incubation (Fig. 29).

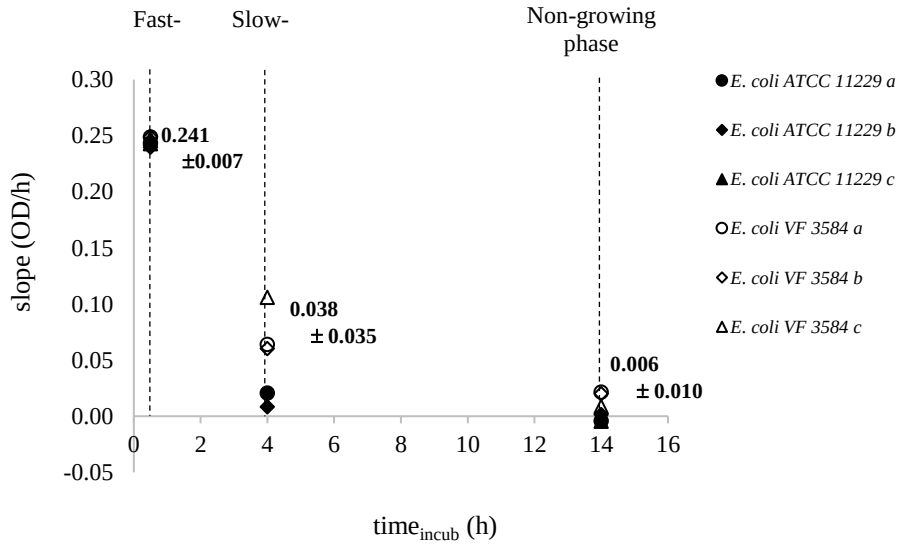

Fig. 29. Slopes of OD growth curves quantified the growth phases of bacteria, measured by three independent replicates

5.4.2 The influence of carvacrol on the fluorescent signal

As a negative control, the effect of carvacrol was measured on F_S of EtBr. In the control experiment, carvacrol was added to the blank (PBS) wells in 25, 75 and 150 mg/L concentrations. The results showed that carvacrol itself had no significant effect (p=0.56) on the fluorescent signal (Fig. 30), however, it has lowered the signal intensity, which was taken into account when the non-treated cultures were analyzed.

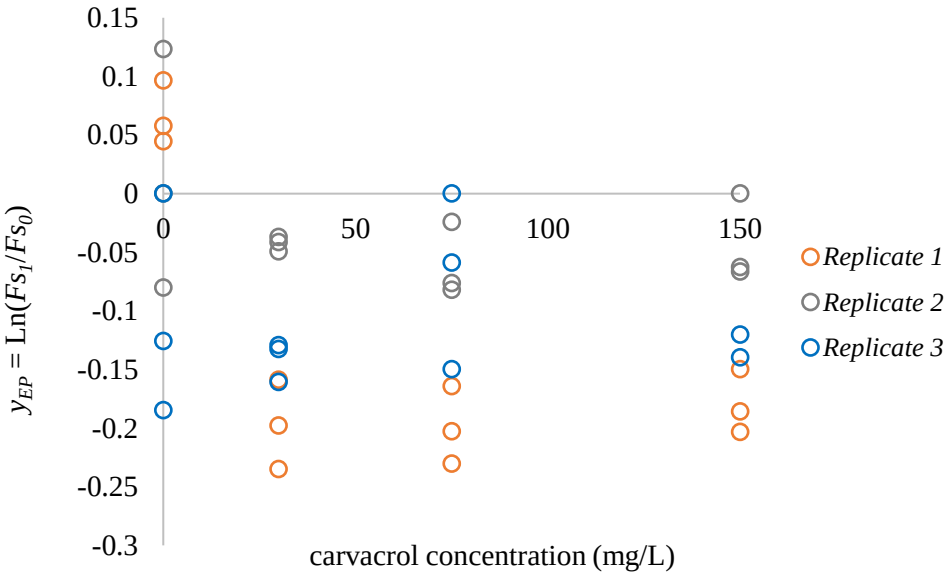

Fig. 30. The concentration dependent effect of carvacrol on EtBr fluorescent signal intensity

5.4.3 Primary model of EP inhibition and membrane integrity

To investigate whether the efflux activity and membrane integrity of *E. coli* are influenced by carvacrol treatments and by the different growth phases, cells were collected after 0.5, 4, and 14 h incubation. Carvacrol treatments were added to the PBS washed cultures, which were diluted to the same concentration (OD=0.2).

The primary models describe how F_S changes over 1 h intervals. The readings set in after about 5 minutes, therefore the data were modelled after only 0.08 h. For clarity and easier comparison, both types of carvacrol-treated F_S curves are shifted to a common starting point with non-treated F_S curves (Fig. 31 and Fig. 32).

Fig. 31 shows the EtBr accumulation in *E. coli* ATCC 11229, derived from the slow-growing phase. The applied carvacrol treatments were 60, 90 and 150 mg/L carvacrol (which correspond to 0.2, 0.3, and 0.5 MIC values). The results of the positive (non-treated culture) and negative (uninoculated sample) controls are also shown to demonstrate that the bacteria had strong intrinsic efflux (EtBr could not accumulate inside the cells, F_S values did not increase over the time) but even the lowest concentration of carvacrol, 60 mg/L, improved the EtBr accumulation rate.

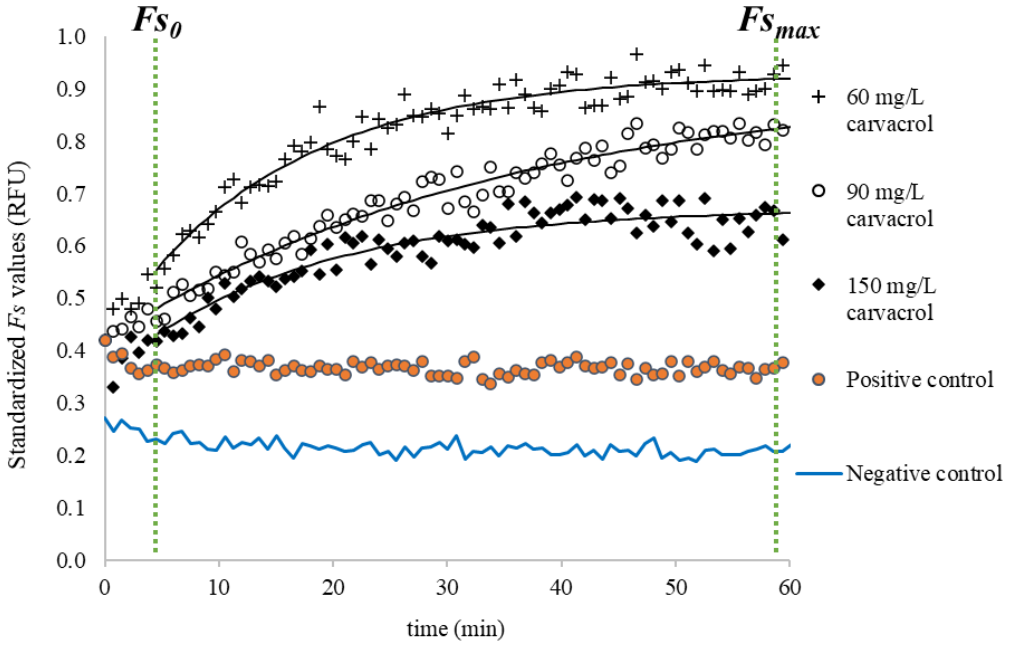

Fig. 31. Saturation model fitted to the standardized F_S curves, demonstrating the accumulation of EtBr at 0.2, 0.3 and 0.5 MIC carvacrol treatments, in slow-growing culture of *E. coli* ATCC 11229. As a negative control, F_S values were measured in uninoculated suspension (in PBS) while the non-treated culture was the positive control.

Symbols represent the experimental values and the black continuous lines show the saturation models fitted to the temporal curves. In the absence of carvacrol treatment, the $F_S(t)$ fluorescent signals can be described as constant values though the F_{S0} and the F_{Smax} parameters increased with the amount of added carvacrol.

The membrane integrity was measured similarly to the EtBr accumulation (Fig. 32). As carvacrol increased, so decreased the membrane integrity of bacteria; this is why the primary model was chosen to be a mirror image of the saturation curve. In the resultant dissipation model the $F_S(t)$ function converges to an F_{Smin} value. As a negative control, F_S values were measured in uninoculated suspension (PBS), and the membrane integrity of heat-treated culture (80 °C, 15 min) is also shown. Non-treated cultures were used as positive controls.

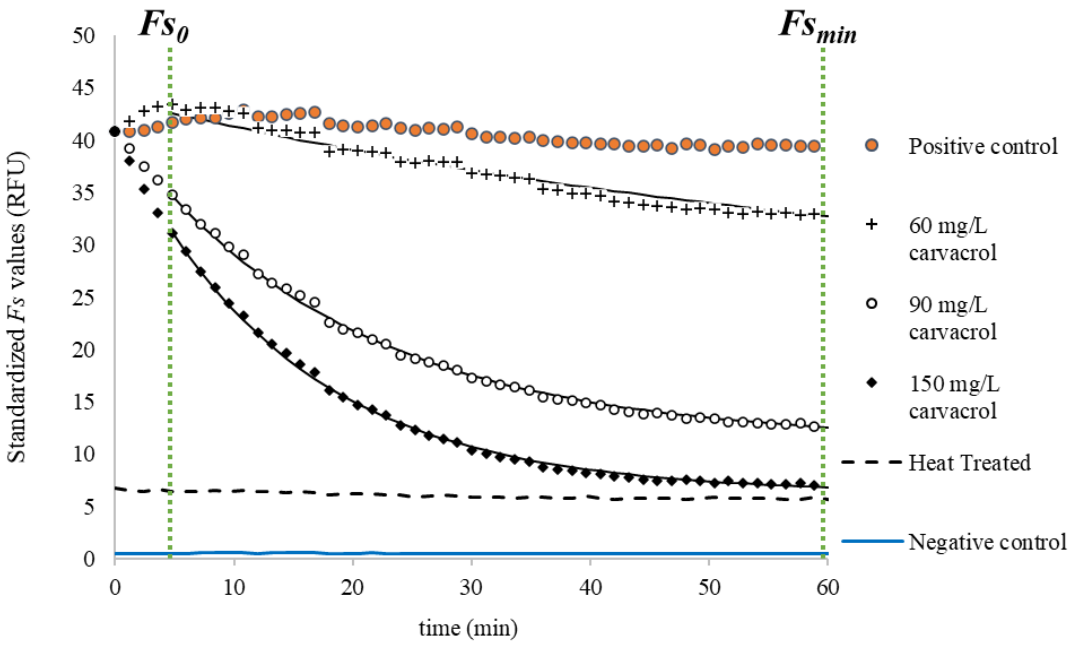

Fig. 32. Dissipation model fitted to the standardized F_S curves, demonstrating the membrane integrity of bacteria at 60, 90 and 150 mg/L carvacrol treatments. *E. coli* ATCC 11229 results are shown using data from the slow-growing phase of the culture

In both cases the ratio between the highest and lowest fitted values of $F_S(t)$ was chosen to quantify the inhibitory effect of carvacrol treatment in the secondary models. Using simply F_{Smax} or F_{Smin} parameters were not suitable response variables for secondary models.

5.4.4 Secondary model of EP inhibition

During the investigation of EtBr accumulation, two groups of the $F_S(t)$ curves could clearly be identified. The first one consisted of curves, which were generated at carvacrol concentrations below a certain level, and the EtBr accumulation rate increased with the treatment concentration. In the second group, the accumulation rate started to decrease (compared to the highest rate) with the increasing carvacrol concentration.

Based on this characteristic, it was found that the bi-linear fit represents significant improvement compared to the linear one to describe the EP inhibition as a function of carvacrol treatment. The convex-from-below model indicated the existence of an optimum. The optimum refers to the concentration of carvacrol where the EtBr accumulation is the highest so does the EP inhibition. At these points the $\ln(F_{S1}/F_{S0})$ ratio reached its highest values (Fig. 33).

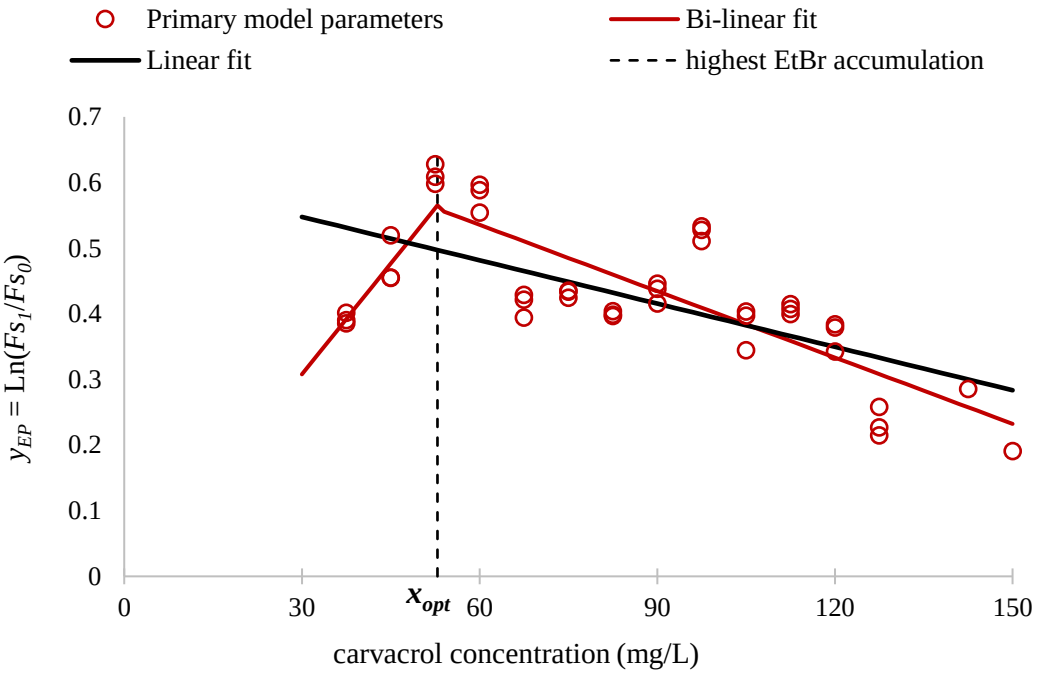

Fig. 31 Bi-linear fit represents improvement over the linear fit to describe the relationship between the primary model parameters $\ln(F_{S1}/F_{S0})$ of EtBr accumulation and carvacrol treatment

For each set of experiment two independent replicates were generated. Fig. 34 represents that the two fitted models are different (blue and red continuous lines) but the location of their maximum can be considered the same.

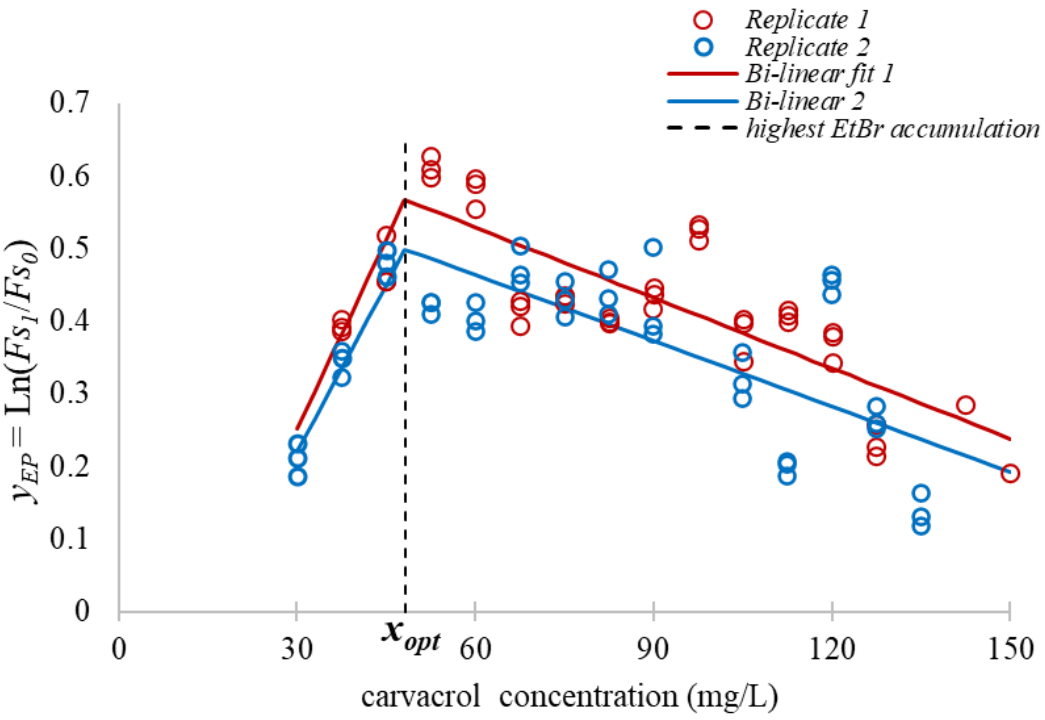

Fig. 32. Secondary model of EP inhibition. EtBr accumulation was measured in exponential phase of *E. coli* VF 3584. The figure demonstrates that the optima of two independent replicates do not differ significantly

In total, six datasets were generated *per* strain (three growth phases, two independent replicates). Secondary models of EP inhibition are shown in Fig. 35, for *E. coli* ATCC 11229.

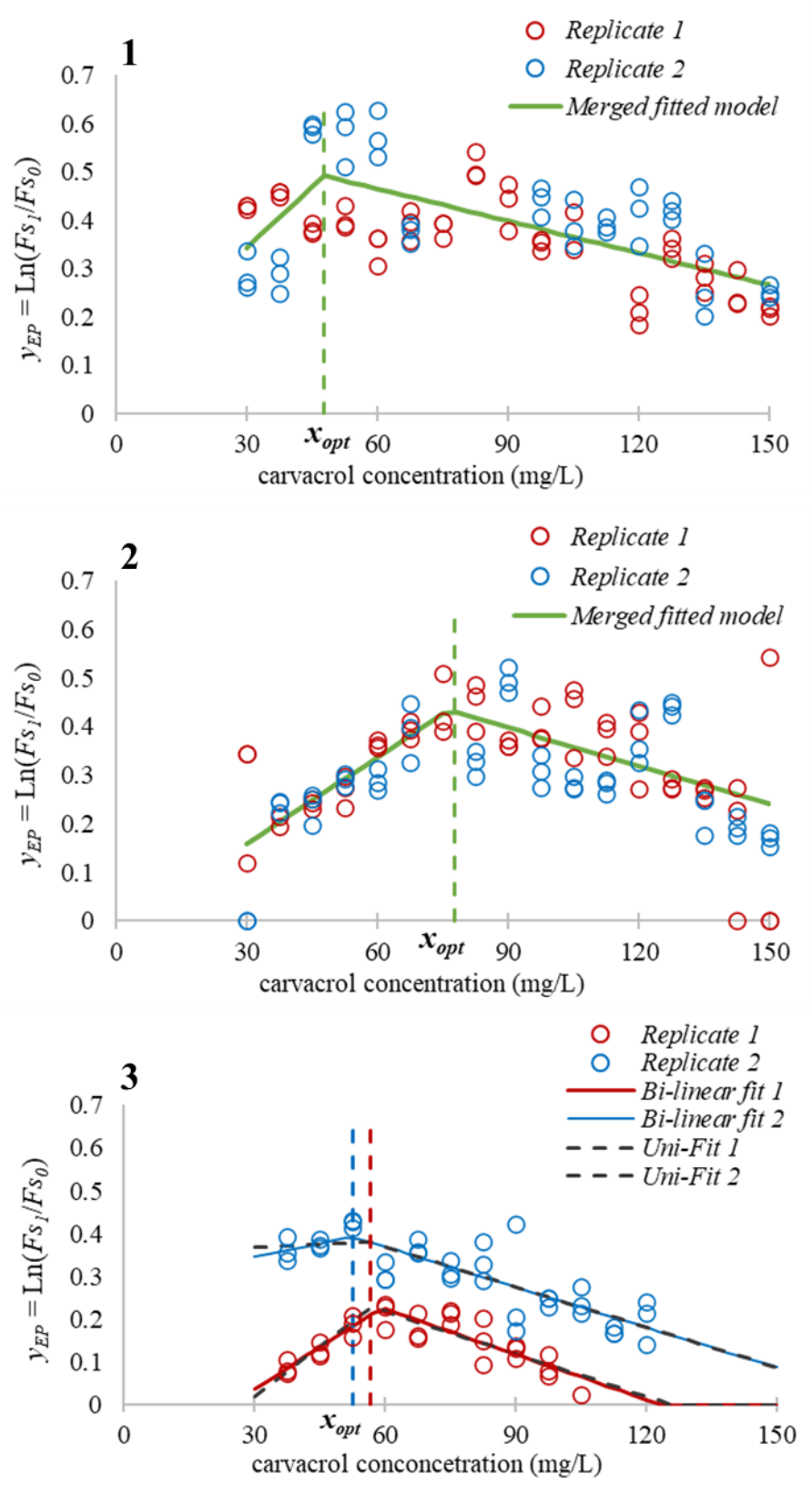

Fig. 35. Bi-linear functions as secondary models of efflux pump inhibition. Numbers indicate the growth phases; 1 - slow-growing, 2 - non-growing, 3 - fast-growing cultures of *E. coli* ATCC 11229. X_{opt} refers to the optimum inhibitor concentration of carvacrol, where the EP inhibition is the highest

On Fig. 35, the two bi-linear models of fast-growing cultures (*E. coli* ATCC 11229) could not be merged, but their optimum points were still close to each other.

In some cases, the F_S values were obvious outliers at the edges of the tested region [30 mg/L, 150 mg/L] of carvacrol concentration. In such cases, the region was narrowed, but never down to an interval that is smaller than 37.5 – 135 mg/L. The use of the bi-linear function in two cases out of the 12 regressions could not have been justified, because it did not provide significant improvement from the linear fit and these experiments were omitted from further analysis.

5.4.5 Secondary model of membrane integrity

The primary model parameter of membrane integrity measurements was simply $y_M = (F_{S1}/F_{S0})$ ratio. It was used to quantify the membrane damage caused by carvacrol. The secondary model of membrane integrity measurements showed a monotone decreasing function, without an optimum. Therefore assuming, the higher the carvacrol concentration, the lower the membrane integrity (Fig. 36).

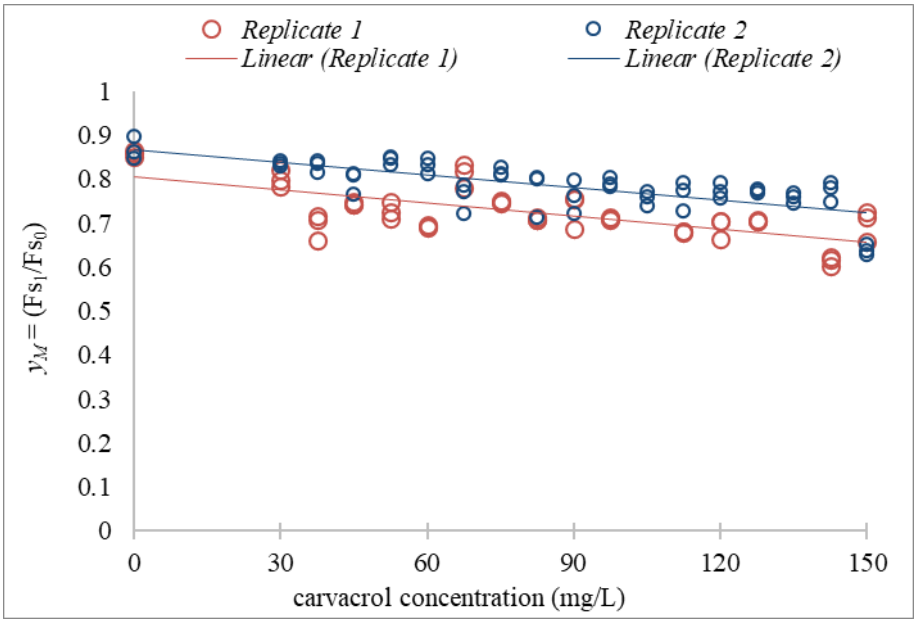

Fig. 36. Linear function fitted to the secondary model of membrane integrity readings according to different concentrations of carvacrol. Results are obtained from the non-growing phase of *E. coli* VF 3584

To express the membrane degradation effect of carvacrol in percentage, y_M values were multiplied by 100 and corrected with the average relative decrease/increase of F_S. It was calculated from the fitted points with the assumption that the membrane integrity of the non-treated cells should be 100%.

5.4.6 Tertiary models: the dependence of secondary model parameters on the growth phase of the culture

The aim of tertiary modelling was to find whether the secondary model parameters of EtBr accumulation (maximum point of y_{EP} values) depended on the growth phase of bacteria. The optimum EPI concentrations of carvacrol (x_{opt}) are shown in Table 8.

Growth phases had significant effect on the optimum EP inhibitor concentration of carvacrol but only if the comparison also included the non-growing cultures. The strain-variability was not significant, however in case of the non-growing cultures it was.

Table 8. Tertiary modelling: Optimum EP inhibitor concentrations of carvacrol (mg/L) against *E. coli* strains and the associated membrane damages (%) as a function of the bacterial growth phases

	***E. coli* ATCC 11229**		***E. coli* VF 3584**	
	Optimum EPI concentration of carvacrol (mg/L)	Membrane damage	Optimum EPI concentration of carvacrol (mg/L)	Membrane damage
Fast-growing phase	55.5 ± 2.4 *	22%	44.7 ± 4.2	10%
Slow-growing phase	47.7 ± 3	21%	52.5 ± 2.7	18%
Non-growing phase	76.2 ± 2.7	18%	93.3 ± 2.1*	11%

** Asterix shows where the optimum concentrations for the two strains are significantly different*

Table 8 summarizes the results of EP inhibition's secondary models, where F-test decided whether the two independent datasets can be merged. An estimate for the optimum was defined at the breakpoint of a bi-linear function. For the conditions, which are denoted by *, the two datasets could not be merged; however, the optimum carvacrol values defined by the two replicates that did not differ significantly ($p>0.05$). Table 8 as well as Fig. 35 demonstrate that the highest carvacrol concentrations to inhibit the efflux of bacteria measured for non-growing cultures.

5.4.7 Growth-phase-induced EP activity in the absence of carvacrol

To describe the growth history-related EP mechanism of the non-treated *E. coli* cultures, the total variation of y_{EP} values (primary model parameters of EtBr accumulation) was examined as a function of the growth phases of bacteria (Fig. 37). The bacterial physiological state was divided into three categories: fast-, slow- and non-growing phase. As a negative control, data of uninoculated suspension (EtBr accumulation measured in PBS) are shown as well.

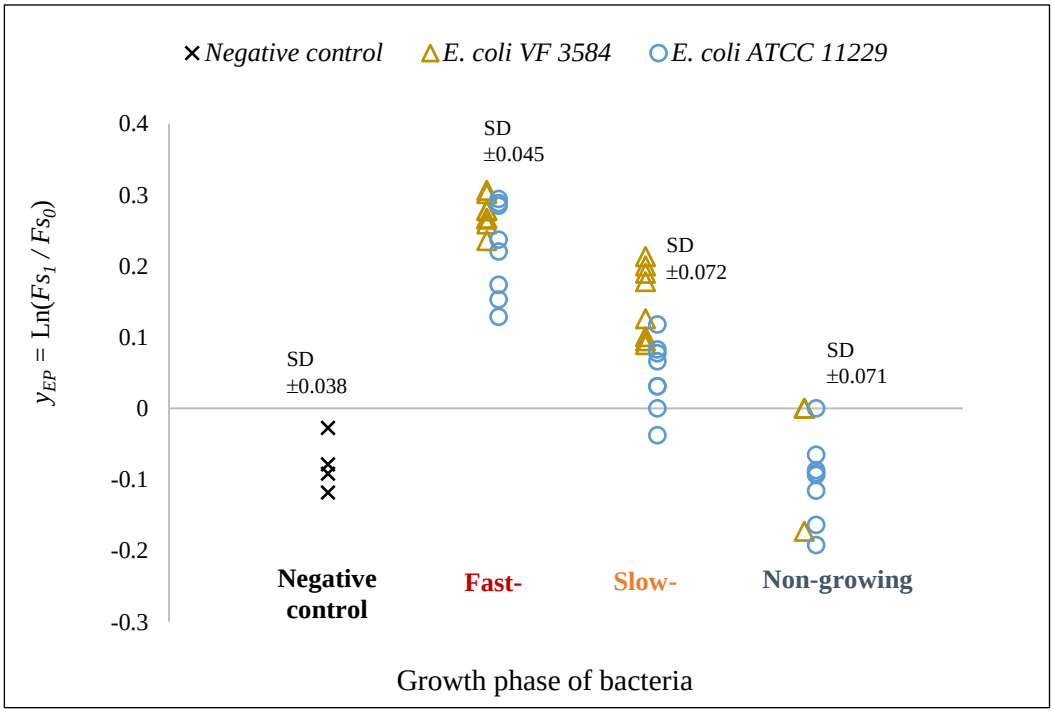

Fig. 37. y_{EP} values of non-treated cultures to describe their intrinsic, growth-phase-related efflux activity

The observations suggest that the EP activity of bacteria strongly depends on their history. In non-growing phase, the y_{EP} values showed a similar pattern to the negative control, only the noise was bigger in the former case. It means that EtBr could not accumulate in these bacteria. No increase was found between the F_{S0} and F_{Smax} values over one hour, which suggests a strong resistance mechanism in the non-growing cultures. This result confirms our expectation that the bacterial resistance mechanism is weaker in the fast- and slow-growing phases than in the non-growing phase. Moreover, in the first two stages, lower carvacrol concentrations were enough to reach the greatest efflux inhibition. The fast- and slow-growing phases are critical points for bacteria, focusing on adaptation and replication. In the non-growing phase, the

resistance mechanism of bacteria is well developed, their cell wall can become less permeable compared to the other two phases and the cells are more stressed.

Metris et al. (2014) showed that the bacterial response to a stress can be coupled to other stresses. The authors found that another stress response was also detectable in the regulatory network of the cells under osmotic stress. At boundary osmotic stress level (4.5% NaCl), the expression of some genes, encoded proteins used in the synthesis of siderophores is increased, which indicated by the expanded import of iron. This finding can be an interpretation of our finding as well. Better understanding of the physiological state induced resistance is a key point in the control of antimicrobial resistance.

5.4.8 Efflux modulation activity of synthetic inhibitors

NMP (synthetic inhibitor) concentration dependent efflux modulation activity was also measured and evaluated similarly to carvacrol. EtBr accumulation was measured at 0, 100, 200 and 300 mg/L NMP treatments in stationary phase cultures. In the primary models, saturation functions could describe the temporal F_S values obtained via EtBr accumulation assays. In the secondary models, to describe the y_{EP} values as a function of NMP treatment, it was found repeatedly that the bi-linear fit represents a significant improvement compared to the linear one (F=5.295, p=0.012 for *E. coli* ATCC 11229 and F=14.908, $p=7*10^{-5}$ in the case of *E. coli* VF 3584) to describe the EP inhibition. However, as shown by Fig. 38, because of the four measurement points (concentrations of NMP) and the four parameters of bi-linear fit (x_{min}, x_{opt}, x_{max}, y_{opt}), the model indicated only a range where the optimum inhibitor concentration of NMP exists and not the exact value of it. Thus, we may assume that to estimate the optimum efflux inhibitor concentration of a component using the currently introduced evaluation approach, at least five concentrations of the tested component should be used.

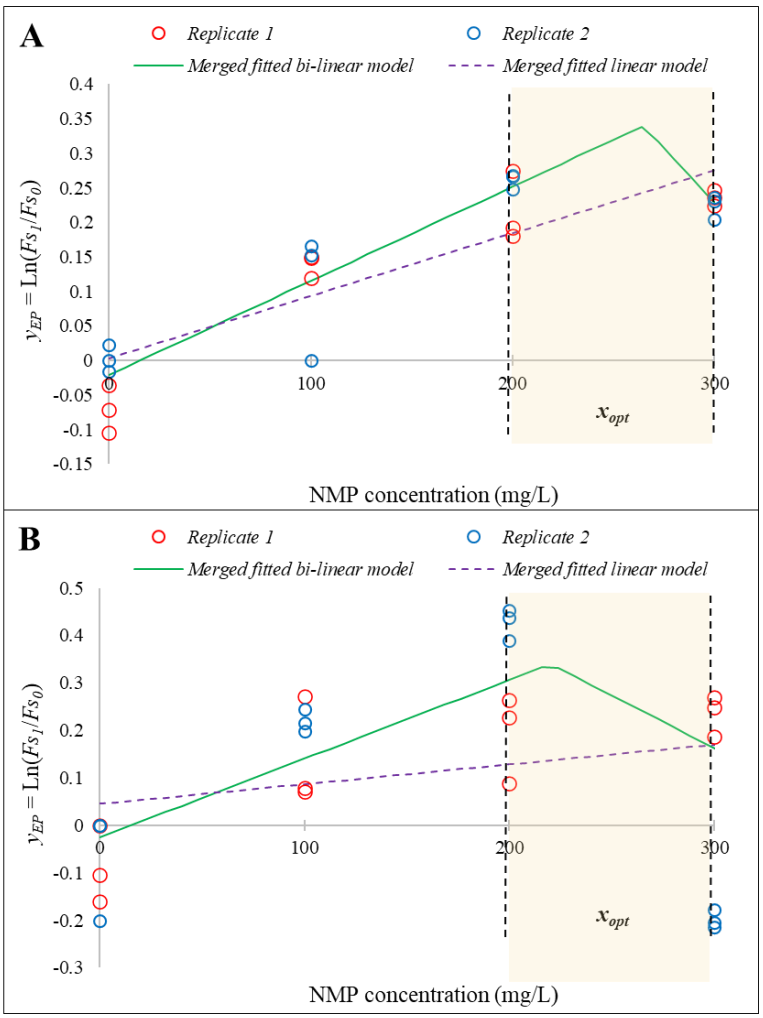

Fig. 38. Bi-linear functions as secondary models of efflux inhibition via a synthetic inhibitor. Bi-linear fit indicates the range of optimum EPI concentration of NMP and represents improvement over the linear fit to describe the EtBr accumulation via the relationship between y_{EP} values and NMP treatment. The figure demonstrates two independent replicates with *E. coli* VF 3584 (A) and *E. coli* ATCC 11229 (B) stationary phase cultures

The optimum inhibitor concentrations of NMP did not differ significantly among the strains, but presumably it is lower for *E. coli* ATCC 11229 as it also required lower inhibitor concentration from carvacrol compared to *E. coli* VF 3584. PaβN was tested only in 22 mg/L concentration. To make comparable the inhibitor efficacy of carvacrol with synthetic inhibitors, Fig. 39 represents the relative y_{EP} values. The relative y_{EP} values were the difference of the highest and lowest primary model parameters of cultures, calculated as: $y_{EP\ at\ optimum\ treatment}$ - $y_{EP\ of\ untreated\ cultures}$.

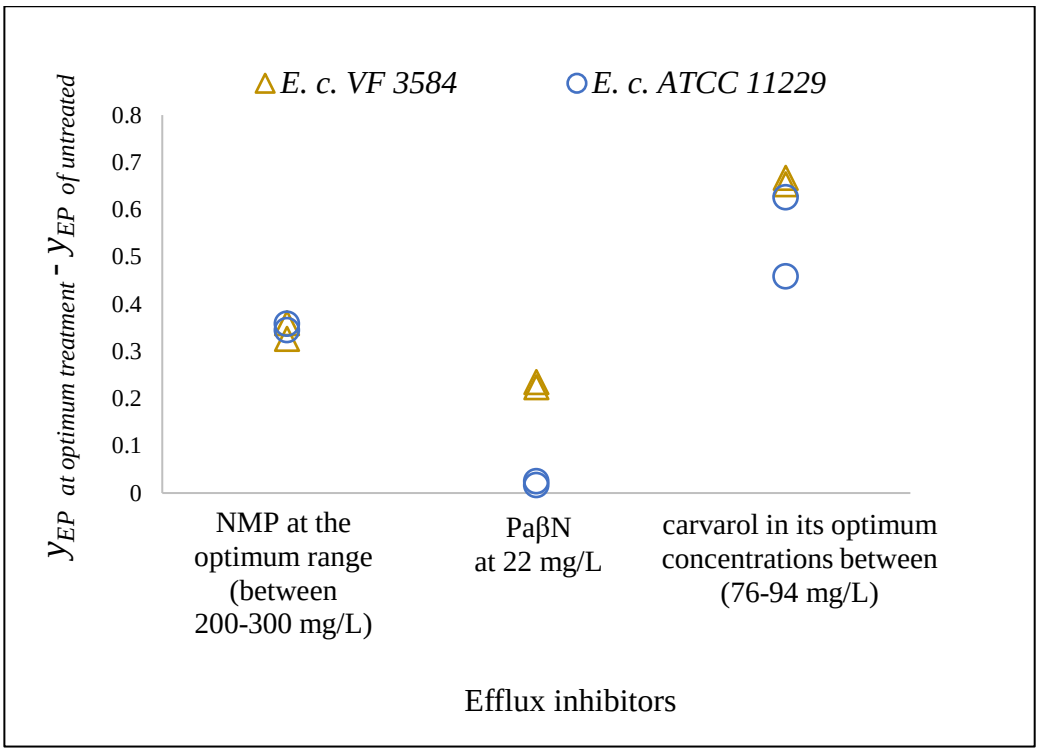

Fig. 39. Relative y_{EP} values to compare the inhibitors' efflux modulation efficacy. Results of stationary phase cultures are represented in two independent replicates for *E. coli* VF 3584 and *E. coli* ATCC 11229

Results suggest that the efflux modulation activity of both NMP and PaβN were lower than the inhibition effect of carvacrol. At the optimum carvacrol treatment, the highest y_{EP} values which infer to the greatest inhibition on the accumulation of EtBr. PaβN seemed to be the least effective efflux inhibitor against these two *E. coli* strains, however this component was tested in the lowest concentration as well.

5.5 Antimicrobial Susceptibility Testing

Carvacrol proved its efflux modulation effect via EtBr accumulation experiments. Furthermore, the region of interest in its optimum inhibitor concentration seemed to be around 50 and 95 mg/L. Thus, the aim of antimicrobial susceptibility testing was to give a practical insight and measure the efficacy of carvacrol (at 75, 90 and 105 mg/L concentrations) with antibiotics. The five tested antibiotics differed not only in their mode of actions but in their molecular sizes as well. Sub-MICs of antibiotics were combined with sub-MIC carvacrol treatments. As a control, the effect of antibiotics at all factors levels were measured without carvacrol. The developed layout (with the aim to have the lowest number of commands) of plates is shown in Fig. 40. The robot delivered 50 µL of antibiotic suspension, twofold serial diluted in Mueller-Hinton

Broth, into the columns of 1 – 10, then 50 µL bacterial culture (*ca*. 10^6 CFU/mL) into each well. The antibiotic concentrations were halved by columns and the MIC of the measured antibiotic was set in the second column. In the last step, the device was programmed to dispense 1 µL carvacrol stock solution (prepared in EtOH) to reach its final concentration *per* well: in row *A* and *B*, 105 mg/L; in *C* and *D*, 90 mg/L and in row *E* and *F*, 75 mg/L. Into the row of *G* and *H* no carvacrol was added, therefore only the MIC of antibiotic was measured. Each tray had 8 growth-control and 8 uninoculated wells (columns 11 and 12).

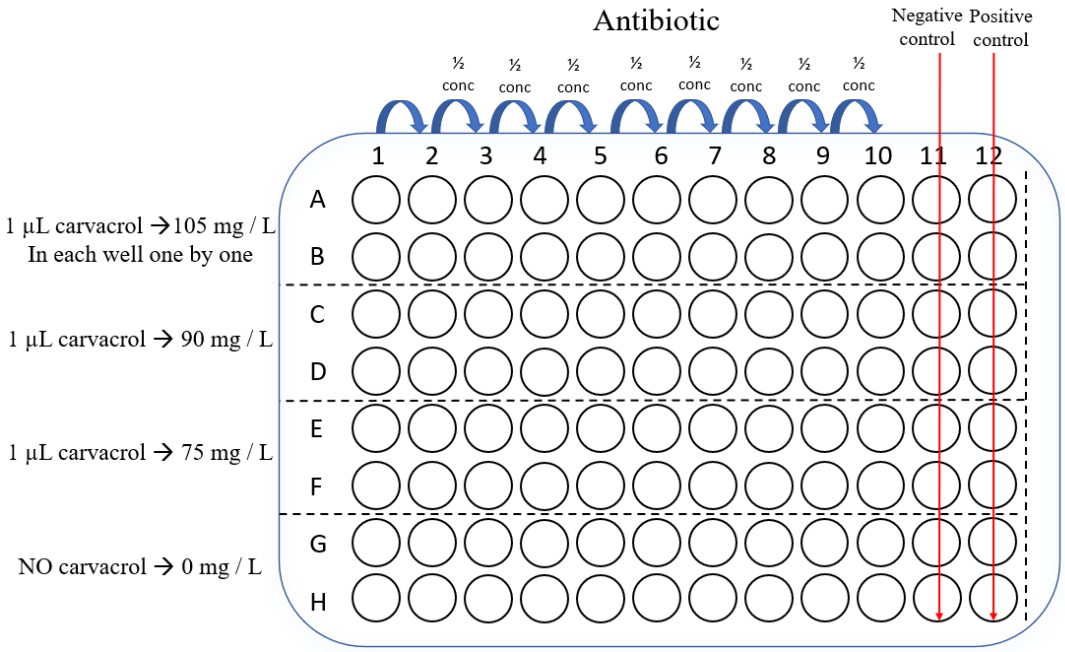

Fig. 40. The developed layout of the microtiter plates prepared for antimicrobial susceptibility testing

Even if the MIC value of an antibiotic was delivered to the wells (and wells did not become turbid), the OD values of antibiotic-containing wells differed from the OD values of uninoculated wells (negative control). Such difference was also highlighted in the EUCAST protocol (European Committee on Antimicrobial Susceptibility Testing 2021). Thus, during data management, results, obtained from the first and second columns (Fig. 40) of the microtiter plates, were used as negative controls.

Multivariate statistical methods were used to assess the multiple factors' effects simultaneously, such as the response of different *E. coli* strains, the effect of five antibiotics and combined carvacrol treatment.

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 significant responses from different strains (Table 9). The least difference between the antibiotics can be made in their lowest concentration, at 0.0625 MIC.

Table 9. Treatment combinations that did not cause significant difference between the responses of strains

Treatment combinations		**Test values of not significant treatments**	
Antibiotic	**carvacrol mg/L**	**F (4;38)**	***p***
0.25 MIC	90	2.34	0.079
0.125 MIC	75	2.44	0.069
	90	1.81	0.154
0.0625 MIC	75	2.10	0.107
	90	1.37	0.27
	105	1.99	0.122

The effect of strains 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 ($F(2;38)=3.319$, $p=0.05$). However, the factor interactions were still significant in six cases, shown in Table 10.

Table 10. Cases where the two factors' (strains and antibiotics) interaction are significant

Treatment combinations		**Test values of significant factors' interaction**	
Antibiotic	**carvacrol mg/L**	**F (8;38)**	***p***
0.5 MIC	105	2.57	0.030
0.25 MIC	0	6.89	<0.001
	75	4.25	0.020
	90	2.45	0.037
0.125 MIC	0	3.99	0.003
0.0625 MIC	0	2.26	0.051

Overall, MANOVA test was significant, therefore DA was used to find the highlighted points. The applied classifications maximized the difference between the strains or antibiotics (five groups).

5.5.1 Classification of *Escherichia coli* strains in terms of their response to combined treatments

The Wilks' lambda gives information about at which percent the variance of the grouping variable is unexplained by the dependent variables, thus the lower test value is the better. Significance of it can be tested by the Chi-square test. In case of not significant result ($p>0.05$), the discriminant function does not differ significantly.

Tests of the dimensionality of DA indicate that out of the five dimensions (five antibiotics), the bacterial responses to some treatment combinations are statistically not significantly different at factor levels. The treatment combinations, where difference between the strain responses could not have been observed, are listed in Table 11.

Table 11. Treatment combinations (MIC proportion of antibiotics) where the growth of *E. coli* strains did not differ significantly

		Test of function 1 through 2		Test of function 2	
MIC prop.	**Antibiotics**	**$\chi 2$ (*df*=8)**	***p***	**$\chi 2$ (*df*=3)**	***p***
0.5	erythromycin	11.62	0.169	0.59	0.899
0.25	cefotaxime	12.7	0.123	3.80	0.285
	gentamicin	13.54	0.095	4.10	0.250
	vancomycin	12.52	0.128	<0.00	>0.999
0.125	cefotaxime	15.04	0.058	3.20	0.360
	ciprofloxacin	7.40	0.495	<0.00	>0.999
	gentamicin	7.00	0.54	2.30	0.516
0.0625	cefotaxime	9.10	0.337	1.60	0.655
	erythromycin	13.80	0.087	1.60	0.662
	gentamicin	5.80	0.673	1.20	0.754
	vancomycin	4.50	0.613	<0.00	>0.999

The highest differences between the strains could be observed at 0.5 MIC antibiotic level, as also highlighted by MANOVA.

Table 12 summarizes the classification power of DA, when the responses of strains were analyzed separately.

Table 12. Classification power of DA at different MIC proportions of antibiotics, when the factor variable was the strains

prop. of MIC	**How many % of the original grouped cases are correctly classified**				
	cefotaxime	**ciprofloxacin**	**erythromycin**	**gentamicin**	**vancomycin**
0.5	100	66.7	100	100	66.7
0.25	88.9	66.7	100	100	66.7
0.125	100	66.7	100	100	66.7
0.0625	77.8	66.7	100	66.7	66.7

5.5.2 Classification of combined treatments in terms of their efficacy against *Escherichia coli* strains

Tests of the dimensionality of DA indicate that out of the three dimensions (*E. coli* strains), in how many cases was not possible to differentiate between the groups of antibiotics. The strains are listed in Table 13. In case of *E. coli* ATCC 11229, the antibiotics' effects were indistinguishable at all the treatment levels. In the growth of *E. coli* ATCC 25922, significant differences were obtained if the drugs were added in 0.5 MIC or 0.25 MIC concentrations. The highest differences between the antibiotics' effects were measurable with the food isolate.

Table 13. Antibiotics treatments levels, where some *E. coli* strains' responses did not differ significantly through the groups of antibiotics

prop. of antibiotic MIC	***E. coli* strain**	**Test of function 1 through 4 (*df*=16)**	**Test of function 2 through 4 (*df*=9)**	**Test of function 4 through 4 (*df*=4)**	**Test of function 4 (*df*=1)**
0.5	ATCC 11229	$\chi2$= 24.81 *p*=0.73	$\chi2$= 11.54 *p*=0.24	$\chi2$=4.54 *p*=0.338	$\chi2$= 1.57 *p*=0.211
0.25	ATCC 11229	$\chi2$=21.10 p=0.18	$\chi2$= 5.63 *p*=0.78	$\chi2$= 1.21 *p*=0.88	$\chi2$=0.003 *p*=0.96
0.125	ATCC 11229	$\chi2$= 26.15 *p*=0.05	$\chi2$=8.57 *p*=0.48	$\chi2$= 1.14 *p*=0.89	$\chi2$= 0.05 *p*=0.82
	ATCC 25922	$\chi2$= 21.22 *p*=0.17	$\chi2$=10.69 *p*=0.3	$\chi2$=3.89 *p*=0.42	$\chi2$=0.48 *p*=0.49
0.0625	ATCC 11229	$\chi2$=18.80 *p*=0.28	$\chi2$=6.58 *p*=0.68	$\chi2$=0.86 *p*=0.93	$\chi2$=0.04 *p*=0.83
	ATCC 25922	$\chi2$=16.60 *p*=0.41	$\chi2$=4.36 *p*=0.89	$\chi2$=0.12 *p*=0.99	$\chi2$=0.01 *p*=0.93

The goodness of DA classification when the effects of five antibiotics were analyzed separately. The lowest separation power of DA was observed at the lowest (0.0625 MIC) antibiotic treatment level (Table 14).

Table 14. Classification power of DA at different antibiotic MIC proportions, when the factor variable was the type of antibiotics

***E. coli* strains**	**Rate of correctly classified original grouped cases (%)**			
	0.5 MIC	**0.25 MIC**	**0.125 MIC**	**0.0625 MIC**
ATCC 25922	92.9	71.4	78.6	50.0
ATCC 11229	73.3	60.0	80.0	53.3
VF 3584	86.7	93.3	86.7	73.3

The sub-MIC concentrations of antibiotics' effect on the bacterial growth is shown in Fig. 41.

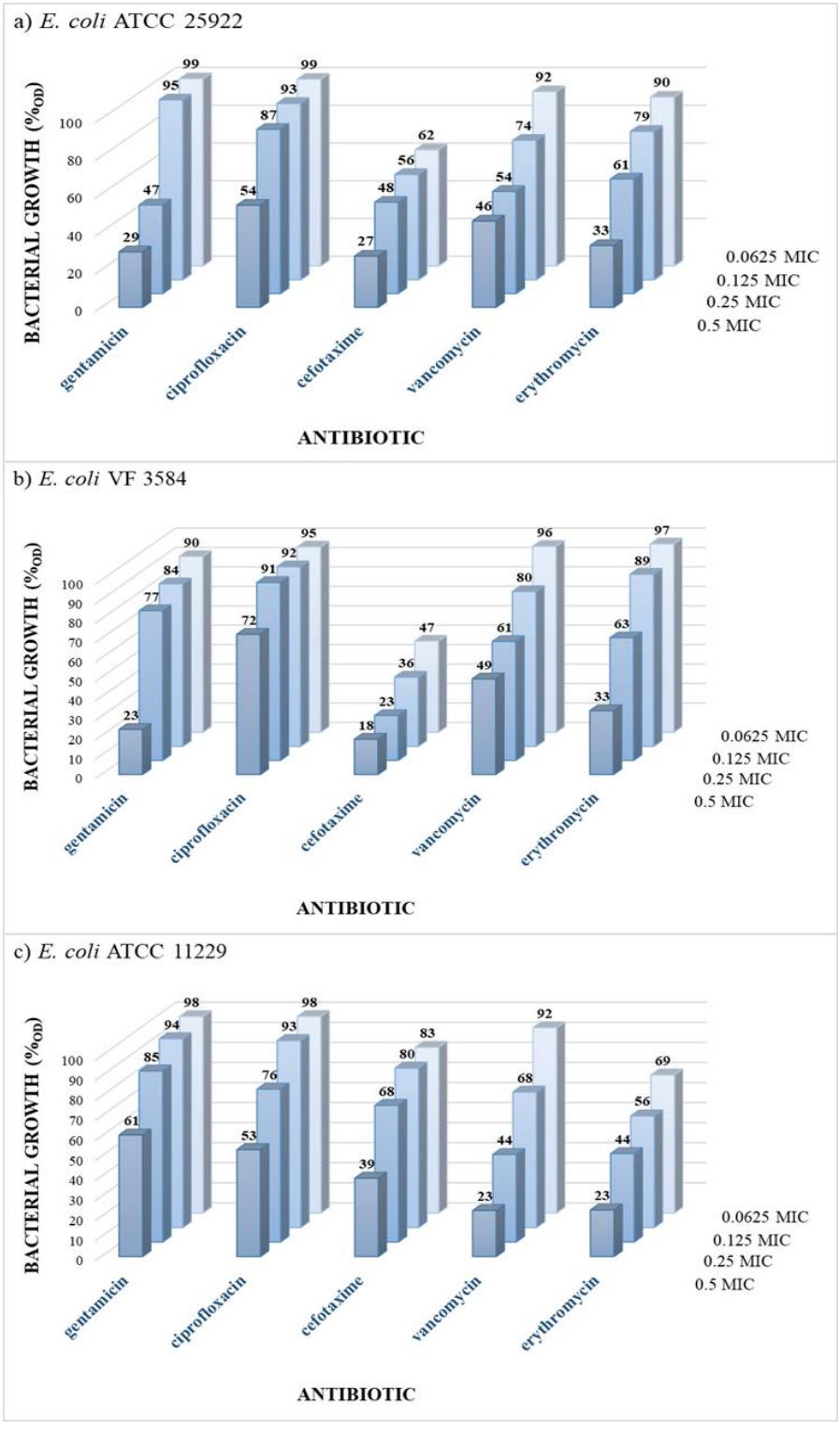

Fig. 41. The bacterial growth (%) depending on sub-MIC of antibiotics in case of three *E. coli* strains (a – ATCC 25922, b – VF 3584 and c – ATCC 11229)

Finally, the results of the combined treatments are represented on Fig. 42. The balloon plot contains four variables: the concentration of carvacrol and antibiotics, the *E. coli* strains and the groups of antibiotics. The size and the color of circles also give information about the inhibition rate (%) of treatments. If there were no added

carvacrol to the sub-MIC of antibiotics, the growth inhibition is considered to be 0%, not counting with the effect of sub-MICs of antibiotics. Inhibition rate describes that by how many percent is the growth inhibition higher when combined treatment used, compared to the singular antibiotic treatment. The median value of three independent replicates was used to analyze the inhibition rate in case of each strain (Fig. 42).

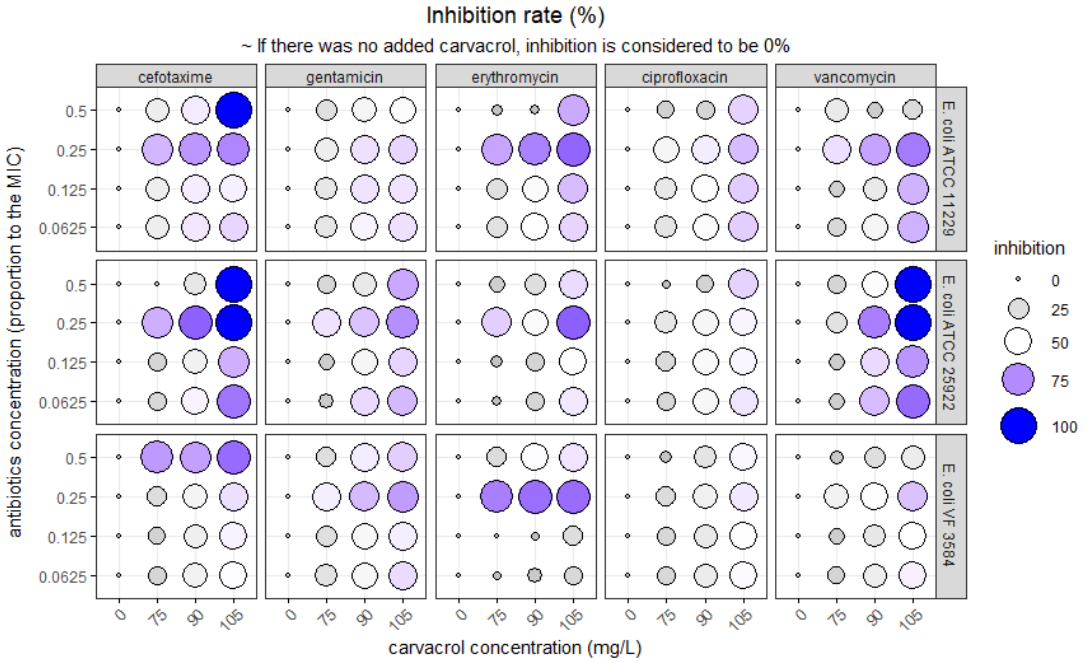

Fig. 42. Balloon plot, indicating the bacterial growth inhibition rate (%) induced by combined treatments. The four variables: (i) carvacrol concentration (axis x), (ii) antibiotic concentration (axis y), (iii) bacteria (rows) and (iv) the group of antibiotics (columns). Markers' size and color illustrate the growth inhibition

There are obvious differences between the types of antibiotics. The source of variance can be their mode of action and their molecular sizes as well. Clearly, 90 and 105 mg/L of carvacrol treatments were more effective than the 75 mg/L. These carvacrol concentrations decreased the bacterial growth, to a rate below 25% in many cases. Moreover, in some cases the highest applied carvacrol concentration (105 mg/L) reduced the bacterial growth to 0%, indicating a reduction in the MIC value of antibiotics. To rank the antibiotics, according to which uptake was helped the most by 105 mg/L carvacrol, the term inhibition rate was introduced.

Based on the inhibition rate values, it is feasible to rank the antibiotics as which one's antimicrobial activity was the highest with the combination of 105 mg/L carvacrol.

Antibiotics were ranked at the levels of 0.5 and 0.25 MIC, with "1" referring to the highest achieved inhibition and "5" to the lowest out of the five groups (Table 15).

Table 15. Ranking the antibiotics based on the efficacy of combined carvacrol (105 mg/L) treatment using the inhibition rate (%) values

Ranking the antibiotics based on their increased activity by 105 mg/L carvacrol						
Antibiotic level	***E. coli* strains**	**1**	**2**	**3**	**4**	**5**
0.5 MIC	ATCC 25922	cefotaxime ≈ vancomycin		gentamicin	ciprofloxacin	erythromycin
	VF 3584	cefotaxime	gentamicin	erythromycin	ciprofloxacin	vancomycin
	ATCC 11229	cefotaxime	erythromycin	ciprofloxacin	gentamicin	vancomycin
0.25 MIC	ATCC 25922	cefotaxime ≈ vancomycin		gentamicin	erythromycin	ciprofloxacin
	VF 3584	erythromycin	gentamicin	vancomycin	cefotaxime	ciprofloxacin
	ATCC 11229	erythromycin	vancomycin	cefotaxime	ciprofloxacin	gentamicin

In case of *E. coli* ATCC 25922, carvacrol worked best with cefotaxime, gentamicin and vancomycin. Similarly, the gentamicin and erythromycin were among the best three combinations in case of *E. coli* VF 3584. In case of the clinical isolate, the activity of cefotaxime and erythromycin increased at both sub-MIC levels by the 105 mg/L carvacrol. At lower (0.125 and 0.0625) sub-MIC concentrations of antibiotics, the treatment of 105 mg/L carvacrol increased their activity by an average of 50 to 60%.

Same ranking was done to evaluate the effect of 90 mg/L carvacrol (Table 16) in the combination with 0.5 and 0.25 MIC of antibiotics. Results were very similar as before, as in the case of *E. coli* ATCC 25922 cefotaxime, gentamicin and vancomycin growth inhibition rate was increased best by carvacrol, in the case of *E. coli* VF 3584 the inhibition effect of gentamicin and erythromycin increased significantly and in case of *E. coli* ATCC 11229 the effect of cefotaxime increased intensively. Using the 90 mg/L carvacrol treatment together with 0.125 and 0.0625 MIC of antibiotics, an average of 40% higher inhibition could be achieved compared to the singular antibiotic treatments.

Table 16. Ranking the antibiotics based on the efficacy of combined carvacrol (90 mg/L) treatment using the inhibition rate (%) values

Ranking the antibiotics based on their increased activity by 90 mg/L carvacrol						
Antibiotic level	***E. coli* strains**	**1**	**2**	**3**	**4**	**5**
0.5 MIC	ATCC 25922	vancomycin	gentamicin	cefotaxime	erythromycin	ciprofloxacin
	VF 3584	cefotaxime	gentamicin	erythromycin	ciprofloxacin	vancomycin
	ATCC 11229	cefotaxime	gentamicin	ciprofloxacin	vancomycin	erythromycin
0.25 MIC	ATCC 25922	cefotaxime	vancomycin	gentamicin	erythromycin	ciprofloxacin
	VF 3584	erythromycin	gentamicin	vancomycin	cefotaxime	ciprofloxacin
	ATCC 11229	erythromycin	cefotaxime	vancomycin	gentamicin	ciprofloxacin

To summarize the efficacy of combined sub-MIC treatments, three factors need to be considered: (i) the mode of action of antibiotics, (ii) the molecular size of antibiotics and (iii) the concentration of carvacrol. It is obvious that using 90 or 105 mg/L carvacrol concentrations together with sub-MIC antibiotics is much more effective than the combination with 75 mg/L carvacrol. Therefore, the activity of 75 mg/L carvacrol was not studied further.

The activity of vancomycin in the presence of carvacrol increased highly but only against *E. coli* ATCC 25922. This strain was sensitive to carvacrol (lower MIC and highest lag time extension), thus probably the treatments of 90 and 105 mg/L carvacrol permeabilized the outer membrane of *E. coli* ATCC 25922, thus the large-scaffold vancomycin could penetrate into the cells thanks to the compromised outer membrane.

For most part, it can be said that carvacrol worked best in combination with cefotaxime, gentamicin and might be erythromycin as well. The molecular weight of cefotaxime and gentamicin are less than 600 Da while the molecular weight of erythromycin is a little bit higher (734 Da). Cefotaxime inhibits the bacterial cell wall biosynthesis, gentamicin inhibits bacterial protein synthesis by binding to 30S ribosomal subunit and erythromycin binds to 50S ribosomal subunit. The efflux mechanism is a membrane-based system within the periplasm and in the cytoplasmic membrane of Gram-negative bacteria with the efflux proteins located in the outer membrane. Since we know that this mechanism is affected by carvacrol (explained by the results of EtBr accumulation and membrane integrity experiments) it is not

surprising that carvacrol worked best with bacterial membrane and protein synthesis inhibiting antibiotics.

Among combined treatments, the least effective was the combined use of carvacrol with ciprofloxacin. Ciprofloxacin, inhibiting DNA synthesis, has the smallest molecular weight among the tested antibiotics. Nevertheless, the inhibition rate of ciprofloxacin was still increased with an average of 50% in the combination with 105 mg/L carvacrol.

6 CONCLUSIONS AND RECOMMENDATIONS

Antibiotics saved millions of lives worldwide. However, bacterial resistance has been accomplished to nearly all antibiotics. Due to the rapid development of bacterial resistance, there is a need for strategies to manage this emergent crisis. Small molecule inhibitors targeting drug efflux from bacterial cells, represent a new solution for combating antibiotic resistance. Moreover, natural volatile compounds not only have antimicrobial activity, but some of them even 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 and ampicillin (Magi et al. 2015; Langeveld et al. 2014; Ventola 2015).

Results obtained in the current thesis suggests the same that 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.

In the first part of my results, the distribution of detection times was considered as a proportional reflection of the distribution of lag times (in which the number of initial cells followed Poisson distribution). The average number of initial cells can be estimated by the proportion of negative wells, which do not become turbid (Poisson parameter denoted by ρ^*); or by the spread plate technique (results denoted by c^*). No significant difference was found between the two estimates. Thus, in further experiments it is enough to estimate Poisson parameter as the initial number of cells considering the cost and time related benefits of this method. Under optimum growth conditions, the probability of growth at single cell level can be considered as one. However, it would be interesting to (i) characterize the single cell kinetics in the presence of carvacrol treatment and to (i) compare the results to the initial cell number estimates of untreated cultures. The difference between them could be used to gain data about the effect of carvacrol on the probability of growth of a single cell. Thereby, the bacterial response could be assessed to the applied carvacrol treatment at single cell level.

In the second part, a well-known bacterial stress response, lag time prolongation was described as a function of carvacrol treatments. Carvacrol concentrations which

prolongated the lag time by 20–40%, are listed in Table 7. At these concentrations, a sharp increase in the power function can be seen as well. However, in the literature there is no information about at which % can be the increase in the lag time considered as significant. Presumably, to define the specific concentration of a treatment, which causes significant lag time prolongation, the increase in lag time duration should be somehow related to the doubling time or the specific growth rate of the microorganism. Furthermore, studies about the adaptation kinetics could be carried out.

In the third part, the fluorescent assays highlighted the efflux modulation and membrane disruption effect of carvacrol. Although, carvacrol was diluted in EtOH, the effect of solvent on the efflux activity was not investigated itself. In the study of Amaral et al. (2011), the addition of 0.2% EtOH promoted the efflux at pH 8. However, there is no monotone increasing tendency in the measured relative fluorescent values induced by the EtOH. The recorded data points return to the initial values within 20 minutes after the EtOH treatment. Based on this fact, this temporary change would not have been perceptible with the evaluation method reported in this thesis. On the other hand, PBS has a slightly lower pH value (7.4) so presumably the efflux was not promoted in this case since it only occurs at high pH when metabolic energy is required for the maintenance of efflux.

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 concentration dependent effect of carvacrol. The breakpoint of the fitted models referred to the optimum value of carvacrol which could be used as an EPI. Similarly, the optimum inhibitor concentration of a synthetic inhibitor, NMP was also presented. Even if the exact concentration of NMP could not be estimated, its optimum y_{EP} values could and were compared to the optimum y_{EP} values of carvacrol. Results suggests that carvacrol has a greater efflux inhibition effect than NMP. Besides, 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 heightened, assuming that the cells adapt to various stress factors during their growth.

As the result of membrane integrity measurements, it was shown that the increasing concentration of carvacrol, 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 colonize the epithelia of various specific hosts. Since carvacrol affects the bacterial cell membrane, additionally the morphological changes of *E. coli* – caused by carvacrol in the region of interest – could be assessed in further experiments using transmission electron microscopy (TEM) or scanning electron microscope (SEM) images to define the effect of carvacrol on the filamentous structures.

In the last part, the effectiveness of the combined treatments was quantified. Measurements were evaluated via comparing the growth inhibition activity of sub-MIC antibiotics to the inhibition efficacy of combined treatments (sub-MIC antibiotics and carvacrol). The tested antibiotics were ciprofloxacin, cefotaxime, erythromycin, gentamicin and vancomycin while carvacrol was added in 75, 90 or 105 mg/L concentrations. Carvacrol increased the bacterial drug sensitivity because the inhibitions were always greater when using the combined treatments. The natural antimicrobial agent 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 the molecular weights of them 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.

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

7 THESES – 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 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 the concentration of *ca.* 20 cells *per* well, 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 the bi-linear function depends 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:

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

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 the treatment of a lower concentration of carvacrol (64-70 mg/L), except in the case of a clinical isolate (*E. coli* ATCC 11229), where a higher concentration of carvacrol (92 mg/L) 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 with erythromycin halving in the value of MICs was also observed. Thereby, the natural agent promoted the antibacterial activity of antibiotics the most which (i) have a molecular weight lower than 800 g/mol and (ii) target the bacterial protein and cell wall synthesis.

8 SUMMARY

Antibiotic resistance is not a future threat as it has been around for a long time and is only getting stronger. Resistant bacteria lost their sensitivity to antibiotics or to the concentration of antibiotics that previously inhibited their growth. Increasing the concentration of treatments is a limited option because of the serious side effects linking to high dose of antibiotics. Bacteria are able to develop resistance via several ways such as (i) altering the sites where drugs are targeted, (ii) via enzymes that inactivate the antibiotics, (iii) decreasing the membrane permeability and (iv) by the active efflux of antimicrobials. Efflux pumps contribute to one of the major bacterial self–protection mechanisms. These systems are accountable for the spread of antibiotic-resistant bacteria via reducing the intracellular concentrations of antibiotics to sublethal levels. Thereby giving a chance to bacteria to adjust to stress factors. Most of the drug–efflux pumps are weakly expressed under normal conditions, however, due to a prolonged exposure to antimicrobial compounds, a higher-level expression is induced.

As for now, we know very little about EPIs. Using synthetic inhibitors for treating bacterial infections in human or veterinary medicine is not allowed because of the significant toxicity of inhibitors. Thus, the development of new EPI molecules can be the key for combating bacterial efflux pumps. EPIs can restore the activity of various antibiotics and control the spread and emergence of MDR bacteria. When studying the efflux mechanism, the blocking efficacy of EPIs can be accomplished through various methods. An indirect one is recording fluorescent signal indicating dye accumulation inside the bacterial cells with the dye acting as a proxy to illustrate the antibiotic accumulation. Furthermore, another common direct method is measuring bacterial susceptibility towards different antibiotics in the absence and presence of inhibitors. Carvacrol is a natural antimicrobial agent, active against pathogens and MDR bacteria. It is recognized as a safe component, but at high concentrations it targets the bacterial cell wall, but its potential efflux inhibitory effect has also been reported.

The main objective of my thesis was to build a comprehensive description about the effects of sublethal carvacrol concentration considering its influence on (i) the bacterial growth and lag time duration, (ii) EtBr accumulation, (iii) membrane integrity and (iv) the bacterial sensitivity towards antibiotics with different molecular weights and modes of action. During the experiments, three *E. coli* strains were used: a

reference (ATCC 25922), a foodborne (VF 3584) and a clinical isolate (ATCC 11229). As a preliminary part, it was justified why *E. coli* is feasible to be used as a model microorganism, even at single cell level.

Distribution of bacterial single cell lag time under optimum growth conditions

An efficient dilution method was developed, based on the recommendation of Buss da Silva et al. (2019), suitable for detecting the lag time variability of bacteria in single cells. The benefit of measuring the lag time duration of single cells is that it can be easily compared to population level data while also revealing important scenarios regarding food safety issues. *E. coli, Shigella sonnei* and *Salmonella enterica* subs. *enterica* strains; 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. By means of the Poisson assumption, the average number of initial cells *per* well was estimated by the parameter of this distribution (ρ^*), obtained via turbidity experiments from the proportion of wells which do not become turbid. On the other hand, the number of initial cells (c^*) were estimated by the traditional plate count method. Between the two estimates no significant difference was found, thus ρ^*, the faster and cost-effective estimate, can be used to assess the initial number of cells in low inoculum level. Moreover, with the newly developed dilution method, I could set very accurately the initial concentration 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 perspective, the presence of pathogenic and indicator bacteria in foods is important. Considering the individual behavior of each cell, the results may have significance in risk estimation and in calculating safe shelf life for foods. The main objective of this part was to set up an efficient method for measuring the variability among the strains.

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 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 described by lag time extension (LE) values, and the increase in lag time duration is expressed in %. It can be concluded that concentrations of 65–112 mg/L carvacrol caused an increase of 20–40% in the lag time duration. However, there were differences among the strains; the clinical isolate seemed to be the most resistant one.

Fluorescent assays to describe the 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 growth phases were identified after 0.5, 4 and 14 h incubation. The cells, tested via fluorescent assays, were used 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 a synthetic inhibitor, NMP. However, the natural agent, carvacrol 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.

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 the concentrations of 75, 90 and 105 mg/L. 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 as well. Multivariate statistical methods were used to evaluate the effect of multiple factors simultaneously. In the combined treatments, there were no dominant distinctions between the three strains' responses. However, when examining the responses of bacteria, differences were observed in terms of the five antibiotics combined with carvacrol. When the antibiotics were combined with the concentrations of 90 or 105 mg/L carvacrol, the treatments significantly reduced the bacterial growth compared to the singular antibiotic treatment. The concentration of 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 antibiotics targeting the bacterial protein and cell wall synthesis such as cefotaxime, gentamicin and erythromycin. 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, even here the inhibition rate of ciprofloxacin still increased with an average of 50% in the combination with the concentration of 105 mg/L carvacrol.

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 only a handful of studies concerning the metabolism, bioavailability of carvacrol and targeted tissues available. However, more studies on these topics are required including animal models and human studies (Sharifi-Rad et al. 2018).

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 over 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.

9 LIST OF PUBLICATIONS IN THE FIELD OF STUDY

Jánosity, A., Vajna, B., Kiskó, G., & Baranyi, J. (2022) 'Distribution of bacterial single cell parameters and their estimation from turbidity detection times', ***Food Microbiology***, 104(2022), 103972, ISSN 0740-0020, https://doi.org/10.1016/j.fm.2021.103972 , **D1**

Jánosity, A., Klančnik, A., Kiskó, G., Možina, S.S., & Baranyi, J. (2021) 'Determining optimum carvacrol treatment as a cardinal value of a secondary model', ***International Journal of Food Microbiology***, 354(2021), 109311. https://doi.org/10.1016/j.ijfoodmicro.2021.109311 , **Q1**

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Jánosity, A., Surányi, B., Ladányi, M., Kiskó, G., Baranyi, J., Burtscher, J., Domig, K. J. (2022) 'Carvacrol makes *E. coli* more susceptible to antibiotics', ***ASM Microbe***, Poster presentation, Washington, D.C., USA

Jánosity, A., Vajna, B., Kiskó, G., & Baranyi, J. (2021) 'Growth Kinetics Of Foodborne Pathogens And The Probability Of Growth At Single Cell Level'. ***World Microbe Forum***, iPoster presentation: https://wmf2021-asm.ipostersessions.com/Default.aspx?s=BF-A9-7E-99-F0-C5-78-65-AA-F6-8A-56-09-EE-E8-FA.

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10 APPENDICES

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