Chapter 3 Biocide resistance of *Listeria* in different physiological state

[3.1]. Antimicrobials and antimicrobial resistance: general background

Biocides are defined as molecules or compounds within a disinfectant product which can control the growth or kill bacteria (SCENHIR 2009). This category include organic and inorganic compounds, which can be used in food processing environments for sanitation of plants and machineries in contact with food and/or foodstuff as well as for decontaminating carcasses. Other compounds with similar activity are used in other application fields, like for veterinary use, as food preservative or for animal husbandry (SCENHIR, 2009). Some of these compounds are listed in Tab. 3.1 along with their field of usage.

Veterinary use	Disinfection of instruments and animal facilities/houses	Disinfection of transporters/trucks	Disinfection of boots and tools
Na-dichloro-isocyanurate	Na-dichloro-isocyanurate	Na-dichloro-isocyanurate	Na-p-toluene-sulfonchloramide (Halamid)
Na-p-toluene-sulfonchloramide (Halamid)	H_2O_2	H_2O_2	H_2O_2 / acetic acid
H_2O_2	Acetic acid	Acetic acid	
Acetic acid	Dideceyl-dimethyl-ammonium chloride (QAC)	Quarternary ammonium chlorides	
Quarternary ammonium chlorides	Alkyl-demethylbenzyl-ammonium chloride (QAC)	QAC + KOH	
Glutaraldehyde (in combinations)	Glutaraldehyde (in combinations)	Glutaraldehyde (in combinations)	
Formaldehyde (in combinations)	Formaldehyde (in combinations)	Formaldehyde (in combinations)	
Isopropanol (in combinations)	Isopropanol (in combinations)	Isopropanol (in combinations)	

Tab. 3.1: Mainly used biocide and their field of applications (SCENHIR, 2009).

[3.2]. Assessment of bactericidal capacity of a biocide

One of the main factors affecting the efficiency of an antimicrobial agent is consisted of applied concentration (Russell and McDonnell, 2000), whose importance is considered relevant for the definition of bacterial resistance in practice. Many reports on emerging bacterial resistance to biocides are based on the determination of the minimum inhibitory concentration (MIC). Using MIC to measure the bacterial

resistance is arguable since much higher concentrations of biocides are used in practice and, therefore, failing to achieve a reduction of bacterial numbers (i.e. lethality) because of elevated MIC is unlikely. Indeed, some studies have shown that bacterial strains showing a significant increase in MIC to some biocides were nevertheless susceptible to higher (in use) concentrations of the same biocides. For this purpose, the determination of minimum bactericidal concentrations (MBC) could be considered a more appropriate methodology (SCEHIR, 2009).

MBC determination allows to conclude if the used concentration is effectively active against tested strains as well as it is able to indicate whether a bacterial strains is insusceptible (i.e. naturally resistant) or resistant (by comparison to a standard strain). From examination of the bacterial inactivation kinetic is possible to obtain information concerning the nature of resistance within a bacterial population and/or the interaction of the biocide with the same population.

Beside MBC, the effectiveness of a biocidal agent is routinely evaluated also through standardized suspension methods, which do not simulate the growth of bacteria attached to surfaces (Wirtanen and Salo, 2004). Several guidelines and standard are produced by the European Committee for Standardization (CEN): the most recent one was the European Standard EN 1040:2006. All disinfectants examined with this protocol were considered effective, if the extent of reduction is more or equal than five logarithms with regard to the vegetative cells and one logarithm concerning the bacterial spores (Wirtanen and Salo, 2004).

Bacteria present in food processing environments are more likely to be found as biofilm, instead of planktonic state. At the moment there is no standardized method to evaluate disinfectant efficacy against bacterial biofilms, although different approaches could be found in the scientific literature. A useful tool for similar investigations could be found in biofilm constructs: poloxamer hydrogels possess demonstrated thermoreversible properties (Wirtanen, et al. 1998), because of which they appear liquid and water-soluble below 15°C and firm gels at temperatures higher than 15°C. At 30°C high bacterial densities can be cultured within the gel and, after the disinfecting treatment, the gel can be simply immersed into the neutralizer/diluents solution.

While disinfectants demonstrated higher bactericidal efficiency against microbial cell suspension, a reduction of biocide bactericidal effectiveness was proved in presence of bacterial biofilms. Of tested sanitizers, only alcohol-based products produced a three logarithmic (or even greater) reduction as well as the formulations containing hydrogen peroxides (Wirtanen and Salo, 2003). Although they did not reflect the effect of used formulations against bacterial contamination *in situ*, biofilm within poloxamer hydrogels, in spite of conventional suspension tests, efficiently discriminated the routinely used concentrations from effectively active dosages.

[3.3]. Involved resistance mechanisms to biocides

Bacterial resistance of biocides has been extensively studied from scientific literature since 1952, when the first paper on this topic was published (Chaplin, 1952). Bacterial resistance to biocides has long been reported with compounds such as: chlorhexidine (Stickler 1974); quaternary ammonium compounds (Gillespie, et al. 1986; Romao, et al. 2005); bisphenol, triclosan (Bamber and Neal, 1999; Heath, et al. 1998; Sasatsu, et al. 1993); iodophor (O'Rourke, et al. 2003); parabens (Flores, et al. 1997; Hutchinson, et al. 2004); and more reactive biocides such as glutaraldehyde (Fraud, et al. 2001; Griffiths, et al. 1997; Manzoor, et al. 1999; Nomura, et al. 2004; Van Klingeren and Pullen, 1993; Walsh, et al. 2001) and peroxides (Dukan and Touati, 1996; Greenberg, et al. 1990; Greenberg and Demple, 1989).

Biocide resistance is hypothesized to occur via mutation or acquisition of resistance determinants from extrachromosomal genetic elements (such as plasmids or trasposons) (Poole, 2002). Few authors reported as possible resistance mechanism biocide inactivation, which is limited to a restricted number of substances such as organomercurials (McDonnel and Russell, 1999; Miller, 1999). Target site mutation, changes in cell envelope permeability or presence of efflux pumps are reported as more commonly and likely reported as effectively active resistance mechanisms (Poole, 2002). Because biocides can be effective against different possible targets, a possibly involved mechanism of resistance could be a modification at the level of the target itself (Mc Donnell and Russell, 1999; Poole 2002). One of the most exploited mechanisms involves modifications of cell envelope and thus alteration of the cell permeability to biocides. This effect is reported as "permeability barrier". This mechanism leads to a limited uptake of the biocide within the cell with a consequent reduction of the effective biocide concentration (Champlin, et al. 2005; Denyer and Maillard, 2002; Lambert, 2002).

The role of the lipopolysaccharides (LPS) as a permeability barrier in Gram-negative bacteria has been well documented (Ayres, et al. 1998; Denyer and Maillard, 2002; Fraud, et al. 2003; McDonnell and Russell, 1999; Munton and Russell, 1970; Stickler, 2004). Different authors have been reported that biocide effectiveness was reduced due to changes in other components of the outer membrane ultrastructure (Braoudaki and Hilton, 2005; Tattawasart, et al. 2000a; Tattawasart, et al. 2000b) including proteins (Brözel and Cloete, 1994; Gandhi, et al. 1993; Winder. et al. 2000), fatty acid composition (Guérin-Méchin, et al. 1999; Guérin-Méchin, et al. 2000; Jones, et al. 1989; Méchin, et al. 1999) and phospholipids (Boeris, et al. 2007). The charge property of the cell surface also plays a role in bacterial resistance mechanisms to positively charged biocides such as QACs (Bruinsma et al. 2006). It is likely that bacterial resistance emerges from a combination of mechanisms (Braoudaki and Hilton, 2005; Tattawasart et al., 2000a; Tattawasart, et al. 2000b), even

though single specific mechanisms are often investigated. Other possibly involved mechanism could be enzymatic degradation of biocides such as peroxides (Valkova, et al. 2001; Demple, 2001), even if it is still not deeply exploited. Although this biological mechanism could lead to an increase in MIC, this could not involve necessarily a decrease in lethal activity of bactericidal compound (SCENHIR, 2009).

[3.4]. Biocide resistance of biofilm and involved mechanism

Biofilm could represent a possible defense against biocidal compounds, even if the protective efficacy can be affected by the examined compound: Minei et al. (2008) demonstrated that peroxyacetic acid could reduce significantly *Listeria monocytogenes* adhered on stainless steel coupons, even if not complete elimination of inoculums was reached. Sodium hypochlorite seem not to be so effective, as shown by Norwood and Gilmour (2000), where only exposure to 1000 μg/ml (or ppm in this article) free chlorine for 20 min was necessary to produce a 2 logarithmic reductive factor, while 30 s with 10 μg/ml free chlorine exhibit a 8 logarithmic reductive effect. This data is confirmed by Hellström et al. (2006), who compared peracetic acid with chlorine in water obtaining 2.1 and 1.21 logarithmic reduction, respectively. Taormina and Beuchat (2002) apply 50-100 mg/ml of cetylpyridinium to have microbial population reduction of more than 5.64 Log.

Benzalkonium chloride is reported by Romanova et al. (2007) to be 1000-fold less effective against *Listeria monocytogenes* biofilms compared to the planktonic cells. Furthermore Romanova, in same report, stated that *Listeria monocytogenes* biofilms were removed at BC concentrations greater than 10 mg/ml, even if slight re-growth was observed after exposure to 10 mg/ml BC. Other possible theories, like plasmid- or efflux pump-mediated resistance, have been proposed (Russell, 1997; Poole 2007), but not conclusive results regarding *L. monocytogenes* were obtained (Mereghetti, et al. 2000), even if better results were found in other species (e.g. *S. aureus*, *E. coli*, *Vibrio* spp. or *Aeromonas* spp.) (Poole, 2007).

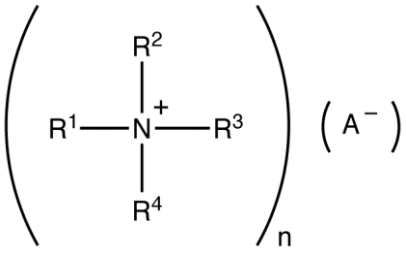
Different explanations for biocide resistance of bacterial biofilms have been produced: (i) limited diffusion of antimicrobial throughout the biofilm thickness, (ii) interaction of biocidal agents with the biofilm matrix, (iii) differentiated metabolic state of cells embedded within the biofilm, (v) presence of efflux pumps and (vii) modifications at the level of outer membrane (Cloete, et al. 2003). It has been hypothesized that glycocalyx could create a barrier able to hinder antimicrobial agent diffusion within the whole thickness of the bacterial biofilm (Giwercman, et al. 1991; Brown, et al. 1995; de Beer, et al. 1994; Chen and Stewart, 1996; Liu, et al. 1998; Stewart, et al. 1998). The biofilm matrix could react with and neutralize the biocide, as demonstrated for iodine (Alexander, 1983; McAvoy, et al. 1989) or chlorine

(Characklis and Dydek, 1976). Because of its polyanionic properties, the glycocalyx could act as an exchange resin able to adsorb the antimicrobial compound.

Hall-Stoodley et al. (2004) attributed this mechanism to extracellular polymeric substances (EPS) matrix, which, beside an overall reduction of penetration ability throughout the whole thickness of the biofilm (Anderson and O'Toole, 2008; Lewis, 2008; Maillard, 2007; Tart and Wozniak, 2008), neutralizes or binds the antimicrobial compound leading to an overall reduction of antimicrobial concentration before it reaches the bacterial cells within the biofilm. Growth within a biofilm shifts the overall cell metabolism towards a starvation physiological state, in which cells become less susceptible to disinfectant agents in comparison with growth in optimal conditions (Gilbert and Brown, 1995; Hall-Stoodley, et al. 2004). Another possibly involved mechanism is related to the presence of persisters within the biofilm matrix: they represent a small subgroup, within a larger microbial population, which expresses adaptive mechanisms to adverse conditions (e.g higher resistance to a certain biocide) and thus a distinct and much more competitive phenotype in comparison with their parental strains.

[3.5]. Quaternary ammonium compounds (QACs)

Their properties find their basis in their chemical structure, which can be generally so described



where n represent a whole number and positive whole number R^1, R^2, R^3, and R^4 could be hydrogen atoms, alkyl groups, aryl groups, or any combination thereof. A^- could be any anion present in the salt. Because they are negatively charged, attraction towards any negatively charged target has been hypothesized as action mechanism and accounts for damages towards material membranes.

Because of the presence of both polar and apolar structural parts, QAC can act as detergent and be efficient in a very broad range of temperatures and they are not affected from influence of organic matter. QACs seem to be effective against several bacterial species, even if it seems that they are more

effective against Gram positive compared with results on Gram negatives. Usually applied concentrations are 200 μg/ml for non porous surface, while 400 ppm are generally applied on permeable material like wooden shelves. *L. monocytogenes* planktonic cells are completely eliminated by exposure to benzalkonium chloride, while 100 ppm can reduce biofilms of same species of just two logarithms explaining persistence of *L. monocytogenes* strains in food transforming plants.

Merenghetti et al. (2000) reported that only 7 of 97 *Listeria monocytogenes* examined strains showed MBC of benzalkonium chloride and cetrimide greater than 7 and 18 μg/ml, respectively, demonstrating that biocide resistance is not homogenously shared among individuals within a certain species. This data was confirmed by Romanova et al. (2002), where quantitatively variable susceptibility to antimicrobial compounds was observed. Best et al. (1990) stated that there are also differences between different species, even if phylogenetically close, such as in the case of *Listeria innocua* and *Listeria monocytogenes*. Also Soumet et al. (2005) grouped 254 isolates in QAC susceptible (MBC< 3.75 μg/ml) and QAC resistant (MBC>7.5 μg/ml). Aarestrup et al. (2007) reported for benzalkonium chloride a bimodal distribution, in which 105 isolates could tolerate until 4 μg/ml

Several research reports indicated that the majority of QAC-resistant isolates were resistant by virtue of possessing one or more efflux pumps, which can be found both in both Gram-positive bacteria and in Gram-negative bacteria, and their substrate specificities differed depending on examined pump. All pumps were able to actively efflux QACs with consequent resistance, although Kucken et al. (2000) reported that clinical isolates bearing the *qacE* gene did not display significant resistance to QAC. Other similar genes, *qacE* and *qacE1,* have been located in the 3 conserved sequence of integrons in Gram-negative bacteria, ensuring diffusion of QAC resistance via horizontal gene transfer. QAC resistance in isolates of *Listeria* was firstly attributed to plasmids, whose transfer frequency is explaining quite realistically the high spreading of this trait. Chromosomally located resistance mechanism was demonstrated in a strain cured of its QAC resistance plasmid and it has been hypothesized that this mechanism could involve an efflux pump with a different substrate profile than *qacG,* in agreement with description of similar mechanism in a *Pseudomonas aeruginosa* adapted to quaternary ammonium compounds by serial transfer. Another possible explanation could be found in cell surface changes, which can also affect sensitivity to biocides, but at the moment no study allowed to give a univocal explanation of this phenomenon (Chapman, 2003).

[3.6]. Peroxyacetic acid or peracetic acid (PAA)

This substance has been revealed more markedly antimicrobial attributes than H_2O_2 because of lesser concentration to use in comparison with H_2O_2. Because it behaves like weak organic acid,

antimicrobial properties of PAA are more effective at lower pH. It can be applied both as liquid or gas and it found large field of application in industry sanitation because of its low metal corrosive capacity and high biodegradability. Another advantage is consisted of its usefulness against bacterial biofilms strongly attached on food contact surfaces. Its action mechanism is based on its ability to generate active radical species

PAA is produced from the reaction between acetic acid and hydrogen peroxide with sulfuric acid as catalyzing agent. This biocidal agent, which is characterized by strong oxidative properties, is mainly used for disinfection of wastewater or in medical and pharmaceutical field as well as within food-processing and beverage industry (Kitis, 2004). A ranking scale of microbial species effectively inhibited from this molecule was established from different authors (Baldry, 1983; Kitis, 2004; Koivunen and Heinonen-Tanski, 2005): most sensitive group was consisted of bacteria, which were followed by virus and bacterial spores and, finally by protozoan and cysts as the latter ones.

Biocidal properties of PAA are related to its strong oxidative characteristics: PAA leads to the formation of radical active oxygen species, which can damage both DNA and lipids (Small, et al. 2007). Other biological effects are related to its capacity to denaturate both structural and functional proteins as well as its ability in increasing cell permeability due to oxidative action towards sulfhdryl and disulfide bonds (Hilgren, et al. 2007; Small, et al. 2007). Damages of cell membranes as well as the arresting ability of microbial transport systems are also attributed to antimicrobial capacity of PAA (Hilgren, et al. 2007; Small, 2007). PAA is often used in combination with other biocidal agent like H_2O_2, which increase its effectiveness against both fungi and spores and reduce negative influence on bactericidal traits due to hard water.

It can be used efficiently against bacteria, yeasts, molds and viruses. Although it can be partially inactivated by organic matter, PAA demonstrated better performances in presence of organic residues than chlorine (Fatemi and Frank, 1999; Hilgren, et al. 2007; Small, et al. 2007) and it can be effective in a broad range of temperatures (Hilgren, et al. 2007). Commonly used level range from 0.5% to 1% and did not corrode stainless steel surfaces, even if higher dosages can produces relevant damages due to higher corrosive capacity. Other disadvantages are related to its chemical instability at high concentrations and its higher cost of usage in comparison with other traditional sanitizers like sodium hypochlorite (Kunigk and Almeida, 2001).

[3.7]. Polyphenolic compounds

In the recent years research has been extensively studying natural compounds which can be effective against harmful microbes both in therapeutic and food preservation fields. Several reports have

reported the bactericidal efficacy of phenolic compound from spices, herb essential oils and fruits (cranberry or grapes). Another important source of these compounds was revised into knotwood, which are part of a branch which is still strongly attached to tree stem. Researchers have focused attention on them, but in particular on softwood knots, due to relevant amounts of phenolic compounds, like lignans, flavonoids and stilbenes. In literature there are several reports, in which their antibacterial attributes are reported and knotwood could represent a valuable source of these natural biocies. Of all species examined, *Pinus* has shown to be the less sensitive to biodeterioration than other wooden species because of higher concentrations of phenolics, with special mention of pinosylvin compound (Lindberg, et al. 2004).

Pinosylvin, which shows strong structural similarities with resveratrol, is a stilbene with anticancerogenic, anti-inflammatory and antioxidant properties (Roupe, et al. 2006). This substance is known for its antifungal and bactericidal properties and its protective action as antioxidant, which play an important role in tree defense against microbial infections, especially in more sensitive points like e.g. when a branch is broken close to the stem. Among tested substances, pinosylvin and its derivates, pinosylvin monomethyl ether and dihydropinosylvin monomethyl ether (PM and DPSME respectively; possible susceptibility order was hypothesized to be PS > PM > DPSM at high level, while at middle concentration DPSM was inverted with PM) possess the highest antimicrobial effect. (Välimaa, et al. 2007). Unfortunately, until now there is still a gap about mechanism involved with regard to polyphenolic molecules, even if there is also a strong interest towards these substances due to their antioxidant properties and their future possible applications (Willför, et al. 2003).

In the present research the bactericidal effectiveness of the two stilbene pinosylvin and resveratrol was compared in parallel with two biocides, benzalkonium chloride and peracetic acid, which are commonly used in food processing environments for food contact surface sanitation. In parallel the antimicrobial susceptibility of planktonic cells was compared to sessile cells attached on stainless steel, on which also the influence of nutrient depletion was assessed. Finally, bacterial biofilms grown in presence of a turbulent flow of milk were evaluated for their increased resistance against selected biocides.

[3.8]. Materials and methods

I. Strains and growth conditions:

Listeria strains were routinely grown in Brain Heart Infusion (BHI; LABM, United Kingdom) for 16-18 h at 37°C and 200 rpm in a shaking bath. Strains were incubated for 24 h at 37°C on Blood Agar plates (Blood Agar Base, LABM, United Kingdom) to evaluate hemolytic activity. Stock cultures were kept at -70°C or in liquid nitrogen. Used strains with relevant properties were reported in Tab. 3.2.

II. Investigated active substances

Benzalkonium chloride (BC, 50% in H_2O, Sigma-Aldrich) and peracetic acid (PAA, 40% in acetic acid: H_2O, Sigma-Aldrich) were diluted with water until 0.47 μM (200 μg/ml) e 0,1% (116 μg/ml), respectively, while pinosylvin (University of Turku, Finland) and resveratrol (99%, Sigma-Aldrich) were dissolved in organic solvents, like DMSO and Ethanol 99%.

Strains	Serotype	Hemolysis	Description
UC 8410	-	-	*L. innocua* Food isolate (cheese brine)
EELA Oulu ba 2392/4	1/2a	+	*L. monocytogenes* Clinical animal isolate (fetal bovine brain)
EELA Oulu ba 1283/2	4b	+	*L. monocytogenes* Clinical animal isolate (sheep brain)
EELA Hki L211	1/2a	+	*L. monocytogenes* Cold smoked rainbow trout foodborne outbreak Food isolate
EELA Hki L627	3a	+	*L. monocytogenes* Butter foodborne outbreak Food isolate
KTL IHD 42526	1/2a	+	*L. monocytogenes* Cold smoked rainbow trout foodborne outbreak Clinical isolate
KTL IHD 42573	3a	+	*L. monocytogenes* Butter foodborne outbreak Clinical isolate
HY/ELTDK LMU 10	1/2a	+	*L. monocytogenes* Cold smoked rainbow trout foodborne outbreak Clinical isolate
KUY/REB 5	1/2a	+	*L. monocytogenes* Environmental isolate (sediment)
KUY/REB 10	1/2a	+	*L. monocytogenes* Environmental isolate (water)
ATCC 7644	1/2c	+	*L. monocytogenes* Sporadic clinical isolate (human spinal fluid)
EGD bapL	1/2a	+	*L. monocytogenes* Clinical isolate (Jordan, et al. 2008)
EELA 133	-	-	*L. innocua* Food isolate (freezer boiler)
EELA 54	-	-	*L. welshimeri* Food isolate (freezer boiler)

Tab. 3.2: Strains employed with relevant characteristics of interest.

III. Assessment of inhibitory effect of biocides

Overnight liquid cultures of *Listeria* strains reported in Tab. 3.2, were harvested by centrifugation for 5 min at 4000 *xg*, washed twice with sterile peptone water and diluted until OD_{600} of 0.16-0.20. Experiments were performed in a 96-well plate, in which serial binary dilutions of medium supplemented with testing antimicrobial were tested to cover range between 0÷512 μg/ml. To each well 100 μl of prepared cell suspension were added as inoculum. After an incubation of 24 hours at 37°C, plates were read at 595 and 630 nm, in order to give a quantitative evaluation of adhered cells and planktonic bacteria, respectively. Test medium were BHI and Iso-Sensi Test Broth (ISB, Oxoid)-BHI broth with 90:10 ratio (LSM medium).

IV. Bactericidal effect of antimicrobial on *Listeria* spp. strains

Subcultures of *Listeria* present in Tab. 3.2 were washed and adjusted with saline solution in order to achieve an OD_{625} of 0.080-0.100. 100 μl of this cell suspension were added into a 100 wells honeycomb plates (Honeycomb, ThermoLabsystems, Finland), where the test medium and the test

antimicrobial were further added. Plates were incubated for 24 h at hours at 37°C with low shaking and readings of optical density were performed every 15 min by using a wide band filter (420-580 nm). At least two independent experiments were performed with 3-5 replicate for each concentration tested. Analysis was conducted using BHI as test medium.

V. Resistance of statically grown biofilm to pinosylvin

Experimental protocol reported in par. III,sect. 2.6, Chapter 2, was performed on 18 h liquid subculture of selected strains, which are reported in Tab. 3.2. In this case dBHI was supplemented with gradually increasing concentrations of pinosylvin ranging from 100 up to 300 μM. Each determination was performed in triplicate.

VI. Bactericidal efficiency of disinfectants on biofilm grown under dynamic condition

Protocol was performed following indications present within UNI EN 1040:2006. The assessment was conducted on biofilm grown through the apparatus described in par. IV, sect. 2.6 of Chapter 2. Tested biocide at defined concentration (BC: 200 μg/ml, PAA: 5 %) were added to bacterial suspension: 100 μl aliquots of inoculated nutrient solution was used to determinate planktonic cells, while 1 cm of wire was taken to evaluate density of sessile cells.). This mixture was kept at room temperature for 30 min. At the end of contact time, a further aliquot was taken from 1.5 ml tube containing the cells/biocide mixture and biocide action was neutralized through addition of a suitable neutralizer solution (200 μg/ml sodium tiosulphate for BC, skimmed milk for PAA) into the tube. The so-obtained mixture was allowed to rest for 5 min at room temperature. After this time, the number of survivors was determined by decimal dilution and plate spreading on ALOA plate.

[3.9]. Results

I. Strains susceptibility to tested biocides

First set of experiments was performed to assess the antimicrobial activity of two natural stilbenes, pinosylvin (P) and resveratrol (R) and the two disinfectants, benzalkonium chloride (BC) and peracetic acid (PAA), which were commonly used in food processing environments, were

evaluated for their bactericidal efficiency. Because of their hydrophobic traits, P and R were dissolved in DMSO, instead of H_2O. In order to achieve the above mentioned goal, for each compound minimum bactericidal concentrations (MBC), whose results are reported in Tab. 3.3, was determined through broth micro-dilution test.

Strain	Code	Pinosilvin	Resveratrol	Benzalkonium chloride	Peracetic acid
L. innocua	UC 8410	128	512	16	16
L. monocytogenes	EELA Oulu ba 2392/4	128	512	8	2
L. monocytogenes	EELA Oulu ba 1283/2	128	512	8	8
L. monocytogenes	EELA Hki L211	128	512	8	16
L. monocytogenes	EELA Hki L627	128	512	4	16
L. monocytogenes	KTL IHD 42526	128	512	8	16
L. monocytogenes	KTL IHD 42573	128	512	8	16
L. monocytogenes	HY/ELTDK LMU 10	128	512	8	16
L. monocytogenes	KUY/REB 5	128	512	8	16
L. monocytogenes	KUY/REB 10	128	512	8	32
L. monocytogenes	ATCC 7644	128	512	8	16
L. monocytogenes	EGD bapL	128	512	16	16
L. innocua	EELA 133	128	512	16	16
L. welshimeri	EELA 54	128	512	8	8

Tab. 3.3: Minimum bactericidal concentrations (MBC) of examined antimicrobials. Data are expressed as µg/ml.

Investigated strains have shown a highly homogeneous behavior against polyphenols P and R, even if P expressed a 3-fold stronger bactericidal effect compared to R (MBC_P = 128 µg/ml, MBC_R = 512 µg/ml). BC was able to inhibit bacterial inoculums at 8µg/ml, although three strains showed tolerance higher by one dilution. Only one isolate (*L. monocytogenes* EELA Hki L627) was sensitive at 4 µg/ml of BC. Wider variability was observed with regard to PAA, to which *L. monocytogenes* Oulu ba 2392/4 was notably susceptible (MBC= 2 µg/ml) while *L. monocytogenes* KUY/REB10 revealed stronger resistance (32 µg/ml, respectively).

Bactericidal effect of P at increasing concentrations was further investigated in parallel with BC and PAA through BioscreenC apparatus, which allows monitoring bacterial growth continually. Strains were selected in order to evaluate influence of source origin on antimicrobial susceptibility. Results are exposed from Fig. 3.4 to Fig. 3.6.

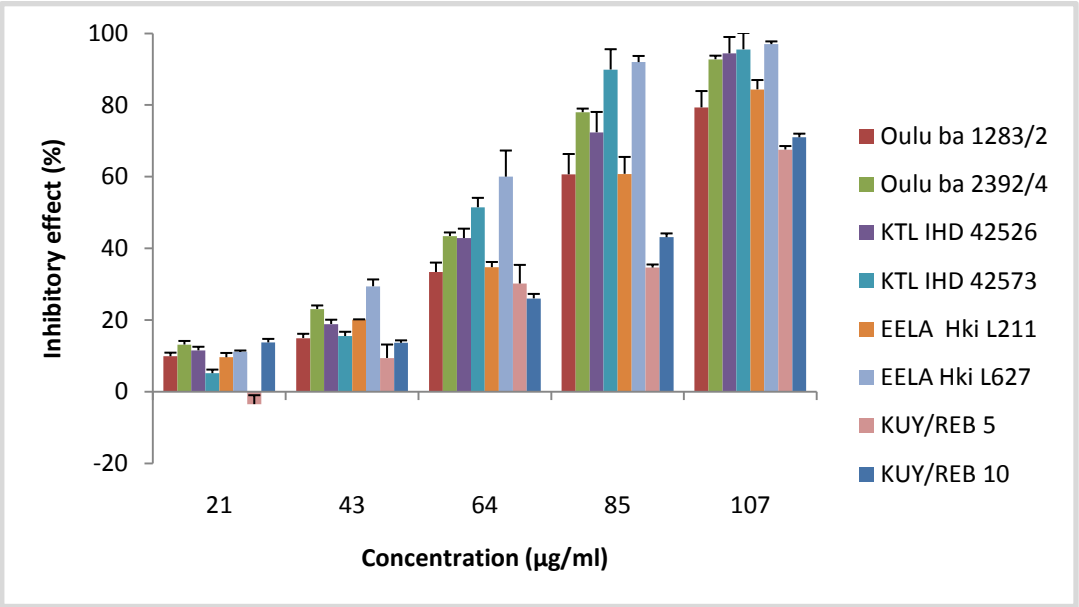


Fig. 3.4: Inhibitory effect of different concentrations of pinosylvin in DMSO on *L. monocytogenes* strains from different sources Data are expressed as average of three replicates from three independent experiments. Tested concentrations are reported in the legend below the graph.

Inhibitory effect of P (Fig. 3.4) was strongly dose-dependent: 200 µM (43 µg/ml) was able to reduce bacterial density more than 50% for all strains (except for *L. monocytogenes* KUY/REB5 and *L. monocytogenes* EELA Oulu ba 1283/2, which were both reduced by 42.2 and 42.1%, respectively), whereas, starting from 300 µM, (64 µg/ml), antimicrobial susceptibility was over 70%. *L. monocytogenes* EELA Hki L211 and EELA Hki 627 (isolated from foods involved in two European listeriosis outbreaks) were less sensitive to P at all tested concentrations than *L. monocytogenes* KTL/IHD 42526 and KTL/IHD 42573 (clinical human isolates from same outbreak), respectively. The environmental strains, *L. monocytogenes* KUY/REB 5 and KUY/REB 10, when exposed to P, were inhibited by 60% at 107 µg/ml, whereas *L. monocytogenes* EELA Oulu ba 1283/2 and *L. monocytogenes* EELA Oulu ba 2392/4, which were isolated from animals, demonstrated sensitivity comparable to both food and clinical strains.

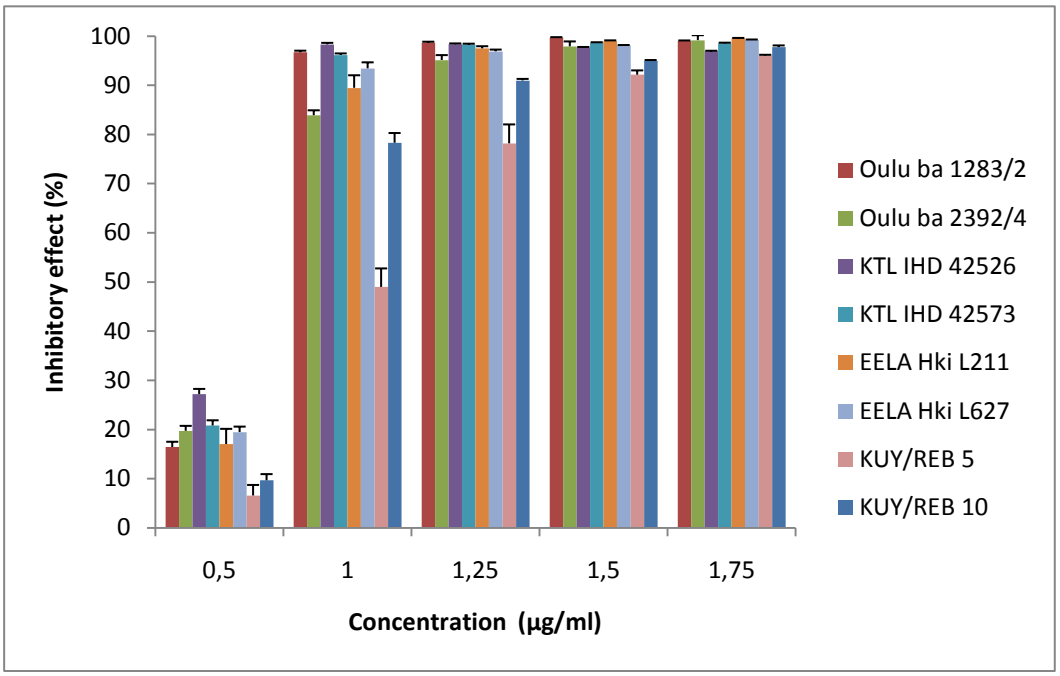


Fig. 3.5: Inhibitory effect of different amounts of benzalkonium chloride on *L. monocytogenes* strains from different sources Data are expressed as average of three replicates from three independent experiments. Tested concentrations are reported in the legend below the graph.

1 µg/ml BC hindered bacterial growth by 49% for *L. monocytogenes* KUY/REB5, while all other isolates were reduced by over 70% at same dosage and bacterial growth was almost totally hindered at 1.75 µg/ml for all tested isolates (Fig. 3.5). Clinical isolates of *L. monocytogenes* were fairly more affected by BC than corresponding food isolates, even if the same strain expressed diversified susceptibility at increasing concentrations of biocide.

Higher concentration of PAA were necessary to achieve the same inhibition values of BC: almost complete growth abolishment was achieved at 240 µg/ml, though 120 µg/ml killed more than 50% of whole bacterial population except for *L. monocytogenes* Oulu ba 1283/2 and for *L. monocytogenes* KUY/REB 5 and KUY/REB 10. In these samples inhibitory effect was 42.5, 44.9 and 48.9, respectively (Fig. 3.6). From these results it was possible to observe that food isolates from two European epidemic listeriosis were less sensitive to examined antimicrobial than corresponding strains isolated by clinical human cases, although EELA Hki L211 was less resistant to PAA than its clinical analogue in the concentration range between 120 and 210 µg/ml.

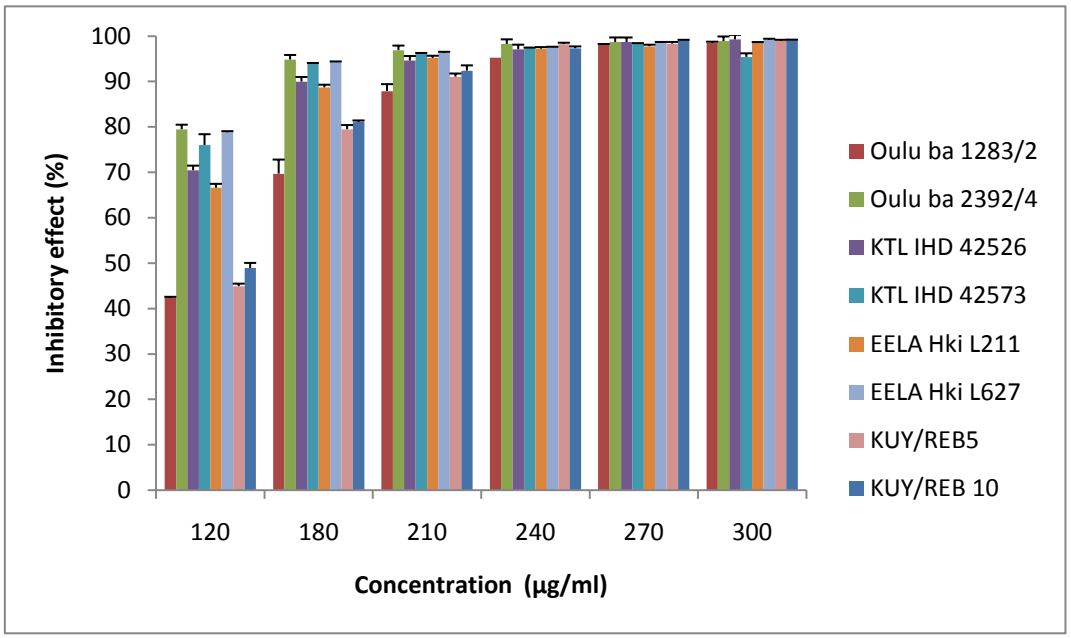


Fig. 3.6: Inhibitory effect of different amounts of peracetic acid on *L. monocytogenes* strains from different sources Data are expressed as average of three replicates from three independent experiments. Tested concentrations are reported in the legend below the graph.

P demonstrated a certain good attitude as antimicrobial when compared to PAA, even if effectively active dosages were still higher than the corresponding ones of BC: basing on this preliminary conclusion, further tests were conducted to improve antimicrobial effectiveness of P. Hypothesizing a possible effect of solvent on the antimicrobial effectiveness, different concentrations of DMSO and of Ethanol 99% were compared for their effect on bacterial growth. *L. innocua* UC 8410 (Fig. 3.7 and Fig. 3.8) was employed for this analysis because of frequently reported use of *L. innocua* as predictive model for its pathogen analogue, while *L. monocytogenes* EELA Hki L211 (Fig. 3.10 and Fig. 3.11) was selected due to its high resistance demonstrated in previous experiments. Percentages were selected in order to minimize any eventual handling difficulties concerning dispensation of very small aliquots of solvent.

In presence of 0.5% and 1.5% of DMSO, lag time of *L. innocua* UC 8410 was not sensibly modified, whereas very slight variations of curve slope were observes suggesting a weak dose dependent effect. The maximal growth was decreased up to 1.3 after 8 h and at 10 h, when DMSO was used at percentage of 0.5 and 1.5%, respectively. Then a gradual decrease, which started after 24 h, was observed, although this phenomenon was more relevant in 0.5%-treated samples. Optical density of 1.0 was the maximal value reached after 18 h exposure to 2.5% and a steady decrease until 0.8 units was observed at 48 h. When exposed were exposed to 3.5%, *L. innocua* UC 8410 grew up to 1.3 units after 6 h and started gradually decreeing until 1.2 units at 48 h, while growth has been kept at 1.4 units since 12 h. Analogue trend was observed when 5% DMSO was used, although maximal density was slightly higher.

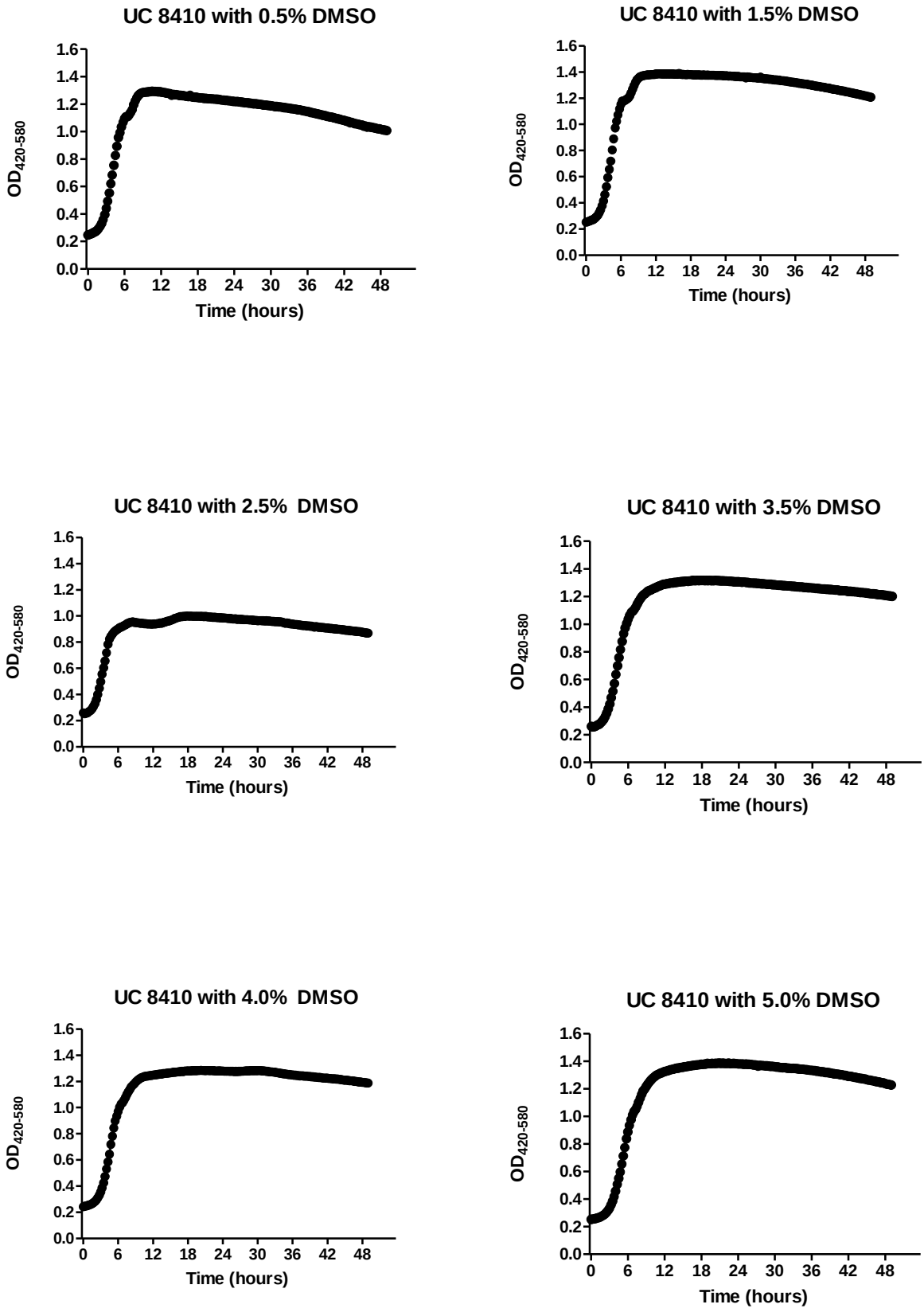


Fig. 3.7: Inhibitory effect of different percentages of DMSO on growth on *L. innocua* UC 8410. Data are expressed as average of five replicates. Error bars are omitted for clarity.

EtOH showed a stronger inhibitory dose dependent effect on *L. innocua* UC 8410 than DMSO (Fig. 3.8): while there was a slight difference between the two lowest percentages, the application of 1.5% EtOH reduced maximal optical density by 0.2 units. 2.5% had an effect comparable with the 1.5%-treated sample, although the curve slope was less sharp. In sample exposed to 3.5% EtOH an increased flattening of log phase was observed along with a reduced maximal growth within 48 h. 4% EtOH, apart from the similar effect on log phase with the above mentioned treatment, led to an overall reduction of the optical density by 0.7 units. Exposure to 5% EtOH caused a prolonged extension of the lag time until 24 h; after this period the bacteria grew regularly to reach the final density of 1.2 units at 40 h. The plateau (corresponding to the stationary growth phase) was observed at 48 h, and then the values started decreasing.

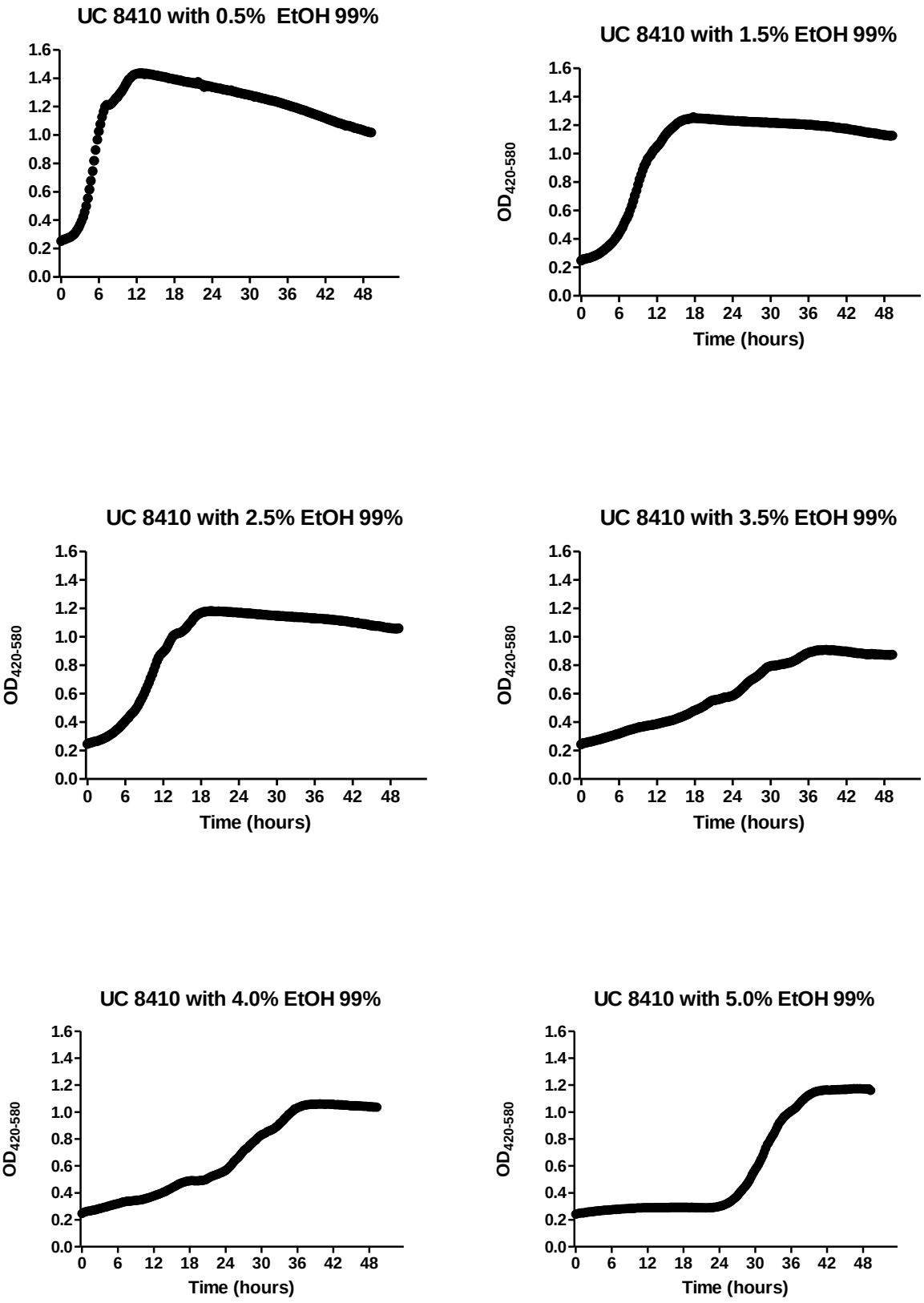


Fig. 3.8: Inhibitory effect of different percentages of EtOH 99% on growth on *L. innocua* UC 8410. Data are expressed as average of five replicates. Error bars are omitted for clarity.

L. monocytogenes EELA Hki L211 showed a behavior close to the one observed in *L. innocua* UC 8410 (Fig. 3.9): in few hours this isolate grew exponentially until 1.4 units at 6 h, then its optical density gradually decreased up to 1.0 at 48 h (Fig. 3.10). DMSO did not significantly modify the growth kinetic of *L. monocytogenes* EELA Hki L211 in terms of growth rate, although both maximal growth and optical density at 48 h were reduced by 0.2 units. Different behavior was observed with EtOH (Fig. 3.11): 0.5% EtOH did not show any significant effect on growth kinetic, although *L. monocytogenes* EELA Hki L211, after 48 h incubation, raised up to 0.9 units. 1.5% of EtOH slightly reduced cell population in the first 6 h of monitoring, while a fairly sharp decrease until 48 h was observed.

Applying 2.5 % EtOH, a weak reduction of growth rate was observed up to 1.0 unit at 12 h, at 40 h a gradual decrease was observed up to 0.8 units, which were kept until 48 h. Increasing the EtOH percentage up to 3.5% a marked decrease of growth rate was detected as well as 0.8 units was the maximal growth reached after 12 h. Optical density decreased slowly and steady up to 0.6 units at 48 h. When EtOH was applied at 4%, a further decrease of the growth rate was observed, while, concerning the maximal population density at the level of 0.7 units was reached after 18 h. The bacterial population gradually decreased up to 0.6 units after 48 h. 5 % further diminished both the growth rate and the final bacterial density, which has been hold at 0.6 units at 30 h.

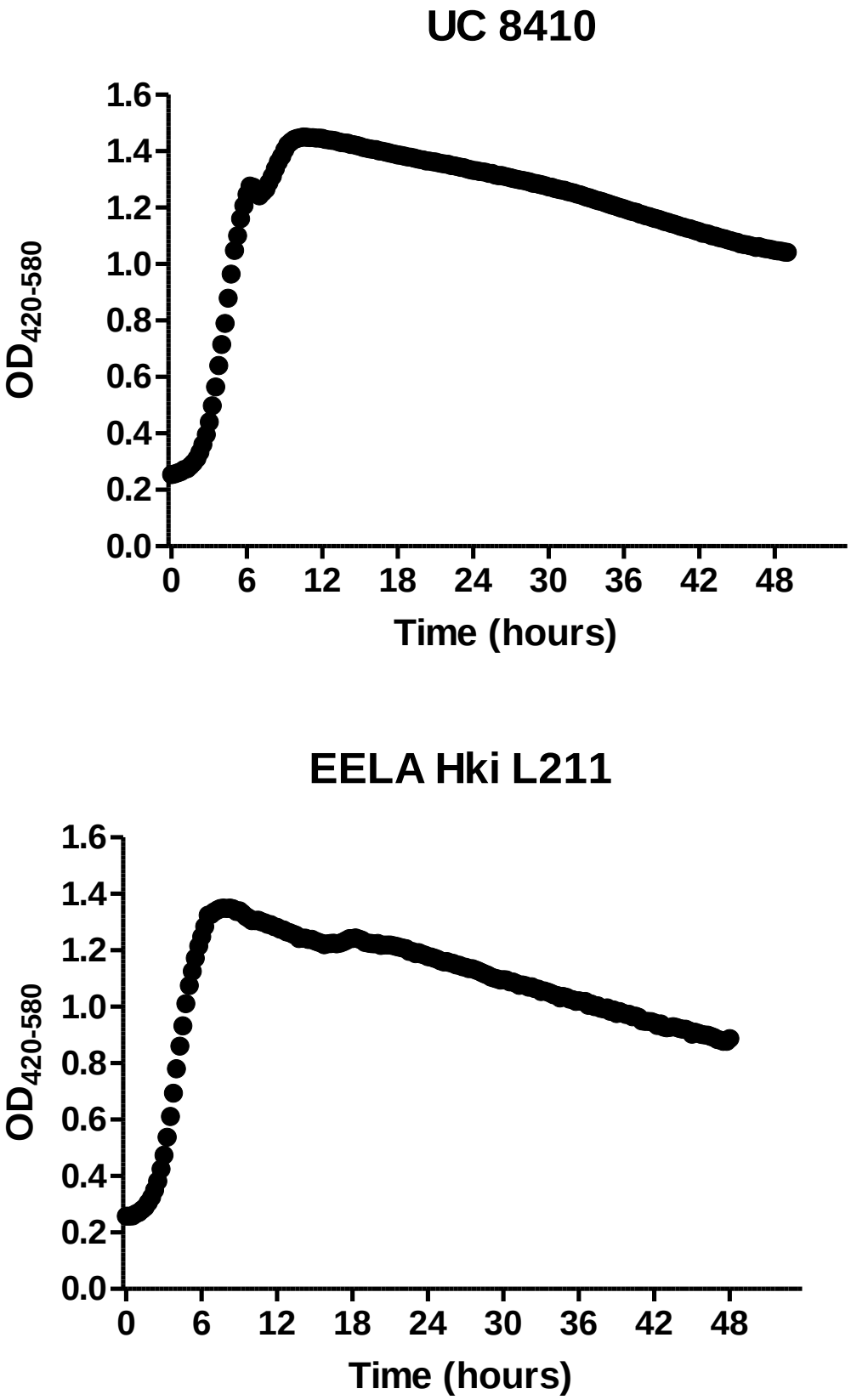


Fig. 3.9: Comparison of growth curves in BHI for 48 h between *L. innocua* UC 8410 (above) and *L. monocytogenes* EELA Hki L211 (below). Data are expressed as average of five replicates. Error bars are omitted for clarity.

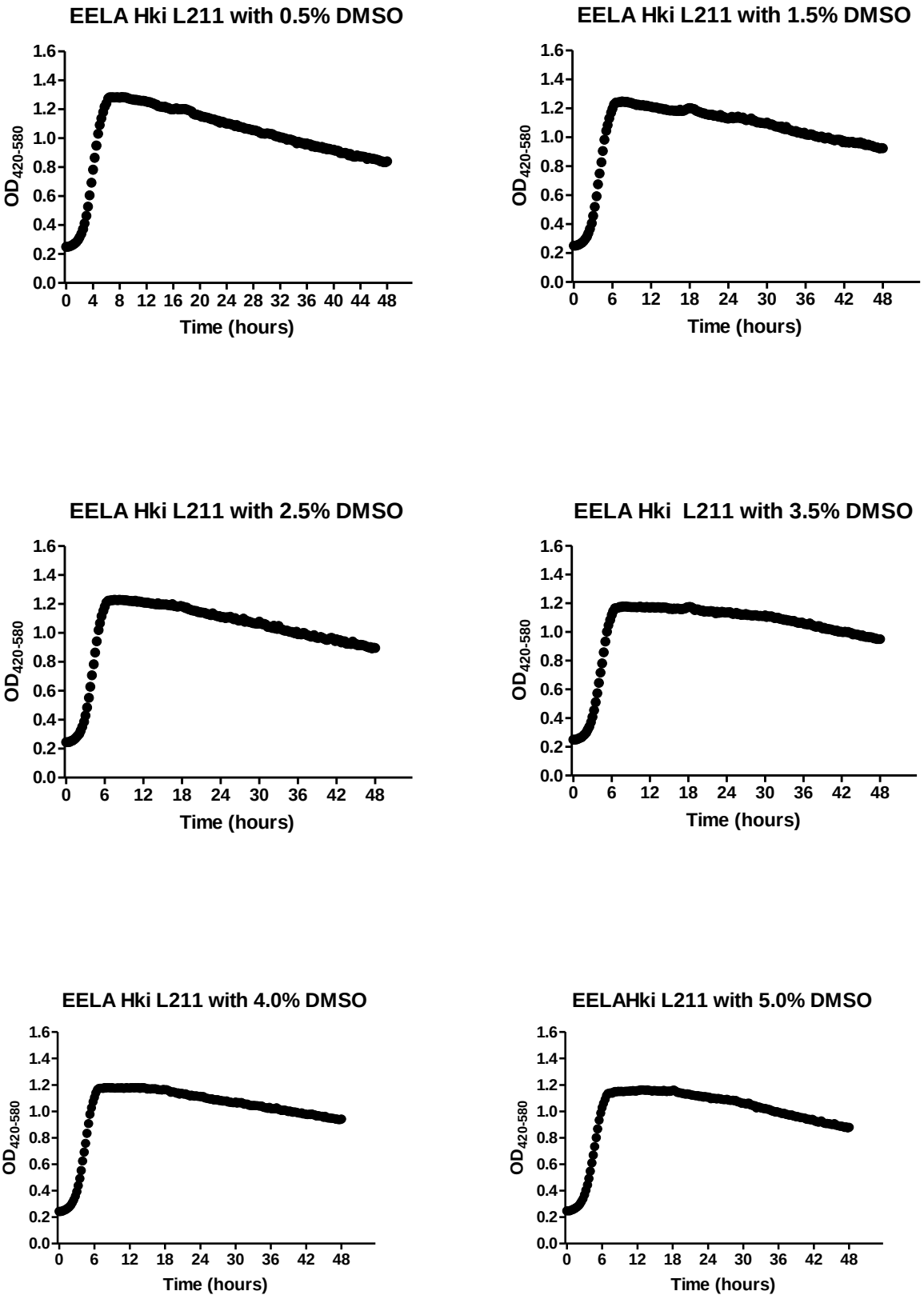


Fig. 3.10: Inhibitory effect of different percentages of DMSO on growth on *L. monocytogenes* EELA Hki L211. Data are expressed as average of five replicates. Error bars are omitted for clarity.

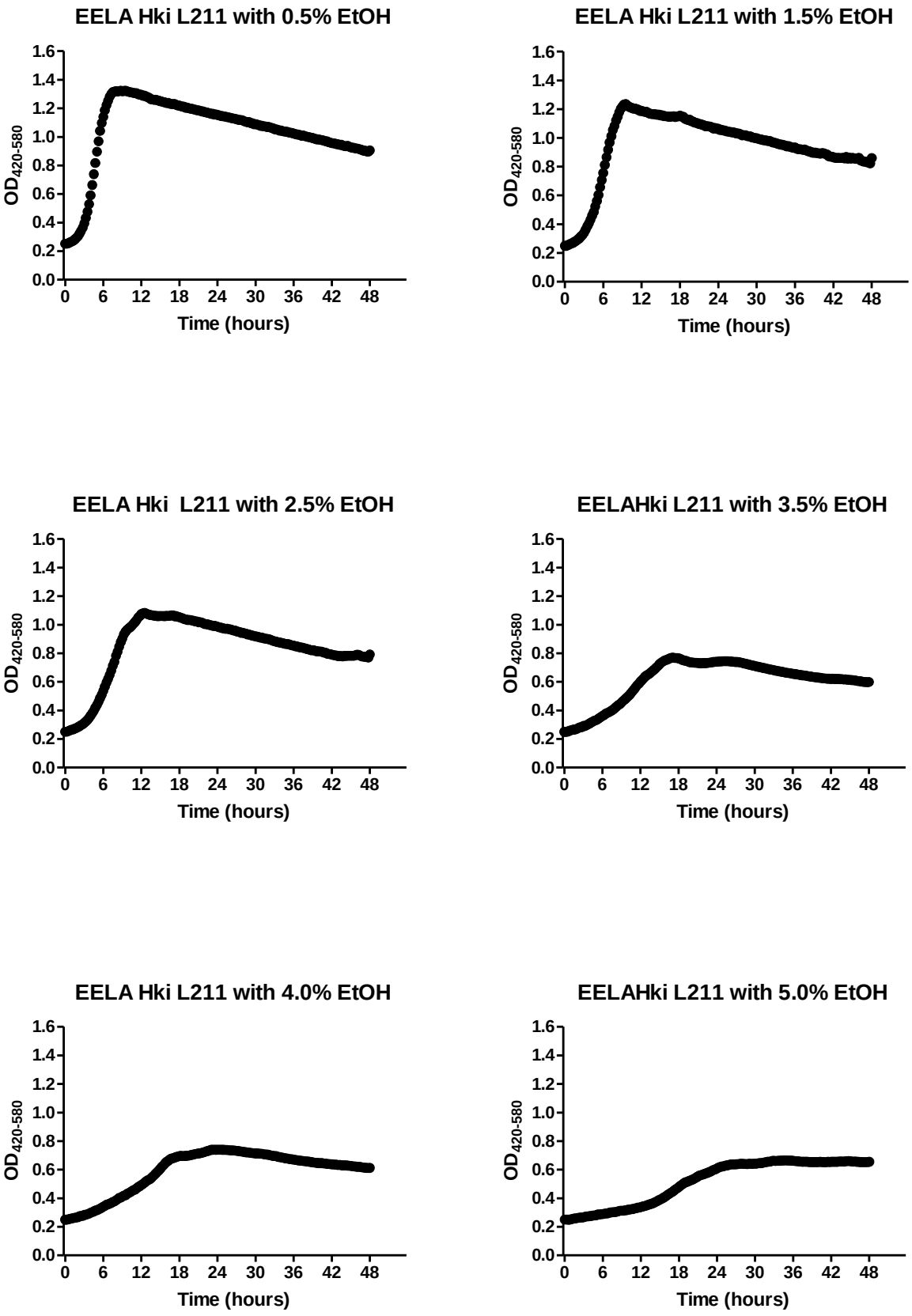
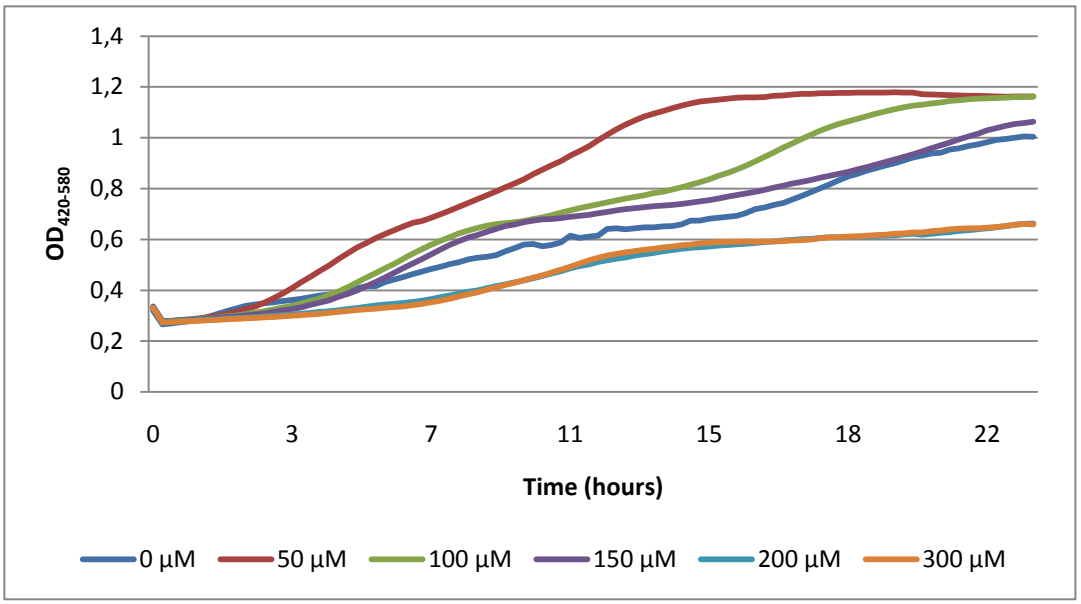


Fig. 3.11: Inhibitory effect of different percentages of EtOH on growth on *L. monocytogenes* EELA Hki L211. Data are expressed as average of five replicates. Error bars are omitted for clarity.

DMSO was demonstrated not capable to alter significantly the growth kinetic of both *L. innocua* UC 8410 and *L. monocytogenes* EELA Hki L211, while EtOH did not affect the growth of both these above mentioned strains up to 2.5%, while higher percentages were demonstrated to have a negatively influence on bacterial growth within 48 monitoring period. Because of its authorized use in food, EtOH was selected for further tests as solvent for P and R. Further purpose of investigation was to observe if the application of the two considered stilbens in EtOH (3% final concentration of this solvent) could markedly modify the growth kinetic parameters, such as the growth rate or the lag phase duration.

In order to have a quantitative evaluation of the inhibitory effect, the area under the curve was obtained through integral calculations and, after referring each calculated value to the one corresponding to the pure solvent (EtOH 99%), percentage of the inhibitory effectiveness of both the stilbenes was calculated (Fig. 3.14 and Fig. 3.15). Results shown from Fig. 3.14 to Fig. 3.16 indicated that up to 150 μM P demonstrate a protective effect towards both the used *Listeria* strains against EtOH added as solvent at a 3% concentration (v/v). The biocidal effect of P was evident on *L. innocua* UC 8410, only when the applied concentration was higher than 200 μM, when the growth rate was lower than control containing 3% EtOH. A similar but more evident effect of inhibition at concentration higher than 200 μM was observed *L. monocytogenes* EELA Hki L211.

When compared to P, R did not exhibit a similar effect on the growth of both *L. innocua* UC 8410 and *L. monocytogenes* EELA Hki L211 (Fig. 3.16). 150 μM R was slightly different from 100 μM and exhibited a protective effect comparable to the one observed with 50 μM. 200 μM and much more 300 μM showed a limited protective effect from 3% EtOH for both the examined strains, although *L. innocua* UC 8410 showed slightly higher sensitivity to R than *L. monocytogenes* EELA Hki L211.



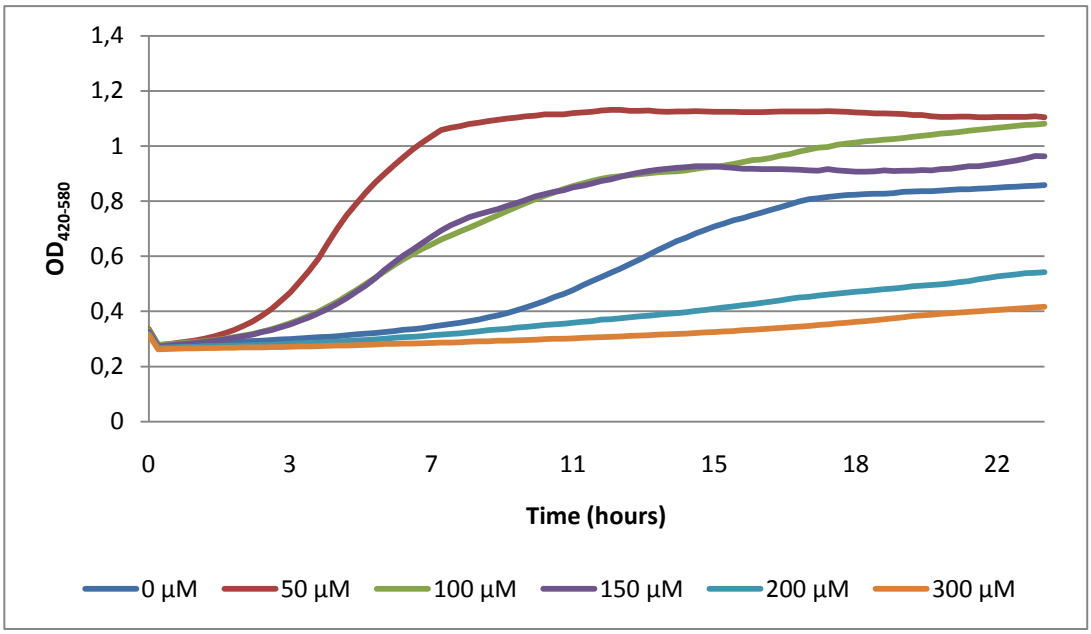


Fig. 3.12: Growth curve of *L. innocua* UC 8410 (above) and *L. monocytogenes* EELA Hki L211 (below) at different concentrations of pinosylvin. Data are expressed as average of five replicates of three independent experiments. Concentrations of pinosylvin are reported in µM. Error bars are omitted for clarity. 50 µM = 10.6 µg/ml, 100 µM = 21.2 µg/ml, 150 µM = 31.84 µg/ml, 200 µM = 42.45 µg/ml, 300 µg/ml.

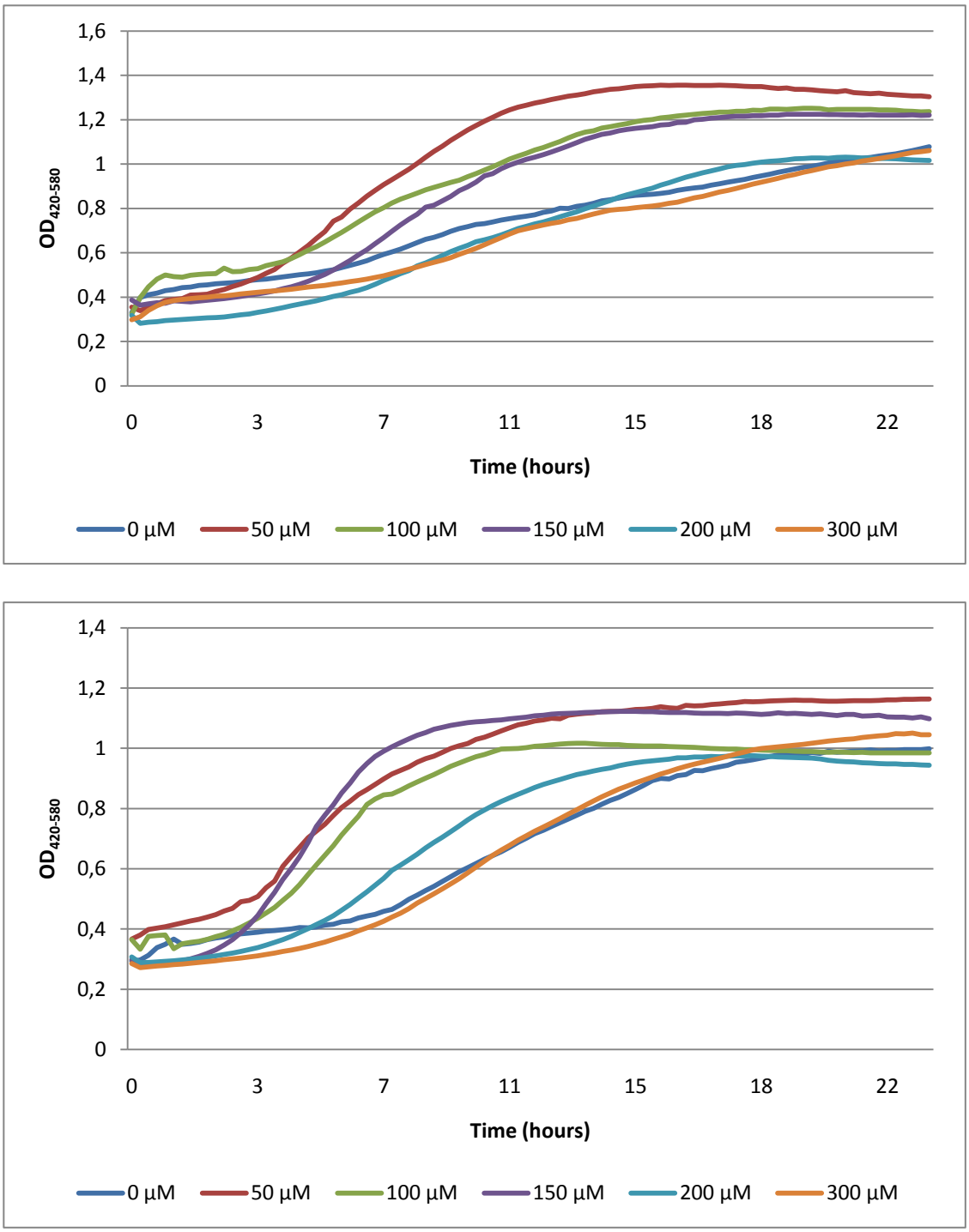


Fig. 3.13. Growth curve of *L. innocua* 8410 (above) and *L. monocytogenes* EELA Hki L211 (below) at different concentrations of resveratrol. Data are expressed as average of five replicates of three independent experiments. Concentrations of pinosylvin are reported in µM. Error bars are omitted for clarity. 50 µM = 10.6 µg/ml, 100 µM = 21.2 µg/ml, 150 µM = 31.84 µg/ml, 200 µM = 42.45 µg/ml, 300 µg/ml.

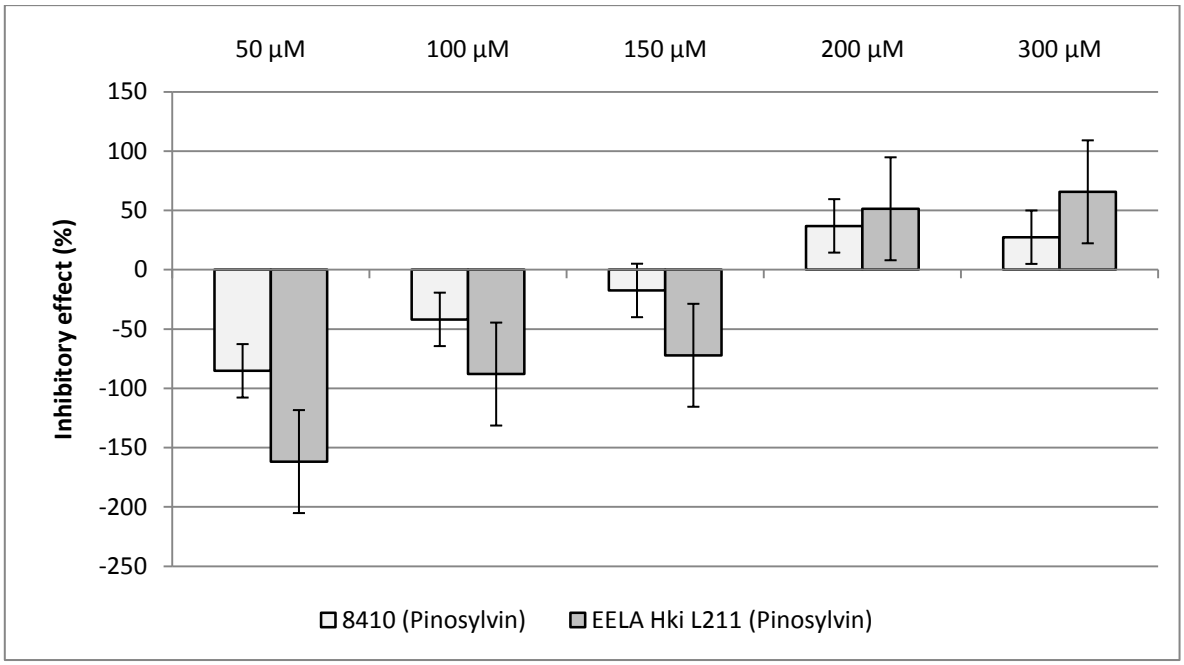


Fig. 3.14: Protective/Inhibitory effect of the stilbene pinosylvin at different concentrations in ethanol 99% through BioscreenC. Data are expressed as fraction of area under curve of examined concentration compared to lowest concentration (0 µM). Measurement was performed on five replicates from three independent experiments. Bars represent average ± standard error mean. 50 µM = 10.6 µg/ml, 100 µM = 21.2 µg/ml, 150 µM = 31.84 µg/ml, 200 µM = 42.45 µg/ml, 300 µg/ml.

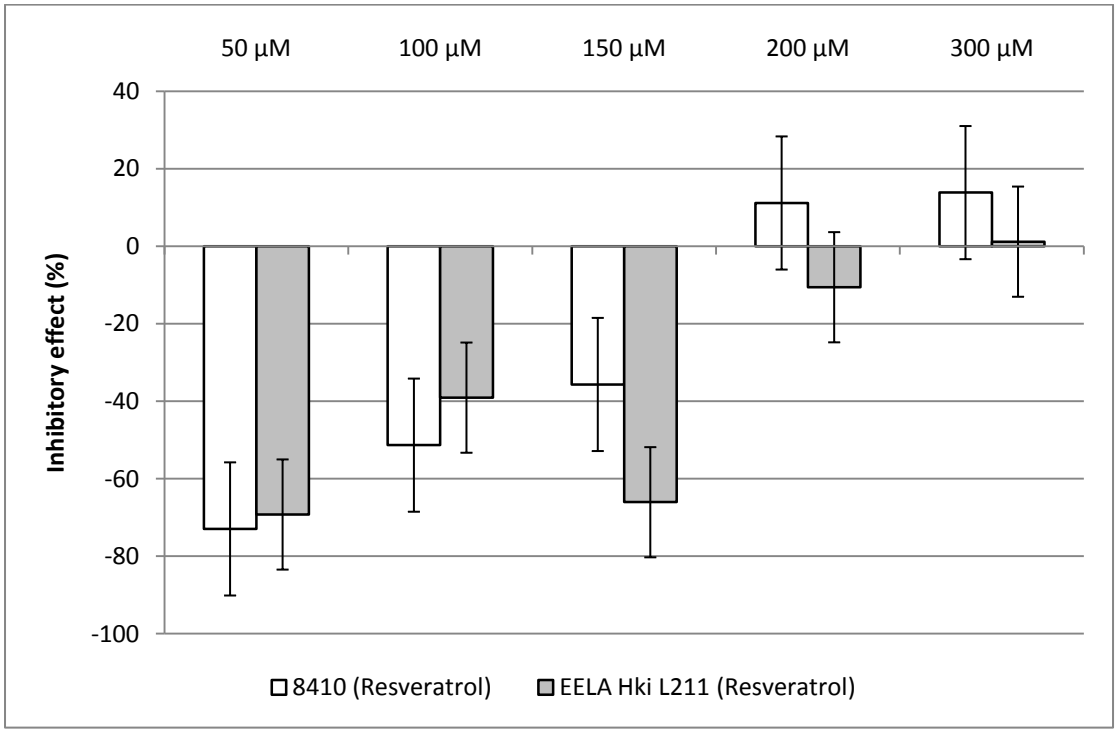


Fig. 3.15: Protective/Inhibitory effect of the stilbene resveratrol at different concentrations in ethanol 99% through BioscreenC. Data are expressed as fraction of area under curve of examined concentration compared to lowest concentration (0 µM). Measurement was performed on five replicates from three independent experiments. 50 µM = 10.6 µg/ml, 100 µM = 21.2 µg/ml, 150 µM = 31.84 µg/ml, 200 µM = 42.45 µg/ml, 300 µg/ml.

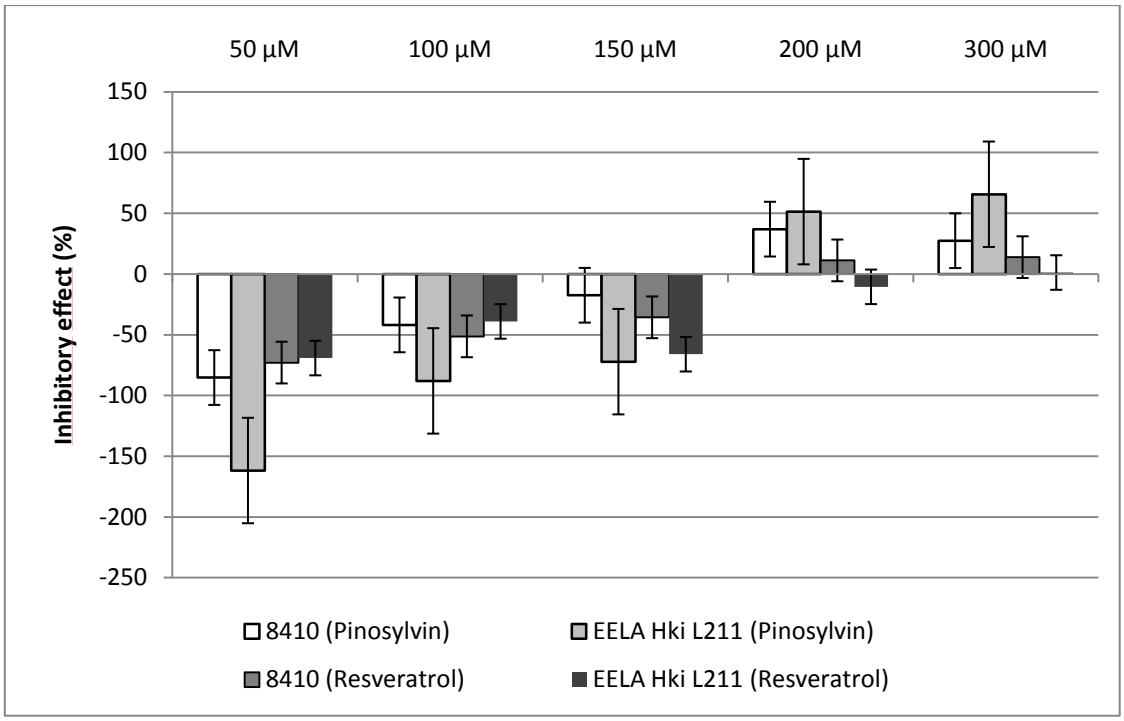


Fig. 3.16: Comparison of bactericidal efficiency of stilbene pinosylvin with resveratrol.

This protocol was applied, in three independent experiments, to other 10 *Listeria monocytogenes* strains reported in Tab. 3.17, including also isolates from sediment and water of fishery factory. No evident difference among employed strains was observed, suggesting that origin of isolation could not be a significant parameter determining a differently expressed antimicrobial resistance. All the strains showed higher susceptibility to P by more than 80% even at 100 µM, even in both the two environmental isolates, which revealed a certain resistance to P in previous tests. Despite the general dose dependent effect, higher concentrations were not quantitatively effective, as shown by testing *L. monocytogenes* ATCC 7644 and *L. monocytogenes* EELA Oulu ba 1283/2 (sporadic human clinical isolate and animal clinical strain, respectively) at 500 µM. One-way ANOVA confirmed that origin source could not account for the observed differences among these isolates, in terms of susceptibility to P, demonstrating the involvement of other factors still not elucidated.

Code	0 μM	100 μM	200 μM	300 μM	400 μM	500 μM
ATCC 7644	47,80 ± 16,45	85,10 ± 4,40	83,18 ± 2,24	83,12 ± 1,84	80,74 ± 3,38	41,03 ± 44,74
EELA Oulu ba 1283/2	58,20 ± 8,14	72,10 ± 6,35	94,71 ± 2,09	92,91 ± 3,55	86,95 ± 2,01	64,62 ± 24,14
HY/ELTDK LMU 10	84,23 ± 7,76	93,26 ± 1,28	89,60 ± 1,29	90,96 ± 0,83	88,72 ± 1,65	89,07 ± 0,31
KUY/REB 5	80,47 ± 6,54	90,64 ± 2,77	88,16 ± 1,58	88,05 ± 1,52	89,34 ± 1,40	90,99 ± 3,52
KTL IHD 42526	89,58 ± 7,74	94,32 ± 2,68	87,75 ± 1,23	89,51 ± 1,50	89,03 ± 0,54	86,66 ± 1,26
KTL IHD 42573	91,41 ± 4,89	90,42 ± 2,28	92,39 ± 1,57	91,68 ± 1,98	88,21 ± 3,04	86,96 ± 2,57
EELA Oulu ba 1283/2	82,59 ± 12,72	93,12 ± 3,99	90,44 ± 2,79	88,78 ± 2,04	88,69 ± 1,96	85,07 ± 0,89
EELA 133	82,73 ± 3,21	88,51 ± 1,21	85,37 ± 2,80	86,41 ± 3,42	87,16 ± 2,37	83,92 ± 1,68
EELA Hki L627	93,67 ± 0,87	93,51 ± 2,43	87,73 ± 1,11	88,22 ± 2,77	85,48 ± 3,06	83,02 ± 1,32
KUY/REB 10	77,62 ± 6,81	89,74 ± 2,34	89,89 ± 2,03	90,46 ± 3,46	84,32 ± 2,41	86,59 ± 1,20

Tab. 3.17: Susceptibility of 10 *Listeria* sp. strains to pinosylvin through 48 h growth monitoring with BioscreenC. Values are calculated from average three replicates from two independent experiments ± standard error mean. 50 μM = 10.6 μg/ml, 100 μM = 21.2 μg/ml, 150 μM = 31.84 μg/ml, 200 μM = 42.45 μg/ml, 300 μg/ml, 400 μM = 63.67, 500 μM = 106.122 μg/ml.

II. Resistance of statically grown bacterial biofilm to antimicrobials

Further step was to assess the resistance of *Listeria* strains under prolonged exposure to pinosylvin dissolved in ethanol of selected strains. First trial was conducted on *L. innocua* UC 8410 in comparison with *L. monocytogenes* EELA Hki L211. As surface, stainless steel coupons were used, while dislodging method was surface swabbing with sterile cotton swabs, previously immerged in sterile peptone water. Growth was monitored under strict nutrient depletion using dBHI (following the experimental protocol mentioned in par. I, Chapter II).

Both *L. innocua* UC 8410 and *L. monocytogenes* EELA Hki L211 were found to adhere on stainless steel more than 10^4 CFU/cm^2 after 168 h in dBHI. Although the tested strains showed strong similarities concerning the kinetic of biofilm formation on stainless steel, the nutrient depletion affected more sensibly the cell recoveries of *L. innocua* UC 8410 in comparison with the ones of *L. monocytogenes* EELA Hki L211: *L. innocua* UC 8410 was reduced by one and two logarithms after 72 and 24 h, respectively, while *L. monocytogenes* EELA Hki L211 was diminished by only one logarithm after 168 h of incubation. Applying 5% EtOH (v/v) to both the above mentioned strains did not lead to any remarkable further reduction of cell amounts on the tested surfaces: only *L. innocua* UC 8410 was affected by this concentration of EtOH, although the reductive effect was increased by less than one logarithm at both 72 and 168 h.

P demonstrated to have a protective effect towards *L. innocua* UC 8410 against EtOH until 300 μM at 72 h (one logarithmic reduction factor), while higher concentrations demonstrated to be efficiently inhibitory at the same time point. 500 μM P was the only inhibitory concentration of P against *L. monocytogenes* EELA Hki L211 after 72 h. 100 μM P was unable to protect *L. innocua* UC 8410 from EtOH after 168 h, while the values obtained at both 200 and 300 μM on the above mentioned strain were almost comparable with the ones observed for the pure solvent as well as for 50 μM (10^4 CFU/cm^2). In comparison with the value referred to the pure solvent (10^6 CFU/cm^2), *L. monocytogenes* EELA Hki L211 was reduced by two logarithms by all concentrations ranging

between 50 and 300 μM at 168h. After 168 h both *L. innocua* UC 8410 and *L. monocytogenes* EELA Hki L211 were completely inhibited by both 400 and 500 μM.

Experiments were extended to other *L. monocytogenes* strains, including both clinical and food isolates from foodborne European outbreaks and a clinical animal strain from bovine brain, in order to evaluate influence of origin about surface adhesion and antimicrobial resistance. Results are summarized in Fig. 3.19. When cell are adhered to stainless steel, susceptibility was completely modified, because even after 168 h of exposure to P counts were higher than 10^2 CFU/cm^2. The most resistant isolates were *L. monocytogenes* EELA Hki L211 and *L. monocytogenes* KTL IHD 42526 (10^4 CFU/cm^2 at 300 μM), while the most susceptible to P was *L. monocytogenes* Oulu ba 2392/4 (10^2 CFU/cm^{2}at the same conditions).

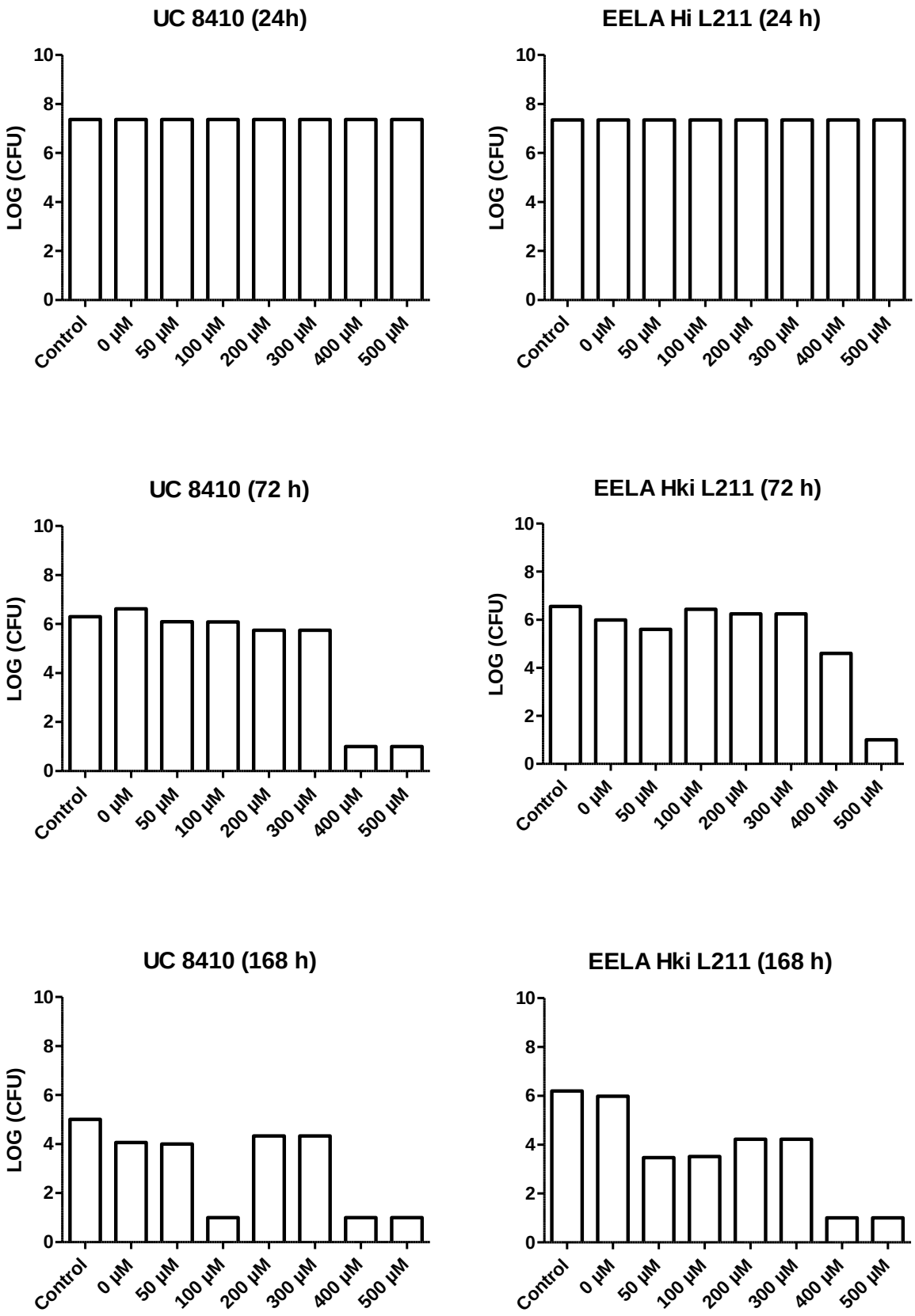


Fig. 3.18: Adhesion on 1 cm^2 stainless steel coupon and exposure to different concentrations of pinosylvin for 7 days. Control is referred to untreated sample, while added volume of ethanol with/without pinosylvin is kept constant. Data are expressed as mean f three replicates. Error bars are omitted for clarity. 1 LOG(CFU) is to be intended as less than 10 CFU/cm^2.

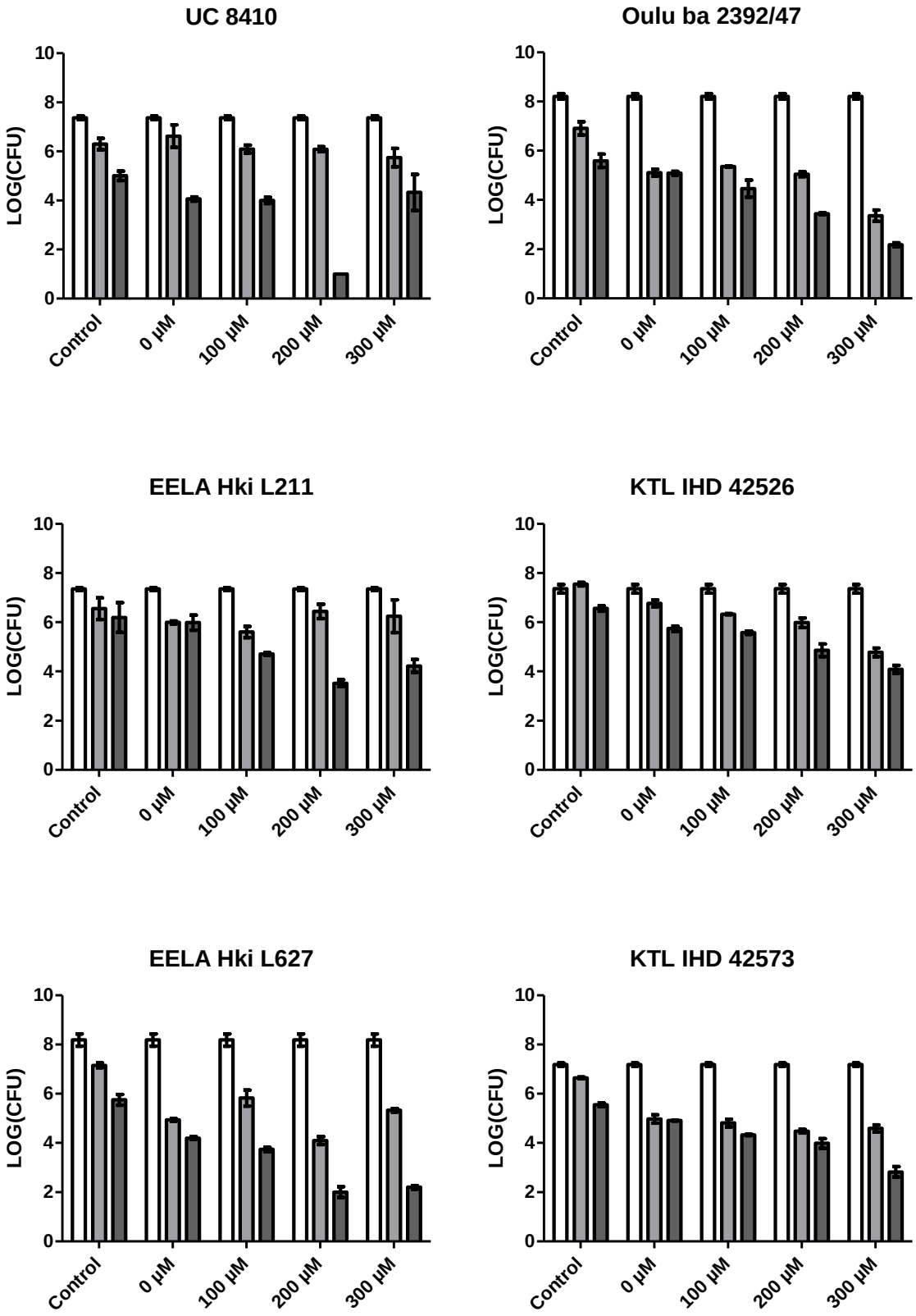


Fig. 3.19: Adhesion on 1 cm^2 stainless steel coupon and exposure to different concentrations of pinosylvin for 7 days. White bars: 24 h, light grey bars: 72 h, dark grey bars: 168 h. Control is referred to untreated sample, while added volume of ethanol with/without pinosylvin is kept constant. Data are expressed as mean of three replicates ± standard error mean. 1 LOG(CFU) is to be intended as less than 10 CFU/cm^2.

III. Biocide resistance of biofilms grown under dynamic conditions

The protective effect of biofilm of *L. innocua* UC 8410, in conditions simulating a dairy food plant, to two biocides, benzalkonium chloride (BC) and peracetic acid (PAA), at the levels routinely used in food processing environments [200 μg/ml and 5% (equal to 5.8 mg/ml), respectively was studied. To achieve this goal different material, such as stainless steel polyethylene terephthalate (PET) and copper (C), were tested in a milk flow. The bactericidal effectiveness of tested biocides was evaluated through the European Standard EN 1040:2006, in which survivors are enumerated by plate counting after neutralization and dilution of the tested biocide

Both examined substances were able to affect significantly bacterial density, although some differences can be observed (Fig. 3.20). BC was able to reduce the plancktonic density by 2.5 logarithms both in SS and PET during the whole period of monitoring, although a reduction up to 10^5 CFU/cm^2 was observed with PET specimen at 6 h. In C inhibitory activity of BC was slightly increased (3 logarithmic reduction factor since 3 h until 24 h). When UC 8410 was adhered to the stainless steel wire, bactericidal efficiency of BC was drastically reduced for 24 h; at the end of 24 h period, both untreated cells and cells exposed to BC had comparable quantitatively levels (above 10^8 CFU/cm^2).

In PET as well as C, no colony was found throughout the whole 24 h monitoring period even at lowest dilution. A possible explanation could be found in the interactions between the milk proteins and the tested surfaces: as observed in par. V of sect. 2.6, skimmed milk was not only able to cover the whole available surface of PET, but, when a certain thickness was reached, the organic matrix left spontaneously the surface material. Concerning C, a very thin layer of organic matter covered homogenously the whole superficial area of the specimen. These considerations suggested that both PET and C were not propitious surfaces for the deposition of organic matter, which acted as building material for the biofilm formation. In this way the above mentioned lower recoveries, after the exposure to BC, of these two surfaces could be explained.

PAA exhibited a stronger antimicrobial effect than BC (Tab. 3.4): compared with untreated sample, PAA reduced both sessile and planktonic cells by 6 logarithms in all tested surfaces. This difference of behavior towards the two investigated chemicals could be explained by strong oxidative properties of PAA, which could be effective in removal of organic matrix within which cells were embedded. Mechanism of action of BC is instead related to damages to membrane surface; resistance to such disinfectant is much more probably due to the chemical's incapacity to completely penetrate the thickness of matrix. Because of its excessive antimicrobial action, PAA was not used in further tests.

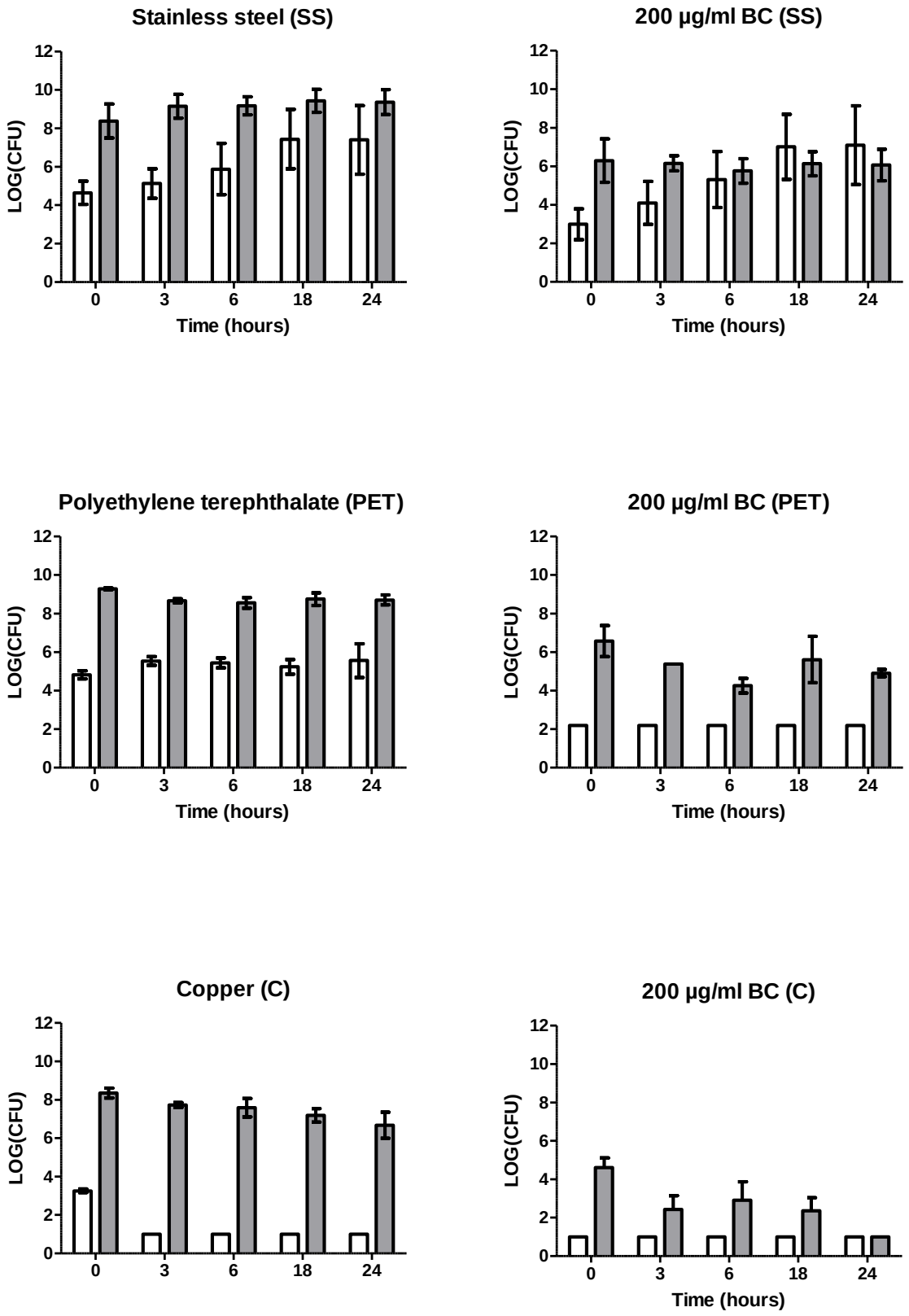


Fig. 3.20: Exposure of bacterial biofilms of *L. innocua* UC 8410 to 200 µg/ml BC on different materials. Data are expressed as average of three replicates ± standard error mean. White bars represent adhered cells, while grey bars are referred to cells which were freely suspended in the skimmed milk flow. 1 LOG(CFU) is to be intended as less than 10 CFU/cm^2.

Time	Stainless steel (SS)	Polyethylene terephthtlate (PET)	Copper (C)
	Sessile cells (CFU/cm2)		
0 h	< 100	< 100	< 100
3 h	< 100	< 100	< 100
6 h	< 100	< 100	< 100
18 h	< 100	< 100	< 100
24 h	< 100	< 100	< 100
	Planktonic cells (CFU/ml)		
0 h	< 100	< 100	< 100
3 h	< 100	< 100	< 100
6 h	< 100	< 100	< 100
18 h	< 100	< 100	< 100
24 h	< 100	< 100	< 100

Tab. 3.4 Exposure of bacterial biofilms of *L. innocua* UC 8410 to 5% PAA on different materials. Data are expressed as average of three replicates ± standard error mean. White bars represent adhered cells, while grey bars are referred to cells which were freely suspended in the skimmed milk flow.

[3.10]. Discussion

In order to give evaluation of bactericidal efficiency of an antimicrobial, most employed protocol in recent research papers was broth micro-dilution test following the NCCLS recommendations, while in Europe similar method is applied according to EUCAST guideline. Generally the method is mainly used to test towards antibiotics, but it can be successfully applied to biocides. Despite the strong similarities in analytical steps, NCCLS standards employed Mueller–Hinton broth, while LSM (mixture of Iso-Sensi Test broth and other growth medium, which varies depending on examined bacterial strains, with a 90:10 ratio) was used according to EUCAST methodology. Scientific literature is plenty of research papers in which both the methods are compared onto different bacterial and fungal species, leading to the conclusion there is not any strong discrepancy between these two methods (even if LSM medium produces slightly higher value than Mueller–Hinton broth), as reported in Koeth et al. (2000).

Four different substances were examined on a panel of 14 *Listeria* spp. strains: two biocides routinely used in food-processing environments (benzalkonium chloride and peracetic acid) were compared to natural polyohenolic compounds, pinosylvin and resveratrol. The reason for similar choosing can be easily found in recent literature reports, in which antimicrobial effectiveness of phenolic and polyphenolic compounds has been extensively described, beside their well known antioxidant capacity and their and thus possible benefits for human health (Roupe, et al. 2006). Most investigated source of phenolic compounds was wine and its raw matter, grapes (Anastasiadi, et al. 2009; Rhodes, et al. 2006; Vaquero, et al. 2007a; Vaquero, et al. 2007b), although it is not the only known source of similar molecules in natural environment.

Taguri et al. (2004) tested plant-extracted polyphenols on food-borne pathogenic bacteria (like *Salmonella* or *E. coli* strains), while Si et al. (2006), after extraction and purification by HSCCC, tested four bioactive polyphenolic molecules, among which epicatechin gallate and epigallocatechin gallate were the most active against *S. aureus* and MRSA. Epigallocatechin gallate is reported by Kohda et al. (2008) capable to inhibit growth of *L. monocytogenes* in macrophages through the inhibition of hemolytic and cholesterol-binding activities of listeriolysin O.

Škrinjar and Nemet (2009) determined MBCof diverse essential oils in vapor phase against foodborne bacteria both in vitro and in selected foodstuffs, whereas Harakudo et al. (2004) examined antibacterial activity of different plant used in cooking applications. Cinnamon stick contains (*E*)-cinnmaldehyde and several other polyphenols, which were able to inhibit five common foodborne bacteria (Shan, et al. 2007). Apple skins from Royal Gala and Granny Smith, beside the several beneficial characteristics for human health, possess a notably high content of phenolic compounds effective on diverse pathogens (mainly against *E. coli*, *P. aeruginosa* and *S. aureus*).

Another quantitatively relevant source of polyphenolic compounds could be found in trees, expecially in knotwoods, where it is possible extracting notable amount of such substances. These so relevant amounts of polyphenolic compounds could account for knotwoods lesser susceptibility to diverse bacteria and fungi. Also antimicrobial capacity of selected and specific compounds was deeply exploited: Välimaa et al. (2007) demonstrated that antimicrobial and citotoxic effect of *Pinus* spp. extracts could be explained by presence of pinosylvin, which was commonly recovered in all examined extract and present strong and promising antimicrobial traits. These properties of pinosylvin were clearly demonstrated by Lee et al. (2005). As limited number of works concerning this specific topic was observe in scientific literature, pinosylvin was selected for the present research activity. Resveratrol is a stilbene present in wine ranging from 0.2 mg/L until10.6 mg/L (Willför, et al. 2003) and, because of strong structural similarity with pinosylvin (clearly shown in

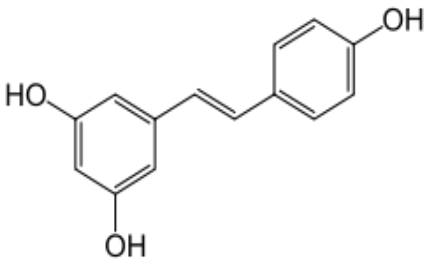


Fig. 3.21and in Fig. 3.22), it was hypothesized to have bactericidal properties, altough it was not elucidated none of studies concerning biocidal properties in wine and grape extracts (Vaquero, et al. 2007 a and b; Rhodes, et al. 2006).

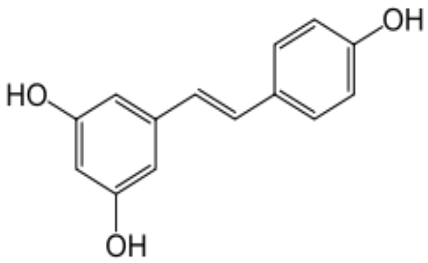


Fig. 3.21: Molecular structure of resveratrol.

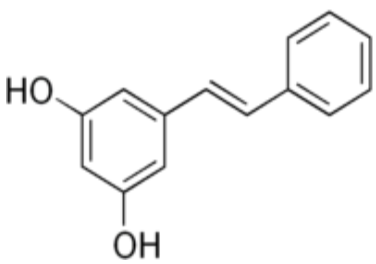


Fig. 3.22: Molecular structure of pinosylvin.

All *Listeria* strains were inhibited by both the two polyphenols examined, even if, despite structural relatedness, the two molecules did not show comparable biological effectiveness (128 versus 512 μg/ml). These two polyphenols did not show equivalent inhibitory activity, when compared to benzalkonium chloride (MBC= 8 μg/ml) and peracetic acid (MBC= 16 μg/ml). While all examined strains expressed same behavior against investigated polyphenols, wider variability of susceptibility was observed in peracetic acid than in benzalkonium chloride. Soumet et al. (2005) and Aarestrup et al. (2007) reported wide variability within *Listeria* spp. to QAC and it cannot be excluded analogue conclusions concerning peracetic acid, although no reference was found in scientific literature. Diversified susceptibility to biocides and other antimicrobial substances has been reported in several papers, although no clear explanation was produced. Moreover, while it has been well known how both peracetic acid and benzalkonium act against microbial cells, action mechanism of phenolic compounds has been not clearly and deeply elucidated, as observed by Välimaa et al. (2007).

Despite its large use, MBC through broth micro-dilution method have some intrinsic limits: evaluation is routinely done before and after 24 h exposure to antimicrobials, but during this period of time any kinetic of adaptation has not been investigated. MBC is defined as the minimal amount starting from which no microbial growth can be observed, but effect of higher or lower concentration has been quantitatively assessed. Therefore BioscreenC apparatus was employed as investigation methodology: this tool is turbidity reader with the possibility to incubate and to monitor periodically cultures at different temperatures in presence/absence of shaking. In literature this instrumentation was related to growth modeling (Begot, et al. 1996; Augustin, et al. 1999; Cheroutre-Vialette, et al. 1998; McClure, et al. 1989), but there are some papers in which BioscreenC was used to monitor antimicrobial susceptibility on several microorganisms, both pathogen/spoilage and useful (Nazer, et al. 2005; George, et al. 2008; Mandalari, et al. 2010) and also for MIC evaluation (Lambert et al. 2000).

Different concentration of pinosylvin (P), benzalkonium chloride (BC) and peracetic acid (PAA) were tested on eight strains of *L. monocytogenes* and their effect on growth curve was monitored for 24 h. Selected isolates were chosen to be representative of most possibly diverse origins: two strains fro food involved in two European listeriosis outbreak were examined in parallel with corresponding human clinical isolates, to which two sporadic clinical isolates were added along with two strains contaminating both water and sediment of fishery processing location. BC was confirmed as most active against all examined isolates both for employed amount and inhibitory capacity, while PAA was able to inhibit all strains used by 80 % at 180 μg/ml. Maximal inhibitory effect of pinosylvin was observed at 107 μg/ml, although environmental strains showed more limited susceptibility to this molecule in comparison with

other isolates (in this case inhibitory efficiency was only slightly above 60%). As reported in Moltz and Martin (2005), Bonaventura et al. (2008) and Lianou et al. (2006), no clear correlation among origin source and antimicrobial resistance was observed.

It was hypothesized that solvent could affect somehow bactericidal effect: for this purpose ethanol 99% was tested in parallel with DMSO on two *Listeria* strains, *L. innocua* UC 8410 (frequently used in scientific literature as a model for the pathogen *L. monocytogenes*) and *L. monocytogenes* EELA Hki L211. While different volumes of DMSO did not significantly affect bacterial growth even at high dosages, 3% EtOH was able to affect markedly kinetic of bacterial growth and it was employed as solvent in further examinations also to simulate any possible food-grade applications. This percentage was selected both to avoid any inhibitory effect due to the solvent and to solve any handling diificulties related to pipetting such small volumes. Both P and resveratrol (R) were tested on the two above-mentioned isolates to investigate any change in bactericidal effectiveness: greater inhibitory capacity was observed in P than R, confirming MBC results. Interestingly minimal inhibitory concentration was 3-fold less than the corresponding observed using DMSO, demonstrating synergic effect of EtOH with P, while lesser extents could have protective ability towards used strains against the solvent itself.

It was hypothesized that biocidal action of P could be dose-dependent and, for this purpose, greater concentrations than 200 μM were investigated: any statistically significant difference was observed until 500 μM, which have comparable effect than previously tested level of P. Extending employed BioscreenC protocol to different *Listeria* strains has allowed both to obtain similar results of above mentioned tests and to show that origin could not affect significantly antimicrobial susceptibility, despite discrepancies among tested isolates.

Subsequent step of survey was evaluating pinosylvin efficiency in preventing microbial contamination on surface. For this purpose we used a system widely employed in literature: stainless steel coupons have been immersed in BHI inoculated with *Listeria* spp. isolates. After a24 h incubation in optimal condition of temperature, coupons were exposed to dBHI, to test survival capacity under strongly limitant conditions of growth. In parallel adhesion capacity, cell viability and susceptibility to pinosylvin of some *Listeria* isolates were investigated. All *Listeria* strains were able to survive and grow, even for long periods, in extremely adverse conditions with not significantly reduction of cellular density. A possible future research topic could be if the transcription of genes of interest could be modified, in terms of entity and direction of transcription, in similar conditions. More surprising results came from resistance to pinosylvin in same conditions: after 7 days inoculums amount was drastically reduced and especially at concentrations of 200 and 300 μM, which have shown a limited efficiency in reducing microbial presence. Despite differences observed among employed strains (clinical isolates have shown an improved tolerance against the animal clinical isolate, which was followed by food isolates), no significant effect related to origin was revealed.

Bacterial susceptibility to sanitizers routinely used in food processing environments was evaluated both on liquid culture and on dynamically grown biofilms on all examined materials. Romanova et al. (2002) screened 19 *L. monocytogenes* isolates for BC resistance finding 4-fold differences among examined samples, although no correlation between serotyping and QAC susceptibility was found in agreement with Mereghetti et al. (2000). To et al. (2002) observed a certain variability among 6 tested strains of *L. monocytogenes* confirming observations of Soumet et al. (2005). Slight discrepancies were described among *Listeria* spp. by Best et al. (1990), although the two *L. monocytogenes* strains did not differ significantly between each other. Only reference coincerning MBCof PAA was Aarnisaalo et al. (2007), where diverse isolates from various food transforming location showed susceptibility ranging between 0.00625 and 0.025%.

In the current investigation BC has demonstrated to possess higher bactericidal efficiency on cultures in growth medium than PAA (MBC= 8 μg/ml), while biocidal effectiveness was significantly reduced after analogue treatments on bacterial biofilms. Bactericidal efficiency of BC was instead significantly changed when exposure of such biocide was performed on sample from grown biofilms: BC bactericidal efficiency on adhered cells ranged between 10 and 80% (referred to 0 and 3 h samples, respectively), although 24 h grown biofilm showed better performances in terms of tolerance to BC (32% bacterial reduction efficiency rate). Holah et al. (2002) reported that *L. monocytogenes* strains did not develop any resistance to QAC in food grade applications for sanitation, while Romanova et al, (2007) clearly demonstrated growth in biofilm occurred at all tested dosages of BC despite the compromised cellular vitality due to BC application. After exposure to PAA, no detectable colony was observed in both aliquots of surface and nutrient solution, confirming previous study of Bore et al. (2005) and Somers and Wong (2004). PAA levels were increased to 5 % (higher concentration than 1% used by Bore et al. and 3% of Somers and Wong), hypothesizing that biofilm multilayered architecture could be assumed as physical defense against biocide penetration, but our results suggested that this level possessed strongly inhibitory action (attributable to its action mechanism based on strong oxidative traits).

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