Aus dem Institut für Mikrobiologie und Tierseuchen

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Development of a broth microdilution method for biocide susceptibility testing of bacteria

Inaugural-Dissertation

zur Erlangung des Grades eines

PhD of Biomedical Science

an der

Freien Universität Berlin

vorgelegt von

Angela Regina Schug

Tierärztin

aus Lebach

Berlin 2021

Journal-Nr.: 4294

Gedruckt mit Genehmigung

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Uwe Rösler
Erster Gutachter:	Univ.-Prof. Dr. Stefan Schwarz
Zweiter Gutachter:	Univ.-Prof. Dr. Maren von Köckritz-Blickwede
Dritter Gutachter:	Prof. Séamus Fanning

Deskriptoren (nach CAB-Thesaurus): laboratory methods, diagnostic techniques, *staphylococcus aureus*, *escherichia coli*, *enterococcus hirae*, *pseudomonas aeruginosa*, benzalkonium chloride, chlorhexidine, glutardialdehyde, isopropanol

Tag der Promotion: 15.09.2021

Table of contents

Table of contents I
List of figures and tables III
Figures and tables of publication I III
Figures and tables of publication II III
List of abbreviations IV
1 Introduction 1
1.1 Biocides and bacterial resistance to biocides 2
1.1.1 The term biocide 2
1.1.2 Use of biocides and critical applications 2
1.1.3 Definition of resistance and related terms 4
1.1.4 Biocides used in this study 5
1.1.5 Resistance mechanisms 7
1.1.6 Occurrence of biocide resistance 8
1.2 Approaches to biocide testing of bacteria 12
1.3 Test organisms 13
1.4 Test parameters 14
1.5 Cell count determination 14
1.6 Aim of the present study 16
2 Publications 17
2.1 Publication I 17
2.2 Publication II 46
3 Discussion 52
3.1 Broth microdilution method for biocide susceptibility testing 52
3.1.1 Evaluation of the test parameters used 52
3.1.2 Comparison with results of broth macrodilution 54
3.2 Comparison of the different methods to determine biocide susceptibility. 54
3.2.1 Different approaches to phenotypic determination of biocide susceptibility 54
3.2.2 Genotype-based approaches to determine biocide susceptibility 56
3.3 Comparison of two methods for cell count determination 57
3.3.1 Correlation of the results from the two methods for cell count determination 59
3.3.2 Correlation of optical density and number of colony forming units 60
3.4 Further requirements and outlook 61
3.4.1 Quality control ranges 61
3.4.2 Interpretive criteria 62
3.5 Concluding remarks 63
4 Summary 64

5 Zusammenfassung 66
References 68
List of publications VI
Danksagung VIII
Finanzierungsquellen X
Interessenskonflikte X
Selbstständigkeitserklärung XI

List of figures and tables

Figures and tables of publication I

Figure 1 Scheme of the preparation of the biocide dilutions and controls 26
Figure 2 Schematic representation of the inoculum preparation 28
Table 1 MIC values within the acceptable range of the overall modal MIC ± one dilution step 28
Table 2 Deviations of more than ± one dilution step from the most common MIC value within one subgroup and inoculum density 29
Table 3 MIC values obtained via broth microdilution method in comparison to values obtained via broth macrodilution method 31
Table S1 MIC values for biocide/strain combinations reflecting all parameters of the comparative tests
a) Inoculum density according to DVG 33
b) Inoculum density according to CLSI 34
Table S2 MIC values for biocide/strain combination for the “subgroups” and “overall” for inoculum density according to DVG
a) Benzalkonium chloride 35
b) Chlorhexidine 37
c) Glutardialdehyde 39
d) Isopropanol 41
Table S3 MIC values for biocide/strain combination for the “subgroups” and “overall” for inoculum density according to CLSI
a) Benzalkonium chloride 43
b) Chlorhexidine 45
c) Glutardialdehyde 47
d) Isopropanol 49
Table S4 Comparison of the deviations observed after evaluation of the acceptable ranges for “overall” and for the “subgroups” 51
Figure S1 Schematic representation of the comparative tests performed in parallel and the parameters used 52

Figures and tables of publication II

Figure 1 Schematic representation of the preparation of the bacterial suspensions and the steps for the cell count determination 55
Figure 2 Bland-Altman plot showing the deviations of the methods and the limits of agreement 56
Figure 3 Bland-Altman plot for the individual reference strains and comparative investigations 57
Figure 4 Correlation of the cell counts with the optical density using linear regression.58

List of abbreviations

AL	Limits of agreement
AST	Antimicrobial susceptibility testing
BAC	Benzalkonium chloride
BET	Biocide efficacy testing
BORSA	Borderline oxacillin-resistant *S. aureus*
BST	Biocide susceptibility testing
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DCS	Direct colony suspension method
DIN	German Institute for Standardization (Deutsches Institut für Normung e.V.)
DVG	German Veterinary Medical Society (Deutsche Veterinärmedizinische Gesellschaft e.V.)
E. coli	*Escherichia coli*
E. hirae	*Enterococcus hirae*
ECOFF	Epidemiological cut off value
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GLU	Glutardialdehyde
ISO	Isopropanol
K. pneumoniae	*Klebsiella pneumoniae*
L. monocytogenes	*Listeria monocytogenes*
MBC	Minimal bactericidal concentration
MDR	Multi-drug resistant
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*

OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
P. stutzeri	*Pseudomonas stutzeri*
PCR	Polymerase chain reaction
QAC	Quaternary ammonium compound
QC	Quality control
S. aureus	*Staphylococcus aureus*
SC	Subculture
spp.	Species pluralis, multiple species
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSD	Tryptone saline diluent
VAH	Association for Applied Hygiene (Verbund für Angewandte Hygiene e.V.)
WHO	World Health Organization

1 Introduction

The exposure of bacteria to antimicrobial agents as well as to biocides may act as a selective pressure, which promotes the development of resistant bacteria (Levy, 2002b; Russell, 2003; Holmes et al., 2015). The emergence of antimicrobial resistance in bacterial pathogens poses an increasing threat to human and animal health and requires consistent and effective measures to limit the development and spread of resistant bacteria (WHO, 2014; EFSA, 2019). Biocides are used to inactivate pathogenic microorganisms and are therefore of particular importance in infection control and to prevent the spread of nosocomial pathogens. Nevertheless, biocides are increasingly used for a variety of other purposes and in a wide range of applications including hospitals, food production, veterinary medicine, animal husbandry and in households (McDonnell and Russell, 1999; SCENIHR, 2009; Maillard et al., 2013). Preventing the development of bacterial resistance and cross-resistance is of fundamental importance to maintain the effectiveness of biocides for infection control and hygiene (SCENIHR, 2009). Biocide resistance has been reported in a significant number of bacterial isolates (Chapman, 1998; D'Arezzo et al., 2012; Kampf, 2016; Hardy et al., 2018; Pidot et al., 2018; Stein et al., 2019). Biocides also contribute to the development and the spread of bacterial pathogens that exhibit resistance phenotypes to both, biocides and antimicrobial agents (SCENIHR, 2010), as a number of resistance mechanisms (e.g. efflux pumps and membrane permeability changes) can result in resistance to both (Russell, 2001; Maillard, 2005; Thomas et al., 2005). Therefore, there is an urgent need to monitor the emergence and determine the extent of biocide resistance (SCENIHR, 2009).

To monitor biocide resistance systematically, it is necessary to use standardized methods that provide (intra- and interlaboratory) reproducible and comparable information (Gould, 1998; Rodloff et al., 2008; Buffet-Bataillon et al., 2012; RKI, 2019). Many different methods have been used for biocide susceptibility testing (Suller and Russell, 1999; Thomas et al., 2000; Couto et al., 2008; Buffet-Bataillon et al., 2012; Condell et al., 2012; Arioli et al., 2013; Finn et al., 2013; Morrissey et al., 2014; Lanjri et al., 2017; Mombeshora and Mukanganyama, 2017). As the methodology largely differs between the protocols used, results of the different studies are hardly comparable. However, no standardized methods for biocide susceptibility testing (BST) existed until first steps were made as we developed a broth macrodilution protocol (Feßler et al., 2018). A convenient method facilitating high-throughput screening, being easy to use and at the same time providing high precision results, is needed. This includes, along with the test method itself, a method to verify that the correct inoculum density is maintained, which is an important test parameter. This project aims to fill this gap by developing a broth microdilution protocol for BST and by evaluating whether cell counting during BST can be simplified without impairing the quality of the results.

1.1 Biocides and bacterial resistance to biocides

1.1.1 The term biocide

The term biocide is derived from ancient Greek *βίος bios*, English “life” and Latin *caedere,* “kill”. Biocide is a general term that is often used very broadly. For example, according to the EU Biocidal Products Regulation 528/2012 (EU, 2012), biocides comprise among others, disinfectants, antiseptics, preservatives, fungicides, algaecides, rodenticides and insecticides. Others use the term biocide to generally describe a chemical agent that inactivates microorganisms, usually with a broad spectrum of activity (McDonnell and Russell, 1999). Hence, biocides can refer to different chemicals, and their efficacy and use also depend greatly on the formulation of the biocidal product (composition of ingredients and additives) (Maillard, 2018). To avoid problems related to the broad use of the term biocide, Maillard et al. (2013) use the terms “microbicide” and “microbistat” when referring to chemicals that can kill microorganisms or inhibit their replication, respectively, and which represent a subgroup of biocides in the broader meaning of the term (Maillard et al., 2013). Thus, microbicides and microbistats include disinfectants, preservatives and antiseptics with activity against all microorganisms, but exclude antimicrobial agents (Maillard et al., 2013). In this study, I decided to stick to the term “biocide” because it is commonly used throughout the scientific community as well as in official documents (Levy, 2002a; Chapman, 2003a; Russell, 2004; EFSA, 2008a; SCENIHR, 2009; SCENIHR, 2010; Condell et al., 2012; Morrissey et al., 2014; Deus et al., 2017; Humayoun et al., 2018; Kampf, 2018; Kernberger-Fischer et al., 2018; Donaghy et al., 2019; Roedel et al., 2020).

The term biocide is used, (1) when referring to chemicals with antiseptic and disinfectant properties and (2) when referring to substances which, unlike antimicrobial agents, are not commonly used to treat bacterial infections in animals or humans, but which nevertheless have an antibacterial effect.

1.1.2 Use of biocides and critical applications

Biocide use is an integral part of hygiene measures to reduce microbial load and transmission in clinical settings, which is crucial in the era of multidrug resistance and with an increasing number of infections that do not respond to antimicrobial treatment (WHO, 2015).

Normally, biocides are used on inanimate objects (hard surface disinfectants), or externally on the skin (antiseptics and topical antimicrobials). Furthermore, biocides are commonly used to prevent or to limit microbial infections, or are used as an ingredient in pharmaceuticals, cosmetics or other products to prevent microbial contamination (Russell, 2003). The manifold fields of application show the versatility of biocidal products (Maillard, 2005). Nowadays, biocides are increasingly incorporated into many consumer products, e.g.,

in household cleaning products, toothpastes, mouthwashes, clothing, underwear, pens and hand cleaning products (Maillard et al., 2013). In the modern food industry, the use of preservatives is decreasing, with the consequence that, aiming to improve hygiene measures and ensure food safety, there has been an increased use of biocides and disinfectants in the production environment (Langsrud et al., 2003).

However, there are concerns related to the increased use of biocides due to the potential correlation of biocide use with the emergence of antimicrobial resistance in bacteria (Maillard, 2007; SCENIHR, 2009; Maillard et al., 2013). The development of inadequate biocidal products and the improper use of biocidal products is a critical aspect (SCENIHR, 2010). Products containing biocides in low (sublethal) concentrations or for which the bioavailability is low, may increase the risk of selection of resistance in target or non-target microorganisms (Maillard, 2005; SCENIHR, 2010). Several laboratory studies have demonstrated the evolution of resistance to biocides as a result of exposure to low concentrations (Thomas et al., 2000; Walsh et al., 2003; Gomez Escalada et al., 2005a; Christensen et al., 2011; Knapp et al., 2013; Mavri and Smole Možina, 2013). In high concentrations, many biocides may be toxic, both for the user and for the environment (Daschner and Dettenkofer, 1997; Daschner and Schuster, 2004). When biocides are released into the environment, they also set the course for a changed microbial ecology (Levy, 2002b). A problematic issue is the fact that biocides are not always fully biodegradable and can therefore persist in sewage over long periods of time. An example for such biocides are quaternary ammonium compounds (QACs), such as benzalkonium chloride, which are only biodegradable under aerobic conditions (Tezel and Pavlostathis, 2015). This can then result in frequent exposure to subinhibitory concentrations of QACs (Martínez-Suárez et al., 2016). Bacteria repeatedly exposed to subinhibitory concentrations of QACs may develop resistance (Ortiz et al., 2014a). A recent example is the COVID-19 pandemic and the accompanying heavily intensified use of biocidal agents for environmental as well as personal disinfection, both inside and outside health-care settings. As described in the Bulletin of the World Health Organization (WHO) by the director of the Department of Global Coordination and Partnership on Antimicrobial Resistance at WHO and his colleagues, this gives rise to concerns about the risk of possible biocide resistance and cross-resistance to antimicrobials and calls for the requirement to use only agents for disinfection with no or low selection pressure for antimicrobial resistance (Getahun et al., 2020). As a consequence of the COVID-19 pandemic, increased concentrations of biocides and antimicrobial agents are expected in wastewater, wastewater treatment plants and receiving waters, which results, for example from an increased use of biocide-containing soaps and disinfectant cleaners. The effects need to be investigated, because there is concern that they may contribute to the development of resistance (Murray, 2020).

When considering the aforementioned factors, it is undeniable that, overall, the use of biocides has brought an invaluable benefit to both animal and human health (Maillard, 2005; SCENIHR, 2009; Siani and Maillard, 2015). Nevertheless, not reflected or inappropriate use of biocidal products must be avoided. There have been proposals to introduce biocide stewardship (Kampf, 2016), limiting biocide use only to applications with clear benefits. Overall, when using biocidal products, a balance must be struck between the clear benefits and the potential risks in terms of emerging bacterial resistances and of environmental pollution and toxicity (Daschner and Schuster, 2004; Rutala and Weber, 2004).

1.1.3 Definition of resistance and related terms

A key issue is the definition of "resistance". A literal interpretation of resistance is the ability of bacteria to resist the effects of a harmful chemical agent and to survive exposure to a defined concentration of harmful agents (SCENIHR, 2009).

Usually, in the context of biocides, the term resistance can indicate that a strain is not killed or inhibited by: (i) a concentration achieved in practice (the in-use concentration), (ii) a concentration to which the majority of strains of that organism are susceptible, or (iii) a concentration that affects the majority of cells in that culture (SCENIHR, 2009). The definition of resistance is also based on in vitro parameters and is therefore linked to the test protocols for resistance measurement (Russell, 2003; EFSA, 2008b; Maillard, 2018).

Several terms and definitions are used in literature, some of which overlap and are often not sufficiently refined, and overall there is no clear consensus within the scientific community (Maillard, 2018). Some definitions of resistance describe only a small decrease in susceptiblity (Chapman, 1998) while, in the context of chemotherapeutic antimicrobial agents, the term resistance reflects the failure of the agent during clinical use (Maillard, 2018), which relates to the survival at an in-use concentration of a biocide that was intended to be bactericidal. According to Maillard, in many papers the term "reduced susceptibility" has been used, referring to minimal inhibitory concentration (MIC) values or the minimal bactericidal concentration (MBC) (Maillard, 2018) indicating that a bacterial isolate can withstand higher concentrations of a biocide than the majority of the population of the same species. The term “tolerance” is likewise used to denote reduced susceptibility based on raised MIC values (SCENIHR, 2009). In addition, it occurs that the terms "tolerance" and "resistance" are used interchangeably, which could lead to misinterpretation of data (Gilbert and McBain, 2003; Gerba, 2015).

Since there is a lack of standardized biocide susceptibility testing methods and interpretation criteria (see also 3.4.2), a breakpoint is often arbitrarily chosen (Soumet et al., 2005). MICs for a species often show a bimodal distribution, where the subpopulation with low

MIC values usually does not possess resistance mechanisms while the other subpopulation shows higher MIC values and often carries resistance mechanisms (Rodloff et al., 2008). Several authors considered bacteria to be resistant towards a biocide based on the fact that their MICs were at least two (Aase et al., 2000) or four (Emslie et al., 1986) times higher than those obtained for the strains of the same species that are the most susceptible, but without taking into account the distribution of MIC values. This is problematic because doubling the MIC value, i.e. varying by one dilution step in a twofold dilution series, is within the expected variance of the test system and is thus, accepted as normal variation (CLSI, 2018c). The variations of ± 1 dilution step were also observed during the repeated tests during this study.

Altogether, the definition of resistance is not straightforward and therefore it is important that the intended meaning of the word "resistance" is meticulously defined in scientific articles with the necessary reference to the measurement method (Cerf et al., 2010). This means that a definition of the terms used to describe the susceptibility of a bacterial strain to a biocide must be provided with the context of its use.

1.1.4 Biocides used in this study

The mode of action of biocides is comparatively unspecific and significantly influenced by the concentration (Russell and McDonnell, 2000; Maillard, 2002). In contrast to antimicrobial agents, most biocides have several different target sites in the bacterial cell (Russell, 1990; Maillard, 2002; Poole, 2002; Condell et al., 2012) and the overall damage to those target sites leads to the bactericidal effect (Maillard, 2002; Maillard et al., 2013). Biocides act through physico-chemical interactions or chemical reactions on cell components of the bacteria (McDonnell and Russell, 1999; Ortega Morente et al., 2013). In general, three levels of interaction mechanisms can be described according to the target structure in the bacterial cell: interaction with external cellular components, interaction with the cytoplasmic membrane and interaction with cytoplasmic components (Maillard, 2002; Ortega Morente et al., 2013). However, a biocide may act at least on one level (Maillard, 2002; Ortega Morente et al., 2013). The modes of action of biocides differ further based on the type of agent. Biocides can be divided into electrophilic, lytic and oxidizing agents. Electrophilic biocides like glutardialdehyde react with nucleophilic functional groups, which leads to clumping of the cytoplasm. Lytic biocides such as QACs are surfactants and dissolve the cell membrane. Ethanol and aldehydes also act at the cell membrane. Oxidizing agents such as sodium hypochlorite destroy the cell by forming free radicals (Denyer and Stewart, 1998).

The biocides used in this study are widely used in health-care settings, veterinary medicine, animal husbandry as well as in the food industry. To test the stability of the applied protocol with different classes of biocides, representatives of QACs (benzalkonium chloride),

cationic compounds (chlorhexidine), aldehydes (glutardialdehyde) and alcohols (isopropanol) were used in this study. The four selected biocides differ in their modes of action:

Benzalkonium chloride (BAC) is a QAC and a cationic biocide. Its mode of action is based mainly on the destabilization of the bacterial membrane via interaction with negative charges (Kramer et al., 2008). BAC has bactericidal activity against Gram-positive and, to a minor degree, Gram-negative bacteria (Kramer et al., 2008). The in-use concentration of BAC in veterinary medicine is highly dependent on the indication and site of application and ranges from 0.01 % up to 0.2 % (EMEA, 1997). Resistance mechanisms include alterations in membrane composition and expression of *qac* genes encoding inducible efflux pumps (Kramer et al., 2008; Ortega Morente et al., 2013; Jennings et al., 2017; Worthing et al., 2018).

Chlorhexidine (CHX) is a cationic biocide that belongs to the bis-biguanides. The effect of CHX is concentration-dependent, being bacteriostatic in low concentrations and bactericidal in higher concentrations (Kramer et al., 2008). Its spectrum includes particularly Gram-positive, but also Gram-negative bacteria, mycobacteria and fungi (Kramer et al., 2008). CHX damages the cell membrane and interferes with membrane-bound enzyme systems (McDonnell and Russell, 1999). Resistance to CHX is acquired by multidrug efflux pumps and changes in the cell membrane that limit the absorption of the biocide (Kampf, 2016; Cieplik et al., 2019). Concentrations for usage in commercial products range most commonly from 0.5 % to 4 % for surface dinsinfection, from 0.02 % to 4 % for antiseptic applications, or from 0.0025 % to 0.01 for preservation purposes (Maillard, 2005; Milstone et al., 2008).

Glutardialdehyde (GLU) (synonym: glutaraldehyde) belongs to the aldehydes and reacts by cross-linking proteins and lipids on the outer surface of the cell (McDonnell and Russell, 1999). GLU’s activity is highly dependent on temperature (van Klingeren and Pullen, 1993). GLU is most commonly used as chemosterilant for heat sensitive medical devices like endoscopes. It is normally used in a concentration up to 2 %, and is sometimes also recommended in concentrations up to 3 % (van Klingeren and Pullen, 1993; McDonnell and Russell, 1999; Nelson, 2002; SCENIHR, 2009). Resistance to GLU is thought to be conveyed through porin formation and upregulating of multidrug efflux pumps (Vikram et al., 2015).

Isopropanol (ISO) (synonyms: isopropyl alcohol or propan-2-ol) is a short-chain alcohol. ISO kills bacteria by disrupting membrane functions and its activity is rapid and of broad spectrum against all bacteria but is not effective against spores (McDonnell and Russell, 1999). ISO has the best antibacterial activity in concentrations between 60 % and 85 % (Ali Y., 2001; Boyce and Pittet, 2002). For hand disinfection, it is typically used at a concentration of at least 70 %. Concentrations above 90 % are less effective because protein denaturation is decreased in the absence of water (Ali Y., 2001; Boyce and Pittet, 2002). Pidot and co-workers described alcohol-tolerant *Enterococcus faecium* isolates that showed mutations in genes involved in carbohydrate uptake and metabolism (Pidot et al., 2018).

Altogether, biocides can be very diverse chemicals and, unlike antimicrobial agents, have rather non-specific modes of action.

1.1.5 Resistance mechanisms

Bacteria express several mechanisms to respond to the harmful effects induced by biocides. Biocides have multiple targets on the bacterial cell and are often regarded as non-specific (Maillard, 2018). Most likely, several mechanisms contribute synergistically to the detected resistance phenotype and the concentration of the biocide plays a major role in whether there is a lethal or inhibitory effect on the bacterial cell (Denyer and Stewart, 1998; McDonnell and Russell, 1999; Maillard and Denyer, 2009). The understanding of biocide resistance mechanisms has generally improved, but overall the subject remains little studied (Maillard, 2018).

The bacterial resistance mechanisms to biocides can be either intrinsic or acquired. Intrinsic resistance is defined as a natural (innate) property of a bacterial cell that enables it to evade the action of a biocide. In many cases, the cell structure prevents or reduces the penetration of the biocide. Examples are the outer membrane of Gram-negative bacteria, or the cell wall composition of mycobacteria that convey intrinsic resistance to many antimicrobial compounds (Ortega Morente et al., 2013). Similarly, many bacterial spores are intrinsically resistant to certain biocidal agents due to their outer layers that represent an effective barrier for biocide action (Russell, 1999). Furthermore, physiological (phenotypic) modifications can increase bacterial tolerance to biocides, e.g. of cells within a biofilm (Gilbert et al., 1993).

Since biocides usually have multiple targets on the bacterial cell, it is unlikely that the development of resistance is caused by a specific modification of a target or by by-passing of a metabolic process (SCENIHR, 2009). Hence, bacteria are more likely to develop very unspecific resistance mechanisms towards biocides (Gnanadhas et al., 2012). At least some of the general mechanisms responsible for resistance overlap and can be applied to biocides as well as to antimicrobial agents, so that there is a possibility of cross-resistance (Russell, 2002a). On the other hand, most of the mechanisms that cause antimicrobial resistance are agent specific and thus induce resistance to a specific antimicrobial agent or class (Ortega Morente et al., 2013).

Acquired bacterial resistance mechanisms against biocides occur through mutation, through the acquisition of genetic material like certain plasmids or transposons by horizontal gene transfer (Paulsen et al., 1996; Russell, 1997; McDonnell and Russell, 1999), or via over-expression of genes or expression of previously silent genes (Chapman, 2003b). Many mechanisms are intended to decrease the concentration of the biocide, so that it can no longer cause damage to the bacterial cell, whereby repair mechanisms are also involved (Maillard,

2018). The most common acquired resistance mechanism is protection due to changes in cell envelope permeability (cellular impermeability) or increased biocide efflux (McDonnell and Russell, 1999; Schweizer, 2001; Poole, 2002). These resistance mechanisms occur for example via mutation in genes that are involved in the formation of the cell wall, membrane lipids, porins, or outer membrane proteins (Gnanadhas et al., 2012). Efflux mechanisms, being drug-specific or multi-drug, are important determinants of resistance towards both antimicrobial agents and biocides (Poole, 2005) as they are capable of assimilating a wide range of structurally unrelated substances. Efflux can be induced by some biocides. QACs, for example, can promote the expression of efflux pumps, or stimulate mutations within the regulators of efflux pump genes (Oethinger et al., 1998; Olliver et al., 2004; Webber et al., 2005; Morita et al., 2006; Warner et al., 2008; Ricci et al., 2014; Buffet-Bataillon et al., 2016). Overexpression of efflux pumps has also been reported following triclosan exposure (Chuanchuen et al., 2003; Sanchez et al., 2005). Environmental isolates with reduced biocide susceptibility, from areas with high biocide use, remarkably biguanides and QACs, showed a high prevalence of efflux genes in several studies (e.g., *qacA/B*, *norA*, *norB*, *smr*) (Heir et al., 1999; Liu et al., 2015; Conceição et al., 2016; Grande Burgos et al., 2016; Hijazi et al., 2016; Maillard, 2018).

A biocide concentration that does not kill the target bacterium triggers a stress reaction that leads to the development of survival mechanisms. Altogether, the bacterial resistance mechanisms toward biocides are manifold and partly very unspecific. This fuels concerns about links between biocide and antimicrobial resistance and cross-resistance. The available and effective concentration of the biocidal agent during use is therefore of utmost importance.

1.1.6 Occurrence of biocide resistance

The multifactorial mode of action and broad target base of biocidal agents and biocidal products led to the belief that development of biocide resistance in bacteria is rather unlikely (Poole, 2002; Russell, 2003; Roedel et al., 2020). Although overall the data is still sparse (Maillard, 2018) and the field of biocide resistance has been only haphazardly investigated (Chapman, 2003a), there are numerous reports on biocide resistance and examples of biocide-resistant bacteria using the same main resistance strategies that can also lead to resistance to antimicrobial agents (Chapman, 2003a). Most of the investigations are *in vitro* studies relating to a few particular biocidal agents and the susceptibility of few isolates.

There are numerous reports of environmental isolates that were less susceptible to a specific biocidal agent. These isolates originated, for example, from health-care, from food production or slaughterhouses and their resistance concerned various biocidal agents. Some examples are the following: Langsrud and Sundheim found resistance to BAC among

Pseudomonas spp. isolated from poultry carcasses in about 30 % of the isolates (Langsrud and Sundheim, 1997). Langsrud and co-workers also found various strains with low-level resistance to QACs isolated from food and food processing industry in *Listeria monocytogenes*, *Staphylococcus* spp., *Pseudomonas* spp., and, less frequently, in lactic acid bacteria and coliforms (Langsrud et al., 2003). Halbedel and colleagues analyzed an exceptionally large cluster of *L. monocytogenes* isolates collected during a listeriosis outbreak associated with blood sausage in Germany that included 134 highly clonal, BAC-resistant isolates which carried the *emrC* gene that most likely caused the decreased susceptibility to BAC (Halbedel et al., 2020). In health-care settings for example, Wisplinghoff and colleagues found *Acinetobacter baumanii* isolates related to a nosocomial outbreak that required longer agent contact times in order to be killed than non-outbreak-related strains of the same species (Wisplinghoff et al., 2007). Bock and colleagues noted that not all investigated CHX formulations were able to kill multi-drug-resistant (MDR) *Klebsiella pneumoniae* isolates after the recommended exposure time (Bock et al., 2016). Dance and colleagues isolated a strain of *Proteus mirabilis* resistant to CHX and cross-resistant to some antimicrobial agents, which caused a hospital outbreak (Dance et al., 1987). Similarly, Stein and colleagues reported nosocomial transmission of carbapenem-resistant *K. pneumoniae* nonsusceptible to CHX from patients who were regularly washed with CHX, and in addition, some of the isolates also showed resistance to colistin, which they linked to efflux in these CHX-adapted isolates and as a result of the CHX exposure (Stein et al., 2019).

Moreover, there are studies that report outbreaks caused by bacterial contamination of a biocide solution. In these biocide solutions, the bacteria had been able to survive or even grow, causing outbreaks or pseudo-outbreaks. The analysis of these incidents has shed light on some of the critical points and hazards related to the biocides used, some common practices and application errors. Weber and co-workers, for example, reported in their review a number of outbreaks associated with contaminated CHX solutions (Weber et al., 2007). Similarly, Farrand and Williams found in 1973 that CHX-based disinfection can fail due to some Gram-negative bacteria that could survive in the solution (Farrand and Williams, 1973). Lanini and colleagues identified clinical isolates of *Pseudomonas aeruginosa* with increased tolerance in a heavily contaminated triclosan soap dispenser (Lanini et al., 2011). Martin and colleagues isolated a *Bacillus subtilis* isolate from a washer-disinfector whose vegetative form was highly resistant to chlorine dioxide and hydrogen peroxide (Martin et al., 2015).

The introduction and extensive use of certain biocides in disinfection routine can cause a decrease in biocide susceptibility, as shown by several studies (Russell, 2002b; Bock et al., 2016; Hardy et al., 2018; Pidot et al., 2018), where an increase in the MIC values mirrors the biocide's introduction in clinical use.

A number of studies analyzed the *in vitro* training of bacterial strains to survive increasing concentrations of a biocide. Once the biocide was removed, the reduced susceptibility could either be maintained or was lost again. The possibility of associated cross-resistance to other biocides or antimicrobials has also been the focus of many of these studies. Although this approach is detached from *in situ* situations and conditions and the clinical relevance of these studies is limited, as stepwise training is likely to result in multiple mutations, which may not occur in practice (Russell, 2003), such studies can provide valuable information on resistance mechanisms. An example is the study by Thomas and co-workers, in which *P. aeruginosa* was trained to become less sensitive to cationic biocides by gradually exposing it to increasing concentrations of CHX or QACs (Thomas et al., 2000). Similarly, Russel and colleagues as well as Tattawasart and colleagues successfully trained *Pseudomonas stutzeri* to become insensitive to the same kind of agents (Russell et al., 1998; Tattawasart et al., 1999; Tattawasart et al., 2000). Some of their *P. stutzeri* strains also showed reduced susceptibility to other biocides, including triclosan, and to some antimicrobial agents to varying extents (Tattawasart et al., 1999). Induction of reduced susceptibility by triclosan was also investigated in *Escherichia coli*, *Staphylococcus aureus* and *Acinetobacter johnsonii* (Cottell et al., 2009). Other classes of biocides, such as isothiazolones (Winder et al., 2000), hydrogen peroxide and peracetic acid (Dukan and Touati, 1996) have also been the subject of studies to reduce the susceptibility of bacterial isolates to them through stepwise training. Similarly, different serotypes of *Salmonella enterica* were trained to survive increasing sub-inhibitory concentrations of three biocides widely used in food industry facilities (Molina-González et al., 2014).

Three studies regarding the effect of biocide exposure on population dynamics and compositional change in a complex microcosm revealed that chronic or repeated sublethal exposure of sink drain biofilms to some common biocides resulted with differing frequency in clonal expansion of some species, and in selection for reduced susceptibility for a proportion of the tested bacteria (Moore et al., 2008), or led to a decrease in species diversity but did not significantly affect the susceptibility to the tested biocides or antimicrobial agents (McBain et al., 2003; McBain et al., 2004).

Since biocides and antimicrobial agents share some resistance mechanisms, e.g. efflux pumps or permeability changes, and those common resistance mechanisms could favor cross-resistance, the use of biocides has raised concerns about the selection of biocide- and antimicrobial-resistant bacteria (SCENIHR, 2010; Maillard et al., 2013). Cross-resistance describes a resistance mechanism that enables bacteria to survive the effects of several antimicrobial molecules with similar or overlapping mechanisms of action. Some of these cross-resistances are conveyed by mutational up-regulation of multidrug efflux systems that mediate reduced susceptibility to both classes of agents (Poole, 2002; Thorrold et al., 2007;

SCENIHR, 2009). Furthermore, it has been found that changes in the cell envelope (reduction in porins and changes in lipids) caused resistance to both, biocides and antimicrobial agents (Denyer and Maillard, 2002; Nikaido, 2003; Tkachenko et al., 2007). The risk for biocide-selected cross-resistance to clinically important antimicrobial agents has been discussed in the literature (Russell, 1999; Schweizer, 2001; Poole, 2002). The opinion appeared to be that cross-resistance between biocides and antimicrobials has rarely been detected (SCENIHR, 2009) and at least clinically, biocide-antimicrobial cross-resistance seemed to be negligible (Poole, 2002). In 2019, Stein and co-workers found evidence for colistin resistance that emerges from wide-ranging exposure to CHX in carbapenem-resistant *K. pneumoniae* involved in a hospital outbreak (Stein et al., 2019). There were also further reports of biocide-antimicrobial cross-resistance in Gram-negative bacteria (Poole, 2001: Russel 1998, Tattawasart, 1999; Moken, 1997, McMurry 1998). Roedel and colleagues investigated *L. monocytogenes* isolates from German food production plants with reduced biocide susceptibility and found no evidence for cross-resistance to clinically relevant antimicrobial agents in these field isolates (Roedel et al., 2019). With increasing research and attention to the problem of cross-resistance between biocides and unrelated chemicals, such as antimicrobial agents, more and more reports are published of its occurrence (Maillard, 2018).

In addition, bacterial biofilm is an important property in conferring resistance to both antibiotics and biocides (SCENIHR, 2009). For example, Smith and Hunter found that some biocides used in hospitals are ineffective against nosocomial pathogens, such as MRSA and *P. aeruginosa*, which grow as biofilms on surfaces, although they may be effective against planktonic populations, thus failing to control this reservoir of hospital-acquired infections (Smith and Hunter, 2008). Tabak and co-workers demonstrated that the concentrations of triclosan used in consumer products might not be effective in killing *Salmonella* when growing within biofilms (Tabak et al., 2007). The induction of changes in susceptibility and biofilm formation in uropathogenic *E. coli* were investigated by Henly and colleagues and they found biocide exposure, especially to triclosan, causing a decrease in susceptibility that correlated with an increase in biofilm biomass in all isolates and they also observed the induction of antimicrobial cross-resistance by triclosan in 6/84 possible combinations of bacteria, biocide, and antimicrobial agents (Henly et al., 2019). Ortiz and colleagues conclude from their studies on the influence of subinhibitory concentrations of BAC on biofilm formation in *L. monocytogenes* that biofilm formation may be an important feature in certain resistant and persistent strains of *L. monocytogenes* (Ortiz et al., 2014b).

Bacteria have vast potential to adapt and develop ways to survive exposure to biocides, especially if used imprudently or incorrectly. In health-care and other professional settings in particular, all those involved must be trained on pitfalls and know critical points in the use and preparation of biocidal products. It is important that studies are carried out which

focus on the biocide susceptibility of individual strains to monitor susceptibility profiles rather than only concentrating on the efficacy of a biocidal product. So far, studies addressing biocide susceptibility have often varied in their methodologies, thus hampering the comparison of results.

1.2 Approaches to biocide testing of bacteria

How to measure the biocide resistance of bacterial isolates is a key question, but the approaches and strategies for doing so can vary widely and yield different results. Biocide efficacy testing (BET) and biocide susceptibility testing (BST) are two approaches with a different focus, that serve different purposes: The goal of BET is to provide information on the performance of a biocidal product under conditions of use, whereas the goal of BST is to determine the susceptibility of an individual bacterial isolate to a biocide. BST is important in resistance surveillance and epidemiological studies for monitoring and early identification of clinical isolates with elevated biocide MICs.

The objective of BET is to verify that the disinfectant achieves the intended purpose, i.e. a specific disinfection task (Kramer et al., 2008). BET includes factors that may influence the performance of a biocidal product during application and compares the biocidal products with a reference substance to ensure that only biocidal products that have shown to be effective are placed on the market (DIN, 2006; DVG, 2017). For BET, there are harmonized protocols from important reference institutions for certification of biocidal products and advice on disinfection procedures (e.g. DVG, VAH, DIN, see below). BET regimes are usually structured in a two-stage procedure of quantitative suspension tests and tests closer to the actual usage, that include, in a standardized way, factors of the application situation (such as organic load, different surface conditions, application temperatures, contact times) (Kramer et al., 2008). BET usually has the following or similar structure: basic bactericidal activity, followed by quantitative suspension test and quantitative surface test for the evaluation of bactericidal activity, and additionally, field tests under practical conditions can be conducted (Kramer et al., 2008). Approved BET methods comparatively test the new compound and a reference compound and compare the reduction of the bacterial growth, usually of two to four reference strains. In Germany, there are several guidelines for performing BET, e.g. those of the German Veterinary Medical Society (Deutsche Veterinärmedizinische Gesellschaft e.V., DVG) (DVG, 2017), the Disinfectants Commission in the Association for Applied Hygiene (Verbund für Angewandte Hygiene e.V., VAH) (VAH, 2015) and the German Institute for Standardization (Deutsches Institut für Normung e.V., DIN) (DIN, 2006).

In addition, there are standardized and harmonized methods for antimicrobial susceptibility testing (AST), for example issued by organizations such as the Clinical and

Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In the absence of standardized methods for BST, many different protocols were used (Suller and Russell, 1999; Thomas et al., 2000; Couto et al., 2008; Buffet-Bataillon et al., 2012; Condell et al., 2012; Arioli et al., 2013; Finn et al., 2013; Morrissey et al., 2014; Lanjri et al., 2017; Mombeshora and Mukanganyama, 2017). Mostly, these approaches are derived from AST or are based on a similar test principle as done during AST.

The adaptation of the AST methods and test principles for BST is in general implementable. In AST, phenotypic susceptibility testing can be done using a variety of different methods and there are several institutions issuing protocols (CLSI, EUCAST, DIN) that provide quantitative (e.g. broth dilution methods) or qualitative (e.g. agar disk diffusion) results. The broth dilution methods may be either in a macrodilution format based on culturing a defined amount of bacteria in liquid cultures ≥ 2 mL containing different concentrations of antimicrobial agents, or in a microdilution format using volumes < 0.5 mL (Lorian, 2005). After incubation, the tests are examined for visible bacterial growth, which is recognized as turbidity (CLSI, 2018c). The lowest concentration of the antimicrobial agent that prevents visible growth is defined as MIC. The advantage of this test method is the generation of a quantitative result (i.e. the MIC). The macrodilution method is a laborious manual work and requires large amounts of reagents and space for each test. The miniaturization of the test principle with the broth microdilution method facilitates some of these points. Standard microtiter plates, homemade or commercially available, contain usually 96 wells that allow several compounds to be tested simultaneously in a single microtiter plate, most commonly in 2-fold dilution steps (Reller et al., 2009; CLSI, 2018a; CLSI, 2020).

No matter which test methodology is used or which protocol is followed, it is indispensable that susceptibility testing follows a harmonized procedure including quality control criteria. However, there was no recognized and standardized method for BST available. Towards harmonization of the protocols, we have investigated the influence of different parameters, commonly used in BET and AST, on the test results. We have developed a broth macrodilution method for determining biocide susceptibility (Feßler et al., 2018), but this method is laborious and time consuming. Therefore, the aim of this study was to develop a broth microdilution method.

1.3 Test organisms

For this study bacterial species, representing both Gram-positive and Gram-negative species were selected, since they have different properties relevant for biocide susceptibility. For BET, defined strains from national or international strain collections are required, which

are, among others, the following reference strains: the two Gram-positive *Staphylococcus aureus* ATCC® 6538 (DSM 799), and *Enterococcus hirae* ATCC® 10541 (DSM 3320), and the two Gram-negative *Escherichia coli* ATCC® 10536 (DSM 682) and *Pseudomonas aeruginosa* ATCC® 15442 (DSM 939) (VAH, 2015; DVG, 2017). Therefore, those four reference strains were selected for the development of the BST method.

1.4 Test parameters

Differences in the test procedure may alter the results obtained and limit the reproducibility between tests. Therefore, to achieve a repeatable and reproducible test method, a rigorous approach must be taken in the specification of the method, in preparation of the test inocula, along with all aspects of the test methodology (Bloomfield et al., 1995). This refers to, among others, incubation time, incubation temperature, inoculum density, inoculum preparation and choice of nutrient broth (Brill et al., 2006; Bock et al., 2018). One of the major sources of error and low reproducibility involves the lack of standardization of conditions for harvesting and preparing test inocula, among other factors known to affect biocide susceptibility (Bloomfield et al., 1995). Arioli and colleagues for example attributed differing test media with the discrepancies in the MIC values they obtained in contrast to another study (Arioli et al., 2013). Harmonization of parameters used during BST is paramount. Since biocide testing of bacteria in BET is performed with different parameters than AST, parameters commonly used during BET and AST were comparatively investigated in this study and the results were analyzed with respect to their reproducibility. To achieve these goals, the reference strains were subjected to comparative investigations and their influence on the test results was evaluated.

1.5 Cell count determination

Inoculum density is a crucial parameter in many microbiological procedures, including AST, BET and BST. Standardization of the inoculum is essential for an accurate and reproducible test. Higher inoculum density may result in higher MIC values for antimicrobial agents or biocides, while fewer bacteria in the inoculum may result in lower MIC values (CLSI, 2018b). It is therefore very important to strictly apply and rigorously monitor the inoculum density recommended in the respective protocols in conjunction with each test run. Inoculum density refers to the number of bacterial cells in the given sample. Consequently, the determination of bacterial cell count is one of the most fundamental laboratory procedures in microbiology (Hazan et al., 2012). There are several methods for cell count determination of bacteria, some of which are quite simple and do not require special equipment, so they can be performed in any laboratory, while others rely on advanced electronic equipment. Examples of

these methods include, among others, spectrophotometry, direct count with a microscope, membrane filtration method, drop plate method or the plate count method (Lee, 2008). Not all of the methods differentiate between dead and living bacteria while some determine only viable cells, like the plate count, drop plate and membrane filtration methods. Also, some only have a low sensitivity (e.g. spectrophotometry) (Hazan et al., 2012).

In the context of susceptibility and efficacy testing, the plate count method is usually used (DIN, 2006; DVG, 2017; CLSI, 2018c). In plate counting assays, a highly diluted volume of the sample is spread over the surface of a nutrient agar plate using a cell spreader. After incubation, the colonies are counted manually. The concentration of bacteria in the original culture can then be calculated based on the assumption that each colony was formed from a single bacterium (colony forming unit, CFU). Cultures usually need to be highly diluted prior to plating or they will overlap and not be countable or be too numerous to count.

To achieve the required range of inoculum density in susceptibility tests, the inoculum is usually adjusted by light absorption using a spectrophotometer or turbidimeter, but in addition the cell density is confirmed by the determination of CFUs. However, the different test methods for cell count determination have varying degrees of complexity.

In the course of this study, two established methods for cell count determination were compared and examined with regard to their accuracy and their suitability for routine use in the laboratory. The two methods considered were the method used in context of AST based on the CLSI (CLSI, 2018a) and the method used in context of BET as described by the DVG (DVG, 2017). The DVG method for determining the cell count requires several dilution steps and the plating of a total volume of 1,000 µL of two dilution steps. The CLSI method requires fewer dilution steps and the plating of only 100 µL of one dilution step.

1.6 Aim of the present study

The aims of the present study were to

I. develop a protocol for biocide susceptibility testing that provides stable test results and allows a high throughput (**Publication I**), and
II. evaluate, whether cell counting can be simplified without reducing the quality of the results (**Publication II**).

To achieve these goals, four reference strains were subjected to comparative studies on the influence of a number of important test parameters (**Publication I**). In addition, two established methods for cell counting were compared and examined with regard to their accuracy and suitability for routine use in the laboratory (**Publication II**).

2 Publications

2.1 Publication I

Schug AR, Bartel A, Scholtzek AD, Meurer M, Brombach J, Hensel V, Fanning S, Schwarz S, Feßler AT. 2020

Biocide susceptibility testing of bacteria: development of a broth microdilution method

https://doi.org/10.1016/j.vetmic.2020.108791

Veterinary Microbiology 2020; 248: 108791

2.2 Publication II

Schug AR, Bartel A, Meurer M, Scholtzek AD, Brombach J, Hensel V, Fanning S, Schwarz S, Feßler AT. 2020

Comparison of two methods for cell count determination in the course of biocide susceptibility testing

https://doi.org/10.1016/j.vetmic.2020.108831

Veterinary Microbiology 2020; 251: 108831

3 Discussion

3.1 Broth microdilution method for biocide susceptibility testing

3.1.1 Evaluation of the test parameters used

To obtain reproducible and comparable results, standardized test methods are mandatory (Gould, 1998; Rodloff et al., 2008). The reproducibility and high precision of a susceptibility test depends on rigorous standardization of factors involved in the test, such as the subculture, the inoculum density, the way to suspend the inoculum, culture medium used, incubation temperature and incubation time (Gilbert et al., 1987; Bloomfield et al., 1995; Langsrud and Sundheim, 1998; Johnston et al., 2000; Kramer et al., 2008). To investigate the influence of different test parameters, four reference strains and four biocides were repeatedly tested by BST in independent tests. As described in **Publication I**, MIC values were determined for the biocides BAC, CHX, GLU (2-fold dilution series) and ISO (2 %-steps) by broth microdilution using seven independent tests. The reference strains used are *S. aureus* ATCC® 6538, *E. hirae* ATCC® 10541, *E. coli* ATCC® 10536 and *P. aeruginosa* ATCC® 15442. They were chosen as they are commonly used during BET. Tryptic soy broth was used as test medium as it is also commonly used during BET. The microtiter plates contained a final volume of 200 µL/well. Bacterial cell counts were performed to ensure, that the acceptable range of 1 - 10 x 10^8 CFU/mL (DVG, 2017; Feßler et al., 2018) was maintained.

The comparative investigations included the following parameters: (i) the use of different subcultures (1st or 2nd subculture, SC), (ii) different ways to suspend the inoculum (direct colony suspension method with or without the use of glass beads), (iii) different inoculum densities (inoculum density according to DVG (DVG, 2017) and inoculum density according to CLSI (CLSI, 2018c), and (iv) reading the results after three different incubation times (24 h, 48 h and 72 h) at 37 °C in ambient air. Since BET and AST use different approaches regarding these parameters, their influence on test results was comprehensively investigated during the development of the BST method.

For each strain/biocide combination, 168 MICs were determined. The results of the comparative investigations were evaluated for their stability by calculating deviations from the acceptable range. The acceptable range was defined as the most common MIC value ± one dilution step (Wallmann et al., 2006; Riesenberg et al., 2016; CLSI, 2018b; Humayoun et al., 2018). Reproducibility was high for all reference strain/biocide/parameter combinations. Overall, most results (86.9 – 100 %) were within the acceptable range.

Comparing the results for the inoculum densities according to the DVG or to CLSI, high reproducibility was observed for both approaches. The inoculum densities according to

the DVG revealed 85.7 - 100 % of results within the acceptable range and the inoculum densities according to CLSI 84.5 – 100 % respectively.

For a more detailed analysis, the deviations within the seven independent tests were analyzed for each parameter combination. In general, there were no major deviations between the parameters and the different comparative investigations showed no significant influence on the test results.

Since CLSI (CLSI, 2018c) allows a fresh 18-to 24-hour old culture for AST, while DVG (DVG, 2017) requires 2nd or 3rd SC for BET, this parameter was included in the comparative investigations. The 1st SC was directly plated from the glycerol stocks, whereas the 2nd SC was subcultured from the 1st SC. Thus, there is one passage when using the 1st SC and two passages when using the 2nd SC. The two different subcultures were used to confirm that there was no difference between them with the aim of saving one day by possibly using the 1st SC. For the different subcultures (1st or 2nd SC), deviations occurred overall randomly. Overall, using the 1st SC yielded ten values (2.2 %) outside the acceptable range, while the 2nd SC only led to five values (1.1 %) outside the acceptable range. Since those deviations were distributed over the different reference strains and biocides and overall it was a low percentage of deviations, use of both subcultures were considered to be equally applicable.

Evaluating the different incubation times, it was shown that longer incubation times led to higher MIC values with the 24-hour MIC values being the most stable. Consequently, a 24-hour incubation time was recommended. This may also reduce time and space in the incubators, is also similar to the incubation times commonly used for AST (CLSI, 2018c), and is in accordance with our previous study about the development of a protocol for broth macrodilution method (Feßler et al., 2018).

Concerning the inoculum densities, the results of the comparative investigations revealed that both are adequate. The preparation of the inoculum density according to CLSI requires a fewer number of dilution steps and is therefore more simple to perform and also requires less culture medium, which makes this method less prone to pipetting and dilution errors.

For the different methods of inoculum suspension preparation (direct colony suspension method with or without the use of glass beads), deviations occurred overall randomly and both methods were considered to be equally applicable. The direct colony suspension method using glass beads complies with the protocols for BET of DIN, DVG and VAH (DIN, 2006; VAH, 2015; DVG, 2017) and potentially facilitates the preparation of a homogeneous inoculum suspension as it is less influenced by the bacterial species tested and the experience of the personnel (Feßler et al., 2018). The direct colony suspension method without glass beads is used in accordance with CLSI-approved AST methods (CLSI, 2018b;

CLSI, 2018c; CLSI, 2020) and has the advantage that smaller amounts of TSD broth (3-5 mL compared to 10 mL) are needed, which also results in less laboratory waste.

The incubation temperature of 37 °C used for this method is usually used for incubation in BET (DIN, 2006; DVG, 2017), while AST is performed at 35 °C ± 2 °C (CLSI, 2018c; CLSI, 2020). Moreover, the temperature of 37 °C was also used in the previous study to develop a BST method using broth macrodilution (Feßler et al., 2018). The commonly tested bacteria grow well at both temperatures, 35 °C and 37 °C.

In conclusion, the following protocol is suggested: use of a fresh overnight culture (1st or 2nd SC), inoculum suspension via direct colony suspension method with or without glass beads, inoculum density according to DVG or CLSI and reading the results after incubation for 24 h at 37 °C.

3.1.2 Comparison with results of broth macrodilution

The results of the present study (**Publication I**) deviated only slightly from the MIC values of the broth macrodilution method (Feßler et al., 2018). The biocides BAC, CHX and ISO were investigated during the establishment of the broth macrodilution method using the strain *S. aureus* ATCC® 6538 (Feßler et al., 2018). Comparing the results of this study (**Publication I**) obtained after 24 h of all subgroups with those of the broth macrodilution method (Feßler et al., 2018), both studies revealed the same modal MIC values for five of the 13 reference strain/biocide combinations, while the remaining eight were within ± one dilution step. The comparability of the two BST methods is confirmed by this agreement.

Advantages of the broth microdilution method are that it does not require the relatively large amount of reagents and therefore less space is required (Jorgensen and Ferraro, 2009). The broth microdilution method is therefore better suited for high throughput and for the commercial production of test plates, which then no longer require the time-consuming, laborious, manual preparation of solutions for each test. However, the broth macrodilution method may be useful for certain studies, for example when only a few concentrations steps are of interest, or only a few isolates or biocides are the subject of the assay, or a specific test range not present on the microdilution plate is to be tested.

3.2 Comparison of the different methods to determine biocide susceptibility.

In general, susceptibility testing can be performed by using phenotypic or genotypic methods.

3.2.1 Different approaches to phenotypic determination of biocide susceptibility

As markers of phenotypic changes in bacterial susceptibility to a biocide, MIC determinations have been used in many studies (Tattawasart et al., 1999; Russell and

McDonnell, 2000; Suller and Russell, 2000; Thomas et al., 2000; Walsh et al., 2003; Thomas et al., 2005; Maillard et al., 2013; Morrissey et al., 2014; Ortiz et al., 2014a; Conceição et al., 2016; Roedel et al., 2019). Of course it is also possible to use other methods than the broth microdilution method recommended in this study to determine the MIC of a bacterial isolate to a biocide, e.g. the broth macrodilution method mentioned above.

Other approaches include investigating inactivation kinetics, the capacity and rate of killing of a biocide over time using time-kill assays (Cookson et al., 1991; Lanker Klossner et al., 1997; D'Arezzo et al., 2012), or determining bacterial growth kinetics in the presence of a defined biocide concentration (Gomez Escalada et al., 2005b; Thomas et al., 2005; Wesgate et al., 2020). These approaches include elaborate and complex procedures that provide very detailed information and that can indicate phenotypic changes in a bacterial isolate or provide insight into biocide-bacterial cell interactions and the nature of resistance within a population (SCENIHR, 2009; Maillard et al., 2013). The MIC approach was chosen in this study to detect the occurrence of reduced biocide susceptibility in bacterial pathogens early and to monitor changes over time (Feßler et al., 2018), which is very relevant because the susceptibility can decrease with further selection pressure, e.g. through repeated exposure (Thomas et al., 2000; Abdel-Malek et al., 2002; Langsrud et al., 2003; Maillard, 2007). In addition, MIC determination using the BST method as proposed in this study is suitable for rapid investigations of larger strain collections and different biocides. However, the reading of MICs after 24 h of contact between bacteria and biocide does not reflect the in-use situation, with usually very short exposure times (Feßler et al., 2018). MIC values are appropriate trend indicators (Russell and McDonnell, 2000; Walsh et al., 2003; Maillard and Denyer, 2009; Maillard et al., 2013; Knapp et al., 2015) but reveal limited information on the susceptibility at in-use concentrations and regular contact times to biocidal agents. Therefore, MIC values determined *in vitro* must be correlated with the bactericidal activity in application situations (Feßler et al., 2018) as the aim of disinfection measures is the killing of microorganisms. Measuring MBCs would be more appropriate for certain research questions, because they can assess the lethality of the in-use concentration of a biocide (SCENIHR, 2010), and are therefore closer to the application scenario. In order to determine the MBC accurately, the biocide must be removed from the assay or neutralized (Cerf et al., 2010; SCENIHR, 2010). The determination of MBCs is hence a valuable complement to MICs, but much more elaborate. It has been proposed to use a combination of biocide MICs, biocide MBCs, AST and stability testing for a bacterial strain as an element for risk assessment and predicting the potential risk of resistance development following biocide exposure (Knapp et al., 2015; Wesgate et al., 2016). Moreover, the need to develop a framework for realistic risk assessment of biocide use impacts for industry, consumers, and regulators was identified (Maillard et al., 2013). An advanced approach via flow cytometric analysis and fluorescence-activated cell sorting (FACS), that can investigate

and separate bacteria depending on their light scattering and fluorescent properties, offers high throughput and insights into the behavior of individual bacterial strains in their response to biocide exposure (Whitehead et al., 2011; Maillard et al., 2013).

The applicability of methods involving a nutrient broth can be limited because for several biocides, precipitation in the nutrient broth has been reported to occur at higher biocide concentrations restricting the test range (Nicoletti et al., 1993; Sundheim and Langsrud, 1995). For example, Nicoletti and co-workers stated that CHX started to precipitate at concentrations above 0.0256 % (Nicoletti et al., 1993), which is comparable with the results of the present study, where CHX started to precipitate at 0.016 %. Langsrud and Sundheim as well as Langsrud and colleagues could not test BAC in nutrient broth above concentrations of 0.02 % because of precipitation (Langsrud and Sundheim, 1997; Langsrud et al., 2003), which is very similar with the results of the present study, where BAC started to precipitate at 0.03 %.

The question of whether *in situ* studies provide better information about biocide susceptibility of bacterial isolates than *in vitro* studies has been the subject of substantial discussions (Maillard et al., 2013). Both, *in vitro* and *in situ* studies, have advantages and disadvantages and each provide valuable results in their respective focus. The implications and relevance of the results of *in vitro*-only studies for *in situ* applications is limited since important parameters that reflect the conditions during in-use settings are lacking. Biocides are often used as part of a formulation, i.e. several active agents are combined or adjuvants are added. However, biocide formulae have been rarely investigated in terms of BST (Maillard, 2018). Nevertheless, *in vitro* protocols are indispensable as they provide reproducible data sets and generate a high degree of confidence in the results as well as that they are much less complex and costly to conduct (Maillard et al., 2013). To evaluate, for example, the changes in the efficacy of biocides upon contact with organic substances and different contact times, BET must be applied.

The need for monitoring bacterial biocide susceptibility profiles on a regular basis requires reliable high throughput screening, low labor and low cost methods that also allow comparison of results between studies, Therefore, one aim of this study was to develop a broth microdilution method for BST.

3.2.2 Genotype-based approaches to determine biocide susceptibility

For a genotype-based approach, molecular methods can be used because resistance characteristics are often genetically encoded, making it possible to test for specific known genes that confer specific resistance phenotypes. However, the presence of a gene associated with a resistance phenotype does not necessarily imply that phenotypic resistance is observed, as this can also depend on the nature and level of expression of these genes (Hughes and

Andersson, 2017). With regard to genotypic detection of biocide resistance, the challenge is that not many genes are currently known to be associated with biocide resistance. Therefore, it is not possible to search for these unknown genotypic resistance traits. The most common molecular technique for detecting DNA sequences associated with resistance is PCR (polymerase chain reaction) (Zou et al., 2014; Wassenaar et al., 2015; Conceição et al., 2016; Deus et al., 2017). In some cases, however, genotype and phenotype correlate and the presence of certain genes could be associated with reduced biocide susceptibility. Nevertheless, in many cases, further studies are necessary to fully clarify the association of these genes with a change in phenotypic biocide susceptibility (Kernberger-Fischer et al., 2018; Worthing et al., 2018). The presence of some plasmids in bacteria is linked with decreased susceptibility to various biocides like CHX, QACs, and triclosan (McDonnell and Russell, 1999). Multidrug efflux pumps encoded by *qac* genes, which are mainly proteins of the Major Facilitator Superfamily and the Small Multidrug Resistance family (SMR) (Wassenaar et al., 2015), have been associated with increased biocide MICs and been frequently reported for methicillin-resistant *S. aureus* (MRSA) (McDonnell and Russell, 1999). A study on MRSA isolates from primates in the United States found five isolates with elevated MICs (0.0004 %) for BAC that carried the *qacC* gene, whereas the isolates that did not harbor the *qacC* gene had BAC MICs of 0.0001 % (Roberts et al., 2018). There have been reports on genes mediating tolerance to biocides in *E. coli,* for example to QACs via increased efflux through the *qacF* and *sugE* genes, among others (Bay et al., 2008; Zou et al., 2014). Some of the genes linked to decreased biocide susceptibility are localized on large conjugative plasmids which increases the risk of co-selection and spread of those properties (Rensing and Grass, 2003; Bay et al., 2008). Deus and colleagues found biocide tolerance mediating genes, such as *qacF*, *qacE∆1*, *qacH* and *sugE(p)*, in extended-spectrum β-lactamase-producing (ESBL) *E. coli* isolates of human and avian origin, with several of them being located on large plasmids (< 20 kb) which might facilitate the spread of them among Enterobacteriaceae (Deus et al., 2017). In 2012, Ciusa and co-workers found a novel resistance mechanism in human clinical isolates of *S. aureus* involving an additional *fabI* allele derived from *Staphylococcus haemolyticus* (*sh-fabI*) which they linked to positive selection by triclosan (Ciusa et al., 2012).

Although useful, genotypic approaches to determine biocide susceptibility cannot always correctly determine the phenotype present and genotype and phenotype do not always correlate in terms of biocide susceptibility.

3.3 Comparison of two methods for cell count determination

The comparison of two methods for cell count determination was intended to analyze them in terms of their results and their practicability during routine use (**Publication II**). Cell

counts refer to the inoculum density, which is an important parameter for many experiments performed with bacteria including AST, BST and BET. The proper inoculum density is usually confirmed using the number of CFUs via plate count tests. The DVG describes a method for cell count determination during BET (DVG, 2017). The CLSI provides a method for cell count determination during AST (CLSI, 2018c). These methods differ in their dilution steps and the plating volume of the bacterial suspension, which is spread on agar plates and from which the grown colonies are counted after incubation. The DVG cell count method requires the plating of 1,000 µL of two dilution steps (which requires six and seven dilution steps made up in a ten-fold dilution series), whereas with the CLSI cell counting method, an aliquot of 100 µL of one dilution step is plated (which requires only three dilution steps).

The procedure for determining CFUs via plate counting is quite time consuming (Hazan et al., 2012), but inexpensive and does not require additional reagents (Beal et al., 2020). Under ideal test conditions, the number of colonies present on an agar plate would equal the number of bacterial cells present in the sample. However, under real conditions, some individual bacterial cells may be so close or attached to each other that they form only one common colony and therefore clumps of bacteria are miscounted as a single colony (Jansson and Prosser, 1997; Auty et al., 2001; Ou et al., 2017). Thus, under the real, non-ideal conditions, the number of colonies is less than the number of individual bacterial cells contained in the sample. Therefore, the results are given as CFU/mL and not as bacteria/mL (Hazan et al., 2012). As there are fewer colonies to count, there is a preference to analyze only high dilutions of the original culture, but unfortunately, at low counts, minor counting errors have a considerable effect on the calculated concentration (Brugger et al., 2012). However, counting plates with many CFUs is also prone to errors, as it requires a high degree of attention on the part of the operator and is time-consuming (Brugger et al., 2012). In addition, a high number of CFUs on a plate can lead to incorrect results due to bacterial overcrowding (Breed and Dotterrer, 1916). Besides, CFU determination is a rather slow approach to quantify bacteria due to the fact that most microorganisms require at least 12 hours to form visible colonies and results are usually available only after one to three days (Hazan et al., 2012). As Jansson and Prosser pointed out, plate counting also does not detect dormant organisms or those with a lag time exceeding the incubation time and colony formation may be constrained by antagonistic interactions with neighboring colonies or by competition for nutrients, leading to underestimation of counts (Jansson and Prosser, 1997). DVG guidelines require an initial reading of the plates after 20-24 h and a final count after another incubation period of 20-24 h (DVG, 2017). CLSI requires that colonies be counted "after incubation" (CLSI, 2018c), which can be correlated with the incubation times used for the respective bacterial species tested. In this study, plates were counted after 24 h because the bacterial species tested were well counted after 24 h, with no relevant changes in colony numbers after another 24 h incubation.

Overall, although CFU determination via plate counting may be time and material intensive, it provides an appropriate estimate of the number of viable bacterial cells despite the possibility of miscounts, does not require highly specialized equipment and is therefore widely used and highly suitable for use in the context of susceptibility tests. In addition, counting colonies on agar plates can be facilitated by the use of colony counters, which can recognize the pressure of marking of the colonies on the plates with a pen.

3.3.1 Correlation of the results from the two methods for cell count determination

Deviations of the DVG and CLSI methods for cell count determinations of the same inoculum suspension were compared using 95 % Bland-Altman limits of agreement (AL) based on the agreement between two quantitative measurements by examining the mean difference, constructing limits of agreement and estimating an agreement interval within which 95 % of the differences of the one method compared to the other, fall (Bland and Altman, 1986; Giavarina, 2015) (**Publication II**). For all tests with the four reference strains using the inoculum preparation according to DVG, the comparative cell count determinations were performed 28 times. They also included the parameters of the comparative investigations 1st or 2nd SC, and direct colony suspension method with or without the use of glass beads.

The Bland-Altman analysis indicates whether more or less CFUs were counted using the CLSI method in comparison to the DVG method, which was used as basis during the analysis. Overall, the deviations between the CLSI and the DVG method were within AL of [-0.52 to 0.27] ($\log_{10}$), with the CLSI method measuring slightly lower CFUs (- 0.12 $\log_{10}$) (**Publication II**). The Bland-Altman analysis indicates that the differences between the two methods for cell count determination appear to be random. No influence could be observed on the correlation of the two cell count determination methods for the parameters 1st and 2nd SC and direct colony suspension with or without glass beads.

The Bland-Altman analysis defines limits of agreement, however the user has to make sure that these limits are acceptable for the respective approach, which must be determined based on objectives, such as clinical needs or biological considerations (Giavarina, 2015). This approach and interpretation was also used in similar way by Pathak and co-workers who investigated a novel method to rapidly and reliably quantify mycobacteria (Pathak et al., 2012). They found good linear correlation between viable colony counts and their novel approach and analyzed the performance of their novel method using the agreement expressed by the Bland and Altman analysis of log(CFU) predicted by the novel method versus log(CFU) that were observed by actual colony count (Pathak et al., 2012). The evaluation of the study, which is similar to the present study in terms of methodological questions, interprets even larger Bland-Altman Limits of agreement as acceptable, as they were satisfied with the overall level of

agreement between the two methods with the largest AL [-0.69 to 0.57] (log_{10}) in *Mycobacterium tuberculosis* (Pathak et al., 2012). Since in the present study, the observed deviations were within one log_{10} -step (i.e. a ten-fold dilution step), the results of the two methods did not differ systematically and both methods can be equally used.

3.3.2 Correlation of optical density and number of colony forming units

The Bland-Altman analysis does not allow the more accurate method to be identified. In order to answer this question, the method with the higher correlation of the CFUs with the optical densities (measured with a spectrophotometer) was determined (**Publication II**). For this, the correlation was evaluated according to Cohen using the Pearson correlation coefficient between the logarithmized CFU and the measured optical density (OD) of the inoculum suspension (Cohen, 1988).

The results were different for the four reference strains, but overall with all strains, a higher correlation between the OD and the cell counts was seen for the CLSI method. One reason for this finding might be due to the lower number of dilution steps used during the CLSI method and consequently fewer sources of error. This is in accordance with a study that states that the dilution/pipetting errors increase with higher number of dilution steps (Jennison and Wadsworth, 1940). In contrast, using the DVG method, sampling of a larger volume is performed and two dilution steps are considered which might lower the distribution or sampling error in comparison to considering a smaller volume of one dilution step, like when using the CLSI method (Jennison and Wadsworth, 1940).

The results showed a large influence of the reference strains/bacterial species on the correlation between OD and cell counts. For *S. aureus* ATCC® 6538, the correlation was large (CLSI method) to medium (DVG method). *P. aeruginosa* ATCC® 15442 showed a large (CLSI method) to moderate (DVG method) correlation. Whereas for *E. hirae* ATCC® 10541, a moderate correlation was seen for the CLSI method, but no correlation was recorded for the DVG method. In addition, for *E. coli* ATCC® 10536 the correlation was between small and non-existent for both methods.

When using different measurement methods, some variation is to be expected (i.e. the correlation may range within the threshold of ± 0.1 (Cohen, 1988)). With the results for *E. coli* ATCC® 10536, that yielded values of 0.143 (CLSI) and -0.122 (DVG), there was a negative correlation between optical densities and cell counts higher than the threshold described by Cohen. The negative value is most likely an artefact, as there is no rationale for this, since biologically no negative correlation between optical densities and cell counts is possible. Previous studies with *E. coli* have indicated that high variability in CFU counts may occur (Jarvis et al., 2007; Beal et al., 2020). When preparing bacterial suspensions, clumping or

forming of aggregates of the bacteria may occur which might lead to lower correlations due to the fact that one colony might be formed by more than one bacterial cell and therefore, does not represent the actual number of bacteria present (Jansson and Prosser, 1997; Auty et al., 2001; Ou et al., 2017). This is in accordance with the findings of Jennison (Jennison, 1937), who found clumping as a possible explanation for the lower correlation between plate count and total counts by microscopy. As we prepared the bacterial suspensions of *E. coli* ATCC® 10536, clumping or formation of bacterial aggregates was visible which might be an explanation of the comparatively low correlation.

The analysis of the correlation between OD and cell count was only performed over a small range of OD values (OD_{620nm} 0.26-0.37), which results in lower correlations than when observed over a larger range. This is because the variations caused by the measurements remain the same regardless of the OD range investigated and therefore have less influence on the correlation when a larger OD range is tested. Francois and colleagues, for example, studied *L. monocytogenes* and observed a large correlation between cell counts and OD over a wide range of OD_{600nm} from about 0.01 to 0.95 (Francois et al., 2005). Similarly, Zhang et al. investigated multiple strains and found a good correlation between the optical densities and the plate count method over the full range of the exponential growth phase (Zhang et al., 2015).

The precision of both cell counting methods, the CLSI method and the DVG method, is sufficient and accurate enough to confirm the desired acceptable range of CFU/mL (1-10 x 10^8 CFU/mL), even though there were higher correlations of CFU/mL with optical density in the CLSI method. Therefore, both methods of cell counting are applicable. The recommendation is to use the CLSI method because it is less complex and less time-consuming.

3.4 Further requirements and outlook

As described in chapter 1.1.3, resistance to biocides is a relative term and, unlike AST, for BST there is currently a lack of quality control ranges and interpretative criteria, which limit the utility.

3.4.1 Quality control ranges

Besides difficulties in interpreting the results, there are also possible complications in conducting the tests. As several parameters (e.g. reagents, equipment, and personnel) can influence the test results, it is crucial to perform quality control (QC) tests.

QC ranges for QC strains with known susceptibility to the antimicrobial agents tested are standard practice and well established for AST. QC ranges are essential in order to ensure day-to-day and interlaboratory test performance and reliable test results (Schwarz et al., 2010; Watts JL, 2018). These QC strains are tested in parallel with the isolates which are the subject

of the test, and if the QC strains are not within their acceptable range, the test must be repeated and further troubleshooting initiated if necessary (Wiegand et al., 2008; CLSI, 2018c; Watts JL, 2018). The CLSI published QC ranges for several distinct QC reference strains and antimicrobial agents for broth microdilution and agar disk diffusion and recommendations how they must be regularly applied in routine diagnostics (CLSI, 2018c; CLSI, 2018b; CLSI, 2020).

Similarly, approved QC ranges are likewise necessary and crucial for BST and need to be established. Therefore, interlaboratory trials are necessary to establish QC ranges for reference strain-biocide combinations in order to validate the test results.

3.4.2 Interpretive criteria

So far, there are no interpretive criteria for the results of the susceptibility tests, such as clinical breakpoints or epidemiological cut-off values and there is no organization that sets breakpoints for biocide susceptibility testing.

As the use of biocides differs significantly from that of antimicrobial agents (for example, used not within an organism but on surfaces of objects), most parameters for interpretation of test results for antimicrobial agents are not necessarily applicable to biocides (Feßler et al., 2018). The fact that the BST results are read after 24 h exposure of the bacteria to the biocide (**Publication I**) does not reflect the conditions of application, where usually only very short exposure times are the general rule (Kramer et al., 2008; Cerf et al., 2010; Condell et al., 2012). The determination of MIC values *in vitro* for a biocide does not determine its bactericidal activity or efficacy for in-use applications, MIC values determined *in vitro* must therefore be correlated with the bactericidal activity of the concentration used and the contact time (Feßler et al., 2018).

Epidemiological cut-off values (ECOFFs) are defined on the basis of the normal distribution of MIC values in a given bacterial species. All isolates which have MIC values within this range are considered as “wild-type”, and those presenting MICs above these values are considered as “non-wild type” (EUCAST, 2011). ECOFFs are determined by mathematical calculation and do not consider clinical aspects. "Non-wild type" isolates usually show acquired resistance mechanisms. The fact that an isolate is “non-wild type” or resistant to a biocide based on ECOFFs does not prove resistance in clinical applications (Russell, 2003).

Since interpretive criteria for the definition of biocide resistance currently do not exist, epidemiological data on biocide resistance is limited (Morrissey et al., 2014). For this purpose, larger collections of isolates of the respective species must be tested with a validated method and the MIC distributions evaluated. A number of ECOFFs has been proposed by Morrissey and colleagues for isolates of different bacterial species (*S. aureus*, *Salmonella* spp., *E. coli*, *Candida albicans*, *K. pneumoniae*, *Enterobacter* spp., *Enterococcus faecium* and

Enterococcus faecalis) to the biocides CHX, BAC, triclosan and sodium hypochlorite, based on the analysis of 3319 clinical isolates and their MIC values (Morrissey et al., 2014).

The broth mircodilution method recommended in this study has already been used in a study to characterize 19 borderline oxacillin-resistant *S. aureus* (BORSA) isolates from an equine clinic for the biocides BAC, GLU, and CHX (Scholtzek et al., 2019). *S. aureus* ATCC® 6538 was tested in parallel to see if the results were reproducible. The MICs of the clinical isolates varied between two to three dilution steps and were similar to those of the reference strain *S. aureus* ATCC® 6538 (MICs of 0.00006 - 0.0005 % for BAC, 0.125 - 0.5 % for GLU, and 0.00006 - 0.00025 % for CHX). (Scholtzek et al., 2019). Studies like this provide valuable information on the biocide susceptibility of field isolates.

Thus, further studies using the broth microdilution method recommended in this study are needed to provide an overview of the biocide susceptibility of bacterial isolates of human and veterinary pathogens and to establish interpretive criteria.

3.5 Concluding remarks

Biocides are a key tool for controlling the spread of bacterial pathogens, and screening bacterial isolates for reduced biocide susceptibility is becoming increasingly important. It is crucial to perform studies that focus on the biocide susceptibility of individual strains to monitor susceptibility profiles rather than focusing solely on the efficacy of a biocidal product. To date, studies addressing biocide susceptibility have often differed in methodology, making it difficult to compare results. The proposed method was developed for screening field isolates in routine diagnostics and surveillance studies to detect reduced susceptibility early.

In the development of the broth microdilution method for BST and the comparison of the methods for cell count determination in this study, the influence of different approaches and different parameters of existing methods for BET and AST were thoroughly investigated.

As a result of the comparative studies, the following protocol is suggested: use of a fresh overnight culture (1st or 2nd SC), inoculum suspension via direct colony suspension method with or without glass beads, inoculum density according to DVG or CLSI and reading the results after incubation at 37 °C for 24 h. Both methods for cell count determination can be used as there were only minor differences between them, but since the CLSI method is less complex and less time consuming, this method is recommended.

The proposed broth microdilution method for BST and the proposed method for cell count determination are easy to perform and provide stable results, which can facilitate and harmonize BST of bacterial isolates.

4 Summary

Development of a broth microdilution method for biocide susceptibility testing of bacteria

Biocides are important for the control of bacterial pathogens in many settings. There are numerous reports of biocide-resistant bacteria, and biocides may also contribute to the development and spread of bacterial pathogens that are cross-resistant to biocides and antimicrobial agents simultaneously. Hence, there is an urgent need to monitor and observe the emergence and extent of biocide resistance for which standardized methods that provide reproducible and comparable information are necessary. A suitable method shall facilitate high-throughput screening, be easy to use, and at the same time provide highly accurate results. This includes not only the test method itself, but also a method for verifying that the correct inoculum density is maintained, which is an important test parameter.

The objective of this study was to develop a broth microdilution protocol for biocide susceptibility testing (BST) and to investigate whether cell counting during BST can be simplified without reducing the quality of the results.

To develop the BST protocol, multiple parameters were investigated to determine the minimal inhibitory concentrations (MICs) for benzalkonium chloride, chlorhexidine, glutardialdehyde (2-fold dilution series) and isopropanol (2 %-steps*)* by broth microdilution using seven independent tests. The reference strains *Staphylococcus aureus* ATCC® 6538, *Enterococcus hirae* ATCC® 10541, *Escherichia coli* ATCC® 10536 and *Pseudomonas aeruginosa* ATCC® 15442 were used with tryptic soy broth as test medium. The microtitre plates contained a final volume of 200 µL/well. Comparative studies were made because existing methods for biocide efficacy testing and antimicrobial susceptibility testing use different parameters. In order to develop an appropriate BST method, the influence of these different parameters was thoroughly investigated. The comparative investigations included the following parameters: (i) the use of different subcultures (1st or 2nd subculture, SC), (ii) different ways to prepare the inoculum (direct colony suspension method with or without the use of glass beads), (iii) different inoculum densities, either according to DVG (Deutsche Veterinärmedizinische Gesellschaft e.V., German Veterinary Medical Society) or according to the CLSI (Clinical and Laboratory Standards Institute), and (iv) reading the results after three different incubation times (24 h, 48 h and 72 h) at 37 °C in ambient air. For each strain/biocide combination, 168 MICs were determined. Overall, most results were within ± one dilution step from the most common value, which was defined as the acceptable range. The results of the comparative investigations were evaluated for their stability by calculating deviations from the acceptable range. Based on these results, the following protocol is proposed: use of a fresh

overnight culture (1st or 2nd SC), inoculum suspension via direct colony suspension method with or without glass beads, inoculum preparation according to DVG or CLSI and incubation at 37 °C for 24 h.

Methods to determine the inoculum density commonly refer to cell counts and have been described for biocide efficacy testing according to DVG and for antimicrobial susceptibility testing according to CLSI. To comparatively investigate the two methods for cell count determination, each of the four reference strains was comparatively tested 28 times using the inoculum preparation according to DVG and CLSI. The results were statistically analyzed using Bland-Altman plots and 95 % limits of agreement and cell counts were correlated with the optical density of the bacterial suspensions used. The variations observed between the two methods were within one log_{10} step and the measured number of colony forming units did not differ systematically. Therefore, both methods proved to be suitable for cell count determination. The CLSI method is preferred as it has less dilution steps.

The proposed broth microdilution method for BST and the proposed method for cell count determination are easy to perform and provide stable results, which can facilitate BST of bacterial isolates and thus, represent a step towards harmonization.

5 Zusammenfassung

Entwicklung einer Bouillon-Mikrodilutionsmethode für die Biozid-Empfindlichkeitsprüfung von Bakterien

Biozide sind für die Kontrolle bakterieller Krankheitserreger in vielen Bereichen wichtig. Es gibt zahlreiche Berichte über biozidresistente Bakterien und zudem können Biozide auch zur Entwicklung und Verbreitung von bakteriellen Krankheitserregern beitragen, die gleichzeitig gegen Biozide und antimikrobielle Wirkstoffe kreuzresistent sind. Es besteht daher die dringende Notwendigkeit, die Entstehung und das Ausmaß von Biozidresistenzen zu überwachen. Dafür sind standardisierte Methoden erforderlich, die reproduzierbare und vergleichbare Informationen liefern. Eine dazu geeignete Methode muss ein Hochdurchsatz-Screening ermöglichen, einfach anzuwenden sein und gleichzeitig sehr präzise Ergebnisse liefern. Hierbei ist nicht nur die Testmethode selbst zu berücksichtigen, sondern auch ein Verfahren zur Überprüfung der korrekten Inokulumdichte, die einen wichtigen Testparameter darstellt.

Das Ziel dieser Studie war es, eine Bouillon-Mikrodilutionsmethode für die Biozid-Empfindlichkeitsprüfung zu entwickeln und zu untersuchen, ob die Bestimmung der Inokulumdichte während der Biozid-Empfindlichkeitsprüfung vereinfacht werden kann, ohne die Qualität der Ergebnisse zu beeinträchtigen.

Zur Entwicklung der Bouillon-Mikrodilutionsmethode wurden mehrere Parameter untersucht, um die minimalen Hemmkonzentrationen (MHKs) für Benzalkoniumchlorid, Chlorhexidin, Glutardialdehyd (2-fache Verdünnungsreihe) und Isopropanol (2 %-Schritte) mittels Bouillon-Mikrodilution in sieben unabhängigen Tests zu bestimmen. Die Referenzstämme *Staphylococcus aureus* ATCC® 6538, *Enterococcus hirae* ATCC® 10541, *Escherichia coli* ATCC® 10536 und *Pseudomonas aeruginosa* ATCC® 15442 wurden mit Trypton-Soja-Bouillon als Testmedium untersucht. Die Mikrotiterplatten enthielten ein Endvolumen von 200 µL/Vertiefung. Es wurden vergleichende Studien durchgeführt, da bestehende Methoden für Biozid-Wirksamkeitstests und antimikrobielle Empfindlichkeitsprüfungen unterschiedliche Parameter verwenden. Um eine geeignete Bouillon-Mikrodilutionsmethode für die Biozid-Empfindlichkeitsprüfung zu entwickeln, wurde der Einfluss dieser verschiedenen Parameter eingehend untersucht. Die vergleichenden Untersuchungen umfassten die folgenden Parameter: (i) die Verwendung unterschiedlicher Subkulturen (1. oder 2. Subkultur, SK), (ii) unterschiedliche Arten der Inokulumsuspension (direkte Kolonie-Suspensionsmethode mit oder ohne Verwendung von Glasperlen), (iii) unterschiedliche Inokulumdichten, entweder gemäß DVG (Deutsche Veterinärmedizinische Gesellschaft e.V.) oder gemäß CLSI (Clinical and Laboratory Standards Institute) und (iv)

Ablesen der Ergebnisse nach aerober Bebrütung bei 37 °C und drei verschiedenen Inkubationszeiten (24 h, 48 h und 72 h). Für jede Stamm-Biozid-Kombination wurden 168 MHK-Werte bestimmt. Insgesamt lagen die meisten Ergebnisse innerhalb ± einer Verdünnungsstufe vom am häufigsten gemessenen Wert, was als akzeptabler Bereich definiert wurde. Die Ergebnisse der vergleichenden Untersuchungen wurden auf ihre Stabilität hin bewertet, indem die Abweichungen vom akzeptablen Bereich berechnet wurden. Basierend auf den Ergebnissen wird folgendes Protokoll vorgeschlagen: Verwendung einer frischen Übernacht-Kultur (1. oder 2. SK), Herstellung der Inokulumsuspension über die direkte Kolonie-Suspensionsmethode mit oder ohne Glasperlen, Inokulumdichte gemäß DVG oder CLSI und Inkubation bei 37 °C für 24 h.

Methoden zur Bestimmung der Inokulumdichte beziehen sich üblicherweise auf Zellzahlen und wurden für die Biozid-Wirksamkeitsprüfung durch die DVG und für die antimikrobielle Empfindlichkeitsprüfung durch das CLSI beschrieben. Um die beiden Methoden zur Zellzahlbestimmung vergleichend zu untersuchen, wurde jeder der vier Referenzstämme 28-mal mit der Inokulumpräparation nach DVG und CLSI vergleichend getestet. Die Ergebnisse wurden mit Hilfe von Bland-Altman-Plots und 95 %-Übereinstimmungsgrenzen statistisch ausgewertet und die Zellzahlen mit der optischen Dichte der verwendeten Bakteriensuspensionen korreliert. Die beobachteten Abweichungen zwischen den beiden Methoden lagen innerhalb eines $\log_{10}$-Schrittes und die gemessene Anzahl der koloniebildenden Einheiten unterschied sich nicht systematisch. Daher erwiesen sich beide Methoden als geeignet für die Zellzahlbestimmung. Die CLSI-Methode ist zu empfehlen, da sie weniger Verdünnungsschritte erfordert.

Die vorgeschlagene Bouillon-Mikrodilutionsmethode für die Biozid-Empfindlichkeitsprüfung und die vorgeschlagene Methode für die Bestimmung der Zellzahl sind einfach durchzuführen und liefern stabile Ergebnisse, was die Biozid-Empfindlichkeitsprüfung von Bakterienisolaten erleichtern kann und somit einen Schritt in Richtung Harmonisierung darstellt.

References

Aase, B., G. Sundheim, S. Langsrud and L. M. Rørvik (2000):
Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*.
Int J Food Microbiol, 62: 57-63. DOI: 10.1016/s0168-1605(00)00357-3.

Abdel-Malek, S. M., I. S. Al-Adham, C. L. Winder, T. E. Buultjens, K. M. Gartland and P. J. Collier (2002):
Antimicrobial susceptibility changes and T-OMP shifts in pyrithione-passaged planktonic cultures of *Pseudomonas aeruginosa* PAO1.
J Appl Microbiol, 92: 729-736. DOI: 10.1046/j.1365-2672.2002.01575.x.

Ali Y. , M. J. D., E. J. Fendler, E. L. Larson, (2001):
Alcohols
In: Disinfection, sterilization and preservation, S. S. Block, Volume 1, chap. 12, pp. 229–252. Philadelphia, PA, USA: Lippincott Williams & Wilkins, ISBN: 9780683307405

Arioli, S., M. Elli, G. Ricci and D. Mora (2013):
Assessment of the susceptibility of lactic acid bacteria to biocides.
Int J Food Microbiol, 163: 1-5. DOI: 10.1016/j.ijfoodmicro.2013.02.002.

Auty, M. A. E., G. E. Gardiner, S. J. McBrearty, E. O. O'Sullivan, D. M. Mulvihill, J. K. Collins, G. F. Fitzgerald, C. Stanton and R. P. Ross (2001):
Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy.
Appl Environ Microbiol, 67: 420-425. DOI: 10.1128/aem.67.1.420-425.2001.

Bay, D. C., K. L. Rommens and R. J. Turner (2008):
Small multidrug resistance proteins: a multidrug transporter family that continues to grow.
Biochim Biophys Acta, 1778: 1814-1838. DOI: 10.1016/j.bbamem.2007.08.015.

Beal, J., N. G. Farny, T. Haddock-Angelli, V. Selvarajah, G. S. Baldwin, R. Buckley-Taylor, M. Gershater, D. Kiga, J. Marken, V. Sanchania, A. Sison, C. T. Workman and iGEM Interlab Study Contributors (2020):
Robust estimation of bacterial cell count from optical density.
Commun Biol, 3: 512. DOI: 10.1038/s42003-020-01127-5.

Bland, J. M. and D. G. Altman (1986):
Statistical methods for assessing agreement between two methods of clinical measurement.
Lancet, 1: 307-310.

Bloomfield, S. F., M. Arthur, H. Gibson, K. Morley, P. Gilbert and M. R. W. Brown (1995):
Development of reproducible test inocula for disinfectant testing.
Int Biodeterior Biodegradation, 36: 311-331. DOI: 10.1016/0964-8305(95)00100-X.

Bock, L. J., C. K. Hind, J. M. Sutton and M. E. Wand (2018):
Growth media and assay plate material can impact on the effectiveness of cationic biocides and antibiotics against different bacterial species.
Lett Appl Microbiol, 66: 368-377. DOI: 10.1111/lam.12863.

Bock, L. J., M. E. Wand and J. M. Sutton (2016):
Varying activity of chlorhexidine-based disinfectants against *Klebsiella pneumoniae* clinical isolates and adapted strains.
J Hosp Infect, 93: 42-48. DOI: 10.1016/j.jhin.2015.12.019.

Boyce, J. M. and D. Pittet (2002):
Guideline for hand hygiene in health-care settings. Recommendations of the healthcare infection control practices advisory committee and the HICPAC/SHEA/APIC/IDSA Hand hygiene task force. Society for healthcare epidemiology of America/ Association for professionals in infection control/ Infectious diseases society of America.
MMWR Recomm Rep 51: 1-45.

Breed, R. S. and W. D. Dotterrer (1916):
The number of colonies allowable on satisfactory agar plates.
J Bacteriol., 1: 321-331. DOI: 10.1128/JB.1.3.321-331.1916

Brill, F., P. Goroncy-Bermes and W. Sand (2006):
Influence of growth media on the sensitivity of *Staphylococcus aureu*s and *Pseudomonas aeruginosa* to cationic biocides.
Int J Hyg Environ Health, 209: 89-95. DOI: 10.1016/j.ijheh.2005.08.007.

Brugger, S. D., C. Baumberger, M. Jost, W. Jenni, U. Brugger and K. Mühlemann (2012):
Automated counting of bacterial colony forming units on agar plates.
PloS one, 7: e33695-e33695. DOI: 10.1371/journal.pone.0033695.

Buffet-Bataillon, S., P. Tattevin, M. Bonnaure-Mallet and A. Jolivet-Gougeon (2012):
Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds - a critical review.
Int J Antimicrob Agents, 39: 381-389. DOI: 10.1016/j.ijantimicag.2012.01.011.

Buffet-Bataillon, S., P. Tattevin, J. Y. Maillard, M. Bonnaure-Mallet and A. Jolivet-Gougeon (2016):
Efflux pump induction by quaternary ammonium compounds and fluoroquinolone resistance in bacteria.
Future Microbiol, 11: 81-92. DOI: 10.2217/fmb.15.131.

Cerf, O., B. Carpentier and P. Sanders (2010):
Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: “Resistance” has different meanings.
Int J Food Microbiol, 136: 247-254. DOI: 10.1016/j.ijfoodmicro.2009.10.002.

Chapman, J. S. (1998):
Characterizing bacterial resistance to preservatives and disinfectants.
Int Biodeterior Biodegradation, 41: 241-245. DOI: 10.1016/S0964-8305(98)00025-0.

Chapman, J. S. (2003a):
Biocide resistance mechanisms.
Int Biodeterior Biodegradation, 51: 133-138. DOI: 10.1016/S0964-8305(02)00097-5.

Chapman, J. S. (2003b):
Disinfectant resistance mechanisms, cross-resistance, and co-resistance.
Int Biodeterior Biodegradation, 51: 271-276. DOI: 10.1016/S0964-8305(03)00044-1.

Christensen, E. G., L. Gram and V. G. Kastbjerg (2011):
Sublethal triclosan exposure decreases susceptibility to gentamicin and other aminoglycosides in *Listeria monocytogenes*.
Antimicrob Agents Chemother, 55: 4064-4071. DOI: 10.1128/AAC.00460-11.

Chuanchuen, R., R. R. Karkhoff-Schweizer and H. P. Schweizer (2003):
High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux.
Am J Infect Control, 31: 124-127. DOI: 10.1067/mic.2003.11.

Cieplik, F., N. S. Jakubovics, W. Buchalla, T. Maisch, E. Hellwig and A. Al-Ahmad (2019):
Resistance toward chlorhexidine in oral bacteria - is there cause for concern?
Front Microbiol, 10: 587-587. DOI: 10.3389/fmicb.2019.00587.

Ciusa, M. L., L. Furi, D. Knight, F. Decorosi, M. Fondi, C. Raggi, J. R. Coelho, L. Aragones, L. Moce, P. Visa, A. T. Freitas, L. Baldassarri, R. Fani, C. Viti, G. Orefici, J. L. Martinez, I. Morrissey and M. R. Oggioni (2012):
A novel resistance mechanism to triclosan that suggests horizontal gene transfer and demonstrates a potential selective pressure for reduced biocide susceptibility in clinical strains of *Staphylococcus aureus*.
Int J Antimicrob Agents, 40: 210-220. DOI: 10.1016/j.ijantimicag.2012.04.021.

CLSI (2018a):
Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th ed. CLSI standard M07.
Wayne, PA, USA, Clinical and Laboratory Standards Institute.

CLSI (2018b):
Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 4th ed. CLSI supplement VET08.
Wayne, PA, USA, Clinical and Laboratory Standards Institute.

CLSI (2018c):
Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 5th ed. CLSI standard VET01.
Wayne, PA, USA, Clinical and Laboratory Standards Institute.

CLSI (2020):
Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100.
Wayne, PA, USA, Clinical and Laboratory Standards Institute.

Cohen, J. (1988):
Differences between correlation coefficients.
Statistical power analysis for the behavioral sciences. New York, Lawrence Erlbaum Associates. 2nd ed.: pp. 109-143.

Conceição, T., C. Coelho, H. de Lencastre and M. Aires-de-Sousa (2016):
High prevalence of biocide resistance determinants in *staphylococcus aureus* isolates from three african countries.
Antimicrob Agents Chemother, 60: 678-681. DOI: 10.1128/aac.02140-15.

Condell, O., C. Iversen, S. Cooney, K. A. Power, C. Walsh, C. Burgess and S. Fanning (2012):
Efficacy of biocides used in the modern food industry to control *Salmonella enterica*, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds.
Appl Environ Microbiol, 78: 3087-3097. DOI: 10.1128/AEM.07534-11.

Cookson, B. D., M. C. Bolton and J. H. Platt (1991):
Chlorhexidine resistance in methicillin-resistant *Staphylococcus aureus* or just an elevated MIC? An in vitro and in vivo assessment.
Antimicrob Agents Chemother, 35: 1997-2002. DOI: 10.1128/aac.35.10.1997.

Cottell, A., S. P. Denyer, G. W. Hanlon, D. Ochs and J. Y. Maillard (2009):
Triclosan-tolerant bacteria: changes in susceptibility to antibiotics.
J Hosp Infect, 72: 71-76. DOI: 10.1016/j.jhin.2009.01.014.

Couto, I., S. S. Costa, M. Viveiros, M. Martins and L. Amaral (2008):
Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide.
J Antimicrob Chemother, 62: 504-513. DOI: 10.1093/jac/dkn217.

D'Arezzo, S., S. Lanini, V. Puro, G. Ippolito and P. Visca (2012):
High-level tolerance to triclosan may play a role in *Pseudomonas aeruginosa* antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation.
BMC Res Notes, 5: 43-43. DOI: 10.1186/1756-0500-5-43.

Dance, D. A., A. D. Pearson, D. V. Seal and J. A. Lowes (1987):
A hospital outbreak caused by a chlorhexidine and antibiotic-resistant *Proteus mirabilis*.
J Hosp Infect, 10: 10-16. DOI: 10.1016/0195-6701(87)90027-2.

Daschner, F. and A. Schuster (2004):
Disinfection and the prevention of infectious disease: no adverse effects?
Am J Infect Control, 32: 224-225. DOI: 10.1016/j.ajic.2003.10.008.

Daschner, F. D. and M. Dettenkofer (1997):
Protecting the patient and the environment - new aspects and challenges in hospital infection control.
J Hosp Infect, 36: 7-15. DOI: 10.1016/S0195-6701(97)90086-4.

Denyer, S. P. and J. Y. Maillard (2002):
Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria.
J Appl Microbiol, 92 Suppl: 35s-45s.

Denyer, S. P. and G. S. A. B. Stewart (1998):
Mechanisms of action of disinfectants.
Int Biodeterior Biodegradation, 41: 261-268. DOI: 10.1016/S0964-8305(98)00023-7.

Deus, D., C. Krischek, Y. Pfeifer, A. R. Sharifi, U. Fiegen, F. Reich, G. Klein and C. Kehrenberg (2017):
Comparative analysis of the susceptibility to biocides and heavy metals of extended-spectrum β-lactamase-producing *Escherichia coli* isolates of human and avian origin, Germany.
Diagn Microbiol Infect Dis, 88: 88-92. DOI: 10.1016/j.diagmicrobio.2017.01.023.

DIN (2006):
DIN EN 1040:2006-03. Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1).

Donaghy, J. A., B. Jagadeesan, K. Goodburn, L. Grunwald, O. N. Jensen, A. D. Jespers, K. Kanagachandran, H. Lafforgue, W. Seefelder and M. C. Quentin (2019):
Relationship of sanitizers, disinfectants, and cleaning agents with antimicrobial resistance.
J Food Prot, 82: 889-902. DOI: 10.4315/0362-028x.Jfp-18-373.

Dukan, S. and D. Touati (1996):
Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress.
J Bacteriol, 178: 6145-6150. DOI: 10.1128/jb.178.21.6145-6150.1996.

DVG (2017):
Prüfrichtlinien MHK IV Ermittlung der Minimalen Hemmkonzentration.
Richtlinien für die Prüfung von Desinfektionsverfahren und chemischen Desinfektionsmitteln. Deutsche Verterinärmedizinische Gesellschaft (German Veterinary Medical Society). 4th Edition.

EFSA (2008a):
Assessment of the possible effect of the four antimicrobial treatment substances on the emergence of antimicrobial resistance - Scientific Opinion of the Panel on Biological Hazards.
EFSA Journal 6: 659. DOI: 10.2903/j.efsa.2008.659.

EFSA (2008b):
Foodborne antimicrobial resistance as a biological hazard - Scientific opinion of the panel on biological hazards.
EFSA Journal 6: 765. DOI: 10.2903/j.efsa.2008.765.

EFSA (2019):
The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017.
EFSA Journal 17: e05598. DOI: 10.2903/j.efsa.2019.5598.

EMEA (1997):
Commitee for veterinary medicinal products benzalkonium chloride summary report, The european agency for the evaluation of medicinal products. EMEA/MRL/306/97.

Emslie, K. R., D. E. Townsend and W. B. Grubb (1986):
Isolation and characterisation of a family of small plasmids encoding resistance to nucleic acid-binding compounds in *Staphylococcus aureus*.
J Med Microbiol, 22: 9-15. DOI: 10.1099/00222615-22-1-9.

EU (2012):
Regulation (EU) No 528/2012 Concerning the making available on the market and use of biocidal products, Official Journal of the European Union, L 167/161 - 167/123.

EUCAST, D. B. (2011).
EUCAST definitions (and breakpoint table, MIC and zone distribution website conventions).
Retrieved on 04.08.2020 at 08:32 CET, from
www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_Presentations/2011/EW1_Brown_Definitionsf2.pdf.

Farrand, R. J. and A. Williams (1973):
Evaluation of single-use packs of hospital disinfectants.
Lancet, 1: 591-593. DOI: 10.1016/s0140-6736(73)90730-7.

Feßler, A. T., A. R. Schug, F. Geber, A. D. Scholtzek, R. Merle, J. Brombach, V. Hensel, M. Meurer, G. B. Michael, M. Reinhardt, S. Speck, U. Truyen and S. Schwarz (2018):
Development and evaluation of a broth macrodilution method to determine the biocide susceptibility of bacteria.
Vet Microbiol, 223: 59-64. DOI: 10.1016/j.vetmic.2018.07.006.

Finn, S., J. C. Hinton, P. McClure, A. Amezquita, M. Martins and S. Fanning (2013):
Phenotypic characterization of *Salmonella* isolated from food production environments associated with low-water activity foods.
J Food Prot, 76: 1488-1499. DOI: 10.4315/0362-028x.jfp-13-088.

Francois, K., F. Devlieghere, A. R. Standaert, A. H. Geeraerd, I. Cools, J. F. Van Impe and J. Debevere (2005):
Environmental factors influencing the relationship between optical density and cell count for *Listeria monocytogenes*.
J Appl Microbiol, 99: 1503-1515. DOI: 10.1111/j.1365-2672.2005.02727.x.

Gerba, C. P. (2015):
Quaternary ammonium biocides: efficacy in application.
Appl Environ Microbiol, 81: 464-469. DOI: 10.1128/AEM.02633-14.

Getahun, H., I. Smith, K. Trivedi, S. Paulin and H. H. Balkhy (2020):
Tackling antimicrobial resistance in the COVID-19 pandemic.
Bull World Health Organ, 98: 442-442A. DOI: 10.2471/BLT.20.268573.

Giavarina, D. (2015):
Understanding Bland Altman analysis.
Biochem ME, 25: 141-151. DOI: 10.11613/BM.2015.015.

Gilbert, P., M. R. Brown and J. W. Costerton (1987):
Inocula for antimicrobial sensitivity testing: a critical review.
J Antimicrob Chemother, 20: 147-154. DOI: 10.1093/jac/20.2.147.

Gilbert, P., D. J. Evans and M. R. W. Brown (1993):
Formation and dispersal of bacterial biofilms in vivo and in situ.
J Appl Bacteriol, 74: 67S-78S. DOI: 10.1111/j.1365-2672.1993.tb04343.x.

Gilbert, P. and A. J. McBain (2003):
Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance.
Clin Microbiol Rev, 16: 189-208. DOI: 10.1128/cmr.16.2.189-208.2003.

Gnanadhas, D. P., S. Marathe and D. Chakravortty (2012):
Biocides - Resistance, cross-resistance mechanisms and assessment.
Expert Opin Investig Drugs, 22. DOI: 10.1517/13543784.2013.748035.

Gomez Escalada, M., J. L. Harwood, J. Y. Maillard and D. Ochs (2005a):
Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*.
J Antimicrob Chemother, 55: 879-882. DOI: 10.1093/jac/dki123.

Gomez Escalada, M., A. D. Russell, J. Y. Maillard and D. Ochs (2005b):
Triclosan-bacteria interactions: single or multiple target sites?
Lett Appl Microbiol, 41: 476-481. DOI: 10.1111/j.1472-765X.2005.01790.x.

Gould, I. M. (1998):
Determinants of response to antibiotic therapy.
J Chemother, 10: 347-353. DOI: 10.1179/joc.1998.10.5.347.

Grande Burgos, M. J., M. L. Fernández Márquez, R. Pérez Pulido, A. Gálvez and R. Lucas López (2016):
Virulence factors and antimicrobial resistance in *Escherichia coli* strains isolated from hen egg shells.
Int J Food Microbiol, 238: 89-95. DOI: 10.1016/j.ijfoodmicro.2016.08.037.

Halbedel, S., H. Wilking, A. Holzer, S. Kleta, M. Fischer, S. Lüth, A. Pietzka, S. Huhulescu, R. Lachmann, A. Krings, W. Ruppitsch, A. Leclercq, R. Kamphausen, M. Meincke, C. Wagner-Wiening, M. Contzen, I. Kraemer, S. Dahouk, F. Allerberger and A. Flieger (2020):
Large nationwide outbreak of invasive listeriosis associated with blood sausage, Germany, 2018-2019.
Emerg Infect Dis, 26: 1456-1464. DOI: 10.3201/eid2607.200225.

Hardy, K., K. Sunnucks, H. Gil, S. Shabir, E. Trampari, P. Hawkey and M. Webber (2018):
Increased usage of antiseptics is associated with reduced susceptibility in clinical isolates of *Staphylococcus aureus*.
mBio, 9 (3) e00894-18. DOI: 10.1128/mBio.00894-18.

Hazan, R., Y.-A. Que, D. Maura and L. G. Rahme (2012):
A method for high throughput determination of viable bacteria cell counts in 96-well plates.
BMC Microbiol, 12: 259-259. DOI: 10.1186/1471-2180-12-259.

Heir, E., G. Sundheim and A. L. Holck (1999):
The *qacG* gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry.
J Appl Microbiol, 86: 378-388. DOI: 10.1046/j.1365-2672.1999.00672.x.

Henly, E. L., J. A. R. Dowling, J. B. Maingay, M. M. Lacey, T. J. Smith and S. Forbes (2019):
Biocide exposure induces changes in susceptibility, pathogenicity, and biofilm formation in uropathogenic *Escherichia coli*.
Antimicrob Agents Chemother, 63: e01892-01818. DOI: 10.1128/aac.01892-18.

Hijazi, K., I. Mukhopadhya, F. Abbott, K. Milne, Z. J. Al-Jabri, M. R. Oggioni and I. M. Gould (2016):
Susceptibility to chlorhexidine amongst multidrug-resistant clinical isolates of *Staphylococcus epidermidis* from bloodstream infections.
Int J Antimicrob Agents, 48: 86-90. DOI: 10.1016/j.ijantimicag.2016.04.015.

Holmes, A., L. Moore, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P. Guerin and L. Piddock (2015):
Understanding the mechanisms and drivers of antimicrobial resistance.
Lancet, 387:176-87. DOI: 10.1016/S0140-6736(15)00473-0.

Hughes, D. and D. I. Andersson (2017):
Environmental and genetic modulation of the phenotypic expression of antibiotic resistance.
FEMS Microbiol Rev, 41: 374-391. DOI: 10.1093/femsre/fux004.

Humayoun, S. B., L. M. Hiott, S. K. Gupta, J. B. Barrett, T. A. Woodley, J. J. Johnston, C. R. Jackson and J. G. Frye (2018):
An assay for determining the susceptibility of *Salmonella* isolates to commercial and household biocides.
PLoS One, 13: e0209072. DOI: 10.1371/journal.pone.0209072.

Jansson, J. K. and J. I. Prosser (1997):
Quantification of the presence and activity of specific microorganisms in nature.
Mol Biotechnol, 7: 103-120. DOI: 10.1007/BF02761746.

Jarvis, B., A. J. Hedges and J. E. L. Corry (2007):
Assessment of measurement uncertainty for quantitative methods of analysis: comparative assessment of the precision (uncertainty) of bacterial colony counts.
Int J Food Microbiol, 116: 44-51. DOI: 10.1016/j.ijfoodmicro.2006.12.037.

Jennings, M., M. Forman, S. Duggan, K. Minbiole and W. Wuest (2017):
Efflux pumps may not be the major drivers of *qac* resistance in methicillin-resistant *Staphylococcus aureus*.
Chembiochem,18: 1573-1577. DOI: 10.1002/cbic.201700233.

Jennison, M. W. (1937):
The relations between plate counts and direct microscopic counts of *Escherichia coli* during the logarithmic growth period.
J Bacteriol, 33 5: 461-477.

Jennison, M. W. and G. P. Wadsworth (1940):
Evaluation of the errors involved in estimating bacterial numbers by the plating method.
J Bacteriol, 39: 389-397.

Johnston, M. D., E.-A. Simons and R. J. W. Lambert (2000):
One explanation for the variability of the bacterial suspension test.
J Appl Microbiol, 88: 237-242. DOI: 10.1046/j.1365-2672.2000.00951.x.

Jorgensen, J. H. and M. J. Ferraro (2009):
Antimicrobial susceptibility testing: a review of general principles and contemporary practices.
Clin Infect Dis, 49: 1749-1755. DOI: 10.1086/647952.

Kampf, G. (2016):
Acquired resistance to chlorhexidine – is it time to establish an ‘antiseptic stewardship’ initiative?
J Hosp Infect, 94: 213-227. DOI: 10.1016/j.jhin.2016.08.018.

Kampf, G. (2018):
Biocidal agents used for disinfection can enhance antibiotic resistance in gram-negative species.
Antibiotics (Basel), 7: 110. DOI: 10.3390/antibiotics7040110.

Kernberger-Fischer, I. A., C. Krischek, B. Strommenger, U. Fiegen, M. Beyerbach, L. Kreienbrock, G. Klein and C. Kehrenberg (2018):
Susceptibility of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates of various clonal lineages from germany to eight biocides.
Appl Environ Microbiol, 84: e00799-00718. DOI: 10.1128/aem.00799-18.

Knapp, L., A. Amézquita, P. McClure, S. Stewart and J.-Y. Maillard (2015):
Development of a protocol for predicting bacterial resistance to microbicides.
Appl Environ Microbiol, 81: 2652-2659. DOI: 10.1128/aem.03843-14.

Knapp, L., L. Rushton, H. Stapleton, A. Sass, S. Stewart, A. Amezquita, P. McClure, E. Mahenthiralingam and J.-Y. Maillard (2013):
The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium.
J Appl Microbiol, 115: 1117-1126. DOI: 10.1111/jam.12320.

Kramer, A., K. H. Wallhäusser and O. Assadian (2008):
Wallhäußers Praxis der Sterilisation, Desinfektion, Antiseptik und Konservierung: Qualitätssicherung der Hygiene in Industrie, Pharmazie und Medizin.
Stuttgart: Thieme. ISBN: 9783131411211

Langsrud, S., M. Sidhu, E. Heir and A. Holck (2003):
Bacterial disinfectant resistance - A challenge for the food industry.
Int Biodeterior Biodegradation, 51: 283-290. DOI: 10.1016/S0964-8305(03)00039-8.

Langsrud, S. and G. Sundheim (1997):
Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound.
J Appl Microbiol, 82: 705-712. DOI: 10.1046/j.1365-2672.1997.00186.x.

Langsrud, S. and G. Sundheim (1998):
Factors influencing a suspension test method for antimicrobial activity of disinfectants.
J Appl Microbiol, 85: 1006-1012. DOI: 10.1111/j.1365-2672.1998.tb05265.x.

Lanini, S., S. D'Arezzo, V. Puro, L. Martini, F. Imperi, P. Piselli, M. Montanaro, S. Paoletti, P. Visca and G. Ippolito (2011):
Molecular epidemiology of a *Pseudomonas aeruginosa* hospital outbreak driven by a contaminated disinfectant-soap dispenser.
PLoS One, 6: e17064. DOI: 10.1371/journal.pone.0017064.

Lanjri, S., J. Uwingabiye, M. Frikh, L. Abdellatifi, J. Kasouati, A. Maleb, A. Bait, A. Lemnouer and M. Elouennass (2017):
In vitro evaluation of the susceptibility of *Acinetobacter baumannii* isolates to antiseptics and disinfectants: comparison between clinical and environmental isolates.
Antimicrob Resist Infect Control, 6: 36. DOI: 10.1186/s13756-017-0195-y.

Lanker Klossner, B., H. R. Widmer and F. Frey (1997):
Nondevelopment of resistance by bacteria during hospital use of povidone-iodine.
Dermatology, 195 Suppl 2: 10-13. DOI: 10.1159/000246024.

Lee, P. S. (2008):
Quantitation of Microorganisms.
In: Practical Handbook of Microbiology. E. Goldman and L. H. Green, Second ed.,11-29.
Boca Raton, FL, USA: CRC Press. ISBN: 9781420009330

Levy, S. B. (2002a):
Active efflux, a common mechanism for biocide and antibiotic resistance.
J Appl Microbiol, 92: 65S-71S. DOI: 10.1046/j.1365-2672.92.5s1.4.x.

Levy, S. B. (2002b):
Factors impacting on the problem of antibiotic resistance.
J Antimicrob Chemother, 49: 25-30. DOI: 10.1093/jac/49.1.25.

Liu, Q., H. Zhao, L. Han, W. Shu, Q. Wu and Y. Ni (2015):
Frequency of biocide-resistant genes and susceptibility to chlorhexidine in high-level mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MuH MRSA).
Diagn Microbiol Infect Dis, 82: 278-283. DOI: 10.1016/j.diagmicrobio.2015.03.023.

Lorian, V. (2005):
Antibiotics in laboratory medicine.
5th edition, Philadelphia, PA, USA: Lippincott Williams & Wilkins. ISBN: 9780781749831

Maillard, J.-Y. and S. Denyer (2009):
Emerging bacterial resistance following biocide exposure: should we be concerned?
Chemica Oggi, 27: 26-28.

Maillard, J. Y. (2002):
Bacterial target sites for biocide action.
J Appl Microbiol, 92 Suppl: 16s-27s.

Maillard, J. Y. (2005):
Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems.
Ther Clin Risk Manag, 1: 307-320.

Maillard, J. Y. (2007):
Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern?
J Hosp Infect, 65 Suppl 2: 60-72. DOI: 10.1016/s0195-6701(07)60018-8.

Maillard, J. Y. (2018):
Resistance of bacteria to biocides.
Microbiol Spectr, 6. DOI: 10.1128/microbiolspec.ARBA-0006-2017.

Maillard, J. Y., S. Bloomfield, J. R. Coelho, P. Collier, B. Cookson, S. Fanning, A. Hill, P. Hartemann, A. J. McBain, M. Oggioni, S. Sattar, H. P. Schweizer and J. Threlfall (2013):
Does microbicide use in consumer products promote antimicrobial resistance? A critical review and recommendations for a cohesive approach to risk assessment.
Microb Drug Resist, 19: 344-354. DOI: 10.1089/mdr.2013.0039.

Martin, D. J. H., R. L. Wesgate, S. P. Denyer, G. McDonnell and J. Y. Maillard (2015):
Bacillus subtilis vegetative isolate surviving chlorine dioxide exposure: an elusive mechanism of resistance.
J Appl Microbiol, 119: 1541-1551. DOI: https://doi.org/10.1111/jam.12963.

Martínez-Suárez, J. V., S. Ortiz and V. López-Alonso (2016):
Potential impact of the resistance to quaternary ammonium disinfectants on the persistence of *Listeria monocytogenes* in food processing environments.
Front Microbiol, 7: 638. DOI: 10.3389/fmicb.2016.00638.

Mavri, A. and S. Smole Možina (2013):
Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides.
Int J Food Microbio, l 160: 304-312. DOI: 10.1016/j.ijfoodmicro.2012.11.006.

McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder, B. B. Price and P. Gilbert (2003):
Exposure of sink drain microcosms to triclosan: population dynamics and antimicrobial susceptibility.
Appl Environ Microbiol, 69: 5433-5442. DOI: 10.1128/aem.69.9.5433-5442.2003.

McBain, A. J., R. G. Ledder, L. E. Moore, C. E. Catrenich and P. Gilbert (2004):
Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility.
Appl Environ Microbiol, 70: 3449-3456. DOI: 10.1128/aem.70.6.3449-3456.2004.

McDonnell, G. and A. D. Russell (1999):
Antiseptics and disinfectants: activity, action, and resistance.
Clin Microbiol Rev, 12: 147-179.

Milstone, A. M., C. L. Passaretti and T. M. Perl (2008):
Chlorhexidine: expanding the armamentarium for infection control and prevention.
Clin Infect Dis, 46: 274-281. DOI: 10.1086/524736.

Molina-González, D., C. Alonso-Calleja, A. Alonso-Hernando and R. Capita (2014):
Effect of sub-lethal concentrations of biocides on the susceptibility to antibiotics of multi-drug resistant *Salmonella enterica* strains.
Food Control, 40: 329-334. DOI: 10.1016/j.foodcont.2013.11.046.

Mombeshora, M. and S. Mukanganyama (2017):
Development of an accumulation assay and evaluation of the effects of efflux pump inhibitors on the retention of chlorhexidine digluconate in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.
BMC Res Notes, 10: 328. DOI: 10.1186/s13104-017-2637-2.

Moore, L. E., R. G. Ledder, P. Gilbert and A. J. McBain (2008):
In vitro study of the effect of cationic biocides on bacterial population dynamics and susceptibility.
Apl Environ Microbiol, 74: 4825-4834. DOI: 10.1128/AEM.00573-08

Morita, Y., L. Cao, V. C. Gould, M. B. Avison and K. Poole (2006):
nalD encodes a second repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*.
J Bacteriol, 188: 8649-8654. DOI: 10.1128/JB.01342-06.

Morrissey, I., M. R. Oggioni, D. Knight, T. Curiao, T. Coque, A. Kalkanci, J. L. Martinez and B. Consortium (2014):
Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms.
PLoS One, 9: e86669. DOI: 10.1371/journal.pone.0086669.

Murray, A. K. (2020):
The novel coronavirus covid-19 outbreak: global implications for antimicrobial resistance.
Front Microbiol, 11: 1020-1020. DOI: 10.3389/fmicb.2020.01020.

Nelson, D. B. (2002):
Effectiveness of manual cleaning and disinfection for the elimination of hepatitis C virus from GI endoscopes.
Am J Gastroenterol, 97: 204-206. DOI: 10.1111/j.1572-0241.2002.05404.x.

Nicoletti, G., V. Boghossian, F. Gurevitch, R. Borland and P. Morgenroth (1993):
The antimicrobial activity in vitro of chlorhexidine, a mixture of isothiazolinones ('Kathon' CG) and cetyl trimethyl ammonium bromide (CTAB).
J Hosp Infect, 23: 87-111. DOI: 10.1016/0195-6701(93)90014-q.

Nikaido, H. (2003):
Molecular basis of bacterial outer membrane permeability revisited.
Microbiol Mol Biol Rev, 67: 593-656. DOI: 10.1128/mmbr.67.4.593-656.2003.

Oethinger, M., I. Podglajen, W. V. Kern and S. B. Levy (1998):
Overexpression of the *marA* or *soxS* regulatory gene in clinical topoisomerase mutants of *Escherichia coli*.
Antimicrob Agents Chemother, 42: 2089-2094. DOI: 10.1128/aac.42.8.2089.

Olliver, A., M. Vallé, E. Chaslus-Dancla and A. Cloeckaert (2004):
Role of an *acrR* mutation in multidrug resistance of *in vitro*-selected fluoroquinolone-resistant mutants of *Salmonella enterica* serovar Typhimurium.
FEMS Microbiol Lett, 238: 267-272. DOI: 10.1111/j.1574-6968.2004.tb09766.x.

Ortega Morente, E., M. A. Fernández-Fuentes, M. J. Grande Burgos, H. Abriouel, R. Pérez Pulido and A. Gálvez (2013):
Biocide tolerance in bacteria.
Int J Food Microbiol, 162: 13-25. DOI: 10.1016/j.ijfoodmicro.2012.12.028.

Ortiz, S., V. López-Alonso and J. Martínez-Suárez (2014a):
Control of *Listeria monocytogenes* contamination in an Iberian pork processing plant and selection of benzalkonium chloride-resistant strains.
Food Microbiol, 39: 81–88. DOI: 10.1016/j.fm.2013.11.007.

Ortiz, S., V. López and J. V. Martínez-Suárez (2014b):
The influence of subminimal inhibitory concentrations of benzalkonium chloride on biofilm formation by *Listeria monocytogenes*.
Int J Food Microbiol, 189: 106-112. DOI: 10.1016/j.ijfoodmicro.2014.08.007.

Ou, F., C. McGoverin, S. Swift and F. Vanholsbeeck (2017):
Absolute bacterial cell enumeration using flow cytometry.
J Appl Microbiol, 123: 464-477. DOI: 10.1111/jam.13508.

Pathak, S., J. A. Awuh, N. A. Leversen, T. H. Flo and B. Åsjø (2012):
Counting mycobacteria in infected human cells and mouse tissue: A comparison between qPCR and CFU.
PLoS one, 7: e34931. DOI: 10.1371/journal.pone.0034931.

Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell and R. A. Skurray (1996):
Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity.
Proc Natl Acad Sci USA, 93: 3630-3635. DOI: 10.1073/pnas.93.8.3630.

Pidot, S. J., W. Gao, A. H. Buultjens, I. R. Monk, R. Guerillot, G. P. Carter, J. Y. H. Lee, M. M. C. Lam, M. L. Grayson, S. A. Ballard, A. A. Mahony, E. A. Grabsch, D. Kotsanas, T. M. Korman, G. W. Coombs, J. O. Robinson, A. Gonçalves da Silva, T. Seemann, B. P. Howden, P. D. R. Johnson and T. P. Stinear (2018):
Increasing tolerance of hospital *Enterococcus faecium* to handwash alcohols.
Sci Trans Med, 10: eaar6115. DOI: 10.1126/scitranslmed.aar6115.

Poole, K. (2002):
Mechanisms of bacterial biocide and antibiotic resistance.
Symp Ser Soc Appl Microbiol, 31: 55s-64s.

Poole, K. (2005):
Efflux-mediated antimicrobial resistance.
J Antimicrob Chemother, 56: 20-51. DOI: 10.1093/jac/dki171.

Reller, L. B., M. Weinstein, J. H. Jorgensen and M. J. Ferraro (2009):
Antimicrobial susceptibility testing: A review of general principles and contemporary practices.
Clin Infect Dis, 49: 1749-1755. DOI: 10.1086/647952

Rensing, C. and G. Grass (2003):
Escherichia coli mechanisms of copper homeostasis in a changing environment.
FEMS Microbiol Rev, 27: 197-213. DOI: 10.1016/s0168-6445(03)00049-4.

Ricci, V., J. M. A. Blair and L. J. V. Piddock (2014):
RamA, which controls expression of the MDR efflux pump AcrAB-TolC, is regulated by the Lon protease.
J Antimicrob Chemother, 69: 643-650. DOI: 10.1093/jac/dkt432.

Riesenberg, A., H. Kaspar, A. T. Feßler, C. Werckenthin and S. Schwarz (2016):
Susceptibility testing of *Rhodococcus equi*: An interlaboratory test.
Vet Microbiol, 194: 30-35. DOI: 10.1016/j.vetmic.2015.11.029.

RKI (2019):
Antibiotika-Resistenz-Surveillance in Deutschland.
Retrieved on 16.07.2019 at 13:27 CET from
https://ars.rki.de/Content/Project/Methodology/Consequence.aspx.

Roberts, M.C., Feßler, A.T., Monecke, S., Ehricht, R., No, D., Schwarz, S., 2018.
Molecular analysis of two different MRSA clones ST188 and ST3268 from primates (*Macaca* spp.) in a United States primate center.
Front Microbiol 9, 2199. DOI: 10.3389/fmicb.2018.02199

Rodloff, A., T. Bauer, S. Ewig, P. Kujath and E. Müller (2008):
Susceptible, intermediate, and resistant - the intensity of antibiotic action.
Dtsch Arztebl Int, 105: 657-662. DOI: 10.3238/arztebl.2008.0657.

Roedel, A., R. Dieckmann, H. Brendebach, J. A. Hammerl, S. Kleta, M. Noll, S. Al Dahouk and S. Vincze (2019):
Biocide-tolerant *listeria monocytogenes* Isolates from German food production plants do not show cross-resistance to clinically relevant antibiotics.
Appl Environ Microbiol, 85. DOI: 10.1128/aem.01253-19.

Roedel, A., R. Dieckmann, O. Makarewicz, A. Hartung, M. Noll, M. W. Pletz, S. A. Dahouk and S. Vincze (2020):
Evaluation of a newly developed vacuum dried microtiter plate for rapid biocide susceptibility testing of Clinical *Enterococcus Faecium* isolates.
Microorganisms, 8. DOI: 10.3390/microorganisms8040551.

Russell, A. D. (1997):
Plasmids and bacterial resistance to biocides.
J Appl Microbiol, 83: 155-165. DOI: 10.1046/j.1365-2672.1997.00198.x.

Russell, A. D. (1999):
Bacterial resistance to disinfectants: present knowledge and future problems.
J Hosp Infect, 43: S57-S68. DOI: 10.1016/S0195-6701(99)90066-X.

Russell, A. D. (2001):
Mechanisms of bacterial insusceptibility to biocides.
Am J Infect Control, 29: 259-261. DOI: 10.1067/mic.2001.115671.

Russell, A. D. (2002a):
Antibiotic and biocide resistance in bacteria: Introduction.
J Appl Microbiol, 92: 1S-3S. DOI: https://doi.org/10.1046/j.1365-2672.92.5s1.14.x.

Russell, A. D. (2002b):
Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria.
J Appl Microbiol, 92: 121S-135S. DOI: 10.1046/j.1365-2672.92.5s1.12.x.

Russell, A. D. (2003):
Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations.
Lancet Infect Dis, 3: 794-803. DOI: 10.1016/S1473-3099(03)00833-8.

Russell, A. D. (2004):
Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon.
J Hosp Infect, 57: 97-104. DOI: 10.1016/j.jhin.2004.01.004.

Russell, A. D. and G. McDonnell (2000):
Concentration: a major factor in studying biocidal action.
J Hosp Infect, 44: 1-3. DOI: 10.1053/jhin.1999.0654.

Russell, A. D., U. Tattawasart, J. Y. Maillard and J. R. Furr (1998):
Possible link between bacterial resistance and use of antibiotics and biocides.
Antimicrob Agents Chemother, 42: 2151. DOI: 10.1128/aac.42.8.2151.

Russell, A. D. & Chopra, I. (1990):
Understanding antibacterial action and resistance.
West Sussex, Ellis Horwood, Chichester - ISBN: 0139469222

Rutala, W. A. and D. J. Weber (2004):
The benefits of surface disinfection.
Am J Infect Control, 32: 226-231. DOI: 10.1016/j.ajic.2004.04.197.

Sanchez, P., E. Moreno and J. L. Martinez (2005):
The biocide triclosan selects *Stenotrophomonas maltophilia* mutants that overproduce the SmeDEF multidrug efflux pump.
Antimicrob Agents Chemotherm, 49: 781-782. DOI: 10.1128/AAC.49.2.781-782.2005

SCENIHR (2009):
Assessment of the antibiotic resistance effects of biocides. SCENIHR (Scientific committee on emerging and newly identified health risks).
Eur. Comm. Consum. Prot. DG. Brussels, Belgium.

SCENIHR (2010):
Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. SCENIHR (Scientific committee on emerging and newly identified health risks).
Eur. Comm. Consum. Prot. DG. Brussels, Belgium. DOI 10.2772/39297

Scholtzek, A. D., D. Hanke, B. Walther, I. Eichhorn, S. D. Stöckle, K.-S. Klein, H. Gehlen, A. Lübke-Becker, S. Schwarz and A. T. Feßler (2019):
Molecular characterization of equine *Staphylococcus aureus* isolates exhibiting reduced oxacillin susceptibility.
Toxins 11: 535. DOI: 10.3390/toxins11090535

Schwarz, S., P. Silley, S. Simjee, N. Woodford, E. van Duijkeren, A. P. Johnson and W. Gaastra (2010):
Assessing the antimicrobial susceptibility of bacteria obtained from animals.
Vet Microbiol, 141: 1-4. DOI: 10.1016/j.vetmic.2009.12.013.

Schweizer, H. P. (2001):
Triclosan: a widely used biocide and its link to antibiotics.
FEMS Microbiol Lett, 202: 1-7. DOI: 10.1111/j.1574-6968.2001.tb10772.x.

Siani, H. and J. Y. Maillard (2015):
Best practice in healthcare environment decontamination.
Eur J Clin Microbiol Infect Dis, 34: 1-11. DOI: 10.1007/s10096-014-2205-9.

Smith, K. and I. S. Hunter (2008):
Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates.
J Med Microbiol, 57: 966-973. DOI: 10.1099/jmm.0.47668-0.

Soumet, C., C. Ragimbeau and P. Maris (2005):
Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production.
Lett Appl Microbiol, 41: 291-296. DOI:10.1111/j.1472-765X.2005.01763.x.

Stein, C., S. Vincze, F. Kipp, O. Makarewicz, S. Al Dahouk and M. W. Pletz (2019):
Carbapenem-resistant *Klebsiella pneumoniae* with low chlorhexidine susceptibility.
Lancet Infect Dis, 19: 932-933. DOI: 10.1016/S1473-3099(19)30427-X.

Suller, M. T. and A. D. Russell (2000):
Triclosan and antibiotic resistance in *Staphylococcus aureus*.
J Antimicrob Chemother, 46: 11-18. DOI: 10.1093/jac/46.1.11.

Suller, M. T. E. and A. D. Russell (1999):
Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*.
J Hosp Infect,43: 281-291. DOI: 10.1016/S0195-6701(99)90424-3.

Sundheim, G. and S. Langsrud (1995):
Natural and acquired resistance of bacteria associated with food processing environments to disinfectant containing an extract from grapefruit seeds.
Int Biodeterior Biodegradation, 36: 441-448. DOI: 10.1016/0964-8305(95)00108-5.

Tabak, M., K. Scher, E. Hartog, U. Romling, K. R. Matthews, M. L. Chikindas and S. Yaron (2007):
Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms.
FEMS Microbiol Lett, 267: 200-206. DOI: 10.1111/j.1574-6968.2006.00547.x.

Tattawasart, U., A. C. Hann, J. Y. Maillard, J. R. Furr and A. D. Russell (2000):
Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*.
J Antimicrob Chemother, 45: 145-152. DOI: 10.1093/jac/45.2.145.

Tattawasart, U., J. Y. Maillard, J. R. Furr and A. D. Russell (1999):
Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility.
J Hosp Infect, 42: 219-229. DOI: 10.1053/jhin.1999.0591.

Tezel, U. and S. G. Pavlostathis (2015):
Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology.
Curr Opin Biotechnol, 33: 296-304. DOI: 10.1016/j.copbio.2015.03.018.

Thomas, L., J. Y. Maillard, R. J. W. Lambert and A. D. Russell (2000):
Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a 'residual' concentration.
J Hosp Infect, 46: 297-303. DOI: 10.1053/jhin.2000.0851.

Thomas, L., A. D. Russell and J. Y. Maillard (2005):
Antimicrobial activity of chlorhexidine diacetate and benzalkonium chloride against *Pseudomonas aeruginosa* and its response to biocide residues.
J Appl Microbiol, 98: 533-543. DOI: 10.1111/j.1365-2672.2004.02402.x.

Thorrold, C. A., M. E. Letsoalo, A. G. Dusé and E. Marais (2007):
Efflux pump activity in fluoroquinolone and tetracycline resistant *Salmonella* and *E. coli* implicated in reduced susceptibility to household antimicrobial cleaning agents.
Int J Food Microbiol, 113: 315-320. DOI: 10.1016/j.ijfoodmicro.2006.08.008.

Tkachenko, O., J. Shepard, V. M. Aris, A. Joy, A. Bello, I. Londono, J. Marku, P. Soteropoulos and M. A. Peteroy-Kelly (2007):
A triclosan-ciprofloxacin cross-resistant mutant strain of *Staphylococcus aureus* displays an alteration in the expression of several cell membrane structural and functional genes.
Res Microbiol, 158: 651-658. DOI: 10.1016/j.resmic.2007.09.003.

VAH (2015):
Association for Applied Hygiene (VAH) Disinfectants Commission. Requirements and Methods for VAH Certification of Chemical Disinfection Procedures. mhp-Verlag GmbH. Wiesbaden.

van Klingeren, B. and W. Pullen (1993):
Glutaraldehyde resistant mycobacteria from endoscope washers.
J Hosp Infect, 25: 147-149. DOI: 10.1016/0195-6701(93)90107-b.

Vikram, A., J. M. Bomberger and K. J. Bibby (2015):
Efflux as a glutaraldehyde resistance mechanism in *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* biofilms.
Antimicrob Agents Chemother, 59: 3433-3440. DOI: 10.1128/AAC.05152-14.

Wallmann, J., A. Böttner, L. Goossens, H. M. Hafez, K. Hartmann, H. Kaspar, C. Kehrenberg, M. Kietzmann, D. Klarmann, G. Klein, P. Krabisch, T. Kühn, G. Luhofer, A. Richter, B. Schulz, S. Schwarz, C. Sigge, W. Traeder, K.-H. Waldmann, C. Werckenthin and E. Zschiesche (2006):
Results of an interlaboratory test on antimicrobial susceptibility testing of bacteria from animals by broth microdilution.
Int J Antimicrob Agents, 27: 482-490. DOI: 10.1016/j.ijantimicag.2005.12.011.

Walsh, S. E., J. Y. Maillard, A. D. Russell, C. E. Catrenich, D. L. Charbonneau and R. G. Bartolo (2003):
Development of bacterial resistance to several biocides and effects on antibiotic susceptibility.
J Hosp Infect, 55: 98-107. DOI: 10.1016/s0195-6701(03)00240-8.
Warner, D. M., W. M. Shafer and A. E. Jerse (2008):
Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE Efflux pump system confer different levels of antimicrobial resistance and *in vivo* fitness.
Mol Microbiol, 70: 462-478. DOI: 10.1111/j.1365-2958.2008.06424.x.

Wassenaar, T. M., D. Ussery, L. N. Nielsen and H. Ingmer (2015):
Review and phylogenetic analysis of *qac* genes that reduce susceptibility to quaternary ammonium compounds in *Staphylococcus* species.
Eur J Microbiol Immunol, 5: 44-61. DOI: 10.1556/EUJMI-D-14-00038.

Watts JL, S. M., Lubbers BV (2018):
Antimicrobial susceptibility testing of bacteria of veterinary origin.
Antmicrobial resistance in bacteria from livestock and companion animals.
Microbiol Spectrum, 6(2): ARBA-0001-2017.

Webber, M. A., A. Talukder and L. J. V. Piddock (2005):
Contribution of mutation at amino acid 45 of AcrR to *acrB* expression and ciprofloxacin resistance in clinical and veterinary *Escherichia coli* isolates.
Antimicrob Agents Chemother, 49: 4390-4392. DOI: 10.1128/AAC.49.10.4390-4392.2005.

Weber, D. J., W. A. Rutala and E. E. Sickbert-Bennett (2007):
Outbreaks associated with contaminated antiseptics and disinfectants.
Antimicrob Agents Chemother, 51: 4217-4224. DOI: 10.1128/aac.00138-07.

Wesgate, R., S. Fanning, Y. Hu and J.-Y. Maillard (2020):
Effect of exposure to chlorhexidine residues at “during use” concentrations on antimicrobial susceptibility profile, efflux, conjugative plasmid transfer, and metabolism of *Escherichia coli*.
Antimicrob Agents Chemother, 64: e01131-01120. DOI: 10.1128/AAC.01131-20

Wesgate, R., P. Grasha and J. Y. Maillard (2016):
Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage.
Am J Infect Control, 44: 458-464. DOI: 10.1016/j.ajic.2015.11.009.

Whitehead, R. N., T. W. Overton, C. L. Kemp and M. A. Webber (2011):
Exposure of *Salmonella enterica* serovar Typhimurium to high level biocide challenge can select multidrug resistant mutants in a single step.
PLoS One, 6: e22833. DOI: 10.1371/journal.pone.0022833.

WHO (2014):
Antimicrobial resistance: global report on surveillance.
Geneva, Switzerland, World Health Organization. ISBN: 9789241564748

WHO (2015):
Global action plan on antimicrobial resistance.
Geneva, Switzerland, World Health Organization. ISBN: 9789241509763

Wiegand, I., K. Hilpert and R. E. Hancock (2008):
Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.
Nat Protoc, 3: 163-175. DOI: 10.1038/nprot.2007.521.

Winder, C. L., I. S. Al-Adham, S. M. Abdel Malek, T. E. Buultjens, A. J. Horrocks and P. J. Collier (2000):
Outer membrane protein shifts in biocide-resistant *Pseudomonas aeruginosa* PAO1.
J Appl Microbiol, 89: 289-295. DOI: 10.1046/j.1365-2672.2000.01119.x.

Wisplinghoff, H., R. Schmitt, A. Wöhrmann, D. Stefanik and H. Seifert (2007):
Resistance to disinfectants in epidemiologically defined clinical isolates of *Acinetobacter baumannii*.
J Hosp Infect, 66: 174-181. DOI: 10.1016/j.jhin.2007.02.016.

Worthing, K. A., A. Marcus, S. Abraham, D. J. Trott and J. M. Norris (2018):
Qac genes and biocide tolerance in clinical veterinary methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* and *Staphylococcus pseudintermedius*.
Vet Microbiol, 216: 153-158. DOI: 10.1016/j.vetmic.2018.02.004.

Zhang, X., Y. Wang, J. Guo, Y. Yu, Y. Li and C. Liu (2015):
Comparing two functions for optical density and cell numbers in bacterial exponential growth phase.
J Pure Appl Microbiol, 9: 299-305.

Zou, L., J. Meng, P. F. McDermott, F. Wang, Q. Yang, G. Cao, M. Hoffmann and S. Zhao (2014):
Presence of disinfectant resistance genes in *Escherichia coli* isolated from retail meats in the USA.
J Antimicrob Chemother, 69: 2644-2649. DOI: 10.1093/jac/dku197.

List of publications

Feßler AT, Schug AR, Geber F, Scholtzek AD, Brombach J, Hensel V, Meurer M, Michael GB, Reinhardt M, Speck S, Truyen U, Schwarz S and the Biocide Susceptibility study group (2018):
Development and evaluation of a broth macrodilution method to determine the biocide susceptibility of bacteria.
Vet Microbiol 223:59-64

Schug AR, Bartel A, Scholtzek AD, Meurer M, Brombach J, Hensel V, Fanning S, Schwarz S, Feßler AT (2020):
Biocide susceptibility testing of bacteria: Development of a broth microdilution method.
Vet Microbiol 248:108791

Schug AR, Bartel A, Meurer M, Scholtzek AD, Brombach J, Hensel V, Fanning S, Schwarz S, Feßler AT (2020):
Comparison of two methods for cell count determination in the course of biocide susceptibility testing.
Vet Microbiol 251:108831

Further aspects of this thesis have been presented at national and international conferences as posters or oral presentations:

Feßler AT, Geber F, Schug AR, Meurer M, Hensel V, Speck S, Reinhardt M, Brenner Michael G, Truyen U, Schwarz S:
Biocide susceptibility testing – development of a new testing method.
Proceedings of the 7th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Braunschweig, Germany (2017)

Schug AR, Feßler AT, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Development of a broth microdilution method for biocide susceptibility testing.
Proceedings of the Conference of the *Deutsche Veterinärmedizinische Gesellschaft* (DVG), division Bacteriology and Mycology, Hannover, Germany (2018)

Schug AR, Feßler AT, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Biocide susceptibility testing of bacteria: Establishment of a broth microdilution method.
Proceedings of the Junior Scientist Zoonoses Meeting, Hamburg, Germany (2018)

Schug AR, Feßler AT, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Determination of biocide susceptibility: Development of a broth microdilution method.
Proceedings of the International Symposium on Zoonoses Research, Berlin, Germany (2018)

Schug AR, Feßler AT, Bartel A, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Biocide susceptibility testing: development of a broth microdilution method.
Proceedings of the 8th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Tours, France (2019)

Schug AR, Feßler AT, Bartel A, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Comparison of two established methods for cell count determination.
Proceedings of the 8th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Tours, France (2019)

Schug AR, Feßler AT, Bartel A, Scholtzek AD, Meurer M, Brombach J, Hensel V, Schwarz S:
Development of a broth microdilution method for biocide susceptibility testing of bacterial isolates using four reference strains.
Proceedings of the International Symposium on Zoonoses Research, Berlin, Germany (2019)

Danksagung

Besonderen Dank möchte ich Herrn Universitätsprofessor Dr. med. vet. Stefan Schwarz aussprechen. Er gab mir die Möglichkeit dieses Projekt am Institut für Mikrobiologie und Tierseuchen durchzuführen und war stets bereit Wege und Möglichkeiten zu finden, es besser und effektiver zu gestalten. Danken möchte ich auch dafür, dass er sich unentwegt für seine Arbeitsgruppe und das Institut einsetzt, was nicht zuletzt maßgeblich dazu beitrug, dass internationale Kontakte entstanden, mein Projekt in der wissenschaftlichen Fachgemeinschaft einen Platz bekam und entscheidend vorangetrieben wurde.

Des Weiteren gilt mein besonderer Dank Dr. med. vet. Andrea Feßler, PhD für die Betreuung. Ihr Interesse, Engagement und Hingabe machten einen äußerst gewinnbringenden Austausch möglich und sorgten für die entscheidenden Anstöße.

Meinem Mentor Prof. Séamus Fanning, BSc, PhD und seinem Team in Irland danke ich ausdrücklich für den Austausch, die zielführenden Anregungen und Hilfestellungen sowie für die Möglichkeit mein Projekt in Irland weiterzuentwickeln und zu verbessern. Hierbei gilt mein besonderer Dank Yujie Hu, sowohl für die methodische und technische Unterstützung als auch den fachlichen und kulturellen Austausch.

Außerordentlich danken möchte ich auch meiner Mentorin Prof. Dr. rer. nat. Maren von Köckritz-Blickwede für den wertschätzenden Austausch, das Feedback und die förderlichen Anregungen.

Ich bedanke mich bei der *Dres. Jutta & Georg Bruns Stiftung für innovative Veterinärmedizin* für die Startfinanzierung sowie bei deren Schirmherrin, der Deutschen Veterinärmedizinischen Gesellschaft e.V. (DVG) für die Verwaltung und Abwicklung. Danken möchte ich insbesondere auch dem Stifterpaar Dres. Jutta und Georg Bruns sowie dem Stiftungsrat-Vorsitzenden Prof. emer. Dr. Dr. h. c. mult. Hartwig Bostedt für die motivierenden Worte und das Interesse. Weiter möchte ich mich beim Bundesministerium für Bildung und Forschung (BMBF) bedanken, welches das Projekt im Rahmen des #1HealthPREVENT-Forschungsverbunds finanziert hat (Förderkennzeichen: 01KI1727A). Hier gilt mein besonderer Dank den Mitgliedern des #1HealthPREVENT-Forschungsverbunds, insbesondere PD Dr. med. Robin Köck für den Austausch, die fachlichen Anregungen, die wertschätzende Atmosphäre und gegenseitige Unterstützung.

Ich möchte mich bei allen Beteiligten der Dahlem Research School, vor allem Angela Daberkow und Christine Gaede, sehr herzlich für die umfangreichen Angebote und den reibungslosen Ablauf bedanken.

Für die Beratung zu Fragen der statistischen Auswertung und Beurteilung stand mir Alexander Bartel unermüdlich mit seinem Fachwissen bei, weshalb ihm mein aufrichtiger Dank gebührt.

Meinen Co-Autorinnen und Co-Autoren danke ich sehr für die stets motivierende und angenehme Zusammenarbeit. Insbesondere gilt hierbei mein Dank Marita Meurer, PhD.

Das Projekt wurde durch die stete Bereitstellung flüssiger und fester Medien in bedeutendem Maße von Monika Feldhan unterstützt. Ihr gilt hierfür meine besondere Wertschätzung und mein herzlicher Dank. Ebenso herzlich danke ich Julian Brombach für die technischen und methodischen Einweisungen.

Besonders hervorheben möchte ich Anissa Scholtzek, PhD, Ihr gilt von Herzen mein außerordentlicher Dank für die hervorragende Zusammenarbeit sowie für die menschliche und fachliche Unterstützung.

Für den wissenschaftlichen Austausch und Diskurs, die ebenso wissenschaftliche, technische, methodische und persönliche Förderung, die das Projekt vorangetrieben und verbessert haben, bin ich folgenden Personen von Herzen sehr verbunden: Katrin Bote, PhD, Judith Pöppe, Beneditta Suwono, Britta Magsik und Dr. med. vet. Lisa Niemann sowie dem gesamten Team des Instituts für Mikrobiologie und Tierseuchen.

Außerdem haben folgende Personen zum Gelingen meines Projekts beigetragen, weshalb ich Ihnen ebenso sehr danke: meine Familie, insbesondere meine Schwester Maike Elisa Schug, Thomas Alan Kern, Dr. phil. Maria Theobald, Dr. med. vet. Isabella Lorenzini und Dr. med. vet. Inga Wolff.

Finanzierungsquellen

Die Arbeiten wurden finanziell unterstützt durch die Dres. Jutta & Georg Bruns Stiftung für innovative Veterinärmedizin und das Bundesministerium für Bildung und Forschung (BMBF) im Rahmen des #1HealthPREVENT-Forschungsverbunds (Förderkennzeichen: 01KI1727A).

Interessenskonflikte

Im Rahmen dieser Arbeit bestehen keine Interessenskonflikte durch Zuwendungen Dritter.

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Ort, Datum, Unterschrift

Berlin, 15.09.2021 Angela Regina Schug