Biocide tolerance and antibiotic cross-resistance in human pathogenic bacteria

DISSERTATION

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“Nur unter Druck entsteht aus Kohle Diamant“

- Danke Kerstin und Olli, ihr habt mir die Grundsteine gelegt -

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Abbreviations

AmpC	AmpC β-lactamase
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli*
ARG	Antibiotic resistance gene
BAC	Benzalkonium chloride
BMRG	Biocide/metal resistance gene
BPR	EU Biocidal Products Regulation
CC	Clonal Complex
C&D	Cleaning and disinfection
CEN/TC	Comité Européen de Normalisation/Technical Committee
CFU	Colony forming units
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
CTP	Cetylpyridinium chloride
DDAC	Didecyldimethylammonium chloride
DLG	Deutsche Landwirtschafts-Gesellschaft/German Agriculture Society
DNA	Desoxyribonucleic acid
DVG	Deutsche Veterinärmedizinische Gesellschaft/German Veterinary Association
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
ExPEC	Extraintestinal pathogenic *E. coli*
EA	Essential agreement
EC	European Commission
ECOFF	Epidemiological cut-off
EN	European standard
ESBL	Extended-spectrum β-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
h	Hour
HACCP	Hazard Analysis and Critical Control Points
I	Susceptible, increased exposure
ICU	Intensive care unit
IfSG	Infektionsschutzgesetz/ German Infection Protection Act
IHO	Industrieverband Hygiene & Oberflächenschutz/Industrial Hygiene and Surface Protection Association
ISO	International Organization for Standardization
KRINKO	Kommission für Krankenhaushygiene und Infektionsprävention/Commission for Hospital Hygiene and Infection Prevention
L. monocytogenes	*Listeria monocytogenes*
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration

MBIC	Minimum biofilm inhibitory concentration
MDR	Multidrug-resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
mL	Milliliter
MPG	Medizinproduktegesetz/German Act on Medical Devices
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAD	Nicotinamide adenine dinucleotide
OCT	Octenidine dihydrochloride
P. aeruginosa	*Pseudomonas aeruginosa*
PAA	Peroxyacetic acid
PCMC	P-chloro-m-cresol
PHMB	Polyhexamethylene biguanide
PT	Product type
QAC	Quaternary ammonium compound
R	Resistant
RKI	Robert Koch-Institute
ROS	Reactive oxygen species
S	Susceptible, standard dosing regimen
S. enteria	*Salmonella enterica*
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
spp.	*species pluralis*, all species of a genus
ST	Sequence Type
VAH	Verbund für angewandte Hygiene/German Association for Applied Hygiene
VRE	Vancomycin-resistant enterococci
VSE	Vancomycin-susceptible enterococci
WGS	Whole genome sequencing
µL	Microliter

Summary

Biocides are applied to control harmful organisms. Especially the use of microbicides as disinfectants is a vital tool to prevent the spread of multidrug-resistant human pathogenic bacteria. Due to the increased use of disinfectants in recent years, concerns have been raised about biocide tolerance and the development of antibiotic cross-resistance. *In vitro* studies indicate biocide use as a risk factor for the emergence of antibiotic resistance. Still, a causal link between biocide usage and antibiotic resistance development in environmental settings needs to be verified.

Thus, this thesis pursued three main objectives (I-III). The controversial current knowledge on the potential of bacteria to develop biocide tolerance and/or antibiotic resistance in response to biocide exposure was analyzed (objective I). Potential associations between biocide tolerance and antibiotic resistance, and underlying genetic determinants in field isolates were examined (objective II). The final aim of this thesis was to develop a ready-to-use test system enabling fast and accurate biocide susceptibility testing of bacteria (objective III).

For the first aim, 78 *in vitro* biocide adaptation studies were evaluated. While literature demonstrated the general linkage between biocide exposure and antimicrobial cross-resistance, the potential of biocide–induced resistance development varied largely between biocides, bacterial target organisms, and experimental settings. Stable adaptation to biocides as well as altered antibiotic susceptibility profiles was frequently reported for biguanides, phenols, and quaternary ammonium compounds (QACs).

For the second aim, comprehensive analyses were conducted of isolates from food (*Listeria monocytogenes*, n=93), livestock (*Escherichia coli*, n=93), and clinical environments (*Enterococcus faecium*, n=90) in Germany. Biocide and antibiotic susceptibility were determined for all isolates by broth microdilution methods according to ISO 20776-1. To differentiate susceptible isolates from those with reduced susceptibility to biocides, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) representing

95% of the tested population (MIC_{95}, MBC_{95} values) served as tentative epidemiological cut-offs. Underlying genetic determinants were detected by whole genome sequencing for *L. monocytogenes* and *E. coli*. The overall proportion of isolates with reduced biocide susceptibility was relatively low, and statistically significant phenotypic associations to antibiotic resistance were not found. However, genotypic associations were observed in *E. coli*. Biocide tolerance conferring genes co-located close to antibiotic resistance genes on plasmids were identified, highlighting the potential for co-selection. Biocide susceptibility of all tested isolates was well below in-use concentrations. That is why they are defined as non-resistant. Identified tolerances were associated with genetic determinants in several cases. Reduced susceptibility to QACs in *L. monocytogenes* could be mainly traced back to genes coding for small multidrug-resistance family efflux pumps. *E. coli* isolates with reduced formaldehyde susceptibility carried the gene coding for the degradative enzyme glutathione- and NAD-dependent formaldehyde dehydrogenase.

In contrast to antibiotic susceptibility testing, no generally accepted, standardized biocide susceptibility test methods are available. Thus, the third aim was to establish a reliable, high-throughput screening system using vacuum dried biocide microtiter plates to identify bacteria resistant to cationic biocides frequently used in hospital settings. The developed test system was validated against the broth microdilution method using freshly prepared stocks of biocides. Biocide plates were evaluated for gram-positive *Enterococcus* spp. as well as gram-negative *E. coli*. This test system has been shown to provide highly reliable results. Subsequently, the test method served for the third susceptibility study of *E. faecium* from the clinical environment. The test system proved to be a fast and easy-to-handle surveillance tool to identify biocide tolerant isolates. Monitoring and early identification of clinical isolates tolerant to disinfectants applied in hospitals could help to adapt hygiene measures and control nosocomial infections while simultaneously reducing the frequency of antibiotic treatment.

Zusammenfassung

Biozide werden zur Bekämpfung von Schadorganismen eingesetzt. Insbesondere der Einsatz von Mikrobiziden als Desinfektionsmittel ist eine wichtige Maßnahme, um die Ausbreitung multiresistenter, humanpathogener Bakterien zu verhindern. Aufgrund des verstärkten Einsatzes von Desinfektionsmitteln in den letzten Jahren sind jedoch Bedenken hinsichtlich Biozidtoleranzen und der Entwicklung von Antibiotikakreuzresistenzen aufgekommen. *In vitro* Studien deuten darauf hin, dass Biozide Antibiotikaresistenzen fördern. Ein kausaler Zusammenhang zwischen der Verwendung von Bioziden und der Entwicklung von Antibiotikaresistenzen in der Umwelt muss jedoch noch verifiziert werden.

Daher verfolgte die These drei Hauptziele (I-III). Es wurde das aktuelle, kontroverse Wissen über das Potenzial von Bakterien Biozidtoleranzen und/oder Antibiotikaresistenz unter Biozidexpositionen auszubilden analysiert (Ziel I). Des Weiteren wurden mögliche Zusammenhänge zwischen Biozidtoleranz und Antibiotikaresistenz sowie die zugrunde liegenden genetischen Determinanten in Feldisolaten untersucht (Ziel II). Zudem sollte in dieser Arbeit ein gebrauchsfertiges Testsystem entwickelt werden, welches eine schnelle und genaue Biozidempfindlichkeitsprüfung von Bakterien ermöglicht (Ziel III).

Zur Erfüllung der ersten Zielstellung wurden 78 *in vitro* Biozidadaptionsstudien ausgewertet. Während die Literatur den allgemeinen Zusammenhang zwischen Biozidexposition und antimikrobieller Kreuzresistenz belegt, variierte das Potenzial der biozidinduzierten Resistenzentwicklung stark zwischen Bioziden, bakteriellen Zielorganismen und experimentellen Bedingungen. Stabile Anpassung an Biozide sowie veränderte Antibiotikaempfindlichkeitsprofile wurden häufig für Biguanide, Phenole und quaternäre Ammoniumverbindungen (QACs) berichtet.

Für das zweite Ziel wurden umfassende Analysen durchgeführt, die Isolate aus Lebensmittel-(*Listeria monocytogenes*, n=93), Nutztier-(*Escherichia coli*, n=93) und klinischer Umgebung (*Enterococcus faecium*, n=90) in Deutschland einschlossen. Die Biozid- und

Antibiotikaempfindlichkeit wurde für alle Isolate mittels Mikrodilutionsverfahren gemäß ISO 20776-1 bestimmt. Um empfindliche Isolate von solchen mit verminderter Biozidempfindlichkeit zu unterscheiden, dienten minimale Hemmkonzentrationen (MIC) und minimale bakterizide Konzentrationen (MBC), die 95% der getesteten Population repräsentieren (MIC95-, MBC95-Werte), als vorläufig abgeleitete epidemiologische Cut-Off-Werte. Zugrundeliegende genetische Determinanten wurden mittels Ganzgenomsequenzierung für *L. monocytogenes* und *E. coli* nachgewiesen. Der Gesamtanteil der Isolate mit reduzierter Biozidempfindlichkeit war relativ gering und es wurden keine statistisch signifikanten phänotypischen Assoziationen zur Antibiotikaresistenz gefunden. Allerdings wurden bei *E. coli* genotypische Assoziationen beobachtet. Biozidtoleranz verleihende Gene wurden in der Nähe von Antibiotikaresistenzgenen identifiziert, die auf Plasmiden kolokalisiert sind, was das Potenzial für eine Ko-Selektion hervorhebt. Die Biozidempfindlichkeit aller getesteten Isolate befand sich deutlich unter den in der Praxis verwendeten Konzentrationen. Daher wurden diese Isolate als nicht resistent definiert. Identifizierte Toleranzen wurden in mehreren Fällen mit genetischen Determinanten in Verbindung gebracht. Eine reduzierte Empfindlichkeit gegenüber QACs in *L. monocytogenes* konnte hauptsächlich auf Gene zurückgeführt werden, die für die Small Multidrug Resistance – Familie kodieren. *E. coli*-Isolate mit reduzierter Formaldehydempfindlichkeit trugen ein Gen, welches für das degradierende Enzym der Glutathion- und NAD-abhängige Formaldehyd-Dehydrogenase kodiert.

Im Gegensatz zur Antibiotika-Empfindlichkeitsprüfung gibt es für Biozidempfindlichkeitstests keine standardisierten Methoden. Daher wurde als drittes Ziel ein zuverlässiges Screening-System etabliert, das auf vakuumgetrockneten Biozidmikrotiterplatten basiert und Bakterien identifiziert, die reduzierte Empfindlichkeiten gegenüber kationischen Bioziden aufweisen. Diese vakuumgetrockneten Biozidplatten wurden mit Hilfe von etablierten Mikrodilutionsmethoden unter Verwendung frisch hergestellter Biozidlösungen validiert. Die Biozidplatten wurden sowohl für gram-positive *Enterococcus* spp. als auch für gram-negative

E. coli ausgewertet. Insgesamt konnte nachgewiesen werden, dass dieses Testsystem sehr zuverlässige Ergebnisse lieferte. Anschließend diente die Testmethode für die dritte Empfindlichkeitsstudie von *E. faecium* aus dem klinischen Umfeld. Das Testsystem erwies sich als ein schnelles und einfach zu handhabendes Überwachungsinstrument zur Identifizierung biozidtoleranter Isolate. Die Überwachung und frühzeitige Identifizierung von klinischen Isolaten, die gegenüber den in Krankenhäusern eingesetzten Desinfektionsmitteln tolerant sind, könnte dazu beitragen, Hygienemaßnahmen anzupassen und nosokomiale Infektionen zu kontrollieren sowie gleichzeitig die Häufigkeit von Antibiotikabehandlungen zu reduzieren.

1 Introduction – Background

1.1 Importance of disinfection in the era of multidrug-resistance

Antimicrobial resistance (AMR) has become a 21st-century global health threat. Limiting its emergence and further spread is one public health priority worldwide. The global action plan on AMR by the World Health Organization in 2015 recommend the prudent use of antimicrobial compounds, preventing their unnecessary use, and reducing the spread of infections through effective prevention, sanitation, and hygiene measures (1). Effective disinfection is considered as one of the key pillars in the multi-barrier approach preventing the dissemination of multi-drug-resistant pathogens (2, 3).

1.2 Biocides as part of disinfectants

Cleaning and disinfection (C&D) are principally considered as combination methods to maintain the hygienic status. In general, cleaning is defined as the removal of soiling. In contrast, disinfection describes chemical, thermal or physical processes aiming to reduce microorganisms to a level (approx. reduction of the microbiological load of 2-4 $\log_{10}$) that the risk of infection or transmission is minimized (4, 5). Adequate cleaning is a crucial prerequisite for disinfection since disinfection without prior cleaning is almost ineffective. Dried films of organic matter (e.g., blood, excreta) may prevent the penetration of a disinfectant and are considered one of the most important environmental factors influencing disinfectant activity (6). Biocides form the basis for chemical disinfectants to ensure the hygiene status of different environments such as food processing, animal husbandry, and clinical settings (7). The authorization of biocides used in different applications is regulated in the EU Biocidal Products Regulation (BPR Regulation (EU) No 528/2012). Biocides used as disinfectants belong to main group 1 and are divided into five product types (PT) such as for human hygiene (PT 1), disinfectants and algaecides not intended for direct application to humans or animals (PT 2),

veterinary hygiene (PT 3), food and feed area (PT 4), and drinking water (PT 5) (ANNEX V, BPR Regulation No 528/2012 (8)).

To implement hygiene measures, different legal frameworks and concepts must be considered for food production facilities, livestock industry, and clinical environments.

1.2.1 Regulations in food production environments

As one impressive example for food processing environments, the U.S. Interagency Retail Lm Risk Assessment estimated that the predicted risk for infection with *Listeria monocytogenes* from the consumption of ready-to-eat products sliced or prepared in retail deli departments would increase by approximately 41% if wiping, washing, and sanitizing activities were not performed (9).

In Germany, three EU Regulations provide the legal framework to regulate important aspects of food hygiene. General hygiene requirements for all food business operators are described in Regulation (EC) No 852/2004 on the hygiene of foodstuffs (10). Regulation (EC) No 853/2004 lays down specific hygiene rules for food of animal origin (11), while (EC) No 854/2004 provides specific rules for the organization of official controls on products of animal origin intended for human consumption (12). Additionally, Regulation (EC) No 2073/2005 provides the basis for microbiological criteria for foodstuffs (13). Regulation (EC) No 852/2004 states, that all businesses in the food industry are obligated to implement a Hazard Analysis and Critical Control Points (HACCP) system. The HACCP system is a quality control and assurance system in all production processes in the food industry and among others, it regulates the use of food industry disinfectants. In Germany, the DIN 10516 - Food hygiene – Cleaning and disinfection (4) is related to Regulation (EC) No 852/2004 and serves as a guide to ensure hygienically safe conditions in food facilities. In general, the workflow follows cleaning, rinsing, disinfection, rinsing, and drying (4). Chemical disinfectants for food and feed areas belong to PT 4 of the EU BPR. The most comprehensive overview on effective chemical

disinfectants for the food sector has been published by the Industrial Hygiene and Surface Protection Association (Industrieverband Hygiene & Oberflächenschutz [IHO]) (14), and the German Association of Veterinary Medicine [DVG] (15). Currently, there are no absolute numbers on the use of various biocides. Frequently, listed disinfectants contain alcohols, aldehydes, chlorine-releasing compounds, quaternary ammonium compounds (QACs), or peracids (14).

1.2.2 Regulations in livestock industry

In modern animal husbandry, hygiene is an indispensable component of operational management. Even though, a total number of infection reduction rates is not published for farm animals it serves primarily to prevent the entry of diseases, to reduce the spread of multi-resistant bacteria, and to ensure optimal performance conditions at the beginning of the food chain from farm to fork (5, 16). In 1995, Fotheringham reviewed that cleaning alone removes 99% of bacteria under experimental conditions, whereas in farm environments, this figure is likely to be approximately 90%. Disinfection removes further 6-7% of bacteria in practice, and yet another 1-2% reduction can be obtained by fumigation (17). Various cleaning and disinfection (C&D) measures are available to implement favorable hygiene on the farm. As the contribution of animals as a reservoir of multi-resistant human pathogens has gained special attention, the application of HACCP principles, initially developed for the food processing plants, is also recommended to ensure safety along the entire food chain (18). The type of measures depends on the company-specific circumstances and the respective area of application. As mentioned before, criteria are laid down in Regulations (EC) 853/2004 and (EC) 854/2004. The German Agricultural Society leaflet 364 (DLG, Deutsche Landwirtschafts-Gesellschaft) (5) provides general instructions on hygiene technology and management for C&D of stable systems. Complete elimination of all germs, so-called sterilization, is neither possible nor necessary in agricultural practice. Almost all routine procedure protocols in animal

husbandry include chemical disinfection (5). Corresponding disinfectants belong to PT 3 of EU BPR. In Germany, approved disinfectants are listed by the DVG (15), the German Agricultural Society (Deutsche Landwirtschafts-Gesellschaft [DLG]) (19), or IHO (14), which are often used as guides in animal husbandry. The most listed active ingredients of these disinfectants include aldehydes, cresols, organic acids, oxidizing agents, and QACs (15).

1.2.3 Regulations in clinical environments

In addition to hand hygiene and proper reprocessing of medical devices, C&D of surfaces is of increasing importance in hospitals as part of a multi-barrier approach for preventing infection (20). Grabsch and colleagues, for example, showed that by implementing a hospital-wide improvement program, including bleach-based C&D measures, significant reduction of newly identified vancomycin-resistant enterococcus (VRE) colonization (25%) and reduction of environmental contamination (66%) could be observed. Furthermore, newly diagnosed VRE bacteremia in patients during hospitalization decreased by 83% (21).

Since 2001, the German Hospital Hygiene and Infection Prevention Commission (KRINKO) is legally anchored in §23 of the German Protection Infection Act (Infektionsschutzgesetz [IfSG]). The KRINKO addresses general requirements for C&D of surfaces in the healthcare sector. Surface C&D measures need to be established individually depending on a) the probability of direct contact, b) the possible contamination with pathogens, and c) the degree of clinically relevant immunosuppression of patients. Workplace-related C&D measures are generally defined in standard operating procedures of hygiene plans providing detailed information on procedures and responsibilities of the staff (22, 23). The procedures of the C&D plan need to be established based on specifications of the IfSG (24) and the German Act on Medical Devices (MPG) (25). Occupational Health and Safety Regulations and versatile regulations complement certain specifications (8, 26-31). Suitable disinfectants recommended for use in health care sectors in Germany are listed by the Association for Applied Hygiene (VAH) (32) and the

Robert Koch-Institute (RKI) (33). These chemical disinfectants belong to PT 2 of the EU BPR. Most often listed surface disinfectants contain alcohols, aldehydes, biguanides, chlorine-releasing compounds, bleach, or oxygen-releasing compounds (32, 33). Furthermore, biocidal agents like QACs, biguanides, phenolic compounds, and bipyridines are common ingredients reported to be used as disinfectants, or antiseptics (34). In contrast to disinfectants, antiseptics are applied to body surfaces to eradicate colonization with pathogens (35).

1.2.4 Modes of action of biocides used in food, livestock, and healthcare industries

General considerations on the efficacy of surface disinfectants include the modes of action of the active substance and its interaction with the organisms. An overview of the modes of action to bacteria is given in Figure 1 (adapted from Merchel Piovesan Pereira et al. (36)) and Table 1 for relevant classes of biocides. So far, biocidal mechanisms of action are not fully understood and appear to be diverse. Most biocides affect multiple targets, whereby effects on the bacterial membrane are most frequently described. Any alteration in the outer membrane of gram-negative bacteria e.g., changing the hydrophobic properties, or porin-related mutations (leading to loss or structural changes) can contribute to resistance. This critical layer is lacking in gram-positive bacteria, which makes gram-negative bacteria less susceptible to antibiotics and biocides (37-39). Some biocides such as biguanides or QACs have lipophilic domains in their molecules, allowing close interactions with cell membrane phospholipids (40). Depending on the concentration, biocides may have bacteriostatic or bactericidal effects. Concentration-dependent effects are marked in Table 1.

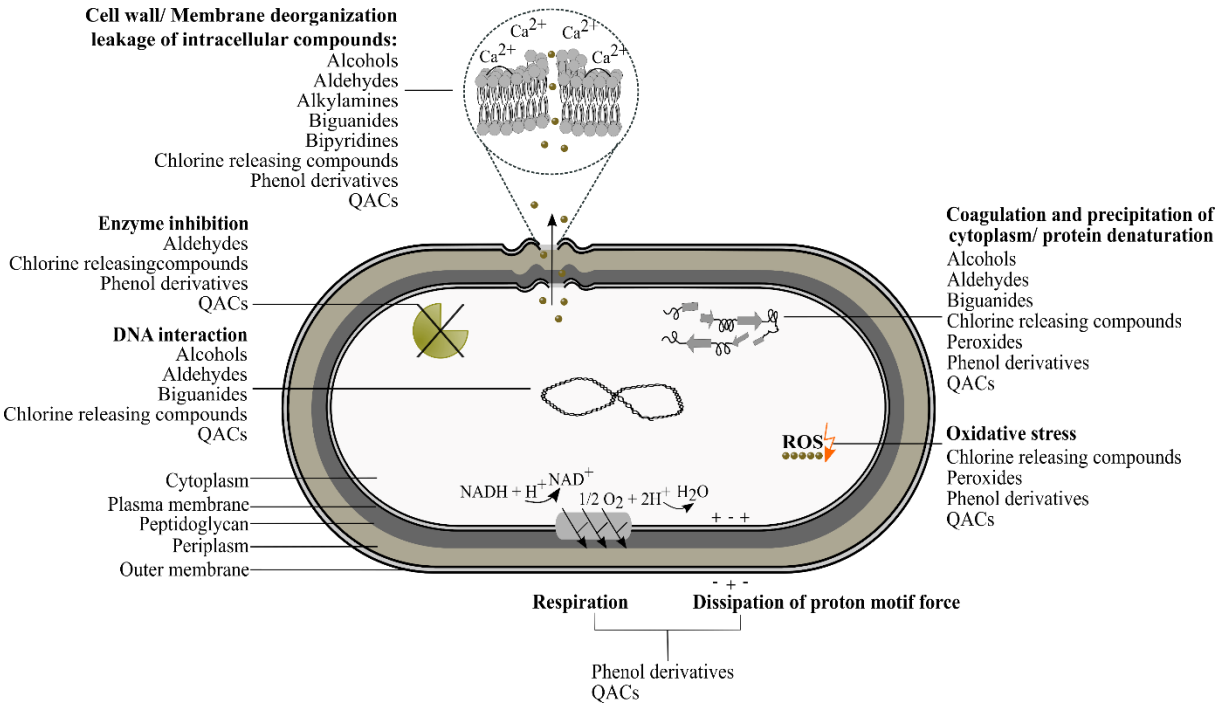

Figure 1: Modes of action for relevant classes of biocides.
The figure was adapted from Merchel Piovesan Pereira et al. (36). Mechanisms are illustrated for gram-negative bacteria. They are also applicable to gram-positive species. QACs = quaternary ammonium compounds, ROS = reactive oxygen species

Table 1: Mode of action reported for relevant biocide classes

Compounds	Mode of action	Cellular response	Reference
Alcohols			
Ethanol Isopropanol Propan-1-ol	Protein denaturation by disruption of hydrogen bonds[2] DNA precipitation by removing hydration shell (hydrogen bonding)[2]	Disruption of cytoplasmic membrane[2] Leakage of intracellular components[2]	(41-43)
Aldehydes			
Formaldehyde Glutaraldehyde	Alkylation and cross-linking of amino, sulphydryl, and hydroxyl groups of proteins and nucleic acids[2]	Loss of essential cell function[2]	(44, 45)
Alkylamines			
N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine	Membrane destabilization by surfactant properties	Increased membrane permeability	(46)
Biguanides			
Chlorhexidine Polyhexamethylene biguanide	Bridge formation between phospholipids Displacement of divalent cations Coagulation and precipitation of the cytoplasm[2] DNA interaction/chromosome condensation	Reduction of membrane fluidity Destruction of membranes Leakage of intracellular components[2]	(47-49)

Compounds	Mode of action	Cellular response	Reference
Bipyridines			
Octenidine dihydrochloride	Binding to lipid cell membrane components, like salts of fatty acid glycerol phosphates Interaction with enzymatic systems and polysaccharides in cell walls	Loss of membrane functionality Leakage of intracellular compounds	(50)
Chlorine-releasing compounds			
Chlorine dioxide Sodium hypochlorite	Potent oxidizers of organic material DNA interaction	Destruction of cell protein activity Disruption of phosphorylation and membrane-associated activities	(35, 51)
Peroxides			
Hydrogen peroxide Peracetic acid	Strong oxidizing effects Production of free hydroxyl radicals Destruction of DNA, proteins, and lipids	Loss of essential cell function	(35)
Phenol derivatives			
Hexachlorophene	Inhibition of dehydrogenase enzymes[1] Protein denaturation[2] Damage or disruption of the membrane[2] Coagulation of cytoplasm[2]	Inhibition of electron transport chain and respiration[1] and cellular metabolism[1] Leakage of intracellular compounds[2] Inhibition of release of intracellular material[2]	(52-54)
P-chloro-m-cresol	Disruption of membrane potential and the membrane permeability of cytoplasmic membrane Cytoplasm coagulation[2]	Loss of membrane functionality Loss of vital cell functions[2]	(55)
Triclosan	Imitation of the natural substrate of type II fatty acid synthase enoyl-reductase[1] Intercalation into the cell membrane[2] Oxidizing effects	Inhibition of bacterial lipid biosynthesis[1] Cell death[2]	(56-59)
Quaternary ammonium compound			
Benzyl group containing compound Benzalkonium chloride Benzethonium chloride *Other QACs* Didecyldimethyl ammonium chloride and bromide Cetrimide Cetylpyridinium chloride Cetyltrimethylammonium bromide	Degradation of proteins and nucleic acids Disorganization of the membrane due to strong positive charge and hydrophobic regions[1] Inhibition of respiratory enzymes[1] Dissipation of proton motive force and oxidative stress[1]	Destruction of membrane osmoregulation[1] Leakage of intracellular compounds[2] Lysis of the cell wall[2]	(35, 49, 60)

1 = low concentration (bacteriostatic activity), 2 = higher concentration (bactericidal activity)

1.3 Bacterial tolerance to biocides

1.3.1 Definitions associated with biocide tolerance

Due to the indiscriminate use of biocides in different settings, the awareness of various hazards such as bacterial adaptation, as well as cross- and co-resistance development, increased (7, 41, 61). This includes reduced susceptibility to used biocidal substances themselves, other biocides, or antibiotics. Furthermore, it is assumed that subinhibitory concentrations of biocides may lead to mutation propagation, horizontal gene transfer, or recombination events within bacterial populations (57, 62, 63).

The term “tolerance” to biocides is associated with phenotypes able to survive biocidal stress. According to the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), tolerance is defined as “reduced susceptibility to an antimicrobial molecule characterized by a raised minimal inhibitory concentration (MIC), or a situation in which a preservative system no longer prevents microbial growth” (7). Such situations are often linked to reversible physiological alterations, including biofilm formation, expression of small colony variants, or slow growth.

In contrast to tolerance, resistance describes a situation where a strain is not killed or inhibited by in-use concentrations (7). The ability to resist such in-use concentration is often linked to genetic mutations. Co-selection describes the selection pressure exerted by one antimicrobial agent on a single organism to different antimicrobial compounds. To survive, the co-selected organism uses strategies of cross- or co-resistance (64). In cross-resistant organisms, resistance to the biocide itself is accompanied by resistance to other biocides or antibiotics due to the same resistance mechanism (40). Co-resistant bacteria display resistance to the biocide itself and unrelated antimicrobial substances due to physical linkage of the genetic resistance determinants or their coordinated expression (65).

1.3.2 Mechanisms leading to biocide tolerance

So far, mechanisms contributing to biocide tolerance are not fully understood. An overview of already described tolerance mechanisms to different biocide classes used in the described settings is given in Figure 2 (adapted from Merchel Piovesan Pereira et al. (36)) and Table 2.

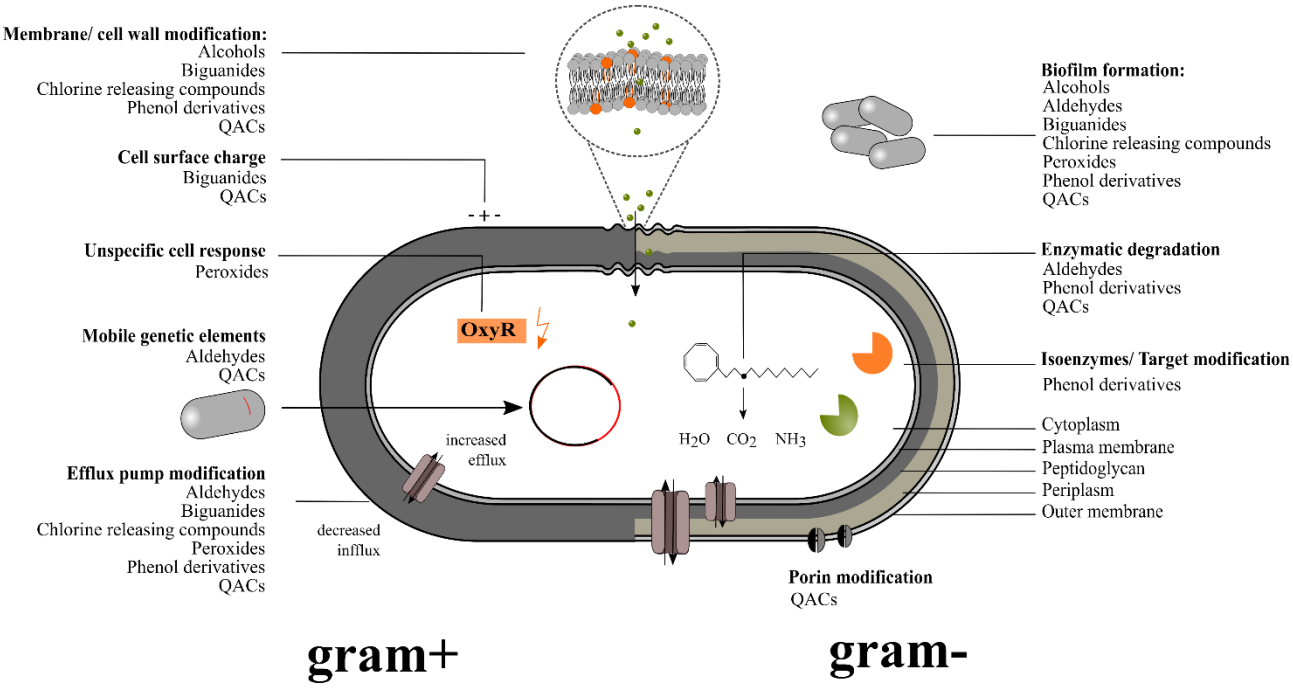

Figure 2: Tolerance mechanisms for relevant classes of biocides in gram-positive and gram-negative bacteria. The figure was adapted from Merchel Piovesan Pereira et al. (36). QACs = quaternary ammonium compounds, OxyR = bacterial peroxide sensor

Table 2: Tolerance mechanisms reported for relevant biocide classes

Compounds	Tolerance mechanism	Cellular response	Reference
Alcohols			
Ethanol	Alteration of the fatty acid composition Enhanced biofilm formation capacity Increased growth rates accompanied by diverse genotypic changes	Decreased uptake	(42, 66-71)
Isopropanol			
Propan-1-ol			
Aldehydes			
Formaldehyde	Biofilm formation Enzymatic degradation Increased expression of multidrug-resistance efflux pumps	Decreased uptake Destruction of biocidal compounds Increased efflux	(72-74)
Glutaraldehyde			

Compounds	Tolerance mechanism	Cellular response	Reference
Alkylamines			
N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine	No information available	No information available	
Biguanides			
Chlorhexidine Polyhexamethylene biguanide	Alteration of the cell envelope Biofilm formation Changes of Cell surface hydrophobicity Membrane rigidity Lipopolysaccharide expression Outer membrane profile including net negative charge Upregulation of efflux pump activity	Reduced uptake Increased efflux	(74-79)
Bipyridines			
Octenidine dihydrochloride	No information available	No information available	
Chlorine-releasing compounds			
Chlorine dioxide Sodium hypochlorite	Biofilm formation Changed cell morphology and ultrastructure Increased cell surface hydrophobicity Modification of efflux pumps	Reduced uptake Increased efflux	(74, 80-82)
Peroxides			
Hydrogen peroxide Peracetic acid	Biofilm formation Unspecific cell responses (e.g., induction of SoxRS system, OxyR-regulon)	Increased efflux Prevention of the formation of radicals Deactivation of free radicals before damage of biological molecules Repairing of biomolecules after damage Reduced uptake	(74, 83, 84)
Phenol derivatives			
Hexachlorophene	Activation of efflux pumps Alteration of the bacterial cell wall and outer membrane composition	Increased efflux Reduced uptake	(85)
P-chloro-m-cresol	No information available	No information available	

Compounds	Tolerance mechanism	Cellular response	Reference
Triclosan	Biofilm formation Enzymatic degradation Increased *fabI* expression due to mutation in the promoter region Less affected isoenzymes like FabK or FabV Overexpression of efflux pumps (sigma factor mutations or mutations in transcriptional regulators such as *ramA*, *marA*) Point mutation in the triclosan specific target encoding gene *fabI*(enoyl-reductase)	Destruction of the biocidal compound Further production of enoyl reductase for fatty acid biosynthesis Increased efflux Reduced uptake	(74, 85-94)
Quaternary ammonium compound			
Benzyl group containing compound Benzalkonium chloride Benzethonium chloride *Other QACs* Didecyldimethyl ammonium chloride and bromide Cetrimide Cetylpyridinium chloride Cetyltrimethylammonium bromide	Acquisition of QAC specific efflux pumps Biofilm formation Change of cell surface charge hydrophobicity structure and density of porins membrane compositions Enzymatic degradation Overexpression of innate efflux pumps	Increased efflux Reduced uptake	(36, 60, 90, 95-98)

1.3.3 Study types assessing biocide tolerance development

Different *in vitro* study types are used to investigate biocide tolerance development. The most common experimental setup is based on multiple exposures of bacterial isolates to low or moderately increasing concentrations of pure biocide substances until a significant increase of the MIC of the tested substance is observed or for a predefined number of passages (85, 99, 100) . Based on *in vitro* studies, it is possible to assess the general bacterial ability to adapt to a biocide of interest. These results need to be verified in field studies since *in vitro* experiments do not cover the complex interplay between biocides and microbes in environmental settings. Latest field studies investigated putative associations between biocide tolerance and antibiotic cross-resistance by comparing susceptibility of bacterial isolates from different environments

to certain antimicrobials. Especially statistical evaluation of biocide susceptibility and antibiotic resistance data was used to identify associations between biocide tolerance and antibiotic resistance (101-105). In contrast to antibiotic resistance, there are fundamental issues assessing biocide tolerance because of the lack of epidemiological data that hampers the establishment of appropriate breakpoints. Only a few studies defined and used epidemiological cut-offs (ECOFFs) similar to antibiotic susceptibility evaluation for specific bacterial species and selected biocides to differentiate between susceptible and tolerant isolates, enabling an evaluation of biocide tolerance development (102, 104-107). In some studies, additional molecular investigations were carried out to identify underlying mechanisms (104, 105, 108, 109).

1.4 Methods used for biocide susceptibility testing

Parameters such as the MIC and the minimum bactericidal concentration (MBC) are used to assess biocide susceptibility. For antibiotic susceptibility testing, the MIC is used together with pharmacokinetic and pharmacodynamic models to determine clinical breakpoints (S - Susceptible, standard dosing regimen, I – Susceptible, increased exposure, R – Resistant) to guide therapy (110). The MIC is defined as the lowest concentration (in mg/L) of an antimicrobial compound that prevents visible growth of a microorganism under defined conditions (111). Regarding biocide susceptibility, the MIC can be used as an indicator of tolerance development as elevated MICs reflect concentrations that would otherwise lead to inhibition of cell growth (7). However, it may provide limited information on the survival of single bacterial cells towards in-use biocide concentrations. It may be helpful in combination with other techniques such as the MBC used to measure biocide resistance (112). The MBC is defined as the lowest concentration of an antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 h under a standardized set of conditions described in CLSI document M26-A (113). The determination of the MBC is considered a convenient method that

allows the comparison of lethality between wild-type (normal susceptibility) strain and potentially resistant strains (112). Thus, MBC is deemed to be an indicator of biocide resistance if compared to in-use concentration (7). While MIC and MBC testing of pure substances provide information on biocide susceptibility in bacterial populations, efficacy tests of disinfectants are carried out to prove the activity of disinfectants for specific conditions such as concentration and contact time. Efficiency tests are mandatory for disinfectants and prerequisites to register the product in the disinfectant lists of VAH, RKI, and DVG.

1.4.1 MIC determination

Techniques used to determine the MIC of biocides are based on antibiotic susceptibility test procedures described in standard protocols such as ISO 20776-1 or guidelines of the Clinical and Laboratory Standards Institute M07 (111, 114). Most frequently, the broth dilution test system is used. Broth dilution uses liquid growth medium containing geometrically increasing concentrations (typically two-fold dilution series) of the antimicrobial agent, which is inoculated with a defined number of bacterial cells (approx. 5×10^5 CFU/mL) (114). The final volume of the test defines whether the method is termed macrodilution (when a final volume of ≥ 2 mL is used) or microdilution (using microtiter plates with a final volume per well of ≤ 500 µL). After incubation, increased turbidity or sediment indicates the growth of the organisms. Dilution methods are contemplated as reference methods for *in vitro* susceptibility testing and are also used to verify the performance of other susceptibility testing methods, such as the agar diffusion method (115). The methods of antibiotic susceptibility testing have shown that the main disadvantages of macrodilution are the effortful preparation of antimicrobial solutions, which is error-prone due to manual agent solution preparation. Furthermore, it requires a comparatively large amount of reagents and space (116). The advantage of the microdilution method is thus the reproducibility due to standardized preparation (mechanization) and the savings in reagents and space that result from the miniaturization of

the test system (116). Nevertheless, in any case, the final result is significantly influenced by the approach, which must be carefully controlled if reproducible results shall be attained (114).

1.4.2 MBC determination

The MBC is identified after broth dilution (macro- or microdilution) by neutralizing and sub-culturing of a sample from wells or tubes without visible microbial growth on non-selective agar plates. When determining the MBC, the use of a neutralizing agent is vital to avoid an overestimation of the lethal concentration (7). The number of surviving cells (CFU/mL) is determined for each sample after 24h of incubation. MBC measurements by microdilution are characterized as simple and straightforward. They permit many of strains/biocidal compounds to be tested at the same time within 24 h, resulting in rapid data collection (112).

1.4.3 Test limitations

Besides a few attempts (117, 118), there are no standardized protocols and methods available to evaluate bacterial susceptibility to biocides. Often the state of knowledge on the methodological approach from antibiotic research is transferred to biocide susceptibility testing. Numerous critical parameters that influence the results of MIC testing for biocides have been reported, including the type of growth medium, plate material, and the inoculum preparation method (112, 119). The lack of standardization impedes comparison of different biocide susceptibility study results of field isolates and points towards the need for standard procedures in the future that allow intra- and inter-laboratory comparability, and reproducibility (7, 120). MBC suspension tests comprising neutralization and enumeration steps finally resulting in a diluted bacterial suspension. Thus, in turn, affect the detection limit of surviving countable organisms (112).

Furthermore, it needs to be considered that experimental conditions such as biocide concentrations, exposure time, dilution, and bioavailability used for MIC and MBC testing do not reflect in-use conditions (117). Nevertheless, the determined MIC and MBC values are

important first indicators of a changing biocide susceptibility development. Pursuing methods like transcriptomic and whole genome sequence analysis can support these test results and explore underlying mechanisms.

2 Synopsis

2.1 Aims and hypotheses

According to EU BPR regulation (528/2012), biocides are supposed to deter, render harmless, or destroy harmful organisms. Especially the use of microbicides as disinfectants is an essential part to prevent the spread of multi-drug resistant human pathogenic bacteria. *In vitro* studies indicate biocide use as a risk factor for the emergence of antibiotic resistance. Still, it remains challenging to demonstrate a causal link between biocide usage and antibiotic resistance development in environmental settings such as food production facilities, livestock industry, and health care settings.

The first aim of this thesis was to analyze the controversial current knowledge on the potential of bacteria to develop biocide tolerance or antibiotic resistance, or both, in response to biocide exposure. The second aim was to examine biocide susceptibility profiles for specific bacteria in chosen settings and to analyze potential associations between biocide tolerance and antibiotic resistance as well as underlying genetic determinants. The third aim of this thesis was to develop a ready-to-use test system enabling fast and accurate biocide susceptibility testing of bacteria for setting-specific substances.

This thesis is finally dealing with the following hypotheses and biological questions.

Hypothesis 1: Bacterial exposure to biocides induces biocide tolerance and antibiotic resistance

In vitro adaptation studies were reviewed to answer the following questions:

i. Which methods are currently described in the literature to investigate the adaptive potential of bacteria to biocides?
ii. Are gram-positive and gram-negative bacteria able to adapt similarly to biocides during biocide exposure experiments?

iii. Are bacterial adaptations to biocides stable over time? What kind of differences can be observed between gram-positive and gram-negative bacteria?

iv. Is it possible to draw general conclusions on the co-occurrence of antibiotic adaptation triggered by *in vitro* biocide exposure experiments?

Hypothesis 2: Biocide tolerance is interlinked with antibiotic resistance in environments with regular disinfection regimes

To gain more knowledge of biocide and antibiotic susceptibility profiles in environmental settings in Germany, field isolates collected from German food (*L. monocytogenes*), livestock (*E. coli*), and clinical environments (*E. faecium*) were investigated with focus to the following questions (2.3.2 Publication 1-3 Biocide tolerance and antibiotic resistance in environments with regular disinfection regimes)

i. Are field isolates collected from environments with regular disinfection regimes less susceptible to relevant biocides and antibiotics in comparison with reported data?

ii. Can reduced susceptibilities to biocides and antibiotics be associated with characteristic genetic determinants?

iii. Do reduced biocide susceptibilities (increased MIC/MBC values) of field isolates correlate with reduced antibiotic susceptibilities (increased MIC values)?

Hypothesis 3: A vacuum-dried microtiter plate system enables fast, accurate, and reproducible routine biocide susceptibility testing

To promote the harmonization of standardized biocide susceptibility testing, a new ready-to-use test system for the identification of cationic biocide susceptibility profiles was developed and tested for gram-positive and gram-negative bacteria. The following questions were addressed (2.3.3 Publication 3, Part 1 The need for reproducible routine biocide susceptibility testing):

i. Are biocide susceptibility profiles obtained with the newly developed vacuum-dried microtiter plate system comparable to results from broth microdilution method with freshly prepared biocide solutions?
ii. Is it possible to interlink biocide susceptibility datasets obtained with the vacuum-dried microtiter plate system with antibiotic susceptibility profiles to determine possible co-occurrence of biocide tolerance and antibiotic co- or cross-resistance in human pathogenic bacteria?
iii. How can such test systems contribute to improve hygiene management regimes?

2.2 Study design

To address the hypotheses and related questions, the thesis was designed as presented in Figure 3.

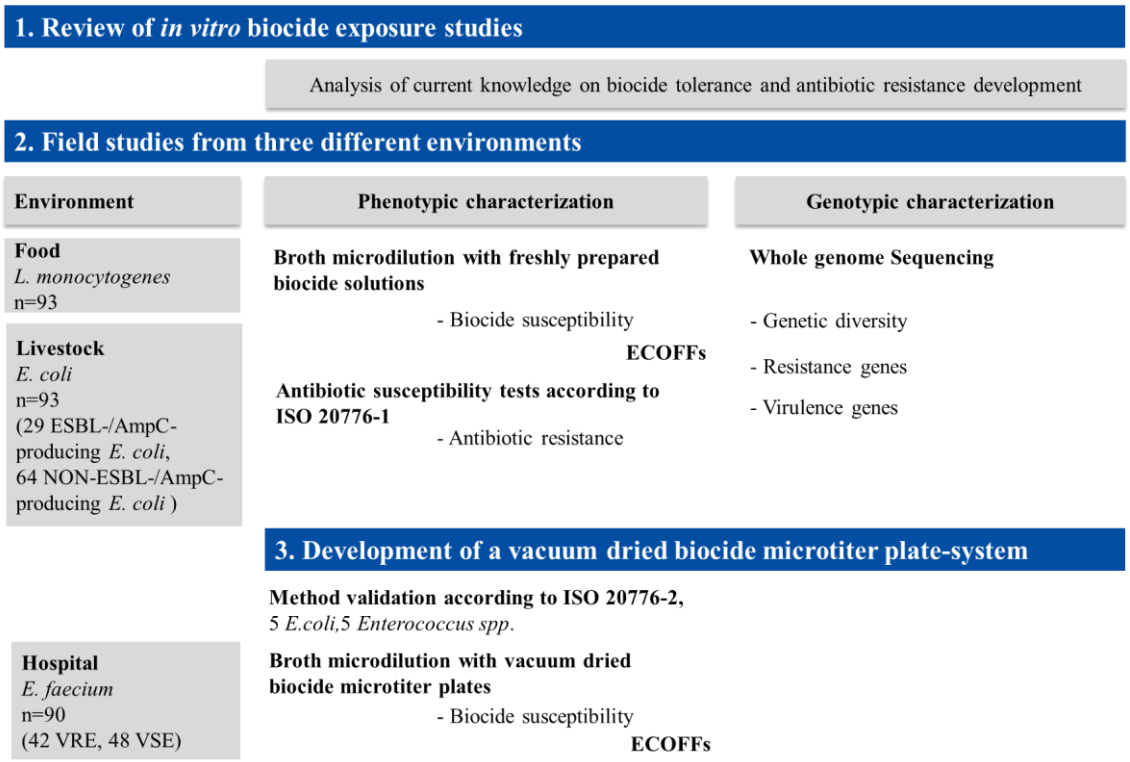

Figure 3: Schematic structure of the study design
The study is divided into three parts. One to review the current state of research (1) and two with own laboratory work and their evaluation (2/3).
ESBL= extended-spectrum ß-lactamase, AmpC = AmpC ß-lactamase, VRE vancomycin-resistant enterococci, VSE= vancomycin-susceptible enterococci

First, the controversial current knowledge on the potential of bacteria to develop biocide tolerance or antibiotic resistance, or both during biocide exposure were analyzed. For this purpose, publications investigating bacterial *in vitro* exposure to biocides were reviewed. In this review 78 biocide adaptation studies providing data of 1369 individual tests for planktonic cells (see Appendix I) were included. All studies were evaluated regarding adaptation capability of gram-positive and gram-negative bacteria to biocides, stability of the adaptation, and adaptation to antibiotics. Analyzed publications investigated exposure to various biocides,

including alcohols, aldehydes, alkylamines, biguanides, chlorine-releasing compounds, peroxides, phenol derivatives, and QACs.

In a second step, potential associations between biocide and antibiotic susceptibility in field isolates collected from German environments following a regular disinfection regime, including food processing plants (*L. monocytogenes*, n=93, Publication 1), animal husbandry (*E. coli*, n=93, Publication 2) and clinical environments (*E. faecium*, n= 90, Publication 3, Part 2) were analyzed. Study populations of *E. coli* and *E. faecium* contained proportions of extended-spectrum ß-lactamase (ESBL, n=16) and AmpC ß-lactamases producing (n=13) *E. coli* as well as vancomycin-resistant *E. faecium* (n=42). Phenotypic biocide and antibiotic susceptibility testing were performed for all isolate sets using broth microdilution assays according to ISO 20776-1. Based on these data, tentative ECOFFs according to the procedures for antibiotics in the EUCAST guidelines (121) for each dataset were defined, because there are currently no criteria available to distinguish between biocide tolerant and susceptible isolates. Substance-specific ECOFFs represented 95% of the tested bacterial population (MIC_{95} or MBC_{95}). Genotypic characterization was carried out for *L. monocytogenes* and *E. coli* using whole genome sequence data. It included the analysis of phylogenetic relationships as well as the determination of virulence, biocide, and antibiotic resistance-associated genes. Furthermore, data gained by the phenotypic and genotypic analysis were statistically analyzed for associations between reduced biocide susceptibility and antibiotic resistance in the different environments.

In the third part of this thesis, a newly developed biocide susceptibility test based on microtiter plates containing vacuum-dried cationic biocides was evaluated. This customized microtiter plate was developed by MERLIN Diagnostika GmbH according to my specifications. During the evaluation, results obtained with the new assay and the standard method (use of freshly prepared biocide solutions in microdilution) under consideration of reproducibility and essential agreement (EA) according to ISO 20776-2 for a set of *E. coli* (n=5) and enterococci

(n=5) (Publication 3, Part 1) were compared. Subsequently, biocide susceptibility for *90 E. faecium* from clinical environments using the herewith published test method was determined. Obtained data served as the basis for the third epidemiological study (Publication 3, Part 2). The application of the developed ready-to-use test system enables the routine surveillance of bacterial tolerance towards disinfectants in hospitals.

2.3 Publications and extended discussions

2.3.1 Literature review: Impact of *in vitro* biocide exposure to bacterial tolerance and antibiotic resistance

An extensive literature search was conducted to obtain the current status on biocide and antibiotic resistance development. The focus was particularly on the ability of bacteria to adapt to biocides, the stability of the adaptation and the adaptability to antibiotics during *in vitro* biocide exposure assays.

2.3.1.1 Bacterial adaptation to biocides and adaptation stability

Data availability differed essentially between study types. However, the investigated datasets indicate that biocide adaptation events and adaptation stability seem to be dependent on several factors such as (i) experimental settings, (ii) tested substances, and (iii) bacterial properties.

(i) Experimental settings

In general, *in vitro* experiments could be classified as follows: a) multiple exposures to pure substances; b) multiple exposures to biocidal products, c) single exposure to pure substances, and d) single exposure to biocidal products (Hypothesis 1-i). Whereas multiple long-term exposure studies intend to induce *de novo* tolerance development due to mutations, single exposure experiments mainly interrogate the potential of an active substance or biocidal product to select for tolerant geno- and phenotypes pre-existing in a bacterial population. Adaptation to biocides during multiple exposures was slightly more frequently reported (33%; n=411) than single exposure experiments (25%; n=31, Appendix I)). However, stable adaptation was substantially more often reported for single exposure (85%, n=23) than multiple exposure experiments (43%, n=158). Interpretation of this observation remains difficult. It needs to be noticed that this outcome may be primarily linked to the tested substances instead of the experimental setting. A high proportion of single exposure experiments tested adaptation to specific biocides such as triclosan, for which stable adaptations are frequently reported (93, 94,

118, 122, 123). Nonetheless, the results might partially reflect the different approaches of both methods as well. Single exposure experiments aim for the selection of pre-existing subpopulations with reduced susceptibility to the tested substance. Thus, it seems explainable that pre-existing subpopulations remain stable in their susceptibility profiles. In contrast, multiple exposure experiments lead to gradual biocide adaptations due to mutations or phenotypic adaptation, or both. As mutational adaptations are frequently accompanied by fitness costs (40) and transient adaptation is often the result of a temporary cellular stress response (124, 125), the lower proportion of stable mutants after multiple exposures compared to single exposure is not astonishing. The stability of the adaptation events is an essential factor for biocide tolerance development. Nevertheless, only a small number of studies investigated this parameter (n=395 out of 1,369 individual tests).

(ii) Tested substances

Exposure to active ingredients of biocidal products helps to understand the effect of the active compound on emerging resistance, whereas the use of biocidal product formulations reflects actual use (126). Thus, the composition of the substance (pure substance vs. biocidal products) is another vital factor to consider in biocide adaptation experiments. In most experiments, bacteria were challenged via exposure to pure substances (82%, 1119 individual tests). This data creates an imbalance with regard to comparison of adaptation outcomes after exposure to pure substances and biocidal products. Overall, exposure to pure substances resulted more frequently in bacterial adaptations (38%, n=423) compared to biocidal products (8%; n=19). Stable adaptations after exposure to pure substances were reported for 174 individual tests (45%). Only a few studies investigated the stability of adapted isolates obtained after exposure to biocidal products (n= 9). Hence, trends cannot be observed. It is assumed that additional ingredients of biocidal products may act synergistically, leading to reduced bacterial viability and adaptation capacity (126). Stable adaptations to pure substances were frequently reported for biguanides, phenolic compounds, and QACs, while they were less often described for

aldehydes, alcohols, oxidizing agents, and chlorine-releasing compounds. Cationic biocides like QACs and biguanides are membrane active agents interacting with the cell surface and integrating into the cytoplasmic membrane (49). In low concentrations, they are bacteriostatic and bacteria may adapt more easily due to cell membrane modifications like alterations of the net negative charge (79), changes in cell membrane permeability (75), or the upregulation of efflux pump activity (76, 127-129). In contrast, biocides with strong oxidizing effects like peroxides act highly nonspecific in intracellular compartments. They are very effective due to the production of free hydroxyl radicals interacting with intracellular DNA, proteins, and lipids leading rapidly to cell death (35). Thus, chances to adapt due to unspecific stress responses may be meager. The number of studies investigating adaptation to highly reactive substances has been comparatively rare yet. This could create a bias concerning data interpretation and assessment of biocide tolerance development.

(iii) Bacterial properties

A total of 756 individual tests of gram-negative and 613 individual tests of gram-positive organisms were considered. While similar biocide adaptation frequencies in gram-positive and gram-negative bacteria (33%; n=202 vs. 32%; n=240 respectively) were observed (Hypothesis 1-ii), the proportion of stability varied remarkably.

Stable adaptations were generally more frequently observed in gram-negative bacteria (Hypothesis 1-iii). While 60% (n=127) of the stability tested gram-negative bacteria remained stable, only 29% (n=54) stable adaptations could be recorded for gram-positive bacteria. Stable adaptations may be attributable to the selection of mutants (130) containing genetic changes without or with a minor impact on fitness costs. Elevated mutation frequencies have been recently reported in natural and pathogenic gram-negative species like *E. coli*, *S. enterica*, and *P. aeruginosa.* They were associated with greater antibiotic resistance levels compared to non-mutators (131). If biocides may be similarly involved establishing mutator populations with reduced antimicrobial susceptibility needs to be elucidated.

Structural differences between gram-positive and gram-negative bacteria influence the sensitivity to most antimicrobials. The outer membrane of gram-negative bacteria that is lacking in gram-positive bacteria contributes to reduced susceptibility to biocidal compounds and antibiotics (38, 39).

Mutations of various regions can contribute to tolerance in bacteria and lead to reduced susceptibility. Prominent examples of biocide tolerance mechanisms in gram-negative bacteria include (over)expression of efflux pumps, reduction of porins, or alterations of the net negative charge of the bacterial cell wall as well as membrane composition (79, 129, 132-135). Often described mechanisms in gram-positive bacteria include transient alterations of thickness and degree of cross-linking of the peptidoglycan or glycocalyx formation, enzyme-mediated inactivation or increased efflux (39).

In gram-positive and gram-negative bacteria, interspecies- as well as intraspecies-specific differences were observed regularly. The gram-positive *Staphylococcus* spp., for example, were frequently described to adapt to benzyl group containing QACs (71%) while they adapted less often to chlorhexidine (CHX, 24%) and triclosan (43%) during multiple exposure experiments with pure substances. In contrast, *Enterococcus* spp. could easily adapt to QACs (92%) and CHX (68%) but not to triclosan (17%).

For the gram-negative *Pseudomonas* spp. adaptation during multiple exposure experiments with pure substances was often described for CHX (79%) but less frequently observed for QACs (21%) and triclosan (33%). In opposition to the findings for *Pseudomonas* spp., approx. 50% of the investigated *Salmonella* spp. adapted to the respective substances. These examples demonstrate the difficulties arising from general statements to adaptive behavior of gram-positive and gram-negative species. As the number of isolates per species varies widely (e.g., *Staphylococcus* spp. 14 isolates investigated for benzyl group containing QACs, and 38 isolates for CHX, 63 isolates for triclosan), species-specific adaptation frequencies could bias the comparison of adaptive capacities for gram-positive and gram-negative species. Ultimately, due

to the diversity of the applied methods and low level of comparability, no general statement whether gram-positive or gram-negative bacteria adapt more easily to specific biocides is possible. More specifically, adaptations might be species and strain-dependent.

2.3.1.2 Antibiotic adaptation

Subsequent antibiotic adaptation following *in vitro* exposure to biocides was investigated for 490 individual tests of gram-positive and gram-negative bacteria. While contact with some substances like aldehydes, chlorine-releasing compounds, or peroxides seems to pose a lower risk, altered antibiotic susceptibility profiles were reported as a result of exposure to biguanides, phenolic derivatives, and QACs. Detailed information is provided in Figure 4.

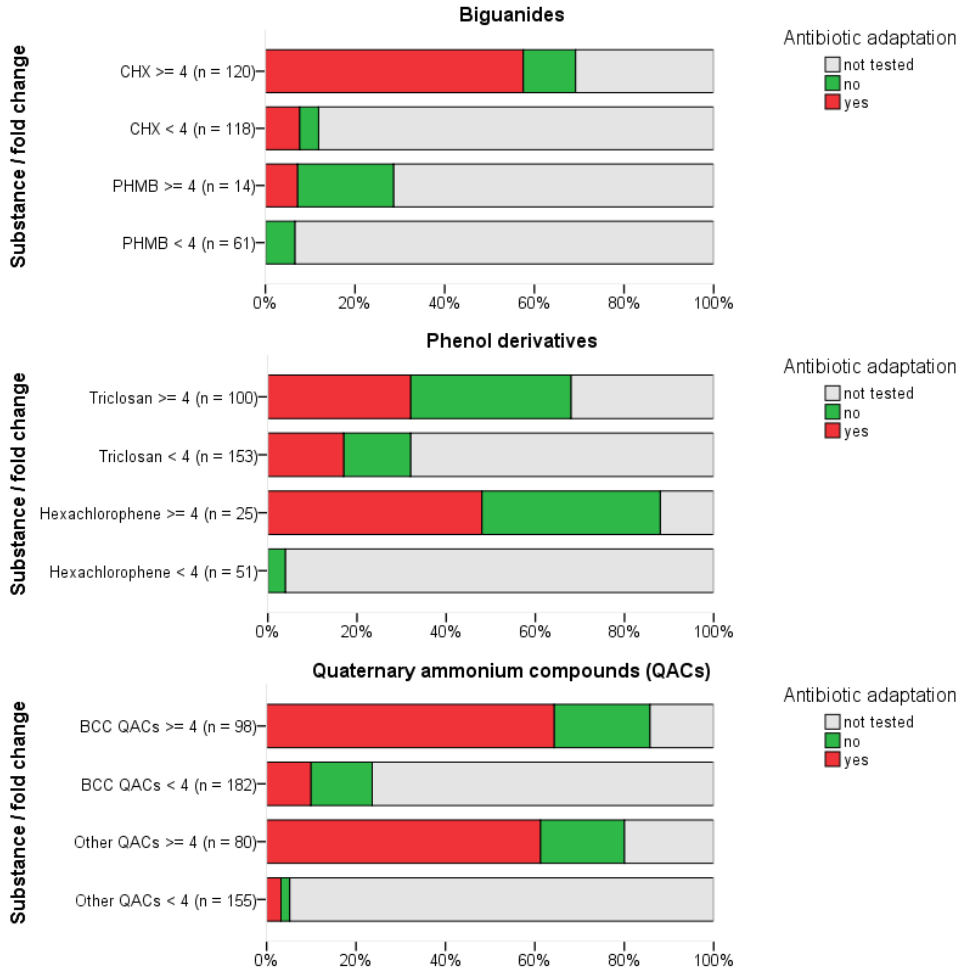

Figure 4: Adaptation of bacteria to at least one antibiotic after exposure to the biguanides chlorhexidine (CHX) and polyhexamethylene biguanide (PHMB), phenol derivatives triclosan and hexachlorophene as well as the quaternary ammonium compounds (QACs) benzylgroup containing (BCC) QACs and other QACs. Results are shown separately for biocide adapted mutants (fold change, FC ≥ 4) and isolates without adaptation to the biocide of interest after exposure to the respective substance (FC < 4). Colors display observed antibiotic adaptation.

Even antibiotic resistance development due to biocide exposure is recognizable, the overall limited number of individual biocide tests and investigated isolates does not allow a predictive, general conclusion on the development of antibiotic resistance in biocide-adapted bacteria and may rather be strain-specific (Hypothesis 1-iv). As one example, Braoudaki was able to show that cross-resistance in triclosan adapted *E. coli* K-12 (n=1) and *E. coli* O55 (n=1) was observed to a lesser extent compared to *E. coli* O157:H7 (n=2). *E. coli* K-12 developed cross-resistance to chloramphenicol, while *E. coli* O55 exhibited resistance to trimethoprim. In comparison, *E. coli* O157:H7 was resistant to chloramphenicol, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride (BAC), and CHX after exposure to triclosan. The authors suggested that genetic variability in *E. coli* O157 and *E.coli* K12 may facilitate differences in cross-resistance profiles (136).

It is important to highlight that observed changes in antibiotic susceptibility following *in vitro* biocide exposure were mainly moderate and seldom defined as clinical resistance according to CLSI and EUCAST guidelines. Nonetheless, even small changes may provide growth advantages under selective pressure and trigger the development of high-level resistance.

In conclusion, the results of the literature review support hypothesis 1 that bacterial exposure to biocides can induce biocide tolerance and antibiotic resistance.

2.3.1.3 Transferability of results obtained from *in vitro* exposure experiments to workaday environments

In situ, bacteria might face selection pressure due to contact with sub inhibitory biocide concentrations for various reasons in different environments. This might occur due to application errors, dilution in the environment, interfering materials, degradation of biocidal products, or wash-off events. In general, in-use concentrations of disinfectants are much higher than the MICs of the microbial species of concern. Still, inevitably the use of concentrations well above the MIC values results in gradients over time and space which will finally overlap

with the sub-MIC levels (62). One example is the relative environmental persistence of many biocides like QACs and triclosan. Their tendency to bind to organic matter and soil provides a potential long-lasting low-level exposure to microorganisms (137, 138). Biocide residues found in different environments like surface water, wastewater, or sediments may force the selection of permanently adapted microorganisms. As a kind of chain reaction, elevated concentrations of biocides are needed to inhibit the growth of adapted bacterial populations, as was shown in an outbreak of *Serratia marcescens* on a neonatology ICU. Isolates exhibited resistance to 0.5% Mikrobac forte® consisting of benzyl-C12-18-alkyl dimethyl ammonium chloride 199 mg/g and N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine 50 mg/g, which was regularly used for disinfection of surfaces before the outbreak. One of the measures to contain the outbreak included increasing the concentration of used Mikrobac forte® to 2% (139). Conceivably, the elevated concentration of disinfection may lead again to the disposition of higher remaining concentrations in the environment.

Due to elevated biocidal selection pressure following risks may arise *in situ*:

(i) Bacterial adaptation to the substance:

Biocides may induce transient adaptation reactions by biofilm formation, expression of small colony variants, or slow growth, selecting for phenotypes with the ability to temporarily survive biocidal stress (40, 61, 140). A prominent example was reported by Sheridan et al. who found triclosan tolerance caused by mingled mechanisms, including growth inhibition, increased biofilm formation, and change in outer membrane proteins in triclosan tolerant *E. coli* 0157:H19 (141).

(ii) Propagation of mutation

Sub-MIC levels of biocides may act as mutagens and increase mutation rates which may influence the rate of de novo biocide, respectively, antibiotic resistance development (57, 142, 143). Mutational antibiotic resistance caused by biocides, or byproducts could even explain

multidrug-resistance in areas lacking antibiotic selection pressure (57, 143). For example, Lv et al. demonstrated mutagenic activities of disinfection byproducts in disinfected drinking water, where antibiotic concentrations are too low to select antibiotic resistant strains effectively. After exposure to disinfection byproducts, resistance to individual antibiotics and multidrug-resistance were both raised in *P. aeruginosa* PAO1 by various levels. Norfloxacin and polymycin B resistances were 10-fold enhanced compared to a control (143).

(ii) Influence on horizontal gene transfer

Sublethal concentrations of biocides can increase or decrease transfer frequencies of MGEs such as plasmids and phages (41, 144, 145). Jutkina et al. were able to show that subinhibitory concentrations of CHX and triclosan (200 times and 20 times below the MIC, respectively) increased the frequency of antibiotic resistance gene transfer for the recipient *E. coli* strain by influencing the exchange rates of mobile genetic elements (62).

(iv) Cross-resistance:

Due to similar resistance mechanisms, biocides can select for bacteria expressing resistance not only to the used biocide but additionally to other biocides or antibiotics. An illustrative example for cross-resistance in clinical environments was described by Stein et al. (146). They have recently described a clonal cluster of carbapenem-resistant *Klebsiella pneumoniae* isolates showing diminished susceptibility to CHX. These isolates were detected on a ward that has implemented routine washing with CHX to reduce the rate of catheter-related infections. Strikingly, CHX tolerance was associated with resistance to colistin, likely caused by increased efflux of both substances via the same route.

(v) Co-resistance:

Due to the presence of physically linked genetic resistance mechanisms or their coordinated expression, biocides can select bacteria resistant to unrelated antimicrobial substances. In a large-scale bioinformatics approach based on analyzing completely sequenced bacterial

genomes and plasmids available in the NCBI repository, Pal et al. identified a higher proportion of plasmids carrying both biocide/metal resistance genes (BMRGs) and antibiotic resistance genes (ARGs) hosted by *Escherichia*, *Staphylococcus*, *Salmonella*, and *Klebsiella* compared to other bacterial genera (147). The BMRGs that commonly co-occurred with ARGs on plasmids were mercury resistance genes and the *qacEΔ1* gene supposed to induce low-level resistance to quaternary ammonium compounds. A previous study has shown that *qac* resistance genes located on class 1 integrons are often present in bacteria exposed to detergents, biocides, or antibiotics (148). Thus, QACs may act as a major driver for the selection of class 1 integrons (149). This evidence and the findings of Pal et al. led to the author`s hypothesis that transposons and integrons are involved in the process of biocide/metal-driven co-selection of antibiotic resistance (147).

In conclusion, i*n vitro* studies support the hypothesis 1 that bacterial exposure to biocides can induce biocide tolerance and antibiotic resistance. However, this knowledge needs to be verified in field studies to explore and understand the possible risks arising in complex environments with regular hygiene measures using biocides as chemical disinfectants.

2.3.2 Publication 1-3: Biocide tolerance and antibiotic resistance in environments with regular disinfection regimes

As shown in 2.3.1, the results of biocide exposure experiments tend to be highly variable. Outcomes are heavily influenced by the chosen experimental setting and by choice of biocides and microorganisms being examined. While being a useful tool to investigate possible outcomes to adaptation or co-selection, the transferability to real-world interactions of such controlled experiments remain questionable (40).

In order to gain more knowledge on bacterial susceptibility to biocides and putative associations with antibiotic resistance in natural settings, I investigated field isolates collected from environments following a regular disinfection regime. According to the literature, carriage of ESBL in *E. coli* and vancomycin resistance in *E. faecium* has been associated with reduced susceptibility to certain biocides (108, 150, 151). To examine published associations, antibiotic resistant and susceptible isolates (ESBL/AmpC ß-lactamases (AmpC)-producing *E. coli* (n=29), and Non-ESBL-/AmpC-producing *E. coli* (n=64) in publication 2 of animal husbandry as well as VSE (n=48) and VRE (n=42) in publication 3 of clinical environments were included. Whole genome sequencing was performed for all *L. monocytogenes* and *E. coli* isolates to analyze phylogenetic relationships and resistance determinants (details are deposited at the National Center for Biotechnology Information database https://www.ncbi.nlm.nih.gov/, under accession numbers MK944275 to MK944277, and JAFMWT000000000-JAFMVF000000000, respectively).

The study population of *L. monocytogenes* showed a broad heterogeneity of MLST clonal complexes (CC). Most CCs isolated from technical equipment and surfaces of German food production facilities included CC2, CC8, and CC9 as predominant genotypes (figure 2 in publication 1). In Germany, CC8 and CC2 isolates are frequently reported as causative agents of human listeriosis (152, 153). The detection of clinically relevant genetic lineages highlights

the importance of contaminated food production environments as transmission routes for virulent *L. monocytogenes*.

The study population of *E. coli* also showed a broad heterogeneity of multilocus sequence types (STs). Most STs isolated from surfaces of grounds, walls, and equipment included ST117 and ST297 belonging to phylogroup F and B1, respectively. While phylogroup B1 comprises commensals or intestinal pathogens (154), phylogroup F is highly associated with extraintestinal pathogenic *E. coli* (ExPEC) (155-157). ExPEC can cause urinary tract infections, meningitis, or sepsis (158). ST 117 is also linked to avian pathogenic *E. coli* (APEC), the avian pathotype of ExPEC, causing colibacillosis. APECs were also isolated from human patients (159). These findings emphasize a zoonotic risk originating from farm environments.

Overall, the datasets revealed a low proportion of biocide tolerant strains (*L. monocytogenes:* 22%, *E. coli:* 10%, *E. faecium:* 0%) based on our definition of tentative ECOFFs (Hypothesis 2-i). In general, susceptibilities to tested substances and proportions of biocide tolerant isolates were comparable to data already published in the literature (for detailed comparisons, see publications (104, 105, 107)) (Hypothesis 2-i). Several reduced phenotypic susceptibilities could be interlinked with genetic determinants (Hypothesis 2-ii). I identified 15 *L. monocytogenes* isolates with reduced susceptibility to BAC. In 13 out of them, genetic determinants coding for efflux pumps of the small multidrug resistance family (SMR) were detected. These included *qacH* (n=10), *bcrABC* (n=1), and *emrC* (n=2). These efflux pumps have been previously associated with increased efflux of BAC in *L. monocytogenes* (160-163). Thus, it is very likely that they were responsible for the observed phenotypes. Interestingly, QAC tolerance was predominantly identified in isolates belonging to genetic lineages without known clinical relevance. Reduced susceptibility to sodium hypochlorite, peroxyacetic acid (PAA), and isopropanol was not associated with specific genetic determinants (104).

Furthermore, I identified three *E. coli* isolates with reduced susceptibility to formaldehyde (increased MIC value). These isolates carried a gene coding for a glutathione-and NAD-

dependent formaldehyde dehydrogenase located on a plasmid, which has been previously described to cause formaldehyde tolerance by enzymatic degradation of the biocide (72). Specific determinants associated with reduced susceptibility to p-chloro-m-cresol (identified in one isolate), BAC (four isolates), or PAA (one isolate) could not be found. Importantly, phenotypic biocide tolerance for FA, PAA, and BAC was found in isolates with and without clinical relevance.

Intriguingly, *qacEΔ1* and *sugE(p)* were identified in 10% and 8% of the isolates, respectively. Both genes encode efflux pumps of the SMR family and have been reported to be involved in QAC tolerance (164-166). However, reduced susceptibility to the investigated QACs, benzalkonium chloride and didecyldimethylammonium chloride, compared to isolates lacking the efflux pump encoding genes could not be observed in my study (105). This might be due to the substrate specificity of the efflux pumps themselves (164, 165) or the applied susceptibility test method influencing the mode of bacterial growth and expression of SMR efflux pumps (165). Interestingly, the QAC tolerance efflux pump determinants *qacEΔ1*, and *sugE(p)* were both located on mobile genetic elements close to antibiotic resistance genes *sul1* and bla_{CMY-2}, respectively. As *qacEΔ1* and *sugE(p)* have been linked to phenotypes with reduced susceptibility to QACs in the past (164-166), co-location with antibiotic resistance genes on mobile genetic elements support the hypothesis of Pal et al. that mobile genetic elements such as integrons play an important role in biocide driven co-selection of antibiotic resistance (147).

For *L. monocytogenes* and *E. coli*, antibiotic susceptibility testing was performed to compare with biocide susceptibility results. In addition, genetic determinants responsible for identified phenotypes were characterized. Detailed information on susceptibility profiles and underlying mechanisms are given in the corresponding publications (104, 105). In general, a low level of antibiotic resistance in *L. monocytogenes* isolated from food production environments in Germany was observed. Only five isolates (5%) were resistant to at least one antibiotic in three or more classes and therefore defined as multidrug-resistant (MDR). In contrast, antibiotic

susceptibility profiles were highly diverse in the tested *E. coli* population. In this study, 34 isolates (37 %) were defined as MDR.

With very few exceptions, antibiotic resistance could be traced back to known underlying genetic determinants (Hypothesis 2-ii). Correlation analyses of biocide susceptibility data and corresponding antibiotic resistance profiles did not reveal any correlation between reduced susceptibility (increased MIC or MBC) to biocides and antibiotics. Furthermore, biocide susceptibility was not significantly influenced by the expression of extended-spectrum ß-lactamases in *E. coli* or vancomycin resistance in *E. faecium* as indicated in the literature (108, 150, 151). This might be due to geographic variability or depend on the frequency at which isolates were previously exposed to biocides. For example, *E. faecium* originated from a clinical ward with low CHX usage, whereas Alotaibi et al. conducted an investigation with Danish VRE and VSE isolated from a hospital ward with heavy use of CHX (150).

In summary, susceptibility profiles to various biocides did not differ between antibiotic susceptible or resistant isolates (Hypothesis 2-iii). Even though I could not generally support hypothesis 2 that biocide tolerance is interlinked with antibiotic resistance in environments with regular disinfection regimes, there is one epidemiological study describing antibiotic cross-resistance. As mentioned before, Stein et al. reported a clonal cluster of carbapenem-resistant *Klebsiella pneumoniae* isolates with reduced susceptibility to CHX. These isolates originated from intensive care unit (ICU) patients on a ward using CHX for routine washing to decrease the rate of catheter-related infections. Alarmingly, reduced CHX susceptibility was associated with colistin resistance, likely caused by increased efflux of both substances via the same route (146).

Given the identified low number of biocide-tolerant isolates in our studies, it is likely that additional factors contribute to the persistence of bacteria in different niches.

Successful adaptation depends on various aspects, including the structure (sessile vs. planktonic), and composition (pure vs. mixed culture) of the bacterial community, temperature,

oxygen, and nutrient access, pH, detergents, or exposure times as these factors influence the growth, metabolism/physiology of the bacterial cells and the division cycle which are critical points for bacterial susceptibility (7, 35). Furthermore, these factors are involved in quorum sensing (transduction of cell-cell signals) and the formation of biofilms (7). Biofilm formation is an intrinsic survival strategy enabling bacteria to withstand harsh conditions like disinfection. The extracellular matrix provides a diffusion barrier protecting both gram-positive as well as gram-negative bacteria. It provides an enhanced medium for bacterial signaling (e.g., quorum sensing), genetic exchange as well as a potential site for neutralization or binding of chemical agents (40). Ten to 1000-fold elevated MIC values have been reported for different biocides such as BAC or oxidizing agents during comparison of biocidal activity against sessile vs. planktonic bacteria, including *Listeria* spp. or *E. coli* (167-169). Thus, biofilms provide a microenvironment in which pathogens might withstand disinfectant concentrations that otherwise kill planktonic cells. Consequently, bacteria can survive regular cleaning and sanitation procedures and persist in food-processing plants, animal husbandry, or clinical environments. In many of these environments, conditions favor attachment and biofilm formation, i.e., flowing water, suitable attachment surfaces, ample nutrients, and a sufficient number of bacteria supplied by the environment (168). Another key factor for biofilm formation is the promotion of microbial communication, also termed quorum sensing, and its role in establishing of resistant phenotypes (7, 140). Microorganisms within the assembled mass produce and receive small signal molecules (e.g., autoinducer-1, autoinducer-2, and peptides) that regulate microbial communication. With increasing densities of microbial cells, the density of different signal molecules increases. As a result, different autoinducers bind to specific receptors to activate or inactivate gene cascades (170). As a prominent example, Hassett et al. demonstrated that the expression of catalase and superoxide dismutase genes coding for protective enzymes against oxidizing stress was under the control of quorum sensing in *P. aeruginosa* biofilms (171).

Efflux pumps are not only essential requisites to expel drugs and toxic metabolites. They may also release other molecules necessary for cell communication, biofilm formation, osmoregulation, or cell protection (172, 173), increasing the ability to persist in food production environments. Furthermore, in each bacterial population, there is a small subset of the population, called persisters, showing phenotypically resistant dormant variants without genotypic features (174). Bacteria may survive in different niches because they are located in areas difficult to reach for disinfectants. For *E. coli* isolates from animal husbandry, for example, it could be demonstrated that biocide tolerant strains isolated from transitions between floor and wall or cracks and crevices were able to survive hygiene measures (105). These are well-known critical locations in husbandry environments. As they are difficult to clean and disinfect (175, 176), exposure to subinhibitory concentrations of biocides due to improper disinfection practice and the exertion of sub-MIC selection pressure on biocide tolerant bacteria in such niches is very likely.

In summary, the results of our susceptibility studies have shown, that indeed bacteria with reduced susceptibility to biocides (albeit few examples) are present in environments supposed to exert a high selection pressure due to the widespread use of disinfectants. This indicates that biocide tolerance might present one bacterial feature to survive disinfection processes. With the acquisition and, or the expression of tolerance determinants, bacteria might be able to persist in different environments for long periods. Theoretically, the increased prevalence of tolerant isolates might furthermore increase the chance of mutation or plasmid accumulation producing a higher level of stable antibiotic resistance (41). Later on, these determinants might be spread through bacterial populations and be detectable in the genomes of these bacteria.

As most of the analyzed isolates were susceptible to investigated biocides, bacteria have to use additional strategies to survive in environments with regular disinfection regimes. Furthermore, the results do not yet support hypothesis 2 that biocide tolerance is per se interlinked with antibiotic resistance. Nonetheless, biocide tolerance determinants adjacent to antibiotic

resistance genes on mobile genetic elements could be identified, indicating that co-selection of biocide tolerant and antibiotic-resistant bacteria might be principally possible.

2.3.3 Publication 3-Part 1: The need for reproducible routine biocide susceptibility tests

Chemical disinfection is a key factor in reducing the spread of resistant bacterial pathogens. However, laboratory settings have extensively demonstrated the possibility of biocide and antibiotic resistance development in bacteria due to exposure to low biocide concentrations. Albeit scarce, *in vitro* findings are supported by epidemiological studies reporting this causality (146). Furthermore, some reports link the introduction of biocides in clinical settings with the identification of bacteria showing reduced biocide susceptibility (146, 177, 178). Hardy et al., for example, have shown significantly decreased CHX and octenidine dihydrochloride (OCT) MIC and MBC values for *Staphylococcus aureus* from a major hospital trust in the UK after the introduction of CHX and OCT for decolonization regimes. Reduced susceptibility to OCT occurred right after the use of the substance in practice between 2013 and 2014 (177). My field studies provide the support that bacteria with reduced biocide susceptibility are detectable in environments with extensive use of disinfectants (104, 105). Within the investigated *E. coli* population, I could identify genetic determinants that have been reported to confer biocide tolerance co-localized with antibiotic resistance genes on mobile genetic elements, indicating the possibility of co-selection of antimicrobial resistance. Taken together, these results imply that antimicrobial resistance development and, or spread might be additionally driven by bacterial exposure to biocides and thus, point towards the need for regular monitoring not only for antibiotic resistance but also for biocide tolerance development. Screening of bacterial isolates for reduced biocide susceptibility becomes more and more important, especially in the light of drastically limited options of efficient antimicrobial therapy of infections caused by multidrug-resistant bacteria (179). Reliable and standardized screening methods, comparable between laboratories, would also be of value during authorization processes of biocides, as the EU BPR regulation requires information on the tendencies of microbicidal products to select for resistant bacteria (8).

In contrast to antibiotic susceptibility testing, generally accepted, standardized methods for biocide susceptibility testing are missing, even though there have been attempts to introduce respective protocols (117, 118, 179). As highlighted in the study by Bock et al., harmonization is of most importance as slight modifications in the choice of nutrient broth or assay plate material influence the outcome of biocide susceptibility profiles (119). Most procedures include broth macro- and microdilution methods to determine MIC and MBC values. These values are also the basis for establishing ECOFFs urgently needed to distinguish between biocide susceptible and tolerant bacteria. MIC values allow for the detection of bacterial isolates with reduced susceptibility to biocides and help to determine tolerance development at an early stage. MBC values, however are of matter where the lethal rather than the inhibitory concentration of the agent is of primary importance. So far, available epidemiological data are limited for this purpose (102, 106).

As part of this thesis, a reliable high-throughput screening system using vacuum dried biocide microtiter plates for rapid biocide susceptibility testing applicable to gram-positive and gram-negative bacteria was established (Publication 3, Part 1 (107)). The method resembles the standardized procedure for antibiotic resistance testing according to ISO standard 20776-1 (111). While conventional methods depend on the time-consuming fresh preparation of biocide stock solutions, which are diluted to a range of concentrations covering MICs and MBCs (applied for the susceptibility studies to L. *monocytogenes* and *E. coli*), the newly developed system contains predefined concentrations of vacuum dried biocidal substances. In this study, the cationic biocides BAC, CHX, cetylpyridinium chloride (CTP), didecyldimethylammonium chloride (DDAC), and OCT were included since they are highly relevant for hospital settings. All substances can be easily de- and rehydrated. CHX and OCT, for example, are frequently used as antiseptics. The conventional microdilution method and the newly developed test system (reference wet plate and dried plate described on page 3 of publication 3 (107)) were used to characterize biocide susceptibility of a collection of *E. coli* and *Enterococcus* spp.

strains. Results were compared under consideration of reproducibility and essential agreement (EA). Whereas the reproducibility reflects the comparability of test results after repetition of applied test methods, the EA is a measure of comparability between the reference method and the method to be evaluated. The overall results have shown that vacuum dried biocide microtiter plates provide a performance level comparable to the reference broth microdilution method using freshly prepared biocide solutions (reference Method ISO 20776-2) (180) (hypothesis 3-i). The EA amounted to 100% for all isolate/substance combinations while the reproducibility varied. For enterococci, reproducibility reached 100% for all measurements. For *E. coli*, reproducibility reached 100% for CTP and DDAC. It was lower for BAC (98%), CHX (96%), and OCT (96%) but still within an acceptable range (≥95%) according to ISO 20776-2 (180). The broad concentration ranges allowed MIC and MBC determination of an exemplarily chosen gram-positive and gram-negative species with the same plate design. Hence, the evaluated microtiter plates are suitable for quick and standardized biocide susceptibility testing of various bacterial species.

The advantages of such a test system are that it is easy to handle, time-saving, and applicable for different purposes. On the one hand, it is valuable for biocide susceptibility monitoring over time to identify trends in biocide tolerance development as early as possible. Thus, bacteria isolated from clinical or processing environments with repeated exposure to biocides could be monitored using this assay. Susceptibility data of routine monitoring, for example, could help to select the most effective and economically suitable antimicrobial agents for decontamination of a particular environment. Furthermore, standardized processes might serve as a basis to collect epidemiological data in order to establish ECOFFs for diverse species/substance combinations similar to the collection of antibiotic ECOFF data accumulated by EUCAST (181). On the other hand, monitoring could also be useful to detect the development of antibiotic cross-resistance at an early stage. Simultaneous testing of antibiotic resistant and susceptible isolates like VRE and VSE enables rapid assessment of differences in biocide susceptibility

profiles for both groups. I have shown that the vacuum dried biocide microtiter plate generates fast, reliable, and accurate results comparable with standardized antibiotic test systems according to EUCAST or CLSI. In general, MIC values of biocide and antibiotic susceptibility tests can be evaluated regarding putative correlations. In this way, early occurrence of cross-resistance might be reliably discovered (hypothesis 3-ii).

Notably, such a test system is precious to standardize testing procedures and hereafter to improve hygiene management regimes. It can be used as a tool for the surveillance of bacterial tolerance in different environments. Based on biocide susceptibility profiles effective disinfectants can be chosen to control the spread of infections. Especially in animal husbandry or clinical environments this could help to reduce the frequency of antibiotic treatment (hypothesis 3-iii).

In conclusion, our results support hypothesis 3 that vacuum-dried plate systems enable fast, accurate, and reproducible routine biocide susceptibility testing urgently needed to produce harmonized, reliable and comparable results. Such test systems are applicable in research and for surveillance programs.

In each of my studies, biocide susceptibility profiles for planktonic cells were characterized. However, additional parameters could also be of valuable importance to determine and understand biocide tolerance development. As subinhibitory biocide concentrations can improve the biofilm formation capacity, like reported for *E. coli*, *Salmonella (S.) enterica,* methicillin-resistant *Staphylococcus aureus* (MRSA), or *L. monocytogenes* (81, 182-184), parameters such as the minimum biofilm inhibitory concentration (MBIC) or the minimum biofilm eradication concentration (MBEC) might help to understand the effects of biocides to bacterial populations and their possibilities to persist in different niches. Other assays such as the “biofilm biocide survival assay” and the “surface-dried cell biocide survival assay” can be used for detailed investigation of activities against surface-dried and biofilm communities (185). In addition, Maillard and the SCENIHR (7, 126) recommend further tests to understand

the underlying mechanisms of reproducible changes in biocide susceptibility. This might include molecular techniques to investigate transcriptomic and proteomic changes resulting from microbicide exposure (95, 142). Genotypic alterations due to biocide exposure might serve as potential resistance markers. Molecular techniques such as WGS, PCR, and microarray technology have been successfully used to identify microbicide resistance mechanisms (88, 105, 186).

3 Conclusion

According to concerns arising from *in vitro* studies that bacteria are able to adapt to biocides, our field studies have demonstrated that biocide tolerant isolates (albeit very few) can occur in environments with regular disinfection regimes. As determined MICs and MBCs were still well below in-use concentrations, reduced susceptibility did not result in resistance to biocides. Although each biocide represents an individual case, so far, there is no conclusive evidence that the use of biocides leads to an increase in antibiotic resistance in everyday life settings. However, even small susceptibility changes may provide growth advantages and may trigger a higher frequency of high-level resistance development over time. The detection of biocide tolerance determinants adjacent to antibiotic resistance genes on mobile genetic elements corroborates the conclusion that co-selection of biocide and antibiotic resistance can occur, and respective susceptibility profiles need to be monitored. For this purpose, reliable and standardized screening methods as our developed vacuum-dried biocide microtiter plate are required. Large data sets will help to identify reliable ECOFFs and to interpret breakpoints. Together with molecular approaches like WGS, underlying mechanisms of biocide tolerance development, and associated antibiotic resistance mechanisms can be uncovered. This knowledge might improve hygiene measures to prevent the spread and further emergence of MDR-resistant pathogens.

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5 List of publications in peer-reviewed journals

Publication 1

Roedel A, Dieckmann R, Brendebach H, Hammerl JA, Kleta S, Noll M, Al Dahouk S, Vincze S. 2019. **Biocide-Tolerant *Listeria monocytogenes* Isolates from German Food Production Plants Do Not Show Cross-Resistance to Clinically Relevant Antibiotics**. Appl Environ Microbiol 85:e01253-19. https://doi.org/10.1128/AEM.01253-19

Publication 2

Roedel A, Vincze S, Projahn M, Roesler U, Robé C, Hammerl JA, Noll M, Al Dahouk S, Dieckmann R. 2021. **Genetic but no Phenotypic Associations between Biocide Tolerance and Antibiotic Resistance in Escherichia coli from German Broiler Fattening Farms**. Microorganisms 9(3),651. https://doi.org/10.3390/microorganisms9030651

Publication 3

Roedel A, Dieckmann R, Makarewicz O, Hartung A, Noll M, Pletz MW, Dahouk SA, Vincze S. 2020. **Evaluation of a Newly Developed Vacuum Dried Microtiter Plate for Rapid Biocide Susceptibility Testing of Clinical *Enterococcus Faecium* Isolates**. Microorganisms 8(4):551. https://doi.org/10.3390/microorganisms8040551

5.1 Additional publication not included in this thesis

Lüth S, Halbedel S, Rosner B, Wilking H, Holzer A, Roedel A, Dieckmann R, Vincze S, Prager R, Flieger A, Al Dahouk S, Kleta S. (2020) **Backtracking and forward checking of human listeriosis clusters identified a multiclonal outbreak linked to *Listeria monocytogenes* in meat products of a single producer**, Emerg Microbes Infect., 9:1, 1600-1608, doi: 10.1080/22221751.2020.1784044.

6 Publications and declaration of contribution

6.1 Publication 1

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FOOD MICROBIOLOGY

Biocide-Tolerant *Listeria monocytogenes* Isolates from German Food Production Plants Do Not Show Cross-Resistance to Clinically Relevant Antibiotics

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ABSTRACT Contamination of food during processing is recognized as a main transmission route of *Listeria monocytogenes*. To prevent microbial contamination, biocides are widely applied as disinfectants in food processing plants. However, there are concerns about the development of antimicrobial resistance in foodborne pathogens due to widespread biocide usage. In our study, 93 *L. monocytogenes* isolates from German food production facilities were (i) tested for biocide and antibiotic susceptibility using broth microdilution assays, (ii) analyzed for links between reduced biocide susceptibility and antibiotic resistance, and (iii) characterized by whole-genome sequencing, including the detection of genes coding for biocide tolerance, antibiotic resistance, and other virulence factors. Fifteen *L. monocytogenes* isolates were tolerant to benzalkonium chloride (BAC), and genes conferring BAC tolerance were found in 13 of them. Antibiotic resistance was not associated with biocide tolerance. BAC-tolerant isolates were assigned to 6 multilocus sequence type (MLST) clonal complexes, and most of them harbored internalin A pseudogenes with premature stop codons or deletions ($n = 9$). Our study demonstrated a high genetic diversity among the investigated isolates including genotypes that are frequently involved in human infections. Although *in vitro* adaptation studies to biocides have raised concerns about increasing cross-resistance to antibiotics, our results do not provide evidence for this phenomenon in field isolates.

IMPORTANCE Foodborne pathogens such as *L. monocytogenes* can persist in food production environments for a long time, causing perennial outbreaks. Hence, bacterial pathogens are able to survive cleaning and disinfection procedures. Accordingly, they may be repeatedly exposed to sublethal concentrations of disinfectants, which might result in bacterial adaptation to these biocides. Furthermore, antibiotic coresistance and cross-resistance are known to evolve under biocide selection pressure *in vitro*. Hence, antimicrobial tolerance seems to play a crucial role in the resilience and persistence of foodborne pathogens in the food chain and might reduce therapeutic options in infectious diseases.

KEYWORDS *Listeria monocytogenes*, antibiotic resistance, biocide susceptibility, virulence factors

Listeriosis is one of the most serious foodborne diseases. Despite the low incidence of listeriosis (0.47 cases per 100,000 population, 2016, European Union), the high hospitalization (98%) and case fatality rate (16.2%) compared to other zoonotic agents render it a serious public health concern (1). The causative agent, *Listeria monocytogenes*, is a Gram-positive, facultative intracellular opportunistic pathogen. Human infections with *L. monocytogenes* predominantly occur after the consumption of contaminated ready-to-eat food products (2). The ubiquitous microorganism may contam-

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inate a wide range of foodstuffs during the various steps of food production and distribution (2). To fulfill hygiene requirements according to EC regulation no. 852/2004 on the hygiene of foodstuffs (3), biocides are widely applied as disinfectants to prevent bacterial contamination. In Germany, the Industrial Hygiene and Surface Protection Association (Industrieverband Hygiene & Oberflächenschutz [IHO]) maintains a list of disinfectants that have been tested according to German (DIN; German Institute for Standardization) and European (EN) standards for use in the health care sector, in animal husbandry, and in food production (https://www.iho.de/). They include quaternary ammonium compounds (QACs), aldehydes, alcohols, chlorine-releasing compounds, or peracids. The awareness of risks related to subinhibitory biocide concentrations triggering antimicrobial resistance in bacteria has substantially increased in the last years (4, 5). In *in vitro* experiments, links between reduced biocidal susceptibility and antibiotic resistance have been described for various substances and bacterial species (6–11), including *L. monocytogenes* (12, 13). Biocide tolerance may be based on similar resistance mechanisms toward different antimicrobial agents (cross-resistance). In the case of coresistance, the mechanisms conferring reduced susceptibility are unrelated but genetically linked, e.g., located on the same genetic element (14). However, the relevance of co- and cross-resistance has not yet been validated in the environment and therefore needs to be verified in field studies.

So far, standardized laboratory methods to investigate biocide susceptibility are not available, and harmonized breakpoints defining biocide tolerance are also lacking. Tolerance is defined as reduced susceptibility of bacteria toward a biocide characterized by a raised MIC (5). Determining epidemiological cutoffs (ECOFFs) for MICs and minimum bactericidal concentrations (MBCs) help interpret susceptibility profiles in a bacterial population. Currently, ECOFF data for biocides are limited to a few bacterial species (15, 16) but do not include *L. monocytogenes*. Epidemiological studies on biocide susceptibility mainly focused on the determination of MICs of QACs (17–19). MIC values provide only limited information on tolerance to in-use concentrations of disinfectants. Hence, MICs can only be interpreted as trend indicators for reduced biocide susceptibility. In addition, MBC values should be determined to evaluate lethality of the in-use concentration of a biocide (5).

Increased tolerance against antimicrobial stress triggered by the application of disinfectants may be an important factor for the persistence of *L. monocytogenes* in food production environments (20, 21). Particularly, members of the small multidrug resistance (SMR) protein family are associated with reduced susceptibility to quaternary ammonium compounds like benzalkonium chloride (BAC). The SMR transporter genes identified in *L. monocytogenes* are *qacH* (22), *emrE* (23), *emrC* (24), and the *bcrABC* cassette (25). The *bcrABC* cassette consists of a transcriptional regulator gene, *bcrA*, and two SMR genes (*bcrB* and *bcrC*). In addition, enhanced expression of efflux pump genes belonging to the major facilitator superfamily (MFS), such as *mdrL*, can contribute to BAC tolerance (26).

To the best of our knowledge, data on the biocide susceptibilities of *L. monocytogenes* isolates originating from Germany are not available, and a link between biocide tolerance and antibiotic resistance in *L. monocytogenes* has not yet been proven. We assume that the selection pressure in food processing plants is high because of the widespread use of disinfectants in hygiene processes. The aim of our study was to examine the biocide susceptibilities of *L. monocytogenes* isolates from food production plants in Germany and to look for potential relationships between biocide tolerance and antibiotic resistance. We therefore tested the susceptibilities to six antimicrobial biocides frequently used in the food industry and to antibiotics relevant for human listeriosis therapy. Further, we analyzed the genetic diversity of the *L. monocytogenes* strains under study and investigated the prevalence of putative biocide tolerance and antibiotic resistance genes as well as virulence genes.

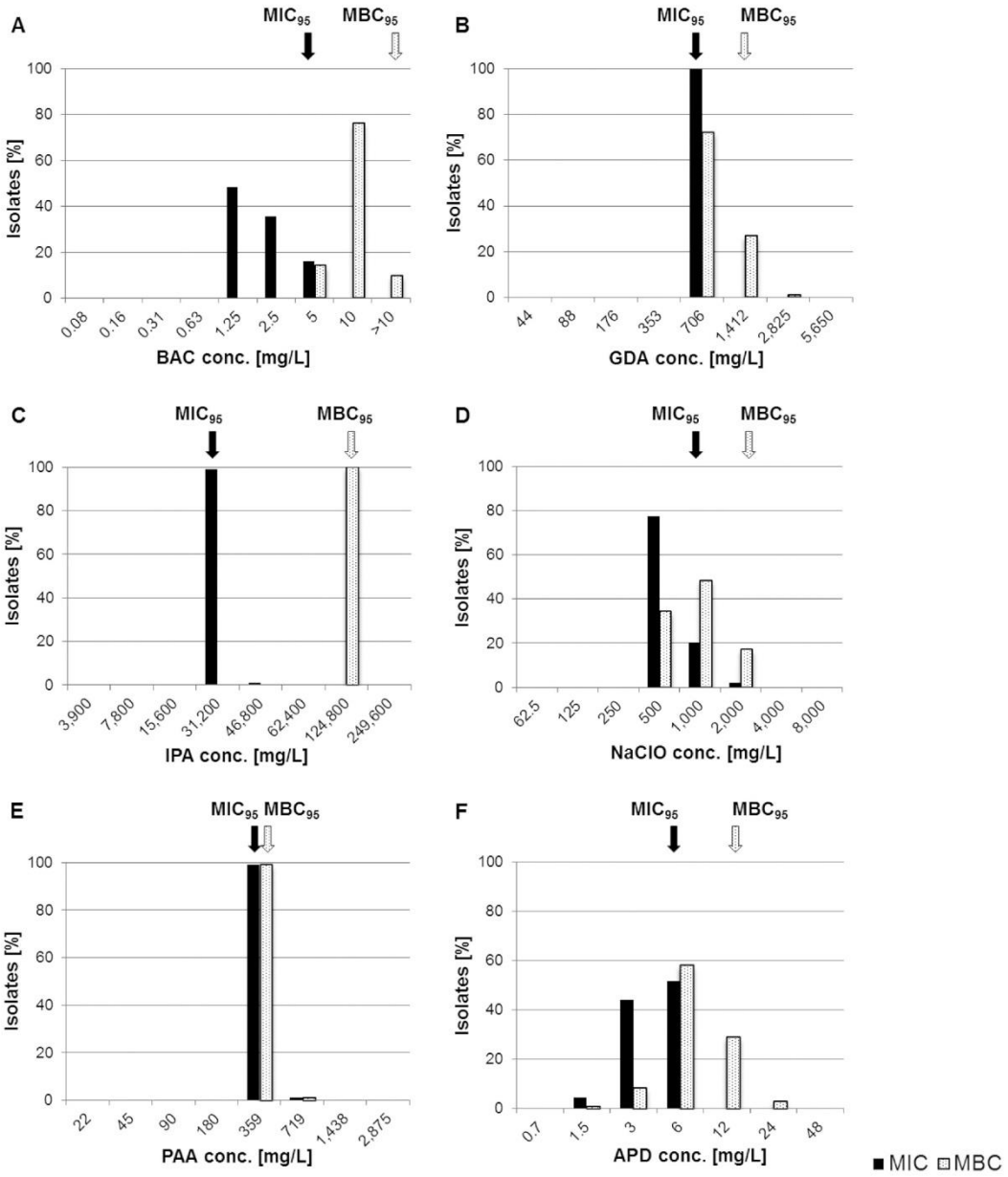

FIG 1 MIC (black bars) and MBC (white bars) distributions of 93 *L. monocytogenes* isolates. Arrows mark MIC_{95} (black) and MBC_{95} (white) values representing tentative ECOFFs. conc., concentration; ECOFF, epidemiological cutoff; BAC, benzalkonium chloride; GDA, glutaraldehyde; IPA, isopropanol; NaClO, sodium hypochlorite; PAA, peracetic acid; APD, biocidal product containing bis(3-aminopropyl)dodecylamine.

RESULTS

Phenotypic analysis. (i) Susceptibility to biocides. In pretests, the neutralizer used for MBC evaluation proved to be effective for all six biocides under investigation and revealed no toxicity (data not shown). An overview on the results of biocide susceptibility testing (MIC and MBC) is given in Fig. 1. MIC and MBC data were not normally distributed. Narrow unimodal MIC and MBC distributions ranging between one and three dilution steps were observed for all biocides. The only exception was bis(3-aminopropyl)dodecylamine (APD), showing a broader MBC distribution of five dilution steps.

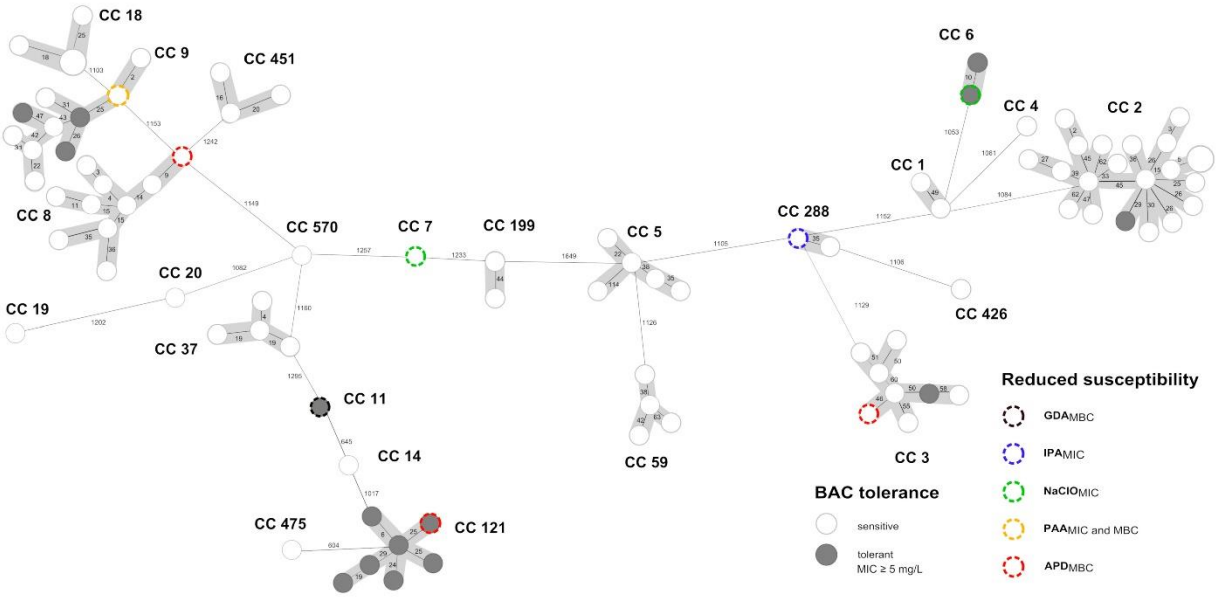

FIG 2 Minimum-spanning tree based on core genome MLST (cgMLST) allelic profiles of 93 *L. monocytogenes* isolates from food production environments in Germany. Each circle represents an allelic profile derived from sequence analysis of 1,701 cgMLST target genes. The size of each circle corresponds to the number of isolates. Numbers on the connecting lines illustrate numbers of target genes with differing alleles in a pairwise comparison. Isolates with reduced biocide susceptibility are color-coded as specified in the legend. Allelic patterns belonging to identical MLST clonal complexes (CC) are shaded in gray. BAC, benzalkonium chloride; GDA, glutaraldehyde; IPA, isopropanol; NaClO, sodium hypochlorite; PAA, peracetic acid; APD, biocidal product containing bis(3-aminopropyl)dodecylamine.

Tentative ECOFFs were empirically set, and isolates with reduced susceptibility toward the tested biocides revealed MIC and/or MBC values above the ECOFFs. Elevated MICs were found for isopropanol (IPA; $n = 1$) and sodium hypochlorite (NaClO; $n = 2$) (Fig. 1). Increased MBCs were detected for glutaraldehyde (GDA; $n = 1$) and APD ($n = 3$). One isolate showed both MIC and MBC values above the ECOFFs for peroxyacetic acid (PAA).

Applying the predefined MIC breakpoint for BAC (≥4 mg/liter) published previously (26, 27), 16% of the isolates ($n = 15$) were classified as BAC tolerant.

(ii) Antibiotic susceptibility testing. All isolates were daptomycin (DPT) resistant but ampicillin (AMP), penicillin G (PEN), vancomycin (VAN), erythromycin (ERY), gentamicin (GEN), linezolid (LIZ), tetracycline (TET), and trimethoprim-sulfamethoxazole (T/S) sensitive. Variable susceptibility patterns were observed for tigecycline (TGC; resistance [R], 76%), meropenem (MER; R, 8%), ciprofloxacin (CIP; susceptible, increased exposure [I], 5%), and rifampin (RAM; I, 1%) (see Table S1 in the supplemental material).

Five isolates (5%) were resistant to three different classes of antibiotics and therefore defined as multidrug resistant. Antibiotic resistance profiles did not differ significantly between biocide-tolerant and -susceptible isolates ($P > 0.05$). Spearman correlation coefficients revealed no association between biocide tolerance and antibiotic resistance (data not shown).

Genotypic characterization. (i) Genetic diversity of *L. monocytogenes*. Core genome multilocus sequence typing (cgMLST) revealed a broad genetic diversity among *L. monocytogenes* isolates from food production environments (Fig. 2). According to classical multilocus sequence typing (MLST), 23 sequence types (STs) belonging to 23 distinct clonal complexes (CCs) were identified. CC2 (23% [$n = 21$]), CC8 (11% [$n = 10$]), CC9 (11% [$n = 10$]), CC3 (9% [$n = 8$]), and CC121 (8% [$n = 7$]) were the most prevalent clonal complexes within this study.

Phenotypically, all isolates belonging to CC121 ($n = 7$), CC6 ($n = 2$), and CC11 ($n = 1$) were BAC tolerant. CC2, CC3, and CC9 isolates were either BAC susceptible or tolerant.

Three out of 15 BAC-tolerant isolates also showed reduced susceptibility to NaClO (CC6), GDA (CC11), or APD (CC121).

(ii) Detection of genes conferring biocide tolerance. In order to detect major determinants of reduced susceptibility or tolerance to biocides in the study population, we screened the translated amino acid sequences against the BacMet database. Five amino acid sequence motifs were exclusively present in BAC-tolerant isolates, whereas isolates tolerant to other biocides did not reveal unique determinants (Table 1). Four of the identified motifs belonged to SMR efflux transporters. Subsequently, the genomes of all isolates were screened for the presence of genes corresponding to the five amino acid sequence motifs, including *qacH*, *bcrABC*, and *emrC* (Fig. 3). *qacH* encoding an SMR efflux transporter was present in 10 out of 15 BAC-tolerant isolates. Single-nucleotide polymorphism (SNP) analysis revealed high similarities between nine of these genes and previously described *qacH* on the nucleotide level (GenBank accession no. HF565366.1) (22). In these cases, *qacH* was located on transposon Tn*6188* (Fig. 3). One isolate, however, carried a gene with 92% similarity to *qacH* but lacked the transposon. According to blastn analysis, the gene was identical to a sequence of an uncultured organism (GenBank accession no. KJ792090).

Both BAC-tolerant CC6 isolates carried *emrC*, a gene encoding another SMR efflux transporter. The *bcrABC* cassette, encoding BcrA, BcrB, and BcrC, was detected in one BAC-tolerant isolate belonging to CC9. The BcrA regulator was found in 10 more isolates lacking BcrBC. The detection of the complete *bcrA* gene sequence was limited to the *bcrABC* cassette carrying a CC9 isolate. The regulator sequences of the remaining 10 isolates revealed only small segments (sequence query coverages, 16 to 33%) with high similarities (≥80%) compared to *bcrA*. The regulator was located upstream of *qacH* in all 10 genomes and showed 85 to 100% similarity to *tetR* family transcriptional regulator genes.

We also screened for other genes that are known to convey BAC tolerance. The *emrE* gene, coding for an SMR efflux pump, could not be detected in our study population. However, the nonspecific efflux pump gene *mdrL* was present in all isolates tested.

(iii) Detection of antimicrobial resistance genes. In whole-genome sequencing (WGS) data, only the fosfomycin resistance gene *fosX* was detected, which was present in all isolates. Analysis of genes that can confer resistance to carbapenems (genes coding for penicillin-binding proteins [PBPs]) or to TGC (*rpsJ*) due to point mutations did not reveal any alterations in the sequence structure that have been previously linked to resistance.

(iv) Detection of virulence genes. We looked for various *Listeria*-specific virulence factors in the tested study population (Fig. 3). The stress survival islets 1 (SSI-1) and SSI-2 were identified in 43% ($n = 40$) and 9% ($n = 8$) of the isolates, respectively. SSI-2 was significantly more often identified in BAC-tolerant isolates than in susceptible isolates ($P < 0.001$). None of the *L. monocytogenes* isolates harbored the *Listeria* genomic island 1 (LG1). In contrast, LG2 was identified in 19 isolates of CC2.

Genes coding for *Listeria* pathogenicity island 1 (LIPI-1) were highly conserved in the study population. While none of the isolates harbored LIPI-2, which is a species-specific pathogenicity island of *Listeria ivanovii*, LIPI-3 was detected in 15% ($n = 14$) of the isolates belonging to CC1, CC3, CC4, CC6, and CC288. LIPI-4 was found in one isolate of CC4.

A full-length internalin A (*inlA*) gene was detected in 85% of the isolates. Most of the BAC-tolerant isolates ($n = 9$) harbored *inlA* genes with premature stop codons or deletions. While all isolates of CC121, four CC9 isolates, and one CC2 isolate harbored *inlA* genes with premature stop codons, both CC6 BAC-tolerant isolates showed a 9-bp deletion.

DISCUSSION

The consumption of contaminated food is the primary source of human listeriosis. *Listeria monocytogenes* can survive harsh conditions in food production facilities, such as low temperature, acidic environments, and disinfection procedures. Thus, contami-

TABLE 1 Unique biocide tolerance genes of BAC-tolerant *L. monocytogenes* isolates

BacMet database protein[a]	Species	UniProt accession no.	blastn similarity to *Listeria monocytogenes* NCBI:txid1639				Sample ID by clonal complex[b]															
			Corresponding gene	GenBank accession no.	Query cover (%)	Identity (%)	CC2	CC3	CC6		CC9			CC11	CC121							
							16-LI00597-0	16-LI00417-0	13-LI00032-0	13-LI00299-0	16-LI00532-0	2010-397-0	2011-31-0	13-LI00159-0	13-LI00147-0	14-LI00078-1	14-LI00080-0	14-LI00084-1	16-LI00145-0	16-LI00445-0	2010-253-0
BcrA	*L. monocytogenes*	I7B1B9	*bcrA*	JX023284.1	16–100	80–100	x	-	-	-	x	x	x	-	x	x	x	x	x	x	x
BcrB	*L. monocytogenes*	I7A797	*bcrB*	JX023284.1	100	100	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-
BcrC	*L. monocytogenes*	I6ZWM1	*bcrC*	JX023284.1	100	99	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-
Multidrug resistance protein	*Enterococcus faecalis*	Q82YU7	*emrC*	LT732640.1	100	100	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-
QAC resistance protein QacC	*S. aureus*	P14319	*qacH*	HG329628.1	100	92–100	x	-	-	-	x	x	-	-	x	x	x	x	x	x	x

[a]QAC, quaternary ammonium compound.
[b]ID, identification. x, present; -, absent.

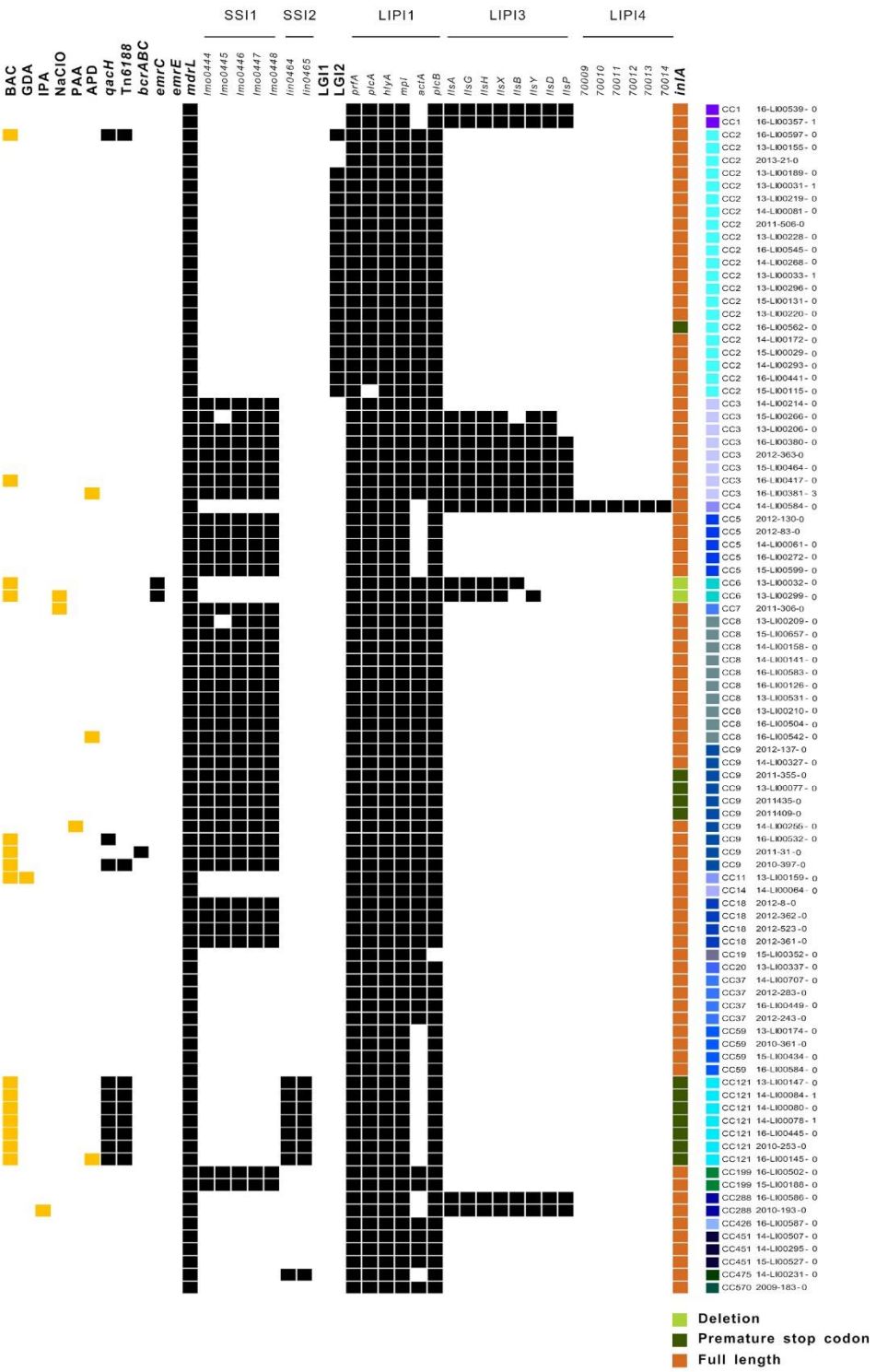

FIG 3 Distribution of biocide tolerance and virulence genes. *Listeria monocytogenes* isolates with reduced susceptibility to biocides are highlighted in yellow. The occurrence of genetic determinants is shown in black. Deletions and truncations of *inlA* are shaded green, whereas a full-length *inlA* is brown. BAC, benzalkonium chloride; GDA, glutaraldehyde; IPA, isopropanol; NaClO, sodium hypochlorite; PAA, peracetic acid; APD, biocidal product containing bis(3-aminopropyl)dodecylamine; SSI, stress survival islet; LIPI, *Listeria* pathogenicity island; LGI, *Listeria* genomic island; CC, clonal complex.

nation of food in the production process is recognized as a major transmission pathway (2). To obtain deeper insight into the properties of *L. monocytogenes* from German food production facilities, we investigated (i) biocide susceptibilities for frequently used substances in food processing plants, (ii) putative associations between reduced susceptibility to biocides and antibiotic resistance, and (iii) the genetic diversity, with a special focus on virulence factors, antibiotic resistance, and biocide tolerance.

Biocide susceptibility. Increased tolerance of *L. monocytogenes* to biocides used in disinfection measures appears to contribute to pathogen persistence, as previously shown for BAC (17, 21). Beside BAC, we examined five additional substances relevant for food hygiene with the aim to broaden the knowledge on the biocide susceptibility of *L. monocytogenes*. Although some isolates showed slightly increased MIC and/or MBC values to several biocides (Fig. 1), the resistance of these isolates under in-use concentrations is unlikely, because the MBC values measured were below the concentrations applied during disinfection (https://www.iho.de/). We determined tentative ECOFFs for all investigated substances to distinguish between susceptible isolates and isolates with reduced susceptibility. In our study, MIC and MBC values were not normally distributed, indicating the need for an increased number and diversity of isolates to be investigated in order to define more reliable ECOFFs. The tentative ECOFFs we defined reveal various limitations because of the lack of standardized biocide susceptibility testing methods, the small sample size investigated, and the fact that ECOFFs typically refer to normally distributed populations (28).

Previous studies applied a MIC breakpoint of ≥4 mg/liter to differentiate BAC-susceptible from -tolerant *L. monocytogenes* (26, 27). According to this definition, a high percentage of the isolates under study (16%) would be considered tolerant. Epidemiological studies from Switzerland and Norway reported similar prevalences of BAC-tolerant *L. monocytogenes* (29, 30). Higher rates ranging from 46% to 79% were observed in Turkey and Spain (31, 32). These data highlight the need for regular surveillance of biocide susceptibility, especially in the case of disinfectants widely used in food production facilities. Since November 2016, BAC has been listed as an unapproved disinfectant and preservative in the European Union (implementation decision 2016/1950). In the future, the reduced BAC application may lead to a decrease in the prevalence of BAC-tolerant *L. monocytogenes* isolates.

It proves difficult to compare epidemiological studies because of the variety of breakpoints defined for BAC tolerance (≥4 mg/liter up to 20 mg/liter) (17, 18, 21). In our study, we were able to show that 13 out of 15 (87%) *L. monocytogenes* isolates with MIC values of ≥5 mg/liter harbor genes which are known to contribute to BAC tolerance, such as *qacH*, *emrC*, and *bcrABC*.

The majority of BAC-tolerant isolates harbored the *qacH* gene located on the transposon Tn*6188*. In previous studies, *qacH* on Tn*6188* was predominantly found in isolates belonging to CC121 and CC9 (18, 21, 32) but was also reported in CC2 (21), which is in line with our results. Interestingly, one CC9 isolate carried a *qacH* gene that was not located on Tn*6188*. Alignment of the sequences revealed 92% similarity to Tn*6188*-carried *qacH* genes. This study reports *L. monocytogenes* harboring *qacH* in the absence of Tn*6188*.

The efflux transporter gene *emrC* was just recently identified in *L. monocytogenes* isolates belonging to CC6 (24). Kremer and colleagues further proved an association between reduced BAC susceptibility due to *emrC* and increased MICs for amoxicillin and gentamicin. In our study, *emrC* was detected in two CC6 isolates, but reduced antibiotic susceptibility was not observed, suggesting that the presence of *emrC* is not necessarily associated with antibiotic resistance. One BAC-tolerant CC9 isolate carried the *bcrABC* cassette (Fig. 2), which has been described before (21). In the two isolates which did not carry unique biocide tolerance genes, reduced susceptibility might have been induced by the overexpression of endogenous efflux pump genes, like *mdrL*, belonging to the MFS family (26).

Besides the known BAC tolerance genes, mechanisms have been described that might contribute to reduced susceptibility to NaClO, QACs, and PAA (27, 33, 34). In this context, biofilm formation or modifications of cell surface properties by alteration of membrane fatty acids and phospholipids that inhibit biocides to enter the cell have been discussed (27). Further, the glutamate decarboxylase system is well known as an acid tolerance system in *L. monocytogenes* (33). To what extent these mechanisms contribute to reduced susceptibility to NaClO, PAA, and other substances tested in our study needs to be elucidated.

Antibiotic susceptibility and cross-resistance. The fact that biocides like disinfectants can be a driver for antibiotic resistance becomes more and more a concern in the scientific community (5). *In vitro* studies demonstrated an association between biocide tolerance and reduced susceptibility to antibiotics in *L. monocytogenes* (12, 13). In our study, biocide tolerance and antibiotic resistance did not correlate, indicating that the mechanisms responsible for the determined BAC tolerance do not necessarily lead to cross-resistance to the tested antibiotics. Overall, antibiotic susceptibility profiles revealed a low level of resistance in *L. monocytogenes* isolated from food production environments in Germany. However, it is alarming that 8% of the isolates in our study were resistant to meropenem because this carbapenem may be used as alternative therapy for bacterial meningitis (35). In an epidemiological study from Poland, the prevalence of meropenem resistance (40%) in *L. monocytogenes* isolates from fish processing plants was even higher (36). In contrast, other studies did not detect meropenem resistance at all among isolates from meat processing plants or human patients (37, 38). In Gram-positive bacteria, carbapenem resistance can be associated with mutation-derived changes in their PBPs (39) which we could not detect in the meropenem-resistant isolates of our study.

All isolates tested were resistant to daptomycin, which is in line with the results from a previous study in our National Reference Laboratory focusing on food isolates (40). However, there are reports that described a lower prevalence of daptomycin resistance in *L. monocytogenes* (41, 42). Nevertheless, daptomycin cannot be recommended for the treatment of human listeriosis because of the reduced susceptibility of *L. monocytogenes* (42).

So far, daptomycin resistance mechanisms of *Listeria* spp. are not fully understood. Other Gram-positive bacteria like *Staphylococcus aureus*, *Enterococcus* spp., and *Streptococcus* spp., developed various strategies to counteract daptomycin, which mainly involve adaptive changes in the cell wall and cell membrane homeostasis (reviewed by Tran et al. [43]).

Resistance to tigecycline was very common in our study population, which was associated neither with the presence of *tetL* and *tetM* genes nor with mutations in *rpsJ*, resistance determinants that have been described for other Gram-positive bacteria (44, 45). The overexpression of unspecific efflux pumps can also be responsible for tigecycline resistance (46). In previous studies, tigecycline-resistant *L. monocytogenes* isolates were found in lower numbers (40, 41).

Genotypic diversity. Molecular typing of *L. monocytogenes* is essential in order to detect disease clusters and to identify food-related sources of infection as early as possible. Pulsed-field gel electrophoresis, the former gold standard for isolate differentiation in outbreak investigations, is increasingly replaced by WGS-based typing methods (47). In this way, the spatial and temporal distribution of *L. monocytogenes* genotypes can be compared. Our data revealed a broad heterogeneity of *L. monocytogenes* MLST clonal complexes in the food production environment, with CC2, CC8, and CC9 as predominant genotypes. In Germany, CC8 and CC2 isolates are frequently reported as causative agents of human listeriosis (48, 49). However, we also identified many isolates that belonged to genotypes of minor clinical importance in Germany, e.g., CC9 and CC121, which were defined as food-associated genotypes (50, 51). Due to limited sample access, our strain collection does not provide comprehensive informa-

tion on the nationwide prevalence of *L. monocytogenes* genotypes in German food production facilities.

Identification of virulence and stress response genes. *Listeria monocytogenes* is a heterogeneous species displaying various degrees of virulence (51). The ability of *L. monocytogenes* to survive harsh environmental conditions is enhanced in isolates carrying SSIs (52, 53). SSI-1 supports survival under acidic conditions and high salt concentrations (53) and can be found equally in isolates from humans, food, and food processing environments (52). Accordingly, we identified isolates of various clonal complexes that carried SSI-1. SSI-2 contributes to the survival of *L. monocytogenes* under alkaline and oxidative stress (52) and is predominantly found in ST121 isolates (belonging to CC121) (52, 54), which is in line with our results. Even though SSI-2 was significantly more frequently identified in BAC-tolerant isolates, Harter and colleagues were able to show that this gene cluster does not mediate tolerance to QACs (52).

LGIs have been associated with increased virulence, heavy metal resistance, and BAC tolerance (23, 55, 56). In our study, isolates only carried LGI2. LGI2 codes for genes involved in pathogenicity and arsenic resistance and seems to be widely present in clinical isolates belonging to CC1, CC2, and CC4 (56). We consistently detected LGI2 in most CC2 isolates (90%).

Carriage of LIPIs promotes virulence. LIPI-1, a pathogenicity island modulating host cell functions, is highly conserved in *L. monocytogenes* (57), and parts of this gene cluster were omnipresent in our isolate collection. LIPI-3 codes for a hemolytic and cytotoxic factor that impacts virulence and is associated with several clonal complexes, including CC1, CC4, and CC6 (58–60). We detected LIPI-3 in all isolates belonging to CC1 and in the single CC4 isolate. In addition, this pathogenicity island was present in all CC3, CC6, and CC288 isolates. We found LIPI-4 only in the single CC4 isolate of our study. LIPI-4 was recently identified in clinical *L. monocytogenes* isolates of CC4 and is linked to hypervirulence (51).

The *inlA* gene codes for a protein that is involved in the invasion of human intestinal epithelial cells and is considered an important virulence factor of *L. monocytogenes*. Premature stop codons resulting in the truncation of *inlA* are associated with attenuated virulence. They are predominantly detected in nonhuman isolates (61). Consistent with previous findings (51), all CC121 and several CC9 isolates (40%) from our study harbored truncated *inlA* genes. Franciosa et al. showed that isolates with a truncated *inlA* gene displayed increased capacity for biofilm formation (62), which may be associated with biocide tolerance and persistence properties.

Listeria monocytogenes from German food production facilities obviously carried virulence factors contributing to human infection. While some of the genes known to be involved in virulence were present in all or most of the isolates under study, others only occurred in specific clonal complexes.

Conclusion. Our study revealed a high genetic diversity among *L. monocytogenes* isolates from technical equipment and surfaces of German food production facilities. The detection of genotypes that are frequently involved in human listeriosis highlights the importance of contaminated food production environments as transmission routes for virulent *L. monocytogenes*. Phenotypic tolerance to BAC was observed in 15 isolates (16%), and efflux pump genes conferring BAC tolerance were identified in 13 of them. Exposure to low concentrations of quaternary ammonium compounds can occur as a result of improper disinfection practices and may enhance the ability of selected isolates to persist in niches within food production environments. However, given the low overall prevalence of biocide-tolerant isolates, it is likely that additional factors contribute to the persistence of *L. monocytogenes*, including the ability to form biofilms.

BAC tolerance and the presence of BAC tolerance genes were not associated with antibiotic resistance, indicating that the mechanisms responsible for reduced BAC susceptibility in the investigated isolates do not confer antibiotic resistance. Moreover, most of the BAC-tolerant isolates harbored internalin A pseudogenes which are known to occur in isolates that exhibit reduced virulence and enhanced biofilm-forming ability.

Altogether, our study does not support significant associations between biocidal selective pressure in food production and antimicrobial tolerance of *L. monocytogenes*. However, from *in vitro* studies, we know that links between biocide tolerance and antibiotic resistance do exist in bacteria. The widespread use of disinfectants might therefore lead to a selection of antibiotic-resistant isolates and needs regular monitoring. Last but not least, a better understanding of the phenotypic traits that contribute to the survival and persistence of *L. monocytogenes* in food processing plants and their underlying genetic determinants is required and a prerequisite for infection control of listeriosis.

MATERIALS AND METHODS

***Listeria monocytogenes* isolates.** Ninety-three *L. monocytogenes* isolates, collected by official food control authorities from 2008 through 2016 in German food production plants and archived at the National Reference Laboratory for *L. monocytogenes* (Germany), were characterized (Table S1). The isolates originated from various surfaces of food processing facilities and equipment, such as slicers, cutting boards, handles, sinks, grinders, cutting tables, derinders, gutters, tubes, and floor drains. Species identification was carried out by biochemical and molecular typing, as previously described (40). Isolates were stored at −80°C until use. Isolates were chosen under consideration of the source and year of isolation, with the main aim of including a highly diverse study population.

Biocides. Susceptibility of the *L. monocytogenes* isolates was tested to six biocides commonly used to sanitize food contact surfaces, namely, the quaternary ammonium compound BAC (≥95%; Sigma-Aldrich, Steinheim, Germany), GDA (50%; Carl Roth, Karlsruhe, Germany), IPA (≥99.9%; Carl Roth), the chlorine-releasing compound NaClO (12% Cl, techn.; Carl Roth), the oxidizing agent PAA (36 to 40% [wt/vol]; Sigma-Aldrich), and a biocidal product (Budenat Intense D443; Buzil-Werk Wagner, Memmingen, Germany) containing APD (7.5% [wt/wt]) as an active ingredient. The biocides were serially diluted in 2-fold steps just before the experiment using standardized hard water as defined in EN 1276, as follows: 10 to 0.08 mg/liter BAC, 5,650 to 44 mg/liter GDA, 249,600 to 3,900 mg/liter IPA, 8,000 to 62.5 mg/liter free chlorine (NaClO), 2,875 to 22 mg/liter PAA, and 48 to 0.7 mg/liter APD in Budenat.

Biocide susceptibility testing. (i) MICs. The MICs of the biocides under study were determined by broth microdilution assays. An overnight culture of each isolate grown on tryptic soy agar (TSA; Merck, Darmstadt, Germany) was adjusted to about 10^6 CFU/ml 2-fold concentrated tryptic soy broth (TSB; Merck). In a 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany), 50 μl of the bacterial solution was added to 50 μl of the double-concentrated biocide. The plate was incubated at 37°C for 20 ± 2 h. Optical density at 595 nm (OD_{595}) was measured after 5 s of shaking using the Mithras^2 multimode reader (Berthold Technologies, Bad Wildbad, Germany; Software MikroWin 2010 v5.18, German UI). Bacterial growth was compared to a negative control (microtiter well containing biocide solution and TSB), and a ΔOD_{595} of 0.1 was considered the cutoff value. The MIC was defined as the lowest concentration of a biocide at which no growth was observed. Biological replicates derived from two independent experiments were conducted on different dates. A MIC variation of one dilution step between the two experiments was accepted. The lower value was defined as the MIC. In case of higher variation, the test was repeated once more, and the median was considered the final MIC.

(ii) Minimum bactericidal concentration. The MBC of each strain and biocide was determined by broth microdilution according to Knapp et al., with minor modifications (63). Dey-Engley neutralizing broth (Sigma-Aldrich) was used to quench biocidal effects for MBC testing. The neutralizer efficacy and toxicity were tested before according to Knapp et al. (64). The MBC was defined as the lowest concentration of the biocide which revealed no visible colonies on TSA.

Determination of tentative ECOFFs. According to EUCAST guidelines (28) tentative ECOFFs can be defined to distinguish between susceptible isolates and isolates with reduced antibiotic susceptibility. Following this approach, tentative ECOFFs of unimodal MIC or MBC distributions were defined for tested biocides at concentrations representing 95% of the bacterial population (MIC_{95} and MBC_{95}, respectively).

Antibiotic susceptibility testing. Antibiotic susceptibilities (S) to AMP (S, ≤2 mg/liter), CIP (S, ≤1 mg/liter; R, ≥4 mg/liter), DPT (S, ≤1 mg/liter), ERY (S, ≤0.5 mg/liter; R, ≥8 mg/liter), GEN (S, ≤4 mg/liter; R, ≥16 mg/liter), LIZ (S, ≤4 mg/liter; R, ≥8 mg/liter), MER (S, ≤0.25 mg/liter), PEN (S, ≤2 mg/liter), RAM (S, ≤1 mg/liter; R, ≥4 mg/liter), TET (S, ≤4 mg/liter; R, ≥16 mg/liter), TGC (S, ≤0.5 mg/liter; R, >0.5 mg/liter), T/S ($S_{T/S}$, ≤0.05/9.5 mg/liter), and VAN (S, ≤2 mg/liter; R, ≥16 mg/liter) were determined using the commercial test system Micronaut S *Listeria* MHK-2 (Merlin Gesellschaft für Mikrobiologische Diagnostika mbH, Bornheim, Germany), as previously described (40). Resistance was assessed using clinical breakpoint guidelines of the Clinical and Laboratory Standards Institute (CLSI) (65, 66). If no breakpoints for *L. monocytogenes* were available, those recommended for *Staphylococcus* spp. were applied. Since CLSI breakpoints for tigecycline were missing, cutoffs defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used (67).

Statistical analysis. Spearman rank coefficients (Rho) were calculated to investigate the correlation of MICs or MBCs between tested biocides and antibiotics using SPSS (IBM SPSS Statistics, v21; IBM Corp., Armonk, NY, USA). Data were tested for normal distribution by the Kolmogorov-Smirnov test. For comparative analysis between two groups of isolates (biocide sensitive versus biocide tolerant), the Mann-Whitney test was applied. *P* values of <0.05 were considered to be significant.

Next-generation sequencing. *Listeria monocytogenes* isolates were cultivated on sheep blood agar (SBA). A single colony was transferred into brain heart infusion (BHI) bouillon and incubated at 37°C for 18 to 20 h while shaking (150 rpm). DNA was extracted from bacterial cells using the PureLink genomic

DNA minikit (Invitrogen, Carlsbad, CA, USA). WGS libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. Paired-end sequencing (2 × 301 cycles) was performed with the MiSeq reagent v3 600-cycle kit (Illumina) on an Illumina MiSeq benchtop sequencer.

First, sequence read quality was analyzed with FastQC v0.11.5 (Babraham Bioinformatics, Cambridge, United Kingdom). Second, sequence reads were assembled using SPAdes v3.10.0 with the options BayesHammer read error correction, postprocessing mismatch corrector with BWA, and an automatic coverage filter (68). Third, assembly quality was analyzed using Quast v4.5 by comparison to the *L. monocytogenes* type strain EGD-e (NCBI:txid169963, NCBI RefSeq accession no. NC_003210.1).

Classical MLST and cgMLST. For phylogenetic comparison of the *L. monocytogenes* isolates, classical MLST and cgMLST were performed on the basis of WGS data. Classical MLST and corresponding clonal complexes were determined according to the scheme of the Institut Pasteur (https://bigsdb.pasteur.fr/listeria/). cgMLST analysis was carried out using the Ridom SeqSphere+ software (v4.0.1; Ridom GmbH, Münster, Germany), according to Ruppitsch et al. (69). The cgMLST scheme relies on a set of 1,701 target genes that are present in >99% of the known genomes of the species. The combination of all alleles in a strain forms a profile that can be used to characterize the phylogenetic relationships among isolates.

***In silico* screening for biocide resistance determinants on protein level.** WGS data of the *L. monocytogenes* isolates under study were screened for the presence of experimentally confirmed resistance proteins recorded in the BacMet database (70) (Antibacterial Biocide and Metal Resistance Genes database, http://bacmet.biomedicine.gu.se/, BacMet v2, last updated 9 December 2017).

The rapid prokaryotic genome annotation software Prokka v1.12 (71) was used to delimit open reading frames (ORFs) in the draft genomes and to annotate protein-coding genes by hierarchical feature prediction at the amino acid sequence level with BLAST+ v2.6.0 and HMMER v3.1b2.

The BacMet database of "experimentally confirmed resistance genes" included 753 amino acid sequences which were uploaded into Prokka as a user-provided set of annotated proteins for the initial round of feature prediction. The annotation of the most significant match (E value, >31) within the BacMet database was transferred to an ORF. BacMet-flagged *Listeria* ORFs were counted and summarized in a genome/feature table for subsequent correlation with phenotypic data (Table S1).

Analysis of biocide tolerance determinants and virulence factors on nucleotide level. Comparative analyses of genes conferring biocide tolerance were carried out using the BioNumerics software v7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium).

We analyzed genes coding for SMR efflux transporters, i.e., *qacH* on the transposon Tn*6188* (GenBank accession no. HF565366), *emrC* (GenBank accession no. LT732640.1), *emrE* (GenBank accession no. CP001602), *bcrABC* (GenBank accession no. JX023284.1), and *mdrL* (GenBank accession no. AJ012115.1) coding for an efflux pump belonging to the MFS.

Furthermore, we looked for the following virulence factors: SSI-1 (GenBank accession no. NC_003210) and SSI-2 (NC_003212.1), LGI1 (CP001602) and LGI2 (CM001159.1), and the *Listeria* pathogenicity islands (LIPI-1, AL591974.1; LIPI-2, AJ004808.1; LIPI-3, AE017262.2; and LIPI-4, CYWW02000024.1). Additionally, the coding sequence for *inlA* (NC_003210) was investigated to determine whether isolates possess a full-length gene, deletions, or truncated sequences indicated by a premature stop codon. A minimum % sequence identity (%ID) threshold of 80% and a minimum length of 80% of the target gene were used for sequence identification.

Investigation of antibiotic resistance genes. Acquired antibiotic resistance determinants were identified by ResFinder 3.0 (Center for Genomic Epidemiology; http://www.genomicepidemiology.org/) (72). *Listeria monocytogenes* penicillin binding protein genes *lmo1892*, *lmo2039*, *lmo1438*, *lmo2229*, *lmo0441*, *lmo2754*, *lmo0540*, *lmo1916*, *lmo1855*, and *lmo2812* (NCBI RefSeq accession no. NC_003210) were analyzed for single-nucleotide polymorphisms which might contribute to meropenem resistance (73, 74). Furthermore, the *rpsJ* gene (NCBI RefSeq accession no. NC_003210) was analyzed for point mutations, which have been previously associated with TGC resistance in *Enterococcus faecium* (44).

Data availability. The sequences of three representative *qacH* genes of 16-LI00597-0, 13-LI00147-0, and 16-LI00532-0 were deposited in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) under accession numbers MK944275 to MK944277, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01253-19.

SUPPLEMENTAL FILE 1, XLSX file, 0.3 MB.

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Supplementary material for

Publication 1

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The separate Excel file for Table S1 containing the isolate information, phenotypic raw data on antibiotic susceptibility and *in silico* screening for biocide resistance determinants can be accessed under:

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6.2 Publication 2

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Genetic but no Phenotypic Associations between Biocide Tolerance and Antibiotic Resistance in *Escherichia coli* from German Broiler Fattening Farms

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Abstract: Biocides are frequently applied as disinfectants in animal husbandry to prevent the transmission of drug-resistant bacteria and to control zoonotic diseases. Concerns have been raised, that their use may contribute to the selection and persistence of antimicrobial-resistant bacteria. Especially, extended-spectrum β-lactamase- and AmpC β-lactamase-producing *Escherichia coli* have become a global health threat. In our study, 29 ESBL-/AmpC-producing and 64 NON-ESBL-/AmpC-producing *E. coli* isolates from three German broiler fattening farms collected in 2016 following regular cleaning and disinfection were phylogenetically characterized by whole genome sequencing, analyzed for phylogenetic distribution of virulence-associated genes, and screened for determinants of and associations between biocide tolerance and antibiotic resistance. Of the 30 known and two unknown sequence types detected, ST117 and ST297 were the most common genotypes. These STs are recognized worldwide as pandemic lineages causing disease in humans and poultry. Virulence determinants associated with extraintestinal pathogenic *E. coli* showed variable phylogenetic distribution patterns. Isolates with reduced biocide susceptibility were rarely found on the tested farms. Nine isolates displayed elevated MICs and/or MBCs of formaldehyde, chlorocresol, peroxyacetic acid, or benzalkonium chloride. Antibiotic resistance to ampicillin, trimethoprim, and sulfamethoxazole was most prevalent. The majority of ESBL-/AmpC-producing isolates carried $bla_{\text{CTX-M}}$ (55%) or $bla_{\text{CMY-2}}$ (24%) genes. Phenotypic biocide tolerance and antibiotic resistance were not interlinked. However, biocide and metal resistance determinants were found on mobile genetic elements together with antibiotic resistance genes raising concerns that biocides used in the food industry may lead to selection pressure for strains carrying acquired resistance determinants to different antimicrobials.

Keywords: *Escherichia coli*; biocide tolerance; antibiotic resistance; biocide determinants; virulence; food safety

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1. Introduction

Escherichia coli is a gram-negative, non-sporulating facultative anaerobe, a widespread gut commensal of vertebrates, and a versatile pathogen [1]. Pathogenic *E. coli* are categorized as intestinal pathogenic (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC) [2]. The latter colonize the gut of healthy hosts without causing disease but by entering extraintestinal sites ExPEC can lead to urinary tract infections, meningitis, skin infections, or sepsis [3]. In addition to affecting humans, avian pathogenic *E. coli* (APEC), the avian pathotype of ExPEC, causes severe economic losses to the poultry industry and may

represent a zoonotic risk [4]. Multidrug-resistant bacteria (particularly those producing extended-spectrum ß-lactamases (ESBL) and/or AmpC ß-lactamases (AmpC)) are a growing threat to food safety [5,6]. ESBL-/AmpC-producing *E. coli* from healthy hosts were classified as commensal strains but recent investigations indicated that they also show characteristics of ExPEC or ExPEC-like strains [3,7]. Humans can be exposed to ESBL-/AmpC-producing pathogens via human-to-human transmission, food, animal, and environmental sources [8]. A high prevalence of ESBL-/AmpC-producing Enterobacteriaceae was previously demonstrated on broiler farms [9–11]. Recent studies suggested that contaminated broiler chicken farms might play an important role in the transmission of ESBL-/AmpC-producing Enterobacteriaceae into the environment [12,13]. Luyckx et al. detected *E. coli* in broiler houses following hygiene measures, highlighting drain holes or floor cracks as critical locations for cleaning and disinfection (C&D) [14,15]. Biocides like quaternary ammonium compounds (QACs), aldehydes, oxidizing agents, organic acids, and cresols are widely used in animal husbandry and food processing plants to prevent microbial growth. However, concerns have been raised that the continued exposure to biocides in industrial settings including food production environments may trigger mechanisms that alter both biocide and antibiotic susceptibility and select for antimicrobial-resistant strains [16,17]. *E. coli* uses multiple pathways to overcome environmental stresses. Acid stress, for instance, is counteracted by a range of physiological, metabolic, and proton-consuming acid resistance mechanisms [18]. Biocide tolerance is a multifactorial process and can include several mechanisms such as target modification [19], biofilm formation [20], changes of cell envelope permeability [21], or the activity of efflux pumps [22]. Proteins involved in tolerance to quaternary ammonium compounds (QACs) include members of the small multidrug resistance (SMR) efflux family such as SugE(c), SugE(p), EmrE, YdgE/YdgF, QacE, QacEΔ1, QacF, QacG, QacH, and QacI as well as members of the major facilitator superfamily (MFS) such as MdfA [23–26].

So far, laboratory methods to investigate biocide susceptibility are not standardized [27,28] and to the best of our knowledge, only one study evaluated epidemiological cutoffs (ECOFFs) for *E. coli* to a limited set of biocidal compounds [29]. As little is known about the link between biocide selection pressure and antibiotic resistance in *E. coli* field isolates in Germany we aimed to characterize a commensal *E. coli* study population including ESBL-/AmpC-producing and NON-ESBL-/AmpC-producing *E. coli* from broiler fattening farms following cleaning and disinfection. Because of the widespread use of disinfectants in hygiene processes, we assumed a high selective pressure in the investigated farm environment. We tested susceptibilities to seven biocides frequently used in farm hygiene and to antibiotics relevant for human and veterinary medicine. In addition, we characterized the genetic diversity of the *E. coli* strains including ExPEC associated virulence genes, and looked for associations between biocide tolerance, antibiotic resistance, and the presence of putative genetic determinants of antimicrobial resistance.

2. Materials and Methods

2.1. E. coli Isolates

A panel of 93 *E. coli* isolates collected in 2016 from three broiler fattening farms after cleaning and disinfection measures were investigated (Table S1). The isolates originated from surfaces of grounds, walls, and equipment such as air inlets, drains, door handles, tractors (for food and litter), electric cables, feeding and drinking troughs from four barns. *E. coli* were isolated from swab samples on MacConkey agar with and without cefotaxime. Species identification and differentiation of ESBL-/AmpC-producing *E. coli* were performed as previously described [30]. In brief, MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) was applied to suspicious isolates for species identification. Beta-lactamase genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, and CIT-type pAmpC genes were identified using a multiplex real-time PCR [31] as well as Sanger sequencing [30]. Isolates were selected from different sources to obtain a highly diverse study population including ESBL-/AmpC- and NON-ESBL-/AmpC-producing *E. coli* (farm 1: barn 1, n = 27 including 13 AmpC-producing

E. coli; barn 2, *n* = 15 including five ESBL-producing *E. coli*; farm 2: barn 3, *n* = 21 including three ESBL-producing *E. coli*; farm 3: barn 4, *n* = 30 including eight ESBL-producing *E. coli*). C&D protocols applied in the barns comprised dry cleaning, wet cleaning, and two disinfection steps. During dry cleaning, bedding and feed were removed. For wet cleaning all-purpose cleaners were used. Disinfection was carried out using formaldehyde-based disinfectants followed by either chlorocresol-based disinfectants (barns 1 and 4) or lime solutions (barns 2 and 3).

2.2. Whole Genome Sequencing

E. coli isolates were cultivated on sheep blood agar. A single colony was transferred into Miller's lysogeny broth (LB) (Merck KGaA, Darmstadt, Germany) and incubated at 37 °C for 19 ± 1 h with shaking at 150 rpm. DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Whole-genome sequencing (WGS) libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end sequencing (2 × 301 cycles) was performed using the MiSeq Reagent v3 600-cycle Kit (Illumina) on an Illumina MiSeq benchtop sequencer. Raw fastq data were trimmed and assembled using the AQUAMIS pipeline (https://gitlab.com/bfr_bioinformatics/AQUAMIS (accessed on 9 July 2018)) based on trimmomatic (version 0.36.), fastp (version 0.19.5), unicycler (version 0.4.4), spades (version 3.11.1), pilon (version 1.22), mash (version 2.1), and quast (version 4.6.3).

2.3. Phylogenetic Analysis

For phylogenetic analysis, multilocus-sequence typing (MLST) was performed using WGS data. The classical MLST scheme defined by alleles of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*, database hosted at the University of Warwick) was applied. MLST types were determined using the MLST 2.0 webtool of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org (accessed on 6 October 2018)) [32]. For phylogroup assignment, a multiplex PCR was conducted as described previously [33] with minor modifications. The total reaction mixture of 25 µL contained 0.2 µM of each primer (except for TspE4C2.1b (0.4 µM) and TspE4C2.2b (0.4 µM)), 12.5 µL of DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, Schwerte, Germany), 5 µL of PCR Water and 2.5 µL of the template DNA. An initial denaturation step of 3 min at 94 °C was followed by 33 PCR cycles with 30 s of denaturation at 94 °C, primer binding for 30 s at 57 °C, and 1 min of elongation at 72 °C, as well as a final elongation step of 5 min at 72 °C. Isolates belonging to phylogroups A and C or E and D were not further differentiated and assigned to phylogroup A/C or E/D, respectively. Furthermore, we determined genetic relatedness between *E.coli* isolates with ParSNP v1.0 [34]. The maximum-likelihood tree was calculated by FastTree2 [35] and visualized with EMBL interactive tree of life, iTOL v4 (https://itol.embl.de/, accessed on 20 September 2019).

2.4. Biocide Susceptibility Testing

2.4.1. Biocides

Susceptibility of the *E. coli* isolates was tested against the two biocides formaldehyde (FA, Carl Roth, Karlsruhe, Germany) and chlorocresol (p-chloro-m-cresol, PCMC, Merck KGaA) used for C&D on the farms under study and five biocides commonly applied in farm hygiene, namely the quaternary ammonium compounds benzalkonium chloride (BAC, Sigma Aldrich, Steinheim, Germany) and didecyldimethylammonium chloride (DDAC, Merck KGaA), hydrogen peroxide (HP, Carl Roth), peroxyacetic acid (PAA, VWR, Dresden, Germany), and acetic acid (AA, Carl Roth). Biocides were serially diluted in 2-fold steps just before the experiment using standardized hard water as defined in EN 1276. The following final concentration ranges were tested: 320 to 5 mg/L BAC, 40 to 0.3 mg/L DDAC, 640 to 5 mg/L FA, 1024 to 8 mg/L HP, 2000 to 16 mg/L PAA, 16,384 to 128 mg/L AA, and 4000 to 63 mg/L PCMC.

2.4.2. Minimum Inhibitory Concentration (MIC)

Biocide MICs were determined using broth microdilution. Overnight cultures grown on tryptic soy agar (TSA; Merck KGaA) were adjusted to about 10^6 CFU/mL in 2-fold concentrated tryptic soy broth (TSB; Merck KGaA). In 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany), 50 µL of the bacterial suspension was added to 50 µL of the double-concentrated biocide solution. Plates were incubated at 37 °C for 20 ± 2 h. Optical density at 595 nm (OD595) was measured after 5 s of shaking using the Mithras2 multimode reader (Berthold Technologies, Bad Wildbad, Germany; Software MikroWin 2010 v5.18, German UI). Bacterial growth was compared to a negative control (microtiter well containing biocide solution and tryptic soy broth, Thermo Fisher Scientific) and a $\Delta OD_{595\,nm}$ of 0.08 was applied as the cut-off value. The MIC was defined as the lowest concentration of a biocide at which no growth was observed. Three independent experiments were performed on different days and the median was considered as the final MIC.

2.4.3. Minimum Bactericidal Concentration (MBC)

The MBC of each strain and biocide was determined by broth microdilution according to Knapp et al., with minor modifications [28]. Dey-Engley neutralizing broth (Sigma-Aldrich) was used to quench biocidal effects for MBC testing. Neutralizer efficacy and toxicity were tested before [36]. The MBC was defined as the lowest concentration of a biocide, which revealed no visible colonies after subculture on tryptic soy agar (TSA, Thermo Fisher Scientific). The reference strain *E. coli* ATCC 25922 was used as internal quality control in both MIC and MBC tests and showed comparable results throughout the experiments.

2.4.4. Determination of MIC_{95}/MBC_{95}

To distinguish between biocide susceptible isolates and isolates with reduced susceptibility, the MIC (or MBC) that encompassed 95% of all MIC (or MBC) values in the distribution was designated as MIC_{95} (or MBC_{95}).

2.5. Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined by broth microdilution using the Sensititre system with EUVSEC/EUVSEC2 plates (Thermo Fisher Scientific) in concordance with the decision 2013/652/EU of the European Union. The following antimicrobial substances were used: Ampicillin, AMP; Azithromycin, AZI; Cefepime, FEP; Cefoxitin, FOX; Ceftazidime, TAZ; Cefotaxime, FOT; Cefotaxime/Clavulanic acid, F/C; Ceftazidime/Clavulanic acid, T/C; Chloramphenicol, CHL; Ciprofloxacin, CIP; Colistin, COL; Ertapenem, ETP; Gentamicin, GEN; Imipenem, IMI; Meropenem, MERO; Nalidixic acid, NAL; Sulfamethoxazole, SMX; Temocillin, TRM; Tetracycline, TET; Tigecycline, TGC; Trimethoprim, TMP. We followed CLSI guidelines and defined resistance using epidemiological cut-offs according to EUCAST.

2.6. Statistical Analysis

Spearman rank coefficients (Rho) were calculated to investigate the correlation of MICs or MBCs between tested biocides and antibiotics using SPSS (IBM SPSS Statistics, Version 21, IBM corp., Armonk, NY, USA). Data were tested for normal distribution by the Kolmogorov-Smirnov test. For comparative analysis between two groups of isolates (e.g., ESBL-/AmpC- versus NON-ESBL-/AmpC-producing isolates) the Mann-Whitney-test was applied. Statistically significant differences between antimicrobial resistance or distribution of virulence determinants in different genetic lineages were tested using the chi2 test and Fisher's exact test. p-values < 0.05 were considered to be significant.

2.7. In Silico Screening for Biocide and Metal Tolerance Determinants at Protein Level

WGS data of the *E. coli* isolates under study were screened for the presence of 753 experimentally confirmed biocide- and metal-resistance proteins recorded in the BacMet database [37] (Antibacterial Biocide and Metal Resistance Genes database; http://bacmet.

biomedicine.gu.se/, BacMet version 2, last updated on 9 December 2017, accessed on 5 December 2018) as described before [38].

2.8. Detection of Biocide Tolerance and Virulence Determinants at Nucleotide Level

The presence of genes conferring biocide tolerance was determined as previously described [38]. The genomes of all isolates were screened for genes encoding for small multidrug resistance (SMR) transporters, i.e., *qacEΔ1*, *qacE*, *qacF*, *qacH*, *qacI*, *qacG*, *emrE*, *sugE(c)*, *sugE(p)*, *ydgE*, *ydgF*, and for the multidrug efflux pump gene *mdfA* of the major facilitator superfamily (MFS). In addition, we screened for genes involved in formaldehyde and acid tolerance. An overview of the investigated genes and corresponding accession numbers is given in Table S2. A minimum sequence identity (%ID) threshold of 80% and a minimum length of 80% of the target gene were defined for the detection of biocide determinants except for *qacEΔ1* and *qacE* (100%ID and 100% minimum length).

In addition, we screened for the presence of 49 virulence genes typically associated with ExPEC including fitness factors that are found in pathogenic and commensal strains (Table S2). Virulence-associated genes (VAGs) were chosen from public databases contained in the *E. coli* functional genotyping plugin (version 1.01) of Bionumerics or from previously published reports [7,39,40]. A minimum sequence identity (%ID) threshold of 90% and a minimum length of 60% of the target gene were used for the identification of VAGs.

2.9. Identification of Antibiotic Resistance Genes

Acquired antibiotic resistance determinants and chromosomal mutations leading to antibiotic resistance were identified using ResFinder 3.0 (Center for Genomic Epidemiology, http://www.genomicepidemiology.org, accessed on 11 January 2019 [41]).

2.10. Accession Numbers of Whole-Genome Sequences

Genome sequence data of the strains under study have been deposited at the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/, accessed on 9 March 2021)) under accession numbers JAFMWT000000000-JAFMVF000000000 (see Table S1).

3. Results

3.1. Phylogenetic Diversity and Virulence-Associated Genes

PCR-based phylotyping of the 93 *E. coli* isolates revealed seven different banding patterns associated with phylogroups A (n = 8), A/C (n = 13), B1 (n = 34), B2 (n = 5), E (n = 2), E/D (n = 7), and F (n = 24). *E. coli* isolates belonged to 30 known and two unknown multilocus sequence types (STs). The most prevalent STs were ST117 (n = 21; two NON-ESBL-/AmpC-producing *E. coli* from barns 2 and 3, 19 ESBL-/AmpC-producing *E. coli* from all barns) and ST297 (n = 10; six AmpC-producing *E. coli* from barn 1, and four NON-ESBL-/AmpC-producing *E. coli* from barn 4) (Figure 1).

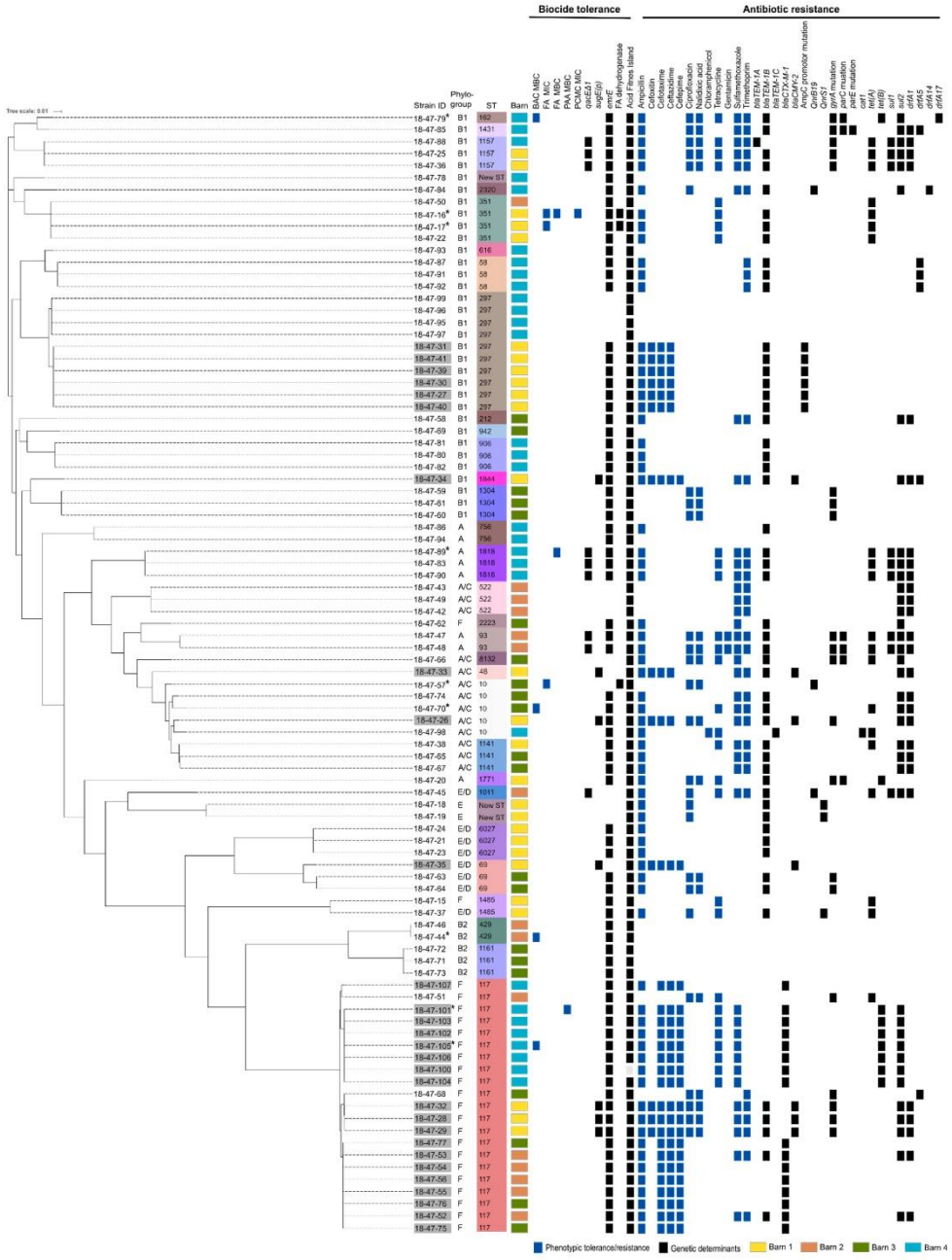

Figure 1. Phylogenetic tree of 93 *E. coli* isolates from broiler fattening farms including their phenotypic biocide tolerance and antibiotic resistance as well as the distribution of biocide tolerance and antibiotic resistance-conferring genes. An asterisk marks biocide tolerant strains. Reduced susceptibility to biocides and antibiotic resistance are indicated for each isolate as blue squares, tolerance, and resistance-conferring genes as black squares. Further information on ESBL-/AmpC-producing *E. coli* phenotype (grey shaded strain ID) and multilocus sequence type (ST) are provided. The affiliation to different barns are highlighted in yellow (barn 1), orange (barn 2), green (barn 3), and blue (barn 4). BAC = Benzalkonium chloride, FA = Formaldehyde, PCMC = Chlorocresol (p-chloro-m-cresol).

Up to 27 ExVAGs (VAGs associated with ExPEC) (55%) were detected in ST117 strains (phylogroup F), up to 23 ExVAGs (47%) in ST429 (phylogroup B2), and up to 20 ExVAGs (41%) in ST69 (phylogroup E/D) (Table S1). All isolates were positive for *fimH* (type 1 fimbriae), *feoB* (ferrous iron transporter, protein B), and *ompA* (outer membrane protein A). The *iss* (increased serum survival protein) and *fimA* (type 1 fimbriae) genes were present in 77 (83%) and 74 (80%) isolates, respectively. Twenty-one VAGs were significantly associated with phylogroup F. Certain genetic determinants such as *papC*, *papEF*, *papG-allele II* (P fimbriae formation), *ireA* (iron-responsive element), and *hlyE* (hemolysin E) exclusively occurred in isolates belonging to ST117 of phylogroup F, whereas *vat* (vacuolating autotransporter toxin) was present in ST429 (phylogroup B2) and some ST117 (phylogroup F) isolates. Iron capture systems were frequently represented in the genomes, but the number of encoding genes varied considerably among isolates from 1 to 11. Iron uptake systems were most prevalent in ST117 and ST429 isolates.

3.2. Susceptibility to Biocides

MIC and MBC data showed non-normal, unimodal distributions ranging between one and three dilution steps for all biocides (Figure 2). MIC and/or MBC values above MIC_{95}/MBC_{95} indicated isolates with reduced susceptibility to the tested biocides.

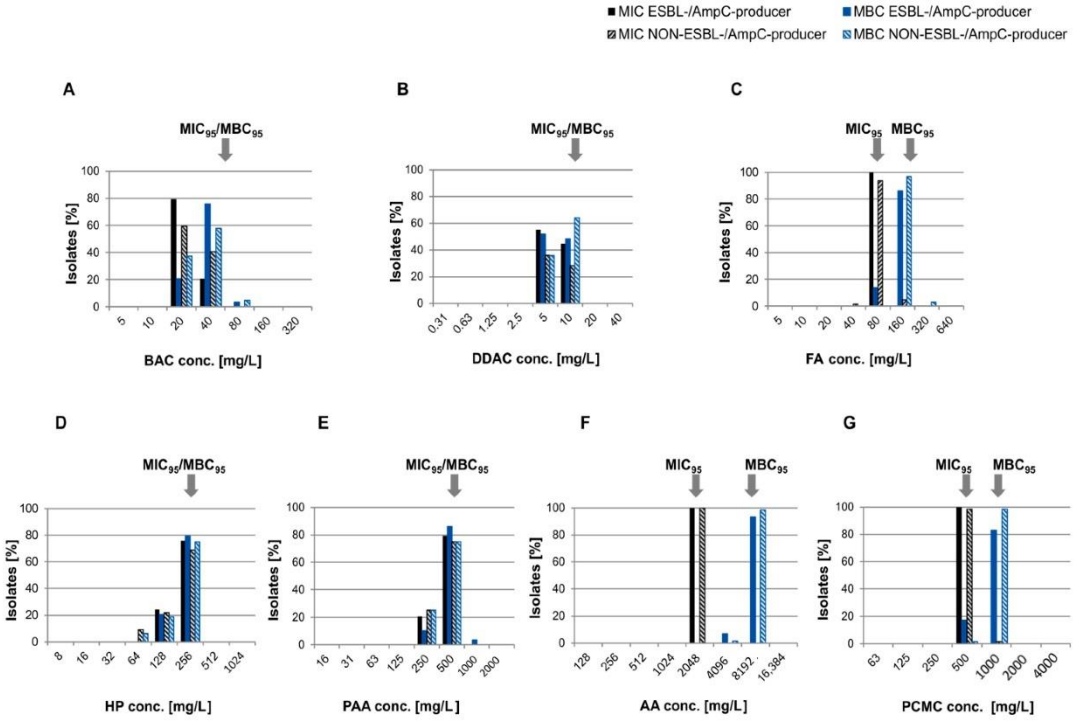

Figure 2. MIC and MBC distributions of ESBL-/AmpC-producing and NON-ESBL-/AmpC-producing *E. coli* isolates for common biocides used in farm hygiene. Black bars = MIC ESBL-/AmpC-producing *E. coli*, black striped = MIC NON-ESBL-/AmpC-producing *E. coli*, blue bars = MBC ESBL-/AmpC-producing *E. coli*, blue striped = MBC NON-ESBL-/AmpC-producing *E. coli*. Arrows mark MIC_{95} and MBC_{95} representing cut-off values for isolates with reduced susceptibility. (**A**) BAC = Benzalkonium chloride, (**B**) DDAC = Didecyldimethylammonium chloride, (**C**) FA = Formaldehyde, (**D**) HP = Hydrogen peroxide, (**E**) PAA = Peracetic acid, (**F**) AA = Acetic acid, (**G**) PCMC = Chlorocresol (p-chloro-m-cresol).

These biocide-tolerant isolates were found in all barns (barn 1: n = 2; barn 2: n = 1, barn 3: n = 2, barn 4, n = 4), and mostly originated from transitions between wall and floor

as well as from cracks and crevices in the ground (Table S1). An individual NON-ESBL-/AmpC-producing *E. coli* isolate (ST351) displayed elevated MIC (160 mg/L) and MBC (320 mg/L) values of FA and an elevated MIC of PCMC (1000 mg/L). Furthermore, three NON-ESBL-/AmpC-producing *E. coli* showed either an elevated MIC (160 mg/L, n = 2, ST10, ST351) or MBC value (320 mg/L, n = 1, ST1818) of FA. Increased MBCs were also detected for PAA (1000 mg/L, n = 1, ESBL-producing *E. coli*, ST117) and BAC (80 mg/L, n = 4, three NON-ESBL-/AmpC-producing *E. coli*, ST10, ST162, ST429, and one ESBL-producing *E. coli*, ST117) (Figure 1).

3.3. Susceptibility to Antibiotics

All isolates were sensitive to carbapenems (ETP, IMI, MERO, COL and TGC). Antibiotic resistance to AMP (100% ESBL-/AmpC-producing *E. coli*, 63% NON-ESBL-/AmpC-producing *E. coli*), SMX (52% ESBL-/AmpC-producing *E. coli*, 36% NON-ESBL-/AmpC-producing *E. coli*), and TMP (28% ESBL-/AmpC-producing *E. coli*, 39% NON-ESBL-/AmpC-producing *E. coli*) were most common in both groups (Figure 1). Thirty-four isolates (37%) were resistant to at least one antibiotic in three or more classes and therefore defined as multidrug-resistant (MDR). Two isolates from barn 2 were resistant to antibiotics in five substance classes including aminoglycosides, ß-lactams, fluoroquinolones, sulfonamides, and tetracyclines.

3.4. In Silico Analysis of Determinants Conferring Biocide and Metal Tolerance

Out of 753 proteins potentially conferring biocide or metal tolerance 249 were identified in our study population (Table S3). Four tolerance determinants were exclusively present in three isolates with increased MIC values of FA (18-47-16 (ST351), 18-47-17 (ST351), and 18-47-57 (ST10). Three of these determinants belonged to an arsenic resistance operon whereas the other one was annotated as nickel/cobalt efflux transporter NcrC that is involved in nickel and cobalt resistance. All isolates under study harbored glutathione- and NAD-dependent formaldehyde dehydrogenase with ≥80% nucleotide identity to the reference (Genbank Acc. No. X73835) found in the formaldehyde-tolerant strain *Escherichia coli* VU3695 [19]. The three isolates with reduced susceptibility to formaldehyde harbored an additional formaldehyde dehydrogenase with 99.6% identity to X73835. Sequence analysis revealed only synonymous mutations compared to the reference (Figure 3).

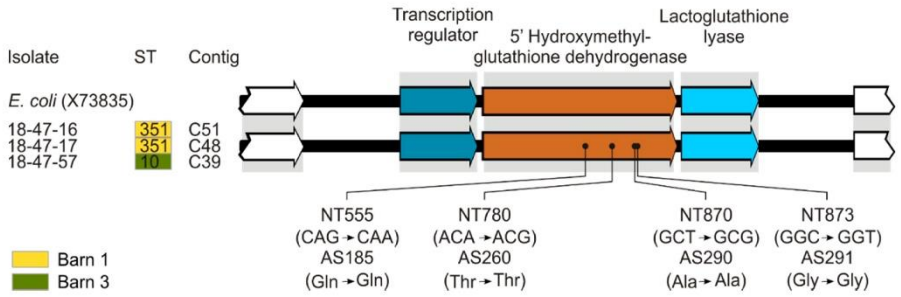

Figure 3. Glutathione-dependent formaldehyde dehydrogenases of *E. coli* isolates compared to the plasmid-encoded reference X73835. The alignment was created using Bionumerics and adjusted by CorelDraw Graphic Suite 3.0 (version 17) for better interpretation. Relevant CDS (arrows) were labeled by protein function based on RAST annotation.

Genes of the *E. coli* acid fitness island were found in all but one isolate of the study population. SMR efflux pump genes *sugE(c)*, *ydgE*, and *ydgF* and the MFS efflux pump gene *mdfA* were always present. We could not detect genes encoding the QAC-specific efflux determinants QacE, QacG, QacF, QacI, and QacH. Seventy-nine isolates (85%) carried *emrE*. The SMR efflux pump gene *qacEΔ1* was detected in nine NON-ESBL-/AmpC-producing *E. coli* isolates (10%) of ST93 (n = 2), ST1011 (n = 1), ST1157 (n = 3), and ST1818 (n = 3) taken at

different sampling sites in the barns 1, 2 and 4 (Figure 4A). *SugE(p)* was detected in seven plasmid-mediated AmpC β-lactamase-(pAmpC-)producing *E. coli* isolates (8%) from barn 1 (ST117 (n = 3), ST10 (n = 1), ST48 (n = 1), ST69 (n = 1), ST1844 (n = 1)) (Figure 4B). However, the presence of efflux determinants was not associated with reduced susceptibility to tested biocides.

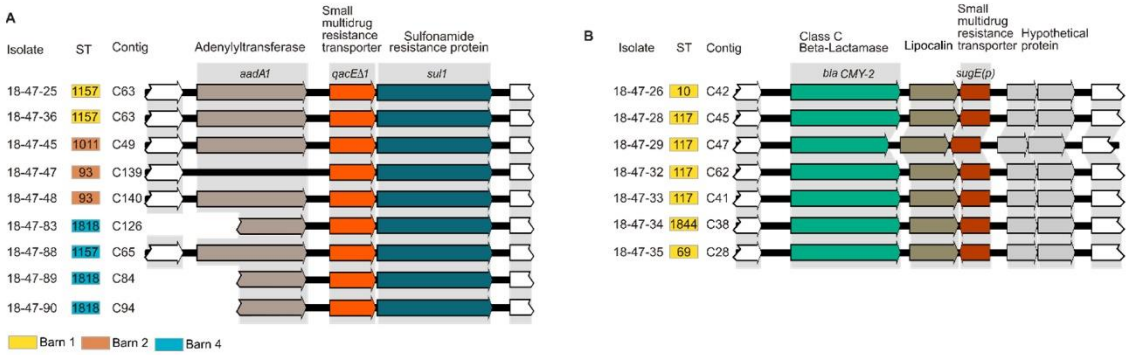

Figure 4. Colocalization of biocide tolerance determinants and antibiotic resistance genes. (**A**) SMR efflux pump encoding gene *qacEΔ1* located between aminoglycoside (*aadA1*) and sulfonamide (*sul1*) resistance genes on the same contig. (**B**) SMR efflux pump encoding gene *sugE(p)* located downstream of class C beta-lactamase. The alignment was created using Bionumerics and adjusted by CorelDraw Graphic Suite 3.0 (version 17) for better interpretation. Relevant CDS (arrows) were labeled by protein function based on RAST annotation.

3.5. In Silico Analysis of Antibiotic Resistance Gene Profiles

Phenotypic antibiotic resistance could be attributed to known genetic resistance determinants except for gentamicin (Figure 1). Identified determinants responsible for beta-lactam antibiotic resistance were $bla_{TEM\text{-}1A}$ (n = 1, ST1157, barn 4), $bla_{TEM\text{-}1B}$ (n = 52, 25 STs from all barns), $bla_{TEM\text{-}1C}$ (n = 1, ST10, barn 4), $bla_{CTX\text{-}M\text{-}1}$ (n = 16, ST117, barns 2, 3, and 4) and $bla_{CMY\text{-}2}$ (n = 7, ST10, ST48, ST69, ST117, ST1844, barn 1) as well as *ampC* promotor mutations (n = 6, ST297, barn 1). Target mutations of *gyrA* (n = 20, 10 STs from all barns), *parC* (n = 6, ST93, ST162, ST1431, ST1771, ST8132, from all barns) and/or *parE* (n = 1, ST1431, barn 4) as well as the resistance genes *qnrB19* (n = 3, ST10, ST1011, ST2320, barns 2, 3, and 4) and *qnrS1* (n = 3, ST1485, unknown ST, barn 1) were found in quinolone resistant isolates. Chloramphenicol resistance could be attributed to the presence of *cat1* (n = 1, ST10, barn 4). All tetracycline resistant isolates were positive for *tet(A)* (n = 20, 10 STs from all barns) or *tet(B)* (n = 9, ST117, ST162, ST1771, barns 1 and 4). In sulfonamide and trimethoprim resistant isolates the resistance genes *sul1* (n = 9, ST93, ST1011, ST1157, ST1818, barns 1, 2, and 4) and *sul2* (n = 38, 16 STs from all barns) as well as *drfA1* (n = 27, 12 STs from all barns), *drfA5* (n = 6, ST58, ST117, ST1431, ST1844, barns 1, 3, and 4), *drfA14* (n = 1, ST2320, barn 4) and/or *drfA17* (n = 1, ST162, barn 4) were present.

3.6. Association Between Reduced Biocide Susceptibility and Antibiotic Resistance and Co-occurrence of Antimicrobial Resistance Genes

Antibiotic resistance was not significantly associated with reduced susceptibility to biocides. There was also no significant difference between isolates from different barns. In addition, ESBL-/AmpC-producing isolates were in general not less susceptible to biocides than NON-ESBL-/AmpC-producing isolates. On the contrary, a higher proportion of NON-ESBL-/AmpC-producing *E. coli* showed reduced susceptibility in terms of MBCs of FA and PCMC compared to ESBL-/AmpC-producing *E. coli* (Figure 2). Interestingly, several isolates carried biocide and metal tolerance genes on mobile genetic elements closely linked to antibiotic resistance genes. For example, eight *qacEΔ1*-positive isolates carried *qacEΔ1*, *sul1*, and *aadA1* on the same contig (Figure 4A). These determinants could

be found downstream of an integron-integrase (*intI1*) gene in four out of nine isolates verifying their localization on a class 1 integron. The same element carried a mercury-resistance operon. Similarly, all *sugE(p)*-positive isolates ($n = 7$) carried *sugE(p)* and bla_{CMY-2} on the same contig (Figure 4B). Sequence data revealed genes associated with conjugal transfer and transcription in close proximity indicating plasmid localization of *sugE(p)* and bla_{CMY-2}.

4. Discussion

Our study aimed at investigating (i) the phylogenetic diversity and virulence determinants, (ii) potential relationships between susceptibilities to biocides and antibiotics, and (iii) genetic determinants of biocide tolerance and antibiotic resistance of *E. coli* isolates from German broiler fattening farms. The study population consisted of 93 isolates sampled after C&D. Most of the field isolates belonged to phylogroup B1 and F. While phylogroup B1 and A mainly comprise commensals or intestinal pathogens [42], phylogroup F are frequently associated with ExPECs in humans, companion animals, and birds [43–45]. Furthermore, ExPEC strains are closely related to avian pathogenic *E. coli* suggesting poultry as a reservoir of zoonotic APEC strains [39,46]. APEC can cause avian colibacillosis, which threatens poultry flocks worldwide. Three of the STs detected in our study, ST10, ST48, and ST117 have been previously linked to APEC strains [47–49] and were also isolated from human patients [50–52], emphasizing a zoonotic risk. ST297, which is known to be highly prevalent in environmental and food samples, and ST69 were also found in our study population and can be pathogenic for poultry and humans [53]. In general, our data revealed a broad heterogeneity of *E. coli* isolates on German broiler fattening farms with variable numbers of virulence-associated genes involved in adhesion, iron uptake, and cytotoxic activity. ST117 (phylogroup F) and ST429 (phylogroup B2) carried the highest number of iron uptake-related genes. Similarly, Projahn et al. observed a high prevalence of determinants involved in iron acquisition in ST117 isolates collected during the years 2014 and 2015 from German broiler meat production chains [7]. *E. coli* can survive extreme acid stress [54] making use of amino acid-dependent and independent resistance mechanisms [55]. One of the amino acid-dependent systems, encoded by 12 genes of the acid fitness island, is highly conserved in *E. coli* and was found in virtually all isolates of our study population.

Escherichia coli can survive hygiene measures, persist over a long period of time, and spread throughout the barns of broiler chicken farms [14,15,56,57]. Overall, phenotypic biocide susceptibility testing did not prove tolerance to disinfectants within our study population since MIC and MBC values of the biocides tested were well below in-use concentrations. Modal MIC values of *E. coli* determined for BAC [58–62], DDAC [63,64], FA [58,60,61], AA [58,65], PAA [66] and PCMC [67,68] in previous studies were similar to our results. In contrast, modal MIC and MBC values of HP reported for avian pathogenic *E. coli* differed by two dilution steps (64 versus 256 mg/L) [61]. So far, breakpoints to distinguish between biocide susceptible and tolerant isolates are missing. Morrissey et al. [29] suggested ECOFFs for the most commonly applied biocides such as BAC, chlorhexidine, triclosan, and sodium hypochlorite considering various species including *E. coli*. According to published MICs (>64 mg/L) and MBCs (>128 mg/L) of BAC, none of our *E. coli* isolates could be defined as tolerant. However, MIC values of biocides are difficult to compare across studies because experimental conditions have not yet been harmonized. In this context, Slipski et al. compared different antimicrobial susceptibility test methods (broth, agar spot colony, and pegged lid biofilms) and showed that the mode of bacterial growth significantly influenced QAC tolerant phenotypes related to SMR over-expression [69]. Thus, standardized methods are urgently needed.

Based on the MIC_{95}/MBC_{95} values determined, nine isolates from our study population showed reduced susceptibility to at least one biocide (Figures 1 and 2). Six of these isolates were taken from transitions between floor and wall or cracks and crevices. These are well-known critical locations in broiler houses because they are difficult to clean and disinfect [14,15], and exposure to subinhibitory concentrations of biocides in such niches is very likely. Three out of the nine isolates showed elevated MICs of formaldehyde

and one isolate additionally had an elevated MIC of chlorocresol. The most widespread bacterial pathway for formaldehyde detoxification involves a glutathione-dependent dehydrogenase catalyzing the reversible formation of S-formylglutathione and NADH from formaldehyde, glutathione, and NAD [70]. Enzymatic degradation of formaldehyde by a plasmid-encoded variant of the enzyme has been previously described as a formaldehyde resistance mechanism in *E. coli* [19,71–73]. In our study, the plasmid-encoded variant of the formaldehyde dehydrogenase was exclusively present in isolates displaying elevated MICs of formaldehyde (160 mg/L) indicating that this enzyme may contribute to the observed phenotype. Interestingly, genes involved in arsenic and nickel/cobalt resistance were also uniquely detected in these formaldehyde tolerant isolates.

SMR efflux pumps are known to confer resistance to a variety of substances, including QACs and antibiotics [23,24,26,74–77], and are commonly found in *E. coli* [59,64,78,79]. Since QACs are frequently used for cleaning and disinfection in the food industry, strains armed with appropriate biocide tolerance mechanisms have an increased ability to persist in food processing environments. Not only drugs and toxic metabolites are expelled from bacterial cells by multidrug efflux pumps, molecules that may be important for cell communication, biofilm formation, and osmoregulation or protection of the cell are also released [76,80].

In our study, all isolates harbored the putative QAC tolerance conferring genes *sugE(c)*, *ydgE*, *ydgF*, and *mdfA*, while *qacE*, *qacF*, *qacG*, *qacH*, and *qacI* were absent. These results are in line with previous findings on the prevalence of *ydgE/ydgF* (87–100%), *mdfA* (86–100%), and *qac* genes (0–18%) in *E. coli* isolates from different sources [64,79]. The SMR transporters *emrE*, *qacEΔ1*, and *sugE(p)* were detected in varying frequencies within our study population. Nevertheless, our data were similar to those obtained from other epidemiological studies on *E. coli* isolated from poultry meat, meat products, and farms in Germany [81], the United States [79], and China [64]. The contribution of *qacEΔ1* as a partially functional derivative of *qacE* [82] on QAC tolerance is controversially discussed [83]. As described before [81,84], we were not able to show an association between the presence of *qacEΔ1* and reduced QAC susceptibility. The SMR efflux pump SugE has its role in QAC tolerance [26,64] with a rather narrow substrate specificity, including cetyltrimethyl ammonium, cetyldimethyl ammonium, cetylpyridinium, and cetrimide cations [69,74], which may explain the phenotypic susceptibility to BAC and DDAC of isolates carrying *sugE(p)* in our study.

Antibiotic resistance profiles were generally consistent with zoonoses monitoring data of commensal *E. coli* from broiler fattening farms in Germany, 2016 [85]. However, 8.3% colistin-resistant isolates were reported in the national monitoring program, whereas colistin resistance was not found in our study population. A significant number of isolates showed resistance to three or more classes of antibiotics including critically important antimicrobials as classified by the World Health Organization such as quinolones and 3rd generation cephalosporins [86]. With the exception of gentamicin, all phenotypic resistances could be traced back to genetic determinants. Different mechanisms are known to confer gentamicin resistance. Most common are enzymes modifying the drug by acetylation (aminoglycoside acetyltransferase, AAC), adenylation (adenylate aminoglycoside nucleotidyltransferase, ANT) or phosphorylation (aminoglycoside phosphotransferase, APH) [87,88]. Mutations in the ribosomal target have also been described [89], but could not be confirmed in our isolates. According to clinical breakpoints of CLSI, *E. coli* is supposed to be resistant to gentamicin if MIC $\geq$ 16 mg/L [90]. As our isolates had MIC values below the clinical but above epidemiological cut-off (ECOFF 2 mg/L), these isolates may have developed resistance. Within the EU, gentamicin is not authorized for use in poultry [91] and resistance is rarely found in conventional broiler stocks in Germany (1.3% in 2016) [85].

In vitro studies showed that antibiotic cross-resistance can occur during bacterial exposure to subinhibitory concentrations of biocides like QACs [92], biguanides [93], and phenolic compounds [94]. The *E. coli* isolates in our study revealed no association between phenotypic biocide tolerance and antibiotic resistance as described before [60,95]. On the contrary, FA and PCMC killed ESBL-/AmpC-producing *E. coli* at slightly lower

concentrations than NON-ESBL-/AmpC-producing *E. coli*. Similarly, lower MICs of DDAC were reported for ESBL-/AmpC-producing *E. coli* in another study [81].

The biocide tolerance determinants *qacEΔ1* and *sugE(p)* were located on mobile genetic elements in close proximity to the antibiotic resistance genes *sul1* and bla_{CMY-2}, respectively. *QacEΔ1* is common in enteric bacteria and is typically associated with the presence of class 1 integrons that carry the sulfonamide resistance determinant *sul1* explaining why all *qacEΔ1* positive isolates showed co-resistance to sulfamethoxazole [96]. On the same genetic element, several mercury resistance genes were observed, which frequently occur on plasmids together with antibiotic resistance genes and the *qacEΔ1* gene [97]. Furthermore, multiple gene cassettes can be arranged in tandem within these elements conferring additional resistance to ß-lactams, tetracycline, gentamicin as well as aminoglycosides [59,64,79]. Worldwide, bla_{CMY-2} is associated with pAmpC-producing *E. coli* from poultry [98]. The genetic element, bla_{CMY-2}-*blc-sugE*, has already been found in IncK plasmids of *E. coli* isolated from humans in Spain and poultry in Norway and Switzerland [99–101]. Plasmids carrying *sugE(p)* and bla_{CMY-2} antibiotic resistance genes have been detected in various STs of *E. coli* [99,101,102] and may be spread by conjugative transfer to different reservoirs. Even though isolates carrying *qacEΔ1* or *sugE(p)* did not show reduced susceptibility to the QACs investigated in our study, the use of QACs in broiler fattening farms may provide selection pressure to strains that carry genes encoding resistance to clinically important antibiotics [64].

5. Conclusions

Our study revealed a high genetic diversity of *E. coli* isolates from German broiler fattening farms including genotypes characteristic of ExPEC strains. Our findings support the hypothesis that poultry farm environments may act as a reservoir of human ExPEC and could play a role in the spread of facultative pathogenic *E. coli*. While the overall prevalence of biocide tolerant strains was low, the detection of isolates carrying formaldehyde tolerance determinants and at the same time showing a reduced MIC to the compound indicates that the use of disinfectants could have provided selection pressure. The QAC tolerance determinants *qacEΔ1* and *sugE(p)* were both located on mobile genetic elements in close proximity to antibiotic resistance genes. In this case, disinfectants may simultaneously select strains with acquired resistance to other antimicrobials. Whether disinfectants can be a driver of antibiotic resistance in zoonotic pathogens from stable to table has to be clarified to assess the consumer risks related to hygiene measures.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/3/651/s1, Table S1: Supplementary information of investigated isolates, Table S2: Biocide tolerance genes and virulence-associated genes (VAGs) screened in this study, Table S3: In silico analysis of determinants conferring biocide tolerance.

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Supplementary material for

Publication 2

Roedel A, Vincze S, Projahn M, Roesler U, Robé C, Hammerl JA, Noll M, Al Dahouk S, Dieckmann R. 2021. **Genetic but no Phenotypic Associations between Biocide Tolerance and Antibiotic Resistance in *Escherichia coli* from German Broiler Fattening Farms. Microorganisms** 9(3),651. https://doi.org/10.3390/microorganisms9030651

Index:

Supplementary Table S2

Biocide tolerance genes screened in this study

Gene	Gene description	Accession no.	Source
qacEΔ1	quaternary ammonium compound efflux SMR transporter	JN596280	(1)
qacE	quaternary ammonium compound-resistance protein	X68232	(1)
qacF	quaternary ammonium compound efflux SMR transporter	JN596279	(1)
qacH	quaternary ammonium compound resistance protein	FJ160769	(1)
qacI	quaternary ammonium compound resistance protein	HQ875011	(1)
qacG	quaternary ammonium compound resistance protein	FJ950725	(1)
emrE	efflux-multidrug resistance protein	AIGY01000024	(1)
mdfa	multidrug efflux pump/Na(+):H(+) antiporter/K(+):H(+) antiporter	Y08743	(1)
sugE(c)	SMR family transporter	X69949	(1)
sugE(p)	SMR family transporter	HQ023864	(1)
ydgE	multidrug transporter subunit	NC_011745	(1)
ydgF	multidrug transporter subunit	NC_011745	(1)
BW690_25775	S-(hydroxymethyl)glutathione dehydrogenase	X73835.1	(2)
slp (Acid Fitness Island)	starvation lipoprotein	NC_000913	(3)
yhiF (Acid Fitness Island)	putative DNA-binding transcriptional regulator	NC_000913	(3)
yhiD (Acid Fitness Island)	inner membrane protein	NC_000913	(3)
hdeB (Acid Fitness Island)	periplasmic acid stress chaperone	NC_000913	(3)
hdeA (Acid Fitness Island)	acid stress chaperone	NC_000913	(3)
hdeD (Acid Fitness Island)	acid-resistance membrane protein	NC_000913	(3)
gadE (Acid Fitness Island)	DNA-binding transcriptional activator	NC_000913	(3)
yhiU (Acid Fitness Island)	multidrug efflux pump membrane fusion protein	NC_000913	(3)
yhiV (Acid Fitness Island)	multidrug efflux pump RND permease	NC_000913	(3)
gadW (Acid Fitness Island)	DNA-binding transcriptional dual regulator	NC_000913	(3)
gadY (Acid Fitness Island)	small regulatory RNA	NC_000913	(3)
gadX (Acid Fitness Island)	DNA-binding transcriptional dual regulator	NC_000913	(3)
gadA (Acid Fitness Island)	glutamate decarboxylase A	NC_000913	(3)

Virulence associated genes (VAGs) screened in this study

Gene	Gene description	Accession no.	Source	Group
afaB/afaC	afimbrial-adhesin-encoding gene	X76688.1	(4)	adhesion
afaE	afimbrial-adhesin-encoding gene	M12868	(5)	adhesion
bmaE	M-agglutinin subunit	M15677	(4)	adhesion
fimA	type-1 fimbrial protein, A chain	NC_000913.3	(6)	adhesion
fimC	periplasm fimbrial chaperone protein	CP004009.1	(7)	adhesion
fimH	mannose-specific adhesin of type 1 fimbriae	AJ225176	(4)	adhesion
focG	F1C fimbriae subunit	S68237	(4)	adhesion
gafD	G-fimbrial lectin protein	L33969	(4)	adhesion
iha	bifunctional enterobactin receptor/adhesin protein	GU725392	(8)	adhesion
papA	fimbrial major pilin protein	X61239	(4)	adhesion
papC	fimbrial major pilin protein	X61239	(4)	adhesion
papEF	fimbrial major pilin protein	X61239	(4)	adhesion
papG allele I	fimbrial major pilin protein	X61239	(4)	adhesion
papG allele I'	fimbrial major pilin protein	X61239	(4)	adhesion
papG-allele-II	fimbrial major pilin protein	M20181	(4)	adhesion
sfa/foc	S and F1C fimbriae subunits	DQ301498	(4)	adhesion
sfaS	S fimbriae minor subunit SfaS	CP000243	(4)	adhesion
tsh	temperature-sensitive hemagglutinin	AY545598/ AF218073	(9), (10)	adhesion
chuA	outer membrane hemin receptor	U67920.1	(11)	iron uptake
feoB	ferrous iron transporter, protein B	GU361604.1	(12)	iron uptake
fyuA	yersiniabactin/pesticin outer membrane receptor	Z38064	(13)	iron uptake
ireA	iron-responsive element	AE014075	(8)	iron uptake
iroD	salmochelin siderophore system, ferric enterochelin esterase	DQ381420.1	(14)	iron uptake
iroN	iron outer membrane receptor	AF449498	(14)	iron uptake
irp-2	yersiniabactin biosynthetic protein	L18881.1	(15)	iron uptake
iucA	N(2)-citryl-N(6)-acetyl-N(6)-hydroxylysine synthase	X76100.1	-	iron uptake
iucD	Iron uptake chelate protein D	M18968.1	(16)	iron uptake
iutA	aerobactin receptor	X05874	(4)	iron uptake
sitA	structural injection transglycosylase	AY126440.1	(17)	iron uptake
flicC (H7)	H7 variant of the Escherichia coli flagellin gene	NC002695	(8)	miscellaneous
ibeA	invasion of brain endothelium	AF289032	(4)	miscellaneous
ompT	Protease 7	41044	(8)	miscellaneous
PAI(malX)	pathogenicity islands (PAIs)	AF00372	(4)	miscellaneous
cvi-cvaC	colicin V immunity protein-colicin V synthesis protein	X57525	(18)	protectins

Gene	Gene description	Accession no.	Source	Group
iss	increased serum survival protein	CP001855	(8)	protectins
kpsMT (K1)	Kl capsular polysaccharide	M57382.1	(4)	protectins
kpsMT II	group 2 capsular polysaccharide units	X53819.1	(4)	protectins
kpsMT III	Group III capsular polysaccharides	AF007777.1	(4)	protectins
neuC	UDP–N-acetylglucosamine (GlcNAc) 2-epimerase	M84026.1	(19)	protectins
ompA	outer membrane protein A	CP004009.1	(7)	protectins
rfc	Escherichia coli O antigen polymerase gene	U39042	(4)	protectins
astA	arginine succinyltransferase	AY545598	(10)	toxins
cdtB	cytolethal distending toxin protein	AJ508930	(20)	toxins
cnf-1	cytotoxic necrotizing factor	U42629	(4)	toxins
hlyA	hemolysin A	M10133	(21)	toxins
hlyD	hemolysin D	2128	(8)	toxins
hlyE	hemolysin E	AF052225	(22)	toxins
hlyF	hemolysin F	14615	-	toxins
vat	vacuolating autotransporter toxin	X16664	(23)	toxins

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The separate Excel file for Table S1 and Table S3 containing the isolate information, phenotypic raw data on biocide and antibiotic susceptibility, virulence determinants, accession numbers of whole genome sequences, and in silico screening for biocide resistance determinants can be accessed under:

https://www.mdpi.com/2076-2607/9/3/651/s1

6.3 Publication 3

Authors: Roedel A., Dieckmann R., Makarewicz O. (O.M.), Hartung A. (A.H.), Noll M., Pletz MW. (M.W.P.), AlDahouk S., Vincze S.

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Article

Evaluation of a Newly Developed Vacuum Dried Microtiter Plate for Rapid Biocide Susceptibility Testing of Clinical *Enterococcus Faecium* Isolates

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Abstract: We investigated the suitability of a newly developed biocide susceptibility test system based on microtiter plates containing vacuum dried biocides as a fast and reliable screening method. The evaluated substances included the cationic biocides benzalkonium chloride (BAC), chlorhexidine dihydrochloride (CHX), cetylpyridinium chloride, didecyldimethylammonium chloride, and octenidine dihydrochloride. Testing a selection of *Escherichia coli* and enterococci, the biocide microtiter plates provided results comparable to those obtained from broth microdilution according to ISO 20776-1. Broad MIC ranges allowed for testing gram-positive and gram-negative species with the same plate design. In the second part of our study, we applied the established method to analyze the susceptibility of 90 clinical *Enterococcus faecium* isolates from a German university hospital, as previous studies have indicated a link between reduced susceptibility to substances such as CHX and BAC and vancomycin resistance. We therefore determined MIC and minimum bactericidal concentrations (MBC) for 48 non-clonal vancomycin susceptible and 42 non-clonal vancomycin resistant isolates, but MIC_{95} and MBC_{95} were quite similar in both groups. Our easy to handle and ready to use test system enables the routine surveillance of bacterial tolerance towards disinfectants in hospitals. As a result, hygiene measures can be adapted and nosocomial infections controlled despite increasing prevalence of antibiotic-resistant bacteria.

Keywords: biocide susceptibility; *Enterococcus faecium*; vancomycin-resistant; VRE

1. Introduction

In the era of multidrug resistance with a rising number of infections unresponsive to antibiotic treatment, the relevance of hygiene measures to reduce bacterial burden and transmission in clinical settings has significantly increased [1]. Most disinfection strategies make use of a mixture of biocides with bacteriostatic and/or bactericidal activities simultaneously affecting different bacterial target sites [2]. The multifactorial mode of action led to the assumption that tolerance development in bacteria is rather unlikely. Nonetheless, numerous in vitro studies demonstrated the adaptation capability of various bacterial species when exposed to different biocidal substances in sublethal

concentrations [3,4]. In addition, recent epidemiological studies provide support that decreasing biocide susceptibility can be caused by the introduction of biocides into the clinical environment. Pidot et al., for example, showed that *Enterococcus faecium* isolates obtained from two major hospitals in Melbourne, Australia after 2010 were 10-fold more tolerant to isopropanol compared to former isolates [5]. Decreasing susceptibility of *Staphylococcus aureus* to biocides such as chlorhexidine (CHX) and octenidine (OCT) over time was observed after increased usage of both substances in hospitals [6]. In several outbreak investigations, we observed reduced susceptibility to biocides of the predominantly used substances in outbreak isolates. We have recently described a clonal cluster of carbapenem-resistant *Klebsiella pneumoniae* isolates with decreased susceptibility to CHX [7]. These isolates were detected by regular screening of intensive care unit (ICU) patients on a ward that has implemented routine washing with CHX to decrease the rate of catheter-related infections. As a matter of concern, reduced CHX susceptibility was associated with resistance to colistin, likely caused by increased efflux of both substances via the same route. Furthermore, we have reported a polyclonal outbreak with *Serratia marcescens* on a neonatology ICU [8]. These isolates exhibited resistance to 0.5% Mikrobac forte® consisting of benzyl-C12-18-alkyl dimethyl ammonium chloride 199 mg/g and *N*-(3-aminopropyl)-*N*-dodecylpropane-1,3-diamine 50 mg/g, which was used for disinfection of surfaces before the outbreak. One of the measures in the bundle that finally successfully contained this outbreak consisted of increasing the concentration of used Mikrobac forte® to 2%. Outcomes of these studies point towards the need for regular monitoring of bacterial biocide susceptibility profiles. For this purpose, reliable high-throughput screening methods are needed that can be easily compared across studies. In contrast to antibiotic susceptibility testing, standardized procedures are missing for biocide susceptibility testing although attempts have been made to introduce respective protocols [9,10]. The need to harmonize biocide susceptibility testing methods was emphasized by a study highlighting the effect of slight modifications in the test procedure, such as choice of nutrient broth or assay plate material, on the results obtained [11]. Currently, biocide susceptibility is frequently tested by broth microdilution as it resembles the standardized procedure for antibiotic resistance testing according to ISO standard 20776-1 [12]. The method includes the fresh preparation of biocide stock solutions, which are diluted to a range of concentrations covering MICs and minimum bactericidal concentrations (MBCs). A defined number of bacterial cells ($2 \times 10^5 - 8 \times 10^5$ cfu/mL) is subsequently exposed to biocides for 18 ± 2 h at 34–37 °C. Concentration ranges for each substance are covered in doubling dilutions. The MIC is defined as the lowest concentration leading to inhibition of bacterial growth, which is determined visually or by measuring optical density. The aforementioned method is time-consuming and error-prone as ranges of biocidal substances need to be prepared prior to each investigation. Hence, a test plate system containing predefined concentrations of biocidal substances would be preferable for routine screening of bacterial susceptibility profiles to biocides of interest. Thus, the first aim of our study was to evaluate the comparability of susceptibility profiles for chosen biocides obtained with broth microdilution method according to ISO 20776-1 and a newly developed microtiter-plate containing vacuum dried cationic biocides (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany). Subsequently, we used the novel test system to determine biocide susceptibility of vancomycin-resistant (VRE) and susceptible enterococci (VSE) as vancomycin resistance has been linked to reduced susceptibility to cationic biocides such as CHX and benzalkonium chloride (BAC) in the past [13,14].

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

In total, 95 field isolates and two reference strains were analyzed comprising of *Enterococcus* spp. and *Escherichia coli*. All enterococci field isolates were sampled at the Jena University Hospital. To verify the suitability of the newly developed test system, an initial strain panel was tested, consisting of four *E. coli* isolates from stable surfaces of broiler fattening farms and the *E. coli* reference strain ATCC 25922,

as well as three clinical isolates of *E. faecium*, one of *Enterococcus faecalis*, and the *E. faecalis* reference strain ATCC 29212. The actual strain panel studied included 42 non-clonal VRE and 48 non-clonal VSE isolates (among them the three pretested *E. faecium*) from blood cultures (n = 73), swabs (n = 7), urine (n = 6), and fecal samples (n = 4; Table S1). Isolates were stored in glycerol stocks. Prior to use they were grown on Mueller–Hinton (MH) agar (Thermo Fisher Diagnostics GmbH Microbiology, Wesel, Germany) overnight at 37 °C.

2.2. Biocides

The cationic biocides BAC (Sigma Aldrich, Steinheim, Germany), chlorhexidine dihydrochloride (CHX; Sigma Aldrich), cetylpyridinium chloride (CTP; TCI, Eschborn, Germany), didecyldimethylammonium chloride (DDAC; Sigma Aldrich), and octenidine dihydrochloride (OCT; Alfa Aesar by Thermo Fisher, Kandel, Germany) were tested including the following concentrations in doubling dilution steps: 256 to 0.5 mg/L (BAC), 128 to 0.25 mg/L (CHX), 256 to 1 mg/L (CTP), 128 to 0.5 mg/L (DDAC), and 32 to 0.125 mg/L (OCT).

2.3. Biocide Susceptibility Testing

2.3.1. MIC Determination by Wet Plate Procedure

MIC values were determined for the initial strain panel using a broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [15] and ISO 20776-1 [12]. Testing was carried out as previously published [16] except for MH broth (Thermo Fisher Diagnostics GmbH Microbiology) used instead of tryptic soy broth. In compliance with EN 1276, standardized hard water was used to freshly prepare all stock solutions and to subsequently adjust the desired concentrations. The MIC was defined as the lowest biocide concentration completely inhibiting bacterial growth, which was determined after additional visual inspection (data not shown). Optical density was measured with a Mithras^2 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany). OD_{595} = 0.04 and 0.08 were considered as cut-off values for enterococci and *E. coli*, respectively. Experiments were repeated on three different days in three technical replicates per day.

2.3.2. MIC and MBC Determination by Dried Plate Procedure

In parallel, susceptibility testing was performed using a broth microdilution method with customized microtiter plates containing vacuum dried biocides (MERLIN Diagnostika GmbH). Briefly, 100 µl MH broth containing approximately 5×10^5 cfu/mL were added to each well and plates were incubated for 20 ± 2 h at 37 °C. OD_{620} was measured with a Multiscan EX microplate photometer (Thermo Scientific, Vantaa, Finland). MIC values were defined after additional visual inspection (data not shown). OD_{620} = 0.08 was considered as the cut-off value for both enterococci and *E. coli*. MICs of the initial strain panel were tested on three different days in three technical replicates per day. Subsequently, MIC testing was conducted for the second strain panel in three biological replicates on three different days using the customized biocide microtiter plate. In addition, MBC tests were performed as previously described [16]. Dey–Engley neutralizing broth (Sigma Aldrich) was used to quench biocidal effects for MBC testing. The MBC was defined as the lowest concentration of the biocide that revealed no visible colonies on MH agar.

2.4. Comparative Analysis of Both MIC Testing Methods

Comparability of results obtained with both methods was assessed by applying two criteria of ISO 20776-2, essential agreement (EA), and reproducibility [17]. According to ISO 20776-2, alternative antimicrobial susceptibility testing methods need to be compared to the reference method for antibiotic resistance testing based on ISO 20776-1. So far, no reference method is available for biocide susceptibility testing. Hence, we compared the results of the MIC testing conducted with customized microtiter plates (dried plate procedure) with data obtained with broth microdilution according to ISO 20776-1 (wet plate procedure). The EA was calculated using the following formula:

$$EA = N_{EA} \times 100/N$$

where N_{EA} = Number of isolates showing a modal value comparable to the modal value obtained with the ISO 20776-1 method (± one doubling dilution step), and N = Total number of tested isolates.

According to ISO 20776-2, the modal MIC value determined with the test system may differ ± one doubling dilution step from the modal value obtained with the ISO 20776-1 method. At least 90% of the data obtained with the test system of interest need to be within this acceptable range (EA ≥ 90%). With these preconditions, at least 95% of the measured data must be reproducible.

3. Results and Discussion

3.1. MIC Values Determined with Vacuum Dried Biocide Microtiter Plates Are in Agreement with Results Obtained with Broth Microdilution According to ISO 20776-1

In our study, MIC values were determined using two independent methods for an initial panel of three *E. faecium*, two *E. faecalis* (Figure 1), and five *E. coli* strains (Figure 2). The MIC values determined with the customized vacuum dried biocide microtiter plate were within the acceptable range of ± one doubling dilution step compared to the modal values obtained with a broth microdilution method according to ISO 20776-1 (EA = 100% for all isolate-substance combinations). For enterococci, data obtained from all replicates were within the acceptable range of ± one doubling dilution step (reproducibility = 100% for all measurements). For *E. coli*, reproducibility reached 100% in CTP and DDAC. It was lower in BAC (98%), CHX (96%) and OCT (96%) but was still within an acceptable range (≥95%) according to ISO 20776-2.

Taken together, vacuum dried biocide microtiter plates provide a performance level comparable with broth microdilution (the ISO 20776-1 reference method). Hence, the evaluated microtiter plates are suitable for quick and standardized MIC testing of cationic biocides. Broad MIC ranges allowed for susceptibility testing of gram-positive and gram-negative species with the same plate design. However, it needs to be noted that this method is restricted to biocidal substances that can be easily de- and rehydrated such as the cationic biocides tested in our study. Customized vacuum dried biocide microtiter plates have been used for biocide susceptibility testing in three independent studies before [18–20]. The microtiter plates were also manufactured by MERLIN Diagnostika GmbH and all contained acriflavine, alkyldiaminoethyl glycin hydrochloride, benzethonium chloride, BAC, and CHX as heavy metal salts. However, none of these studies reported on the comparability of results with data obtained by broth microdilution according to ISO 20776-1.

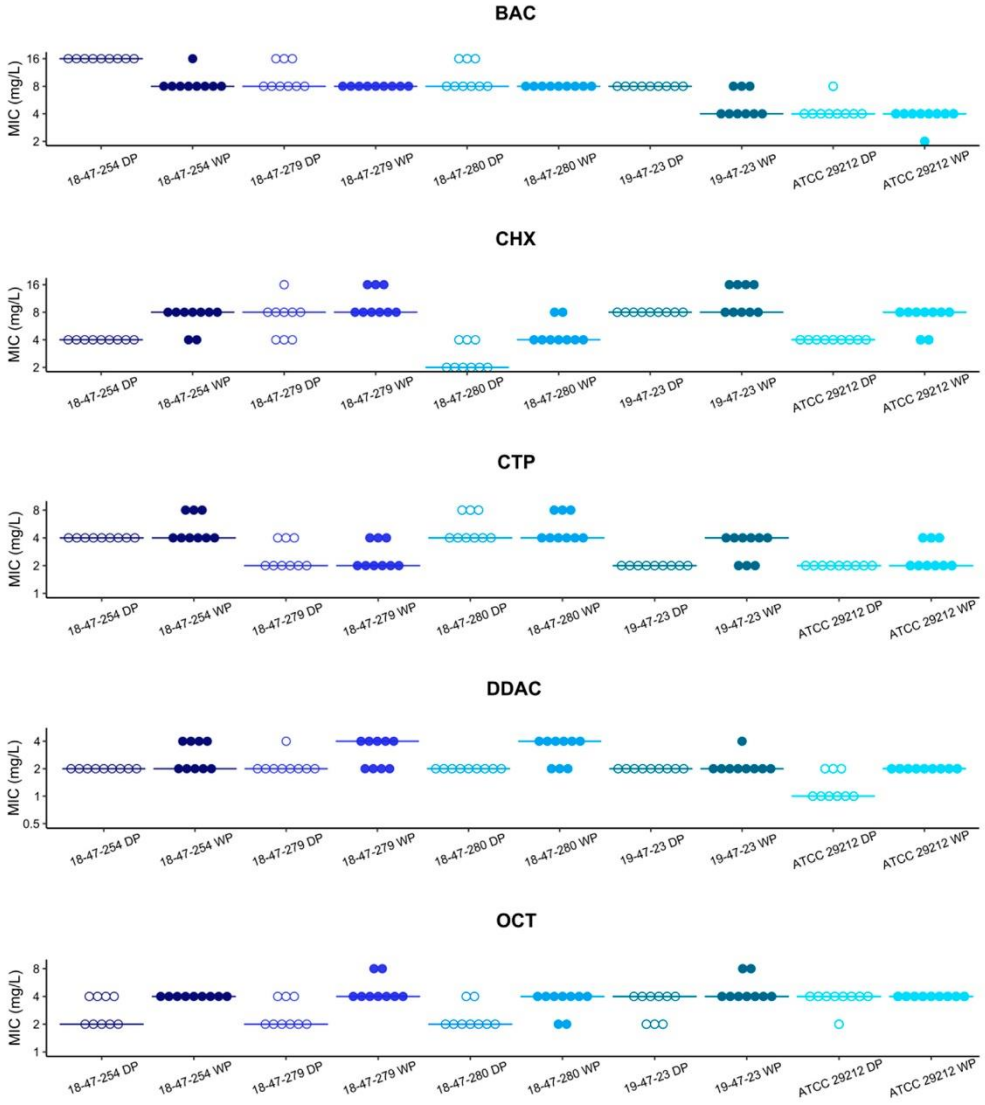

Figure 1. Susceptibility of three *Enterococcus faecium* (18-47-254, 18-47-279, 18-47-280) and two *E. faecalis* (19-47-23, ATCC 29212) strains to cationic biocides. Minimum inhibitory concentrations of benzalkonium chloride (BAC), chlorhexidine dihydrochloride (CHX), cetylpyridinium chloride (CTP), didecyldimethylammonium chloride (DDAC), and octenidine dihydrochloride (OCT) determined by wet plate procedure (WP; filled dots) and dried plates (DP; empty dots) are shown. Lines represent the respective modal values.

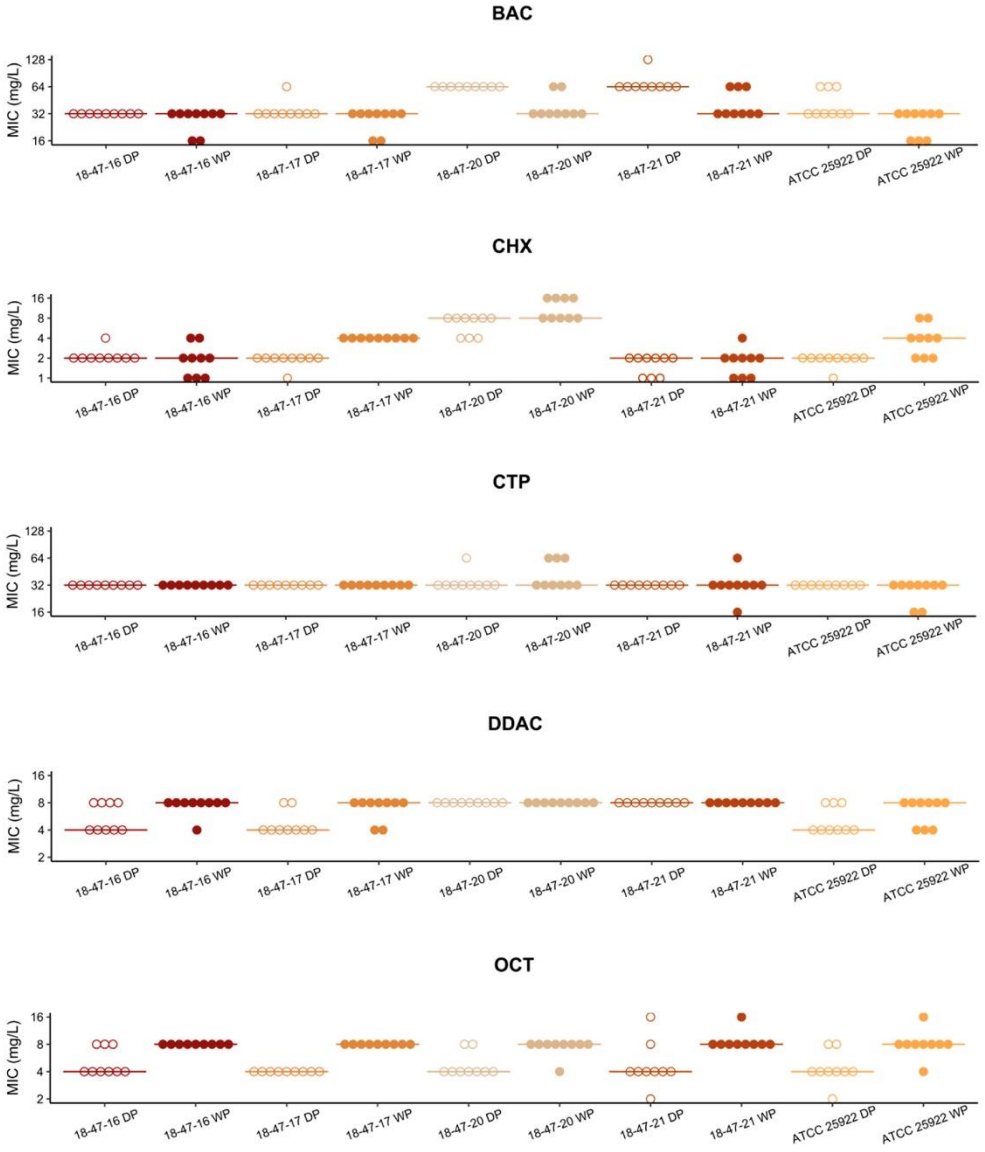

Figure 2. Susceptibility of five *Escherichia coli* strains to cationic biocides. Minimum inhibitory concentrations of benzalkonium chloride (BAC), chlorhexidine dihydrochloride (CHX), cetylpyridinium chloride (CTP), didecyldimethylammonium chloride (DDAC), and octenidine dihydrochloride (OCT) determined by wet plate procedure (WP; filled dots) and dried plates (DP; empty dots) are shown. Lines represent the respective modal values.

3.2. Cationic Biocide Susceptibility Profiles of Vancomycin Resistant and Susceptible E. faecium Are Similar

The vacuum dried biocide microtiter plate system was used to determine the susceptibility of 90 *E. faecium* strains to cationic biocides (Table 1). Reproducibility reached 100% for each substance by accepting a variability of ± one doubling dilution step. The high reproducibility points towards the reliable description of susceptibility patterns for cationic biocides by applying a test system based on vacuum dried plates. Overall, MIC and MBC values of BAC, DDAC, and CHX were in accordance with previously reported data on *E. faecium*, which were generated by broth microdilution according to ISO 20776-1 [13,21], modified broth microdilution and subsequent macrodilution [22,23], or agar dilution [14]. Interestingly, compared to data published by Morrissey et al. [21], MIC values of CHX were quite low (2–4 mg/L vs. 16 mg/L) in our subpopulation of *E. faecium* isolates, which might display geographic variability. Unfortunately, data on susceptibility to CTP and OCT are not available so far. MIC_{95} and MBC_{95} values of OCT, CTP, and DDAC in VRE and VSE were concordant. The MBC_{95} of BAC and the MIC_{95} of CHX were twice as high in VSE compared to VRE. This observation is in contrast to previous findings, where vancomycin resistance was associated with reduced susceptibility to cationic biocides, such as BAC and CHX [13,14]. While our study focused on clinical *E. faecium* isolates from wards with low CHX usage, the study conducted by Alotaibi et al. investigated isolates from Danish hospital wards, where CHX is heavily used [13], which might be one reason for the observed difference in study outcomes. Last but not least, BAC and CHX susceptibility of VRE and VSE differed only in one doubling dilution step, which is within the acceptable range in terms of comparability of results according to ISO 20776-2. Mechanisms mediating reduced biocide susceptibility in enterococci are still not well understood. However, in some studies reduced susceptibility to substances such as CHX and BAC was linked to increased efflux pump activities [13,24,25], which is in line with common biocide tolerance mechanisms described for various bacterial species [26]. Although increased efflux pump activity can be associated with resistance to certain antibiotics in enterococci, e.g., streptogramins, tetracyclines and quinolones [27], there is no evidence that efflux pumps contribute to vancomycin resistance. In enterococci, identified vancomycin resistance mechanisms include target modification and removal of high affinity precursors that are usually synthesized in the cell. Both mechanisms result in reduced binding of vancomycin to the bacterial cell [27,28]. It has been shown that adaptation to biocides can result in modification of bacterial cells. The adaptation of *K. pneumoniae* to CHX, for example, was linked to the upregulation of genes involved in modification of the outer membrane [29]. Whether adaptation to cationic biocides like CHX leads to an alteration of the enterococcal cell wall, which might consequently affect the binding of vancomycin, needs to be investigated in future studies. Results of our study do not provide evidence of an association between reduced susceptibility to cationic biocides and vancomycin resistance.

Table 1. Susceptibility of *Enterococcus faecium* to cationic biocides tested by a customized microtiter plate (MERLIN Diagnostika GmbH).

Biocide (Concentration Range Tested)	Species	Number of Isolates with MIC Value (mg/L) of							MIC_{95}	Number of Isolates with MBC Value (mg/L) of							MBC_{95}
		0.25	0.5	1	2	4	8	16		0.25	0.5	1	2	4	8	16	
BAC (0.5–256 mg/L)	VSE			1		11	36		8						41	7	16
	VRE					15	27		8						42		8
CHX (0.25–128 mg/L)	VSE		1	2	35	10			4				2	1		45	16
	VRE			1	41				2				3	1	3	35	16
CTP (1–256 mg/L)	VSE			3 *	17	28			4					48			4
	VRE				11	31			4					42			4
DDAC (0.5–128 mg/L)	VSE		1 #	25	22				2				48				2
	VRE			26	16				2			2	40				2
OCT (0.125–32 mg/L)	VSE		1	8	39				2				22	26			4
	VRE			4	38				2				22	20			4

Biocide concentrations which have not been tested are shaded in gray. BAC = benzalkonium chloride, CHX = chlorhexidine dihydrochloride, CTP = cetylpyridinium chloride, DDAC = didecyldimethylammonium chloride, OCT = octenidine dihydrochloride, VRE = vancomycin resistant *Enterococcus faecium*, VSE = vancomycin susceptible *Enterococcus faecium*, # MIC ≤ 0.5, * MIC ≤ 1.

4. Conclusions

In our study, we explored the suitability of a newly developed biocide susceptibility test system based on microtiter plates containing vacuum dried biocides as a screening method to identify bacteria resistant to cationic substances frequently used in hospital settings. We were able to show that this test system provides reliable results similar to the broth microdilution method according to CLSI guidelines and ISO 20776-1. Based on the data collected, the test system is appropriate for both, gram-positive and gram-negative species and may, therefore, serve as a fast and easy-to-handle surveillance tool for biocide-tolerant bacterial isolates. In a clinical application trial, we determined the susceptibility of 90 clinical *E. faecium* isolates to cationic biocides. Our results revealed no association of biocide tolerance with vancomycin resistance in the strain collection under study. In summary, monitoring and early identification of clinical isolates tolerant towards disinfectants applied in hospitals will help to adjust hygiene measures and to control nosocomial infections while simultaneously reducing antibiotic consumption.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/4/551/s1, Table S1: *E. faecium* study population.

Author Contributions: Conceptualization, R.D., M.N., S.A.D., and S.V.; methodology, A.R., R.D., and S.V.; validation, R.D., S.A.D., and S.V.; formal analysis, A.R., O.M., and A.H.; investigation, A.R.; resources, O.M. and M.W.P.; writing—original draft preparation, A.R.; writing—review and editing, R.D., O.M., A.H., M.N., M.W.P., S.A.D., and S.V.; visualization, A.R. and S.V.; supervision, R.D., O.M., M.N., M.W.P., S.A.D., and S.V.; funding acquisition, R.D. and S.A.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary material for

Publication 3

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Table S1: *E. faecium* study population

Isolate ID	species	Vancomycin resistance	Isolation source
18-47-214	*E. faecium*	VSE	urine
18-47-215	*E. faecium*	VSE	swab
18-47-216	*E. faecium*	VSE	swab
18-47-217	*E. faecium*	VSE	swab
18-47-218	*E. faecium*	VSE	swab
18-47-219	*E. faecium*	VSE	swab
18-47-220	*E. faecium*	VSE	blood culture
18-47-221	*E. faecium*	VSE	blood culture
18-47-222	*E. faecium*	VSE	blood culture
18-47-223	*E. faecium*	VSE	blood culture
18-47-254	*E. faecium*	VRE	blood culture
18-47-255	*E. faecium*	VRE	blood culture
18-47-256	*E. faecium*	VRE	blood culture
18-47-257	*E. faecium*	VRE	blood culture
18-47-258	*E. faecium*	VRE	blood culture
18-47-259	*E. faecium*	VRE	blood culture
18-47-260	*E. faecium*	VRE	blood culture
18-47-261	*E. faecium*	VSE	blood culture
18-47-262	*E. faecium*	VRE	blood culture
18-47-263	*E. faecium*	VSE	blood culture
18-47-264	*E. faecium*	VSE	blood culture
18-47-265	*E. faecium*	VSE	blood culture
18-47-266	*E. faecium*	VRE	blood culture
18-47-267	*E. faecium*	VSE	blood culture
18-47-268	*E. faecium*	VRE	blood culture
18-47-269	*E. faecium*	VSE	blood culture
18-47-270	*E. faecium*	VSE	blood culture
18-47-271	*E. faecium*	VSE	blood culture
18-47-272	*E. faecium*	VSE	blood culture
18-47-273	*E. faecium*	VSE	blood culture
18-47-274	*E. faecium*	VSE	blood culture
18-47-275	*E. faecium*	VSE	blood culture
18-47-276	*E. faecium*	VSE	blood culture
18-47-277	*E. faecium*	VSE	blood culture
18-47-278	*E. faecium*	VSE	blood culture
18-47-279	*E. faecium*	VSE	blood culture
18-47-280	*E. faecium*	VRE	blood culture
18-47-281	*E. faecium*	VRE	blood culture
18-47-282	*E. faecium*	VRE	urine
18-47-283	*E. faecium*	VRE	fecal samples
18-47-284	*E. faecium*	VRE	blood culture
18-47-285	*E. faecium*	VRE	blood culture
18-47-286	*E. faecium*	VRE	blood culture
18-47-287	*E. faecium*	VRE	blood culture
18-47-288	*E. faecium*	VRE	blood culture
18-47-289	*E. faecium*	VRE	blood culture
18-47-290	*E. faecium*	VRE	blood culture
18-47-291	*E. faecium*	VRE	blood culture
18-47-292	*E. faecium*	VRE	blood culture
18-47-293	*E. faecium*	VRE	blood culture
18-47-294	*E. faecium*	VSE	blood culture

Isolate ID	species	Vancomycin resistance	Isolation source
18-47-295	*E. faecium*	VSE	blood culture
18-47-296	*E. faecium*	VSE	blood culture
18-47-297	*E. faecium*	VSE	blood culture
18-47-298	*E. faecium*	VSE	blood culture
18-47-299	*E. faecium*	VSE	blood culture
18-47-300	*E. faecium*	VSE	urine
18-47-301	*E. faecium*	VSE	urine
18-47-302	*E. faecium*	VSE	urine
18-47-303	*E. faecium*	VSE	fecal samples
18-47-304	*E. faecium*	VRE	swab
18-47-305	*E. faecium*	VRE	fecal samples
18-47-306	*E. faecium*	VSE	blood culture
18-47-307	*E. faecium*	VSE	blood culture
18-47-308	*E. faecium*	VSE	blood culture
18-47-309	*E. faecium*	VSE	blood culture
18-47-310	*E. faecium*	VRE	blood culture
18-47-311	*E. faecium*	VRE	blood culture
18-47-312	*E. faecium*	VRE	blood culture
18-47-313	*E. faecium*	VRE	blood culture
18-47-314	*E. faecium*	VRE	blood culture
18-47-315	*E. faecium*	VRE	blood culture
18-47-316	*E. faecium*	VRE	blood culture
18-47-317	*E. faecium*	VRE	blood culture
18-47-318	*E. faecium*	VRE	blood culture
18-47-319	*E. faecium*	VRE	blood culture
18-47-320	*E. faecium*	VSE	blood culture
18-47-321	*E. faecium*	VSE	blood culture
18-47-322	*E. faecium*	VSE	blood culture
18-47-323	*E. faecium*	VSE	blood culture
18-47-324	*E. faecium*	VSE	blood culture
18-47-325	*E. faecium*	VSE	blood culture
18-47-326	*E. faecium*	VRE	urine
18-47-327	*E. faecium*	VRE	fecal samples
18-47-328	*E. faecium*	VRE	swab
18-47-329	*E. faecium*	VRE	blood culture
18-47-330	*E. faecium*	VSE	blood culture
18-47-331	*E. faecium*	VSE	blood culture
18-47-332	*E. faecium*	VRE	blood culture
18-47-333	*E. faecium*	VRE	blood culture

Abbreviations: *E.* = *Enterococcus*, VRE = vancomycin resistant *E. faecium*, VSE = vancomycin susceptible *E. faecium*

7 Appendix I

7.1 Table 1: Overview on reviewed individual tests for each substance according to exposure frequency and use of pure substance / biocidal product

Number (n)	Biguanides		Phenol derivatives		QACs		Aldehydes	Alkylamines	Alcohols	Chlorine-releasing agents	Peroxides	Σ
	CHX	PHMB	Triclosan	Hexachloro-phene	Benzyl-group compounds	other QACs						
A: Multiple exposure to pure substances												
Individual tests	216	59	233	76	186	202	n.t.	16	n.t.	23	8	1019
Adapted isolates (FC ≥ 4)	110	14	81	25	86	76	n.t.	0	n.t.	0	0	392
Stable adaptations	44	3	49	6	26	23	n.t.	0	n.t.	0	0	151
Unstable adaptations	53	9	19	19	59	49	n.t.	0	n.t.	0	0	208
B: Multiple exposure to biocidal products												
Individual tests	4	16	n.t.	n.t.	57	33	16	19	27	21	34	227
Adapted isolates (FC ≥ 4)	4	0	n.t.	n.t.	6	4	0	5	0	0	0	19
Stable adaptations	4	0	n.t.	n.t.	0	0	0	3	0	0	0	7
Unstable adaptations	0	0	n.t.	n.t.	0	0	0	2	0	0	0	2
C: Single exposure to pure substances												
Individual tests	18	n.t.	20	n.t.	37	n.t.	n.t.	4	n.t.	15	6	100
Adapted isolates (FC ≥ 4)	6	n.t.	19	n.t.	6	n.t.	n.t.	0	n.t.	0	0	31
Stable adaptations	4	n.t.	19	n.t.	0	n.t.	n.t.	0	n.t.	n.a.	n.a.	23
Unstable adaptations	2	n.t.	0	n.t.	2	n.t.	n.t.	0	n.t.	n.a.	n.a.	4
D: Single exposure to biocidal products												
Individual tests	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	8	6	n.t.	n.t.	9	23
Adapted isolates (FC ≥ 4)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0	0	n.t.	n.t.	0	0
Stable adaptations	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0	0	n.t.	n.t.	0	0
Unstable adaptations	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0	0	n.t.	n.t.	0	0
Summary: Total number of individual tests (n) per substance class												
Individual tests	238	75	253	76	280	235	24	45	27	59	57	1369
Adapted isolates (FC ≥ 4)	120	14	100	25	98	80	0	5	0	0	0	442
Stable adaptations	52	3	68	6	26	23	0	3	0	0	0	181
Unstable adaptations	55	9	19	19	61	49	0	2	0	0	0	214
References (see 7.2)	1-25	1,3,4,6,19,26	3,4,6,8,16,18,22,27-44	30	1-4,8,16,17,20,21,23,24,30,38,45-59	1,3-7,11,14,45,60-63	8,38,64-67	2,51,60,65,68,69	8,46,70,71	8,24,51,68,72-78	8,16,22,38,39,51,66,67,75,76,78	

FC= fold change, n.t. = not tested

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(Eidesstattliche) Versicherungen und Erklärungen

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Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

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Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden habe.

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Hiermit erkläre ich, dass ich Hilfe von gewerblichen Promotionsberatern bzw. -vermittlern oder ähnlichen Dienstleistern weder bisher in Anspruch genommen habe noch künftig in Anspruch nehmen werde.

(§ 9 Satz 2 Nr. 7 PromO BayNAT)

Hiermit erkläre ich mein Einverständnis, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung unterzogen werden kann.

(§ 9 Satz 2 Nr. 8 PromO BayNAT)

Hiermit erkläre ich mein Einverständnis, dass bei Verdacht wissenschaftlichen Fehlverhaltens Ermittlungen durch universitätsinterne Organe der wissenschaftlichen Selbstkontrolle stattfinden können.

…….
Ort, Datum, Unterschrift