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Salmonella Infantis - Link between application of disinfectants and resistance to antimicrobial compounds

DIPLOMA THESIS

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Abbreviations

spp.	-	subspecies
EFSA	-	European Food Safety Agency
WHO	-	World Health Organization
EU	-	European Union
MS	-	member states
H2S	-	hydrogen sulfide
WHOCC-Salm	-	WHO Collaborating Centre for Reference and Research on *Salmonella*
MDR	-	Multidrug – resistance
AMR	-	Antimicrobial resistance
ml	-	millilitre
LBA	-	lysogeny broth agar
PBS	-	phosphate-buffered saline
CFU	-	colony forming units
MIC	-	minimum inhibitory concentration
CLSI	-	Clinical and Laboratory Standards Institute
EUCAST	-	European Committee of Antimicrobial Susceptibility Testing
pESI	-	plasmid of emerging *Salmonella enterica* serovar Infantis
ESBL	-	extended-spectrum β-lactamase

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1 Introduction and aim of the study

Salmonella is a widespread bacterial genus, which is able to cause diseases in both humans and animals. Salmonellosis is a septic and enteric disease of animals and humans after oral intake and colonization of the gut with the bacteria (Sarma 2008).

The pathogen is found in both domestic and in wild animals as carriers and source of infection. Therefore, animal-based food is the main transmission route to humans for *Salmonella.* The symptoms after an infection are acute fever, nausea, diarrhoea and stomach cramps. Salmonellosis is the third leading cause of death due to foodborne diseases worldwide (Ferrari et al. 2019).

Classification of salmonellosis in humans and animals can be divided in two groups such as enteric fever (typhoidal) and non-typhoidal salmonellosis (Barrow and Methner 2013). The division can be continued due to host-specificity (Lan et al. 2009, Stevens and Kingsley 2021, Tanner and Kingsley 2018). Strains which are highly adapted to humans and have no nonhuman hosts like *Salmonella* Typhi and *Salmonella* Paratyphi types A, B and C cause enteric (typhoidal) fever. Strains that are adapted to nonhuman hosts like *Salmonella* Dublin (cattle), *Salmonella* Arizonae (reptiles), or *Salmonella* Choleraesuis (swine) cause disease almost only in their animal host, but also have the ability to cause disease in humans. Those strains with a broad host range like *Salmonella* Enteritidis, *Salmonella* Typhimurium or *Salmonella* Infantis cause mostly no symptoms of disease in animals as they become latent carriers but are of great importance in transmission to humans (Evangelopoulou et al. 2013, Kingsley and Bäumler 2000, Lan et al. 2009).

The zoonotic impact becomes increasingly problematic along the global food chain. Farmers, veterinarians, workers in food production as well as consumers are at risk of an infection by working with farm animals or by contact with contaminated food products. The World Health Organization (WHO) deals amongst others with foodborne disease outbreaks and classifies *Salmonella* as one of the four key global causes for diarrhoeal diseases. The WHO emphasizes the significance of food hygiene practices to reduce the chance of transferring emerging resistant serotypes of *Salmonella* which affect the food chain and represent a public health issue (WHO - World Health Organization 2018).

In 2019 Salmonellosis was the second most reported zoonosis in humans after Campylobacteriosis according to the monitoring activities in 36 European countries. The

European Food Safety Authority (EFSA) reported 87,923 confirmed cases of Salmonellosis in humans in 2019 with an EU notification rate of 20.0 cases per 100,000 population, the same level as in 2018. Over the last five years the trend for Salmonellosis in humans has been stable after a long period of a declining trend. 926 Salmonellosis food-borne outbreaks were reported by 23 EU member states in 2019 in total. Thereof 9,169 illnesses, 1,915 hospitalisations and seven deaths. *Salmonella* caused 17.9% of all food-borne outbreaks during 2019 in the EU. The great majority of outbreaks were caused by *Salmonella* Enteritidis. The five most frequently reported serovars in the EU which cause human Salmonellosis are *Salmonella* Enteritidis, *Salmonella* Typhimurium, monophasic *Salmonella* Typhimurium, *Salmonella* Infantis and *Salmonella* Derby. *Salmonella* Infantis represents the 4th most commonly reported serovar in the years 2017 – 2019 after *Salmonella* Enteritidis, *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium. Actually, broilers and broiler meat are the main source for *Salmonella* in the EU with more than 70% beside bovine animals and cattle meat, pigs and pig meat, turkeys and turkey meat and laying hens of *Gallus gallus* and eggs. Thereof *Salmonella* Infantis was reported the most with 29.7% among 17,176 serotyped isolates within the top-five serovars that were responsible for human infections from food-animal sources in 2019 in the EU. The most frequently reported serovar in broilers and their derived carcases is by far *Salmonella* Infantis. The geographical distribution of *Salmonella* Infantis within the EU member states shows at the top rank Italy, the Netherlands, Austria, Slovakia, Spain, Croatia and Romania. The recent epidemiological success of *Salmonella* Infantis is related to its ability to enter and persist along the poultry food chain presenting a growing risk for public health. *Salmonella* Infantis with its worldwide emergence and the enhanced survival fitness of some clones is increasingly of public interest for further investigations. Furthermore, it is assumed that the acquisition of a conjugative megaplasmid provides the bacteria with new resistance features, virulence – associated properties, high tolerance to disinfectants and resistance to heavy metals (The European Union One Health 2019 Zoonoses Report, 2021).

In Austria *Salmonella* Infantis was the most common nonhuman serovar in 2018 with 960 cases. The National Reference Center for *Salmonella* implements antibiotic resistance tests and valuations for all isolates corresponding to European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) standards. In 2018 one strain of *Salmonella* Infantis was resistant to high

– level Ciprofloxacin and another one was resistant to third generation cephalosporines (Bundesministerium für Arbeit, Soziales, Gesundheit und Konsumentenschutz 2019).

Its elimination from affected broiler farms represents a difficult task as the susceptibility against disinfectants varies between field strains (Drauch et al. 2020).

It was previously reported that cross-resistance to disinfectants and antibiotics occur in different bacterial species (Braoudaki and Hilton 2004, Braoudaki and Hilton 2005, Cadena et al. 2019, Chuanchuen et al. 2008, Condell et al. 2012, Deng et al. 2018). So far, this was not shown for *Salmonella* Infantis.

Therefore, in the following study different Austrian *Salmonella* Infantis isolates were investigated with the aim to elucidate a possible link between the application of disinfectants and the development of antibiotic resistance.

2 Salmonella

2.1 Historical Background

Bacterial species which are nowadays known as *Salmonella* have been obviously existing for a long time already. A *typhoid bacillus* was first cultivated in the year 1884 from spleen sections and in mesenteric lymph nodes from a patient whose death was caused by typhoid in 1880 (Le Minor 1994).

These bacteria isolated from pigs by Salmon and Smith 1886 were named *Salmonella* Choleraesuis and considered to trigger hog cholera. A decade later, the characteristic serum agglutination was discovered by Widal (1896).

The appearance of more and more new typhoid bacteria serovars - now known as *Salmonella* spp. - followed subsequently each year (Wray and Wray 2000).

2.2 Taxonomy, Classification and Grouping

The genus *Salmonella* belongs to the family *Enterobacteriaceae*. *Salmonellae* are gram-negative, facultative anaerobic, catalase-positive, oxidase-negative, rod-shaped, non-

spore forming bacteria that are usually motile. *Salmonellae* have a size of 0.7 - 1.5 µm x 2.0 – 5.0 µm and produce colonies with a diameter of 2 – 4 mm (Liu 2010).

So far more than 2600 serotypes (also called serovars) are known. The WHO Collaborating Centre for Reference and Research on *Salmonella* (WHOCC-Salm) occupies a historical role in maintaining the comprehensive list of known *Salmonella* serovars. WHOCC-Salm (Institut Pasteur, France) is also responsible for the validation of new serovars, working together with laboratories in Hamburg (Institut für Hygiene und Umwelt, Germany) and in Atlanta (Centers for Disease Control, USA) (GRIMONT and Weill 2007).

Based on the Kauffmann-White-Le Minor-Scheme (Popoff et al. 1997, Popoff et al. 2000) these serotypes (serovars) are defined. This scheme represents a list of antigenetic formulae to split up all *Salmonella* serovars which are presently known. They are divided in O (somatic lipopolysaccharide), H (flagella) and Vi (capsule) antigens. Molecular investigations have demonstrated that the genus *Salmonella* basically consists of the two taxonomic species *Salmonella enterica* and *Salmonella bongori (V)* (Brenner et al. 2000). *Salmonella enterica* comprises six subspecies called *Salmonella enterica* subsp. *enterica (I)*, *Salmonella enterica* subsp. *salamae (II)*, *Salmonella enterica* subsp. *arizonae (IIIa)*, *Salmonella enterica* subsp. *diarizonae (IIIb)*, *Salmonella enterica* subsp. *houtenae (IV)* and *Salmonella enterica* subsp. *indica (VI)* (Crosa et al. 1973, Ewing et al. 1970, Ewing 1972). More than 99.5% of isolated *Salmonella* strains belong to *Salmonella enterica* subsp. *enterica*. Serovar names possibly denote syndromes, host-specificity or geographical origin of the first strain of the new serovar. To avoid wrong consideration as a species name, compound serovar names shortened in simple names are written italicized. Grouping was defined due to characteristic serological reaction patterns. Each group was described through a capital letter. *Salmonella* Paratyphi A, *Salmonella* Nitra, *Salmonella* Kiel and *Salmonella* Koessen belong to group A. *Salmonella* Paratyphi B, *Salmonella* Abortusovis, *Salmonella* Derby, *Salmonella* Abortusequi, *Salmonella* Typhimurium and others belong to group B. *Salmonella* Paratyphi C, *Salmonella* Choleraesuis, *Salmonella* Typhisuis, *Salmonella* Virchow and *Salmonella* Infantis belong beside others to group C. Group C is subdivided in C1, C2 and C3. *Salmonella* Typhi, *Salmonella* Enteritidis, *Salmonella* Dublin, *Salmonella* Pullorum and *Salmonella* Gallinarum and others belong to Group D, which is also subdivided in D1, D2 and D3. The grouping continues until letter “Z”. As there were not enough letters for all serovars,

numbers were used instead and letters kept in brackets (Brenner et al. 2000, GRIMONT and Weill 2007, Lan et al. 2009, Le Minor and Popoff 1987).

2.3 Pathogenicity

Pathogenicity is defined as the ability of microorganisms to induce pathologic conditions and diseases in animals and humans (Pschyrembel 2013).

Host-specificity is an important feature for pathogenicity (Gerlach and Hensel 2007).

There are host-specific *Salmonella* serovars like *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Arizona, which are highly adapted to poultry and birds. *Salmonella* Pullorum and *Salmonella* Gallinarum cause severe clinical symptoms in poultry. The illness is called fowl typhoid or pullorum disease. In poultry flocks, sporadic appearance is common. There is a transovarian transmission route from subclinical infected parentals to chickens as well as horizontal transmission, airborne or vectors. Main clinical symptoms are high mortality rates in chickens and in brown layers, severe general disorder with difficulty in breathing, diarrhoea with chalk white feces and drop in laying performance. There is often a septic disease progress from peracute to chronic clinical symptoms (Siegmann and Neumann 2012).

Non-host-specific *Salmonella* serovars are able to induce severe disease in humans but no recognizable clinical symptoms in their animal host, e.g. *Salmonella* Enteritidis*, Salmonella* Typhimurium or *Salmonella* Infantis (Siegmann and Neumann 2012). The zoonotic potential of *Salmonella* Infantis is a reasonable cause for further microbial investigations as it represents a global health issue due to foodborne infections of poultry meat origin (Antunes et al. 2016, Nagy et al. 2020).

2.4 Virulence

The strength of pathogenicity is called virulence. Virulence or toxicity describe the degree of aggressiveness of microorganisms inside macroorganisms. It is a quantitative property in contrast to pathogenicity (Pschyrembel 2013).

There are so called virulence factors which are built from microorganisms themselves to increase or maintain their virulence or infectiousness. Examples would be adhesins for better tissue adherence, invasiveness for penetration in host cells, capsular antigens to inhibit phagocytosis or toxins (Pschyrembel 2013).

The existing of a heterogenous population of *Salmonella* Infantis including different clones and clusters is accompanied by crucial differences in antibiotic resistance profiles (Cohen et al. 2020, Gal-Mor et al. 2010).

This leads to the association of multidrug resistant (MDR) strains obviously going along with the possession of specific virulence genes and therefore the ability of higher persistence in farms and slaughterhouses (Aviv et al. 2014, Drauch et al. 2021).

It is commonly known that *Salmonella* spp. are able to carry virulence factors that are encoded on mobile elements. (Alba et al. 2020, Condell et al. 2012, Gal-Mor et al. 2010, Hensel 2004, Johansson et al. 2021, Moreno Switt et al. 2012, Suez et al. 2013)

Interestingly the presence of specific virulence factors can vary drastically between different serovars. Even across isolates within the same serovar there can appear huge differences in virulence (Hensel 2004, Suez et al. 2013).

The gain of functions by horizontal gene transfer represents a key feature in bacterial evolutionary pathways and genomic diversity (Cohen et al. 2020, Gal-Mor et al. 2010, Lawrence 2005).

To enable transmission of genetic information between bacteria there are vehicles like plasmids, pathogenicity islands, integrons, bacteriophages, transposons or integrative and conjugative elements (Aviv et al. 2014, Aviv et al. 2019, Gal-Mor et al. 2010, Lawrence 2005).

DNA elements which are acquired via horizontal gene transfer frequently affect the bacterial fitness, antimicrobial resistance (AMR) and hence the lifestyle and tenacity of the bacterial host itself (Lawrence 2005).

In emergent *Salmonella* Infantis strains it is proven that adaptive chromosomal mutations and a unique megaplasmid with the designation *pESI* (plasmid of Emerging *Salmonella enterica* Infantis) lead to an increasing resistance to multiple drugs, mercury and disinfectants (Alba et al. 2020).

Emerging pESI conjugated *Salmonella* Infantis strains show the ability to improved biofilm formation as well as adhesion and invasion into mammalian and avian host cells in vitro and higher pathogenicity by new virulence-associated phenotypes and increased intestinal inflammation in vivo (Aviv et al. 2014).

Recently it was also shown that infection dynamics of *Salmonella* Infantis strains with or without pESI-like plasmid vary considerably and therefore great differences in virulence was shown by obviously phylogenetically different backgrounds of the strains (Drauch et al. 2021).

2.5 Control measures

To avoid persisting infections of *Salmonella* Infantis, usage of control measures as well as permanent surveillance programmes are indispensable. Microbiologically faultless food is a matter auf concern for public health. Hence the risk of surviving bacteria with increasing fitness and virulence must be minimized along the food chain (Hotes et al. 2011).

2.5.1 Disinfection

Actually, proper disinfection is the most common applied measure to fight bacteria like *Salmonella* Infantis (Denyer 1995, McDonnell and Russell 1999).

Biocides have been used for hundreds of years by humans in hospitals and other health care settings to generally inactivate microorganisms (Block 1991, Hernández-Navarrete et al. 2014, McDonnell and Russell 1999).

At the beginning they were used to prevent nosocomial infections. Antiseptics and disinfectants usually have a broad spectrum of microorganisms against which they have an effect and seem to have multiple targets (Hernández-Navarrete et al. 2014, McDonnell and Russell 1999). In contrast to antibiotics which work very specifically against particular intracellular targets (Mohr 2016, Vázquez-Laslop and Mankin 2018).

Interestingly some bacterial species possess an intrinsic membrane barrier which can detain biocides by low-permeability (Nikaido 1994).

Antiseptics are used in or on living tissues to either kill or inhibit the growth of bacteria or other microorganisms. Disinfectants have the same purpose but are used on inanimate objects or surfaces (Block 1991, McDonnell and Russell 1999).

Biocides in general contain a great amount of active chemical agents (Block 1991, Denyer 1995, McDonnell and Russell 1999).

In biocides the mode and the mechanisms of action like interactions with the cell surface and penetration into the cell are less understood than the mechanisms and activity of antibiotics so far. 13 groups of chemical substances which show different chemical structures and differences in their effects as biocides are described. In particular there are alcohols, aldehydes, anilides, biguanides, bisphenols, diamidines, halogen-releasing agents, halophenols, heavy metal derivates, peroxygens, phenols and cresols, quaternary ammonium compounds and vapor-phase sterilants (Denyer 1995, McDonnell and Russell 1999).

In Austria it is stipulated in *§§8,9 Geflügelhygieneverordnung 2007* to take strict hygiene precautions and biosecurity measures in all poultry farms and production. It is forbidden to enter stables and breeding places without hygienically flawless clothes, shoes and head cover. There must be separate clothing for each production area. Reusable clothes must be cleaned and disinfected after each usage. Visitors are not allowed to enter stables without the farm manager`s permission and only by sticking to hygiene instructions. In poultry production all in all out management is practiced. The stable must undergo thorough wet and dry cleaning and disinfection measures before setting a new flock. Additionally, several samplings for microbiological investigations must be performed by a veterinarian. A new flock can come in after seven days at the earliest and 14 days if presence of *Salmonella* was proven (RIS - Geflügelhygieneverordnung 2007 - Bundesrecht konsolidiert, Fassung vom 26.08.2022, 26/08/2022).

2.5.2 Bacteriophages

In the early 20th century an innovative treatment of bacterial infections was first discovered. It was the usage of so called bacteriophages (Hanlon 2007, Maura and Debarbieux 2011).

As more and more multidrug resistant bacteria arise and the development of new antibiotics is not sufficient, bacteriophages might be an evolving alternative against resistant bacteria (Hanlon 2007, Kutateladze and Adamia 2010).

Bacteriophages (also: phages) occur ten times more often naturally in the environment than bacteria (Kutter and Sulakvelidze 2005). They developed in co-evolution especially in aquatic conditions, and represent bacterial viruses (Hanlon 2007).

Bacteriophages are mainly summarized in three big families. These are siphoviridae, myoviridae and podoviridae comprising 15 genera and ten further families each with only a small amount of members left (Hanlon 2007, Kutter and Sulakvelidze 2005).

The lytic cycle of bacteriophages and effect on bacteria is based on encountering the bacterial host in the environment by random motion, attachment and penetration in the bacterial cell and synthesis of viral bacteriophage components by bacterial DNA replication (Maura and Debarbieux 2011).

After that the lysis of the bacterial cell and the release of the bacteriophage progeny is induced by virions which are built through early bacteriophage mRNA elements overtaking the metabolic processes in the bacterial host (Hanlon 2007).

2.5.3 Vaccines

In Austria there are vaccines available to provide a better management of *Salmonella* in poultry industry and less danger for public health and therefore minimizing the risk of spreading resistant bacteria (MacLennan et al. 2014).

The decrease of 81.6% since 2002 of Salmonella infections in humans in Austria is lead back to a strict management of surveillance programmes relating to the national “Bundesgesetz zur Überwachung von Zoonosen und Zoonoseerregern (Zoonosengesetz)” as well as restrictions in layers. Laying hen flocks comprising more than 350 birds must get vaccinated against *Salmonella* Enteritidis (Bundesministerium für Arbeit, Soziales, Gesundheit und Konsumentenschutz 2019, RIS - Zoonosengesetz - Bundesrecht konsolidiert, Fassung vom 27.08.2022, 27/08/2022).

According to the law *§10 Geflügelhygieneverordnung 2007* farmers must ensure vaccination against *Salmonella* Enteritidis in rearing farms for breeding chickens and

pullets (RIS - Geflügelhygieneverordnung 2007 - Bundesrecht konsolidiert, Fassung vom 26.08.2022, 26/08/2022).

Concerning the efficacy of *Salmonella* vaccines in poultry flocks to prevent disease and intestinal colonization and therefore contamination of eggs and environment and the resulting burden for public health it is important to have a look at the vaccine used as well as the application scheme. There are live attenuated *Salmonella* vaccines beside inactivated *Salmonella* vaccines available (Desin et al. 2013, Fuche et al. 2016, Gantois et al. 2006, Groves et al. 2016, QGV Österreichische Qualitätsgeflügelvereinigung 20/09/2021, Woodward et al. 2002).

2.6 Possible influences of usage of disinfectants for *Salmonella* Infantis and mechanisms of resistance

The industrial use of disinfectants in food industry is an essential tool to avoid bacterial colonization on surfaces and decreasing the risk of cross contamination, but it also contributes to the selection of antibiotic resistant bacteria (Condell et al. 2012, Gantzhorn et al. 2014, Webber et al. 2015).

Additionally, it was found that food-associated bacteria like *Escherichia coli* or *Listeria monocytogenes* tend to yield isolates that are more tolerant towards biocides to which they were exposed to as well (Arvaniti et al. 2021, Du et al. 2015, Guérin et al. 2021, Kode et al. 2021, Merchel Piovesan Pereira et al. 2021, Roedel et al. 2021, Soumet et al. 2016, Xu et al. 2021).

This correlation is less significant in *Salmonella,* even if it is observed in a few isolates. (Soumet et al. 2016)

Environmental biocide challenge, plays an important role in the development of antimicrobial resistance in surviving bacteria (Karatzas et al. 2007, Karatzas et al. 2008, Randall et al. 2007, Whitehead et al. 2011).

A decline in the efficacy of biocides against bacteria is observed since the 1950s which is lead back to a decrease in the susceptibility to several active compounds (Davin-Regli and Pagès 2012, Gnanadhas et al. 2013, Lowbury 1951, Russell 2002).

Furthermore, biofilm formation is an effective way for bacteria to survive as the efficacy of biocide activity is reduced (Høiby et al. 2010, Soto 2013, Venkatesan et al. 2015). The structure of biofilm growth is of great importance by hindering the diffusion of specific biocidal active agents through the bacterial envelope and therefore alleviating the survival of bacteria and accelerating possible changes in gene expression to provide further resistance (Davin-Regli and Pagès 2012, Gnanadhas et al. 2013, Soto 2013)

Moreover, it is commonly known that the expression of efflux systems in bacteria enables the export of both antibiotics and biocides, disinfectants, antiseptics, and preservatives that are all frequently used in medical practice (Davin-Regli and Pagès 2012, Gnanadhas et al. 2013, Soto 2013, Webber et al. 2015).

Efflux pumps make their intracellular concentration decrease, which provokes adaption and selection of bacteria being more tolerant towards biocides and antimicrobial compounds. In summary the mechanisms of resistance to biocides and antibiotics are either intrinsic or acquired (Davin-Regli and Pagès 2012, Gnanadhas et al. 2013, Soto 2013).

Bacterial physiology and morphology is influenced drastically by exposure to biocides as the result are changes in gene expression, modification of growth rates or the emergence of small colonies (Seaman et al. 2007).

Interestingly the chemical biocide and preservative Triclosan, which occurs in cosmetics, tooth paste or consumer goods as well, contributes to the occurrence of bacteria with a decline in susceptibility to antimicrobials and biocides. In this regard, a correlation between resistance to Triclosan and the integration of the active efflux pump in *Salmonella* spp. was found (Hernández et al. 2011, Webber et al. 2008).

Furthermore, Triclosan can cause an alteration in membrane permeability as it is able to improve the characteristics of the bacterial biofilm by expressing genes encoding for the major efflux pump or other genes activating the genetic cascade for multidrug resistance (MDR) control (Tabak et al. 2007).

3 Material and Methods

3.1 Bacterial strains

In the present study four Austrian *Salmonella* Infantis isolates were investigated. Strains MRS17/00712 and PA19/26029 comprised four and two variants, respectively. The phenotypic growth features were determined based on their growing ability on modified semi-solid Rappaport-Vassiliadis agar (MSRV), Xylose-Lactose-Deoxycholate-Agar (XLD), and MacConkey Agar (NEOGEN Culture Media, Lansing, USA). Additionally, the presence of a pESI plasmid was determined (Table 1).

Table 1: Strains of *Salmonella* Infantis

Characteristics	Strain							
	MRS16/01939	MRS17/00712	variants of MRS17/00712			MRS17/02046	PA19/26029	
			small colony	medium colony	large colony		variant 1 (yellow)	variant 2 (black)
origin	Carinthia	Upper Austria	Upper Austria	Upper Austria	Upper Austria	Styria	Lower Austria	Lower Austria
growth (MRSV)	typical	no swarming	no swarming	no swarming	typical	typical	typical	typical black
growth (XLD)	typical	typical	small yellow	medium yellow	typical black	delayed black	colourless	typical black
growth (MacConkey)	yes	yes	yes	yes	yes	no	yes	yes
pESI plasmid	no	no	no	no	yes	yes	no	no

3.2 Disinfectants

Four commercial disinfectants were used in this study. Beside the disinfectants, Table 2 comprises the active compounds, the concentrations tested and the recommended exposure time. The recommended concentration as well as the tested concentrations were made by diluting the disinfectant (dilutant) in lysogeny broth (LB, dilution medium) (Oxoid, ThermoFisher, Vienna, Austria). The exposure times used in the present *in-vitro* testing were 30min, 3h and 5h.

Table 2: Disinfectants used in this study

Disinfectant	Active compounds	Recommended concentration	Concentrations tested	Recommended exposure time
Calgonit DS 680	Aldehyde & quarternary ammonium	2,0%	0,125%; 0,25%; 0,5%; 1,0%; 2,0%	30 min
Calgonit sterizid P12 DES	Aldehyde & quarternary ammonium	0,5%	0,03125%; 0,0625%; 0,125%; 0,25%; 0,5%	15 min
Calgonit sterizid Ecokok	Cresol	0,5%	0,03125%; 0,0625%; 0,125%; 0,25%; 0,5%	30 min
Virkon™ S	Peroxygen	1,0%	0,0625%; 0,125%; 0,25%; 0,5%; 1,0%	30 min

3.3 Application of disinfectants to *Salmonella* Infantis

All *Salmonella* Infantis isolates were stored in cryopreservation fluid (2 ml of 40% glycerol/10 ml brain heart infusion broth (Oxoid, ThermoFisher Scientific, Vienna, Austria)) at -80°C before investigations started. The isolates were thawed and bacterial suspensions were made.

For this, 10ml of LB were inoculated with 100µl of the thawed bacterial isolate and put in the shaking incubator at 200 rpm overnight at 37°C under aerobic conditions (Figure 1).

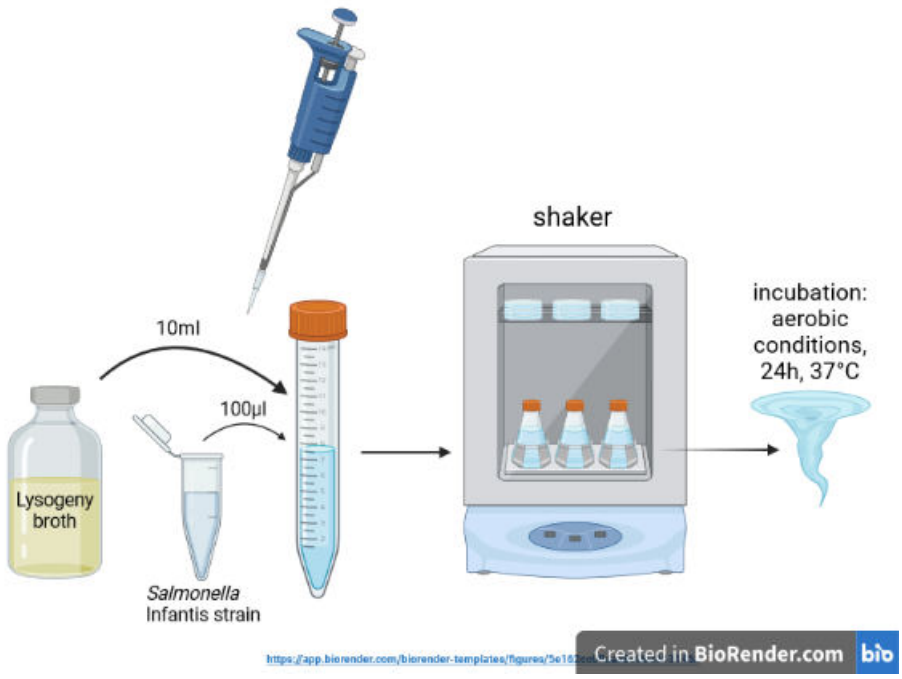

Figure 1: preparation of bacterial suspension

Afterwards, the colony forming units (CFU) count was prepared in serial dilutions in phosphate-buffered saline (PBS, GIBCO, Paisley, UK) up to a dilution of 10^{12} CFU/ml in duplicate. 100 µl of the dilutions were inoculated on MacConkey agar plates by using one-way drigalski spatula (VWR, Vienna, Austria). The plates were incubated for 24h at 37°C (Figure 2). The CFU counts were calculated by determination of the mean value of each dilution in duplicate.

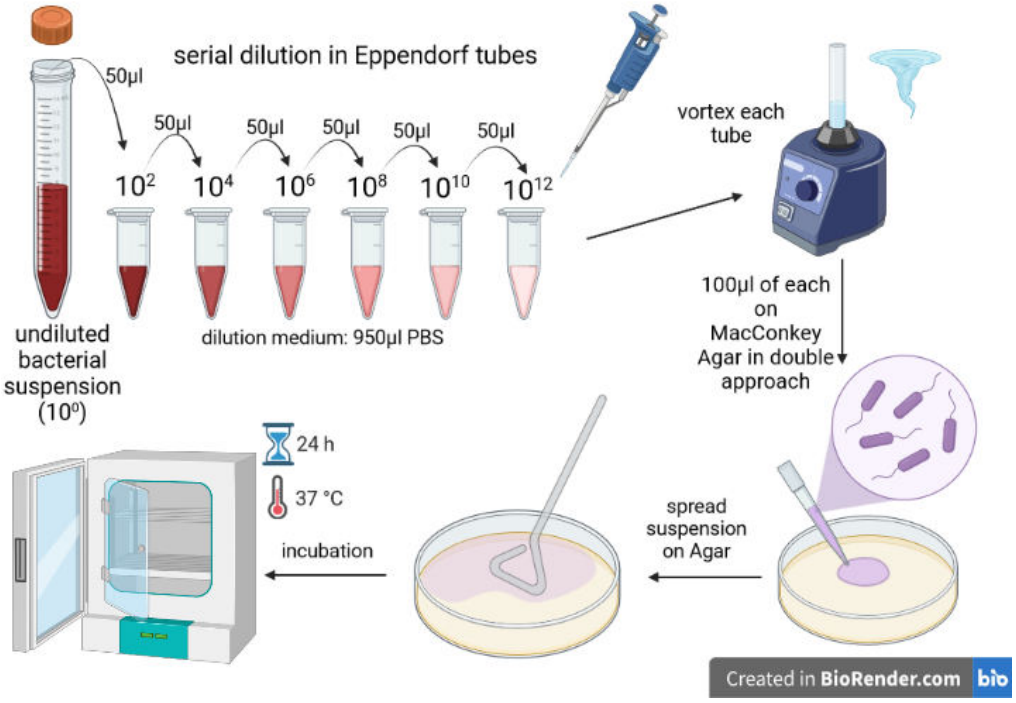

Figure 2: Preparation of CFU count

Dilutions of disinfectants were prepared for macrodilution assay. For this, 9 ml of disinfectant and 1ml of the bacterial suspension were brought together in a 15 ml tube (Sarstedt, Vienna, Austria) and incubated at 37°C under aerobic conditions. After 30min, after 3h and after 5h the tubes were taken out of the incubator and 100µl of each tube were plated on lysogeny broth agar (Oxoid, Thermo Fisher, Vienna, Austria) in duplicate. These plates were incubated for 24h at 37°C (Figure 3).

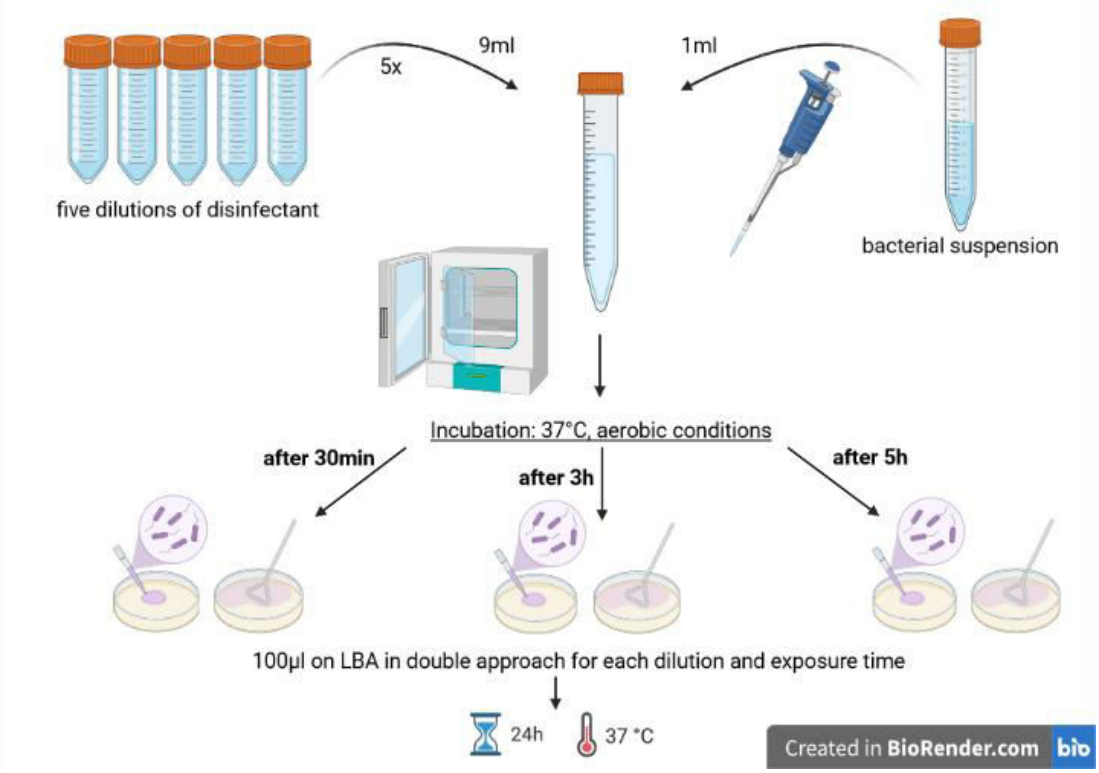

Figure 3: Macrodilution/Application of disinfectants

To analyse the possible impact of stepwise low-level exposure of disinfectants on *Salmonella* Infantis five colonies were picked using a sterile one-way loop (VWR, Vienna, Austria) from each plate in the respective sublethal concentration (MIC) of disinfectant and struck out on MacConkey Agar plates for subcultures. These plates were incubated aerobically overnight at 37°C. Afterwards these pure clones were stored at -80°C in brain-heart infusion broth until their antimicrobial susceptibility testing.

3.4 Microdilution

For antibiotic resistance testing individually designed MICRONAUT-S Veterinary plates (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany) were used. Evaluation of susceptibility was based on the following CLSI protocols: CLSI supplement VET06, CLSI supplement M100, CLSI standard VET01. Furthermore, CASFM recommendations were also included.

In Table 3 the antimicrobial classes and antimicrobial substances with their concentrations used in MICRONAUT-S microtiter plate wells are shown.

A schematic overview of the test setting is shown in Figure 4.

Table 3: MICRONAUT-S plate assignment

	antimicrobial class	*antimicrobial substance*	concentrations (µg/mL)									
1	Penicilline and penicilline combination	Amoxicillin	4	8	16	32						
		Amoxicillin/Clavulanate	4/2	8/4	16/8	32/16						
		Ampicillin	0.25	0.5	1	2	4	8	16			
		Oxacillin	0.25									
2	Cephalosporine	Cefazolin (first generation)	2	4								
		Cefoxitin (second generation)	4									
		Cefotaxime (third generation)	0.25	0.5	1	2	4	8	16	32		
		Ceftazidime (third generation)	0.25	0.5	1	2	4	8	16	32		
3	Chloramphenicol	Chloramphenicol	4	8	16	32						
4	Polypeptide	Colistin	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16
5	Quinolone	Enrofloxacin	0.125	0.25	0.5	1	2					
		Nalidixic acid	4	8	16	32	64					
6	Aminoglycoside	Gentamycin	1	2	4	8	16					
		Neomycin	4	8	16	32						
		Streptomycin	8	16	32	64						
7	Carbapenem	Imipenem	1	2	4							
8	Tetracycline	Tetracycline	0.25	0.5	1	2	4	8	16			
9	Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	8	16								
		Sulfamethoxazol	256	512								
		Trimethoprim/Sulfamethoxazole	0.5/9.5	1/19	2/38	4/76						
10	Macrolide	Tylosin	1	2	4	8	16					

3.5 Agar diffusion test

Agar diffusion testing was performed according to the Kirby-Bauer disk diffusion method (Bauer et al. 1966).

The results were expressed as susceptible, intermediate or resistant according to CLSI guidelines. Table 4 lists the tested antibiotics, their concentration and their CLSI reference levels.

A schematic overview of the test setting is shown in Figure 4.

Table 4: concentrations and reference level for antibiotics used in this study

antibiotics	concentration (µg)	CLSI reference level		
		sensitive	intermediate	resistant
AMPICILLIN	10	≥ 17	14 - 16	≤ 13
CHLORAMPHENICOL	30	≥ 18	13 - 17	≤ 12
ENROFLOXACIN	5	≥ 23	17 - 22	≤ 16
TETRACYCLIN	30	≥ 15	12 - 14	≤ 11

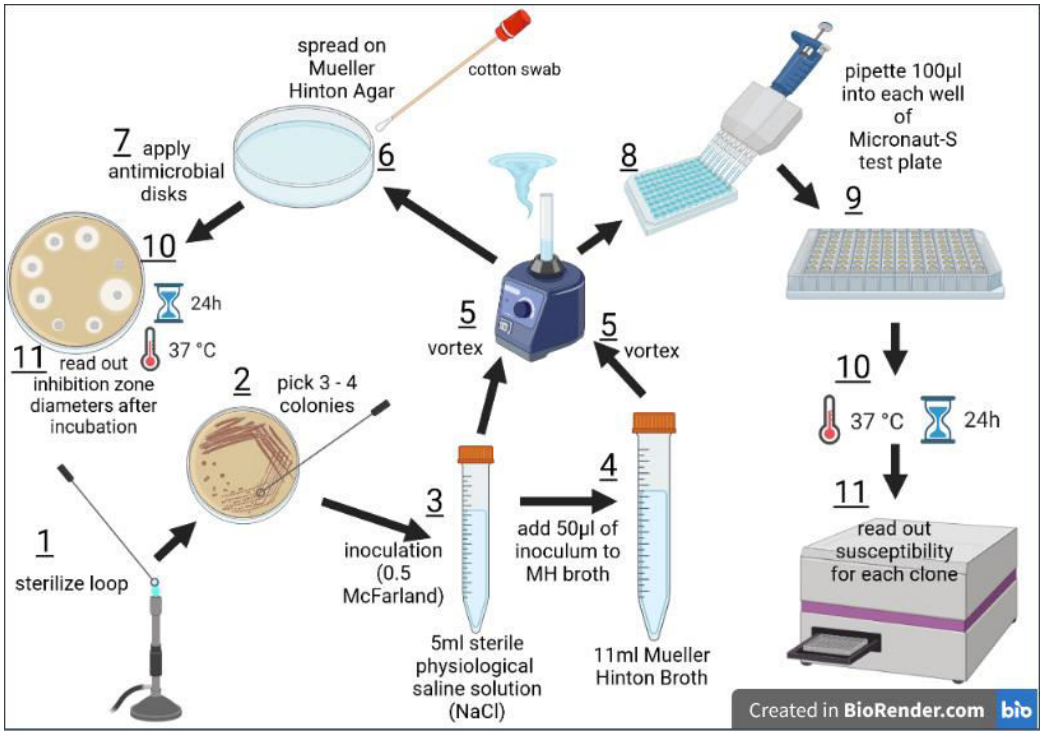

Figure 4: Schematic overview of the antibiotic susceptibility test setting

3.6 Statistics

All calculations were done in Excel (Excel©, Microsoft Corporation, United States of America). The raw data was delivered by the MICRONAUT Software and manually transferred to Excel. All graphs were plotted with Excel, using bar charts, grouped bar charts and XY-Scatter plots. Figures concerning Material and methods were created in BioRender.com (BioRender, 14/07/2022).

4 Results

4.1 Application of disinfectants and selection of antibiotic resistant bacteria

Minimum inhibitory concentrations (MIC) were determined for each strain and each disinfectant by exposure to sublethal concentrations. The collection of surviving bacteria under sub-inhibitory concentrations identified mutants. Table 5 presents an overview of

the application of disinfectants to bacterial strains, showing the number of clones that could be picked for each strain, the exposure time, MIC and disinfectant.

Table 5: Application of disinfectants

Nr.	*Salmonella* Infantis strain	disinfectant	minimum inhibitory concentration (MIC)	exposure time	clones picked
1	MRS16/01939	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,125%	3h	5
		calgonit sterizid Ecokok	0,125%	5h	5
		calgonit sterizid P12 DES	0,03125%	30min	5
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
2	MRS17/00712	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,125%	3h	5
		calgonit sterizid Ecokok	0,125%	5h	5
		calgonit sterizid P12 DES		no growth	
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
3	MRS17/00712 small colony variant	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	2
		calgonit sterizid Ecokok	0,0625%	30min	5
		calgonit sterizid Ecokok	0,0625%	3h	5
		calgonit sterizid Ecokok	0,0625%	5h	5
		calgonit sterizid P12 DES		no growth	
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
4	MRS17/00712 medium colony variant	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,125%	3h	5
		calgonit sterizid Ecokok	0,125%	5h	5
		calgonit sterizid P12 DES	0,03125%	30min	5
		calgonit sterizid P12 DES	0,03125%	3h	5
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
5	MRS17/00712 large colony variant	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,125%	3h	5
		calgonit sterizid Ecokok	0,125%	5h	5
		calgonit sterizid P12 DES	0,03125%	30min	5
		calgonit sterizid P12 DES	0,03125%	3h	5
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
6	MRS17/02046	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,0625%	30min	5
		calgonit sterizid Ecokok	0,0625%	3h	5
		calgonit sterizid Ecokok	0,0625%	5h	5
		calgonit sterizid P12 DES		no growth	
		Virkon™ S	0,25%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
7	MRS19/26029 yellow	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,0625%	3h	5
		calgonit sterizid Ecokok	0,0625%	5h	2
		calgonit sterizid P12 DES	0,0625%	30min	5
		calgonit sterizid P12 DES	0,03125%	3h	5
		calgonit sterizid P12 DES	0,03125%	5h	3
		Virkon™ S	0,5%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5
8	MRS19/26029 black	calgonit DS 680		no growth	
		calgonit sterizid Ecokok	0,125%	30min	5
		calgonit sterizid Ecokok	0,125%	3h	5
		calgonit sterizid Ecokok	0,0625%	5h	5
		calgonit sterizid P12 DES	0,03125%	30min	5
		calgonit sterizid P12 DES	0,03125%	3h	1
		Virkon™ S	0,5%	30min	5
		Virkon™ S	0,25%	3h	5
		Virkon™ S	0,25%	5h	5

4.2 Microdilution

Hereafter, antimicrobial susceptibilities of field isolates as well as their clones received after exposure to the disinfectants are sorted by a) disinfectants, b) MICs and c) exposure times. For a better overview isolates are listed one after the other.

Furthermore, the amount of resistances per clone and resistances sorted by antimicrobial classes are demonstrated in comparison.

4.2.1 Negative Control

According to the lack of growth after exposure to the disinfectant Calgonit DS 680 in all isolates, all exposure times and all concentrations tested, Calgonit DS 680 is assumed to be highly effective in bacterial eliminiation and therefore announced as negative control in this study.

4.2.2 *Salmonella* Infantis MRS16/01939

4.2.2.1 Field strain

The field strain MRS16/01939 proved resistant against seven antimicrobial substances, these are cefoxitin, nalidixic acid, oxacillin, sulfamethoxazole, streptomycin, tetracycline and tylosin. This covers seven antimicrobial classes as well, namely penicilline, cephalosporine, quinolone, aminoglycoside, tetracycline, sulfamethoxazole and macrolide (Figure 5, Table 6).

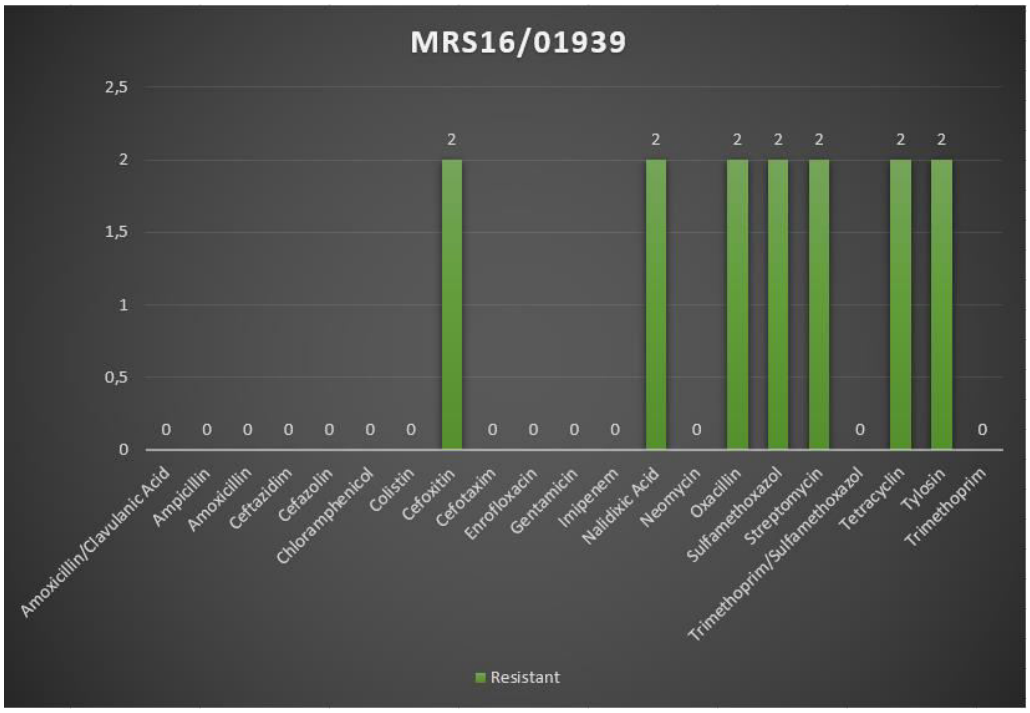

Figure 5: Resistance profile of MRS16/01939

Table 6: Antimicrobial classes of MRS16/01939

antimicrobial class	***antimicrobial substance***	
Penicilline and penicilline combination	Amoxicillin/clavulanic acid	
	Ampicillin	
	Amoxicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin	
	Cefoxitin	**R**
	Cefotaxim	
	Ceftazidim	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	**Nalidixic acid**	**R**
	Enrofloxacin	
Aminoglycoside	Gentamicin	
	Streptomycin	**R**
	Neomycin	
Carbapenem	Imipenem	
Tetracycline	**Tetracyclin**	**R**
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Trimethoprim/Sulfamethoxazole	
	Sulfamethoxazole	**R**
Macrolide	**Tylosin**	**R**

4.2.2.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S 5 clones were picked at a MIC of 0.25%. Increasing resistance was detected in clone I and clone V. Clones II, III and IV showed the same resistance profile as the parental strain MRS16/001939. Clone I tested intermediate for cefazolin and enrofloxacin, and Clone V intermediate for enrofloxacin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0,25%. Increasing resistance could be detected in clones I, III, IV and V. Clones I, IV and V became intermediate to enrofloxacin. Additionally, clones III and IV turned intermediate to cefazolin. There were no deviations from the parental strain MRS16/01939 in clone II.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0,25%. Increasing resistance could be detected in clones I, III and IV. Clone I became intermediate to enrofloxacin. Clones III and IV turned intermediate to cefazolin. There were no deviations from the parental strain MRS16/01939 in clone V.

In total there were eleven increasing resistances detected, which were divided into two antimicrobial classes (Table 7).

Table 7: Number of single increased resistances and number of antimicrobial classes detected for MRS16/01939 after exposure to Virkon™ S

Strain	Disinfectant	%	Time	Clone	Number of increasing resistances	Number of antimicrobial classes
MRS16/01939	Virkon™ S	0,25	30min	I	2	(2) cephalosporine and quinolone
MRS16/01939	Virkon™ S	0,25	30min	II	0	0
MRS16/01939	Virkon™ S	0,25	30min	III	0	0
MRS16/01939	Virkon™ S	0,25	30min	IV	0	0
MRS16/01939	Virkon™ S	0,25	30min	V	1	(1) quinolone
MRS16/01939	Virkon™ S	0,25	3h	I	1	(1) quinolone
MRS16/01939	Virkon™ S	0,25	3h	II	0	0
MRS16/01939	Virkon™ S	0,25	3h	III	1	(1) cephalosporine
MRS16/01939	Virkon™ S	0,25	3h	IV	2	(2) cephalosporine and quinolone
MRS16/01939	Virkon™ S	0,25	3h	V	1	(1) quinolone
MRS16/01939	Virkon™ S	0,25	5h	I	1	(1) quinolone
MRS16/01939	Virkon™ S	0,25	5h	II	0	0
MRS16/01939	Virkon™ S	0,25	5h	III	1	(1) cephalosporine
MRS16/01939	Virkon™ S	0,25	5h	IV	1	(1) cephalosporine
MRS16/01939	Virkon™ S	0,25	5h	V	0	0
					11	2 (cephalosporine and quinolone)

4.2.2.3 Exposure to calgonit sterizid Ecokok

30 min, 3h and 5h after exposure to calgonit sterizid Ecokok 5 clones were picked at a MIC of 0,125%. All clones at all times - except clone III at 30min - turned to sensitive to cefoxitin.

In total there was no increasing resistance detected.

4.2.2.4 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 five clones were picked at a MIC of 0,03125%. Increasing resistances could be detected in clones III and IV. Clones III and IV became intermediate to enrofloxacin. Clones III, IV and V proved sensitive to cefoxitin. There were no deviations from the parental strain MRS16/01939 in clone I and II.

No bacterial growth was present 3h and 5h after exposure to calgonit sterizid P12 DES.

In total there were two increasing resistances detected, which belong to one antimicrobial class (Table 8).

Table 8: Number of single increased resistances and number of antimicrobial classes detected for MRS16/01939 after exposure to calgonit sterizid P12 DES

Strain	Disinfectant	%	Time	Clone	Number of increasing resistances	Number of antimicrobial classes
MRS16/01939	calgonit sterizid P12 DES	0,03125	30min	I	0	0
MRS16/01939	calgonit sterizid P12 DES	0,03125	30min	II	0	0
MRS16/01939	calgonit sterizid P12 DES	0,03125	30min	III	1	(1) quinolone
MRS16/01939	calgonit sterizid P12 DES	0,03125	30min	IV	1	(1) quinolone
MRS16/01939	calgonit sterizid P12 DES	0,03125	30min	V	0	0
					2	1 (quinolone)

4.2.3 *Salmonella* Infantis MRS17/00712

4.2.3.1 Field strain

The field strain MRS17/00712 is naturally resistant to oxacillin and tylosin, and intermediate to streptomycin. This represents three antimicrobial classes, namely penicilline, aminoglycoside and macrolide (Figure 6, Table 9).

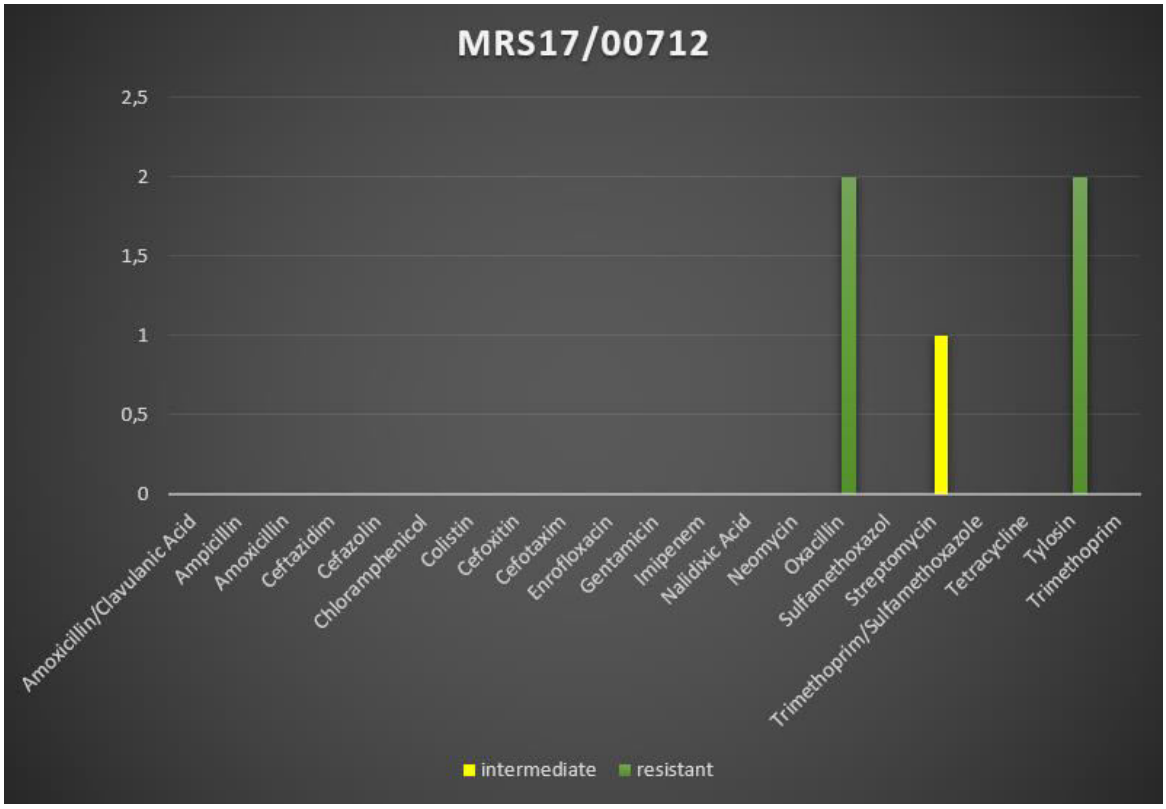

Figure 6: Resistance profile of MRS17/00712

Table 9: Antimicrobial classes of MRS17/00712

antimicrobial class	***antimicrobial substance***	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanic Acid	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	**I**
Carbapenem	Imipenem	
Tetracycline	Tetracycline	
Diaminopyrimidine, sulfamethoxazole, and combinations	Tetracycline, Trimethoprim, Sulfamethoxazol, Trimethoprim/Sulfamethoxazol	
Macrolide	**Tylosin**	**R**

4.2.3.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clones I, II, IV and V. Clone III presented the same resistance profile as the parental strain MRS17/00712. Clones I, II, IV and V proved resistant to nalidixic acid, streptomycin, sulfamethoxazole and tetracycline. Additionally, clone II turned resistant to cefoxitin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0,25%. Increasing resistance was detected in clones I and II. Clones III, IV and V showed the same resistance profile as the parental strain MRS17/00712. Clones I and II acquired resistance to nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and cefoxitin.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0,25%. Increasing resistance was detected in clones I and V. They acquired resistance to nalidixic acid, streptomycin, sulfamethoxazole and tetracycline. Additionally clone I became resistant to cefoxitin. No deviations from the parental strain MRS17/00712 were found for clones II, III, and IV, but they proved sensitive to Streptomycin.

In total there were thirty-six increasing resistances detected, which were divided into five antimicrobial classes (Table 10).

Table 10: Number of single increased resistances and number of antimicrobial classes detected for MRS17/00712 after exposure to Virkon™ S

Strain	Disinfectant	%	Time	Clone	Number of increasing resistances	Number of antimicrobial classes
MRS17/00712	Virkon™ S	0,25	30min	I	4	(4) quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	30min	II	5	(5) cephalosporine; quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	30min	III	0	0
MRS17/00712	Virkon™ S	0,25	30min	IV	4	(4) quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	30min	V	4	(4) quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	3h	I	5	(5) cephalosporine; quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	3h	II	5	(5) cephalosporine; quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	3h	III	0	0
MRS17/00712	Virkon™ S	0,25	3h	IV	0	0
MRS17/00712	Virkon™ S	0,25	3h	V	0	0
MRS17/00712	Virkon™ S	0,25	5h	I	5	(5) cephalosporine; quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
MRS17/00712	Virkon™ S	0,25	5h	II	0	0
MRS17/00712	Virkon™ S	0,25	5h	III	0	0
MRS17/00712	Virkon™ S	0,25	5h	IV	0	0
MRS17/00712	Virkon™ S	0,25	5h	V	4	(4) quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations
					36	**5 (cephalosporine; quinolone; aminoglycoside; tetracycline; diaminopyrimide, sulfamethoxazole and combinations)**

4.2.3.3 Exposure to calgonit sterizid Ecokok

30 min after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Clones IV and V showed the same resistance profile as the parental strain MRS17/00712. Surprisingly clones I, II and III turned sensitive to streptomycin.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was only detected in clone III. It acquired resistance to

sulfamethoxazole. Clone II showed the same resistance profile as the parental strain MRS17/00712. Clones I, III, IV and V became sensitive to Streptomycin.

5h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. There were no increasing resistances detected compared with parental strain MRS17/00712 in all clones, except clones I, II and IV turned sensitive to streptomycin.

In total there was one increasing resistance detected.

4.2.3.4 Exposure to calgonit sterizid P12 DES

No bacterial growth was present 30 minutes, 3h and 5h after exposure to calgonit sterizid P12 DES.

4.2.4 Variants of *Salmonella* Infantis MRS17/00712

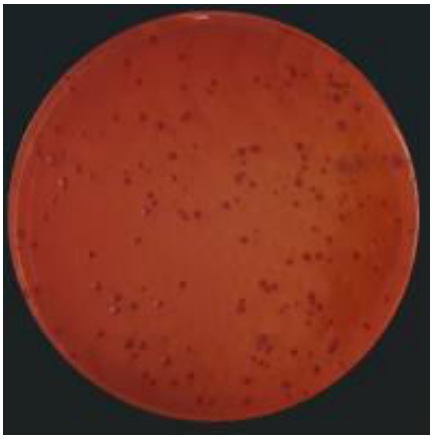

Figure 7: different variants of *Salmonella* Infantis strain MRS17/00712 on MacConkey Agar

4.2.4.1 Variant MRS17/00712 small colony

Cultures of the small colony were naturally resistant to oxacillin and tylosin, and intermediate to streptomycin. This represents three antimicrobial classes, namely

penicilline, aminoglycoside and macrolide. There are no deviations from the field strain MRS17/00712.

4.2.4.1.1 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistances were detected in clones III and IV. Clones I, II and V showed the same resistance profile as the parental strain MRS17/00712 small colony. Clone III became resistant to sulfamethoxazole and clone IV acquired resistance to streptomycin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. All clones showed the same resistance profile as the parental strain MRS17/00712 small colony.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Clones I and III turned sensitive to streptomycin. All other clones showed the same resistance profile as the parental strain MRS17/00712 small colony.

In total there were two increasing resistances detected.

4.2.4.1.2 Exposure to calgonit sterizid Ecokok

30 min after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. Clone II and III turned sensitive to streptomycin. All other clones showed the same resistance profile as the parental strain MRS17/00712 small colony.

30 min after exposure to calgonit sterizid Ecokok two clones were picked at a MIC of 0.125%. One increasing resistance was detected in clone I, that turned resistant against streptomycin. Clone II presented the same resistance profile as the parental strain MRS17/00712 small colony.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. There was no increasing resistance present. All clones show the same resistance profile as the parental strain MRS17/00712 small colony, except clones II, IV and V which turned sensitive to streptomycin.

5h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. There was no increasing resistance present. All clones show the same resistance profile as the parental strain MRS17/00712 small colony, except clones III and V which turned sensitive to streptomycin.

In total there was one increasing resistance detected.

4.2.4.1.3 Exposure to calgonit sterizid P12 DES

No bacterial growth was present 30 minutes, 3h and 5h after exposure to calgonit sterizid P12 DES.

4.2.4.2 Variant MRS17/00712 medium colony

The medium colony was naturally resistant against six antimicrobial substances. These are nalidixic acid, oxacillin, sulfamethoxazole, streptomycin, tetracycline and tylosin. This represents six antimicrobial classes, namely penicilline, quinolone, aminoglycoside, tetracycline, diaminopyrimidine/sulfamethoxazole/combinations and macrolide. Compared to the field strain MRS17/00712 four deviations were present. The MRS17/00712 strain comprising medium colony harbourd four further resistances. These are namely against nalidixic acid, sulfamethoxazole, streptomycin and tetracycline (Figure 8, Table 11).

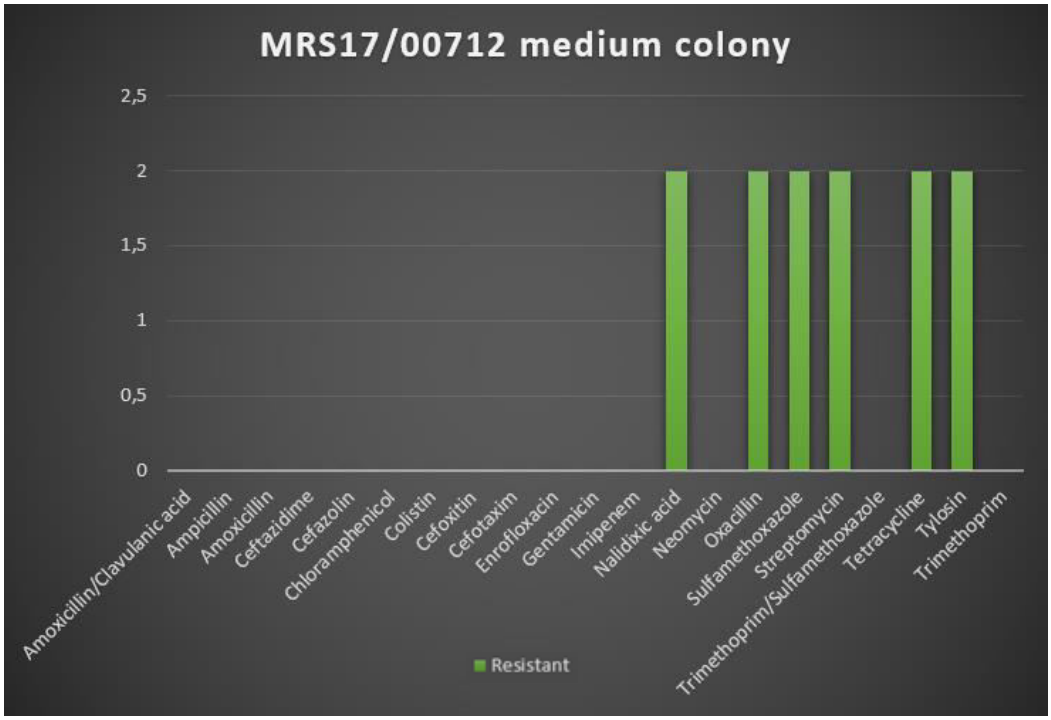

Figure 8: Resistance profile of MRS17/00712 medium colony

Table 11: Antimicrobial classes of MRS17/00712 medium colony

antimicrobial class	***antimicrobial substance***	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanic acid	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	**R**
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	**R**
Carbapenem	Imipenem	
Tetracycline	**Tetracycline**	**R**
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Sulfamethoxazole	**R**
	Trimethoprim/Sulfamethoxazole	
Macrolide	**Tylosin**	**R**

4.2.4.2.1 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in all clones. Each clone acquired resistance to cefoxitin. Furthermore, clones I, II, III and IV were tested intermediate to cefazolin. Additionally, clone I became intermediate to enrofloxacin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Again, increasing resistance was detected in all clones. Similar results as for MIC 0.25% after 30min were found. All five clones acquired resistance to cefoxitin. Clones III and V turned intermediate to cefazolin. Clones IV and V became intermediate to enrofloxacin.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clones I, II, III and V. Clone IV showed the same resistance profile as the parental strain MRS17/00712 medium colony. Clones I, II and V acquired resistance to cefoxitin. Clones II, III and V turned intermediate to cefazolin. Clone III and V additionally became intermediate to enrofloxacin.

In total there were twenty-seven increasing resistances detected, which were divided into two antimicrobial classes (Table 12).

Table 12: Number of single increased resistances and number of antimicrobial classes detected for variant MRS17/00712 medium colony after exposure to Virkon™ S

Strain	Disinfectant	%	Time	Clone	Number of increasing resistances	Number of antimicrobial classes
MRS17/00712 medium colony	Virkon™ S	0,25	30min	I	3	(2) cephalosporine and quinolone
MRS17/00712 medium colony	Virkon™ S	0,25	30min	II	2	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	30min	III	2	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	30min	IV	2	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	30min	V	1	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	3h	I	1	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	3h	II	1	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	3h	III	2	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	3h	IV	2	(2) cephalosporine and quinolone
MRS17/00712 medium colony	Virkon™ S	0,25	3h	V	3	(2) cephalosporine and quinolone
MRS17/00712 medium colony	Virkon™ S	0,25	5h	I	1	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	5h	II	2	(1) cephalosporine
MRS17/00712 medium colony	Virkon™ S	0,25	5h	III	2	(2) cephalosporine and quinolone
MRS17/00712 medium colony	Virkon™ S	0,25	5h	IV	0	0
MRS17/00712 medium colony	Virkon™ S	0,25	5h	V	3	(2) cephalosporine and quinolone
					27	2 (cephalosporine and quinolone)

4.2.4.2.2 Exposure to calgonit sterizid Ecokok

30 minutes after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistances were detected in all clones. Each clone acquired resistance to cefoxitin.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected in all clones, except clone V which surprisingly turned sensitive to streptomycin. Clones I, II, III and IV acquired resistance to cefoxitin.

5h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected in clones III, IV and V. Clones I and II showed the same resistance profile as the parental strain MRS17/00712 medium colony. Clones III, IV and V became resistant to cefoxitin.

In total there were twelve increasing resistances detected, which belong to one antimicrobial class (Table 13).

Table 13: Number of single increased resistances and number of antimicrobial classes detected for variant MRS17/00712 medium colony after exposure to calgonit sterizid Ecokok

Strain	Disinfectant	%	Time	Clone	number of increasing resistances	number of antimicrobial classes
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	30min	I	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	30min	II	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	30min	III	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	30min	IV	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	30min	V	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	3h	I	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	3h	II	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	3h	III	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	3h	IV	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	3h	V	0	0
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	5h	I	0	0
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	5h	II	0	0
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	5h	III	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	5h	IV	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. Ecokok	0,125	5h	V	1	(1) cephalosporine
					12	1 (cephalosporine)

4.2.4.2.3 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in all clones, except clone II. Clones I, III and V acquired resistance to cefoxitin. Clone I turned additionally intermediate to neomycin. Surprisingly clone IV became resistant to colistin. Clone II showed the same resistance profile as the parental strain MRS17/00712 medium colony.

3h after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in all clones. Clones I, IV and V acquired resistance to cefoxitin. Clones I, II and V became intermediate to enrofloxacin. Clone III turned intermediate to cefazolin.

5h after exposure to calgonit sterizid P12 DES there was no bacterial growth.

In total there were twelve increasing resistances detected, which belong to four different antimicrobial classes (Table 14).

Table 14: Number of single increased resistances and number of antimicrobial classes detected for variant MRS17/00712 medium colony after exposure to calgonit sterizid P12 DES

Strain	Disinfectant	%	Time	Clone	number of increasing resistances	number of antimicrobial classes
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	30min	I	2	(2) cephalosporine and aminoglycoside
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	30min	II	0	0
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	30min	III	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	30min	IV	1	(1) polypeptide
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	30min	V	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	3h	I	2	(2) cephalosporine and quinolone
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	3h	II	1	(1) quinolone
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	3h	III	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	3h	IV	1	(1) cephalosporine
MRS17/00712 medium colony	calg. ster. P12 DES	0,03125	3h	V	2	(2) cephalosporine and quinolone
					12	4 (cephalosporine, quinolone, aminoglycoside and polypeptide)

4.2.4.3 Variant MRS17/00712 large colony

Cultures of the large colony were naturally resistant against seven antimicrobial substances. These are cefoxitin, nalidixic acid, oxacillin, sulfamethoxazole, streptomycin, tetracycline and tylosin. This represents six antimicrobial classes, namely

cephalosporine, quinolone, penicilline, diaminopyrimidine + sulfamethoxazole and combinations, aminoglycoside, tetracycline and macrolide. Compared to the field strain MRS17/00712 five deviations were present. The MRS17/00712 strain comprising large colony harbourd five further resistances. These are namely to cefoxitin, nalidixic acid, sulfamethoxazole, streptomycin and tetracycline (Figure 9, Table 15).

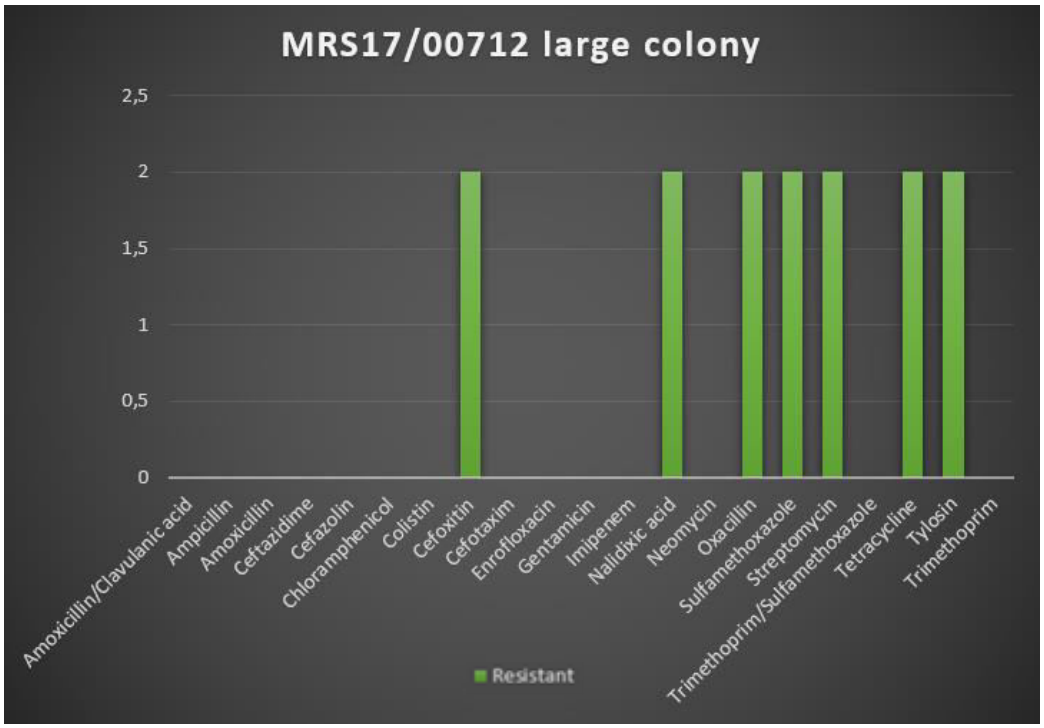

Figure 9: Resistance profile of MRS17/00712 large colony

Table 15: Antimicrobial classes of MRS17/00712 large colony

antimicrobial class	*antimicrobial substance*	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanic acid	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	**R**
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	**R**
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	**R**
Carbapenem	Imipenem	
Tetracycline	**Tetracycline**	**R**
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Sulfamethoxazole	**R**
	Trimethoprim/Sulfamethoxazole	
Macrolide	**Tylosin**	**R**

4.2.4.3.1 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. All clones showed the same resistance profile as the parental strain MRS17/00712 large colony, except clone IV that became intermediate to enrofloxacin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance were detected in all clones. Clone V acquired resistance to colistin. Clones I, II, III and IV became intermediate to enrofloxacin. Additionally, clones I and III proved intermediate to cefazolin.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in all clones. Each clone became intermediate to cefazolin. Clone II additionally turned intermediate to enrofloxacin and neomycin. Clones II and III surprisingly turned sensitive to cefoxitin.

In total fifteen increasing resistances were detected, which belong to four different antimicrobial classes (Table 16).

Table 16: Number of single increased resistances and number of antimicrobial classes detected for variant MRS17/00712 large colony after exposure to Virkon™ S

Strain	Disinfectant	%	Time	Clone	Number of increasing resistances	Number of antimicrobial classes
MRS17/00712 large colony	Virkon™ S	0,25	30min	I	0	0
MRS17/00712 large colony	Virkon™ S	0,25	30min	II	0	0
MRS17/00712 large colony	Virkon™ S	0,25	30min	III	0	0
MRS17/00712 large colony	Virkon™ S	0,25	30min	IV	1	(1) quinolone
MRS17/00712 large colony	Virkon™ S	0,25	30min	V	0	0
MRS17/00712 large colony	Virkon™ S	0,25	3h	I	2	(2) cephalosporine and quinolone
MRS17/00712 large colony	Virkon™ S	0,25	3h	II	1	(1) quinolone
MRS17/00712 large colony	Virkon™ S	0,25	3h	III	2	(2) cephalosporine and quinolone
MRS17/00712 large colony	Virkon™ S	0,25	3h	IV	1	(1) quinolone
MRS17/00712 large colony	Virkon™ S	0,25	3h	V	1	(1) polypeptide
MRS17/00712 large colony	Virkon™ S	0,25	5h	I	1	(1) cephalosporine
MRS17/00712 large colony	Virkon™ S	0,25	5h	II	3	(3) cephalosporine, quinolone and aminoglycoside
MRS17/00712 large colony	Virkon™ S	0,25	5h	III	1	(1) cephalosporine
MRS17/00712 large colony	Virkon™ S	0,25	5h	IV	1	(1) cephalosporine
MRS17/00712 large colony	Virkon™ S	0,25	5h	V	1	(1) cephalosporine
					15	4 (cephalosporine, quinolone, aminoglycoside, polypeptide)

4.2.4.3.2 Exposure to calgonit sterizid Ecokok

30 min after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected only in clone V. Clones I, II and IV showed the same resistance profile as the parental strain MRS17/00712 large colony. Clone III turned intermediate to streptomycin. Clone V became intermediate to cefazolin, but sensitive to cefoxitin.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected only in clone I, which became intermediate to enrofloxacin. In contrast, clones II and IV became sensitive to cefoxitin, and clones III and V turned sensitive to streptomycin.

5h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistances were detected in clones I and II. Clone I turned intermediate to enrofloxacin and clone II became intermediate to cefazolin. Clones III and IV showed the same resistance profile as the parental strain MRS17/00712 large colony. Surprisingly clones I and V turned sensitive to Cefoxitin.

In total there were four increasing resistances detected.

4.2.4.3.3 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in clones I and IV. Clone I turned intermediate to enrofloxacin and clone IV became intermediate to cefazolin. Surprisingly all five clones turned sensitive to cefoxitin.

3h after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in clones III, IV and V. Clones I and II showed the same resistance profile as the parental strain MRS17/00712 large colony. Clones III, IV and V became intermediate to enrofloxacin. Clone IV additionally turned intermediate to cefazolin, but surprisingly sensitive to cefoxitin.

No bacterial growth was present 5h after exposure to calgonit sterizid P12 DES.

In total there were six increasing resistances detected.

4.2.5 *Salmonella* Infantis MRS17/02046

4.2.5.1 Field strain

The field strain MRS17/02046 is naturally resistant to oxacillin, nalidixic acid, streptomycin, sulfamethoxazole and tylosin, and intermediate to tetracycline. These are representing six antimicrobial classes, namely penicilline, quinolone, aminoglycoside, diaminopyrimidine + sulfamethoxazole and combinations and macrolide (Figure 10, Table 17).

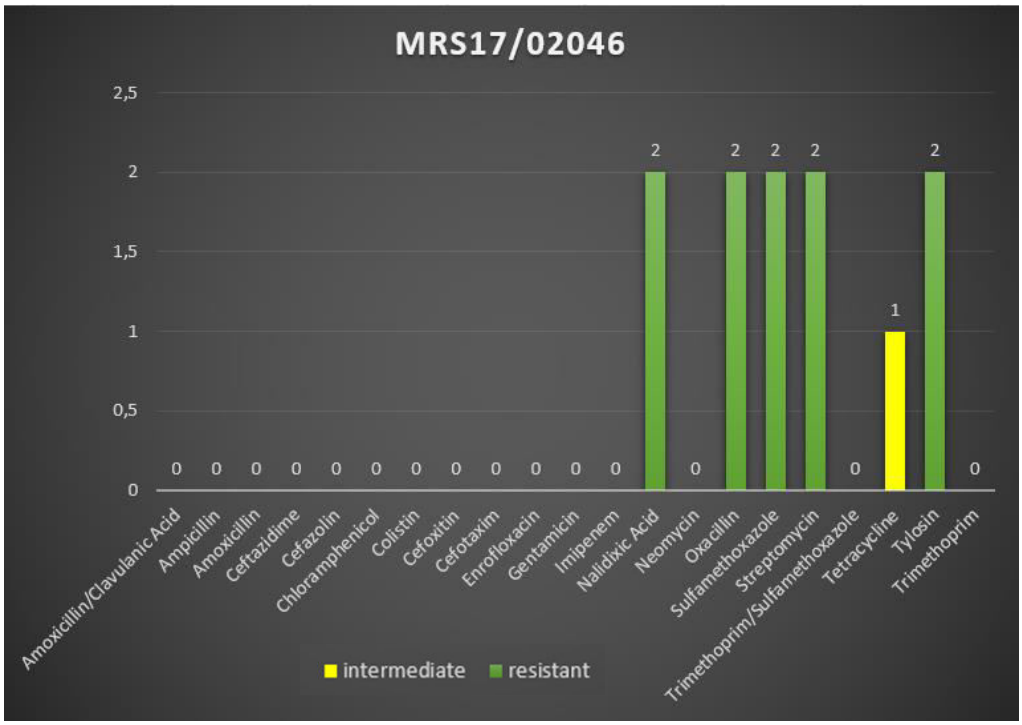

Figure 10: Resistance profile of MRS17/02046

Table 17: Antimicrobial classes of MRS17/02046

antimicrobial class	***antimicrobial substance***	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanate	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	**R**
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	**R**
Carbapenem	Imipenem	
Tetracycline	**Tetracycline**	**I**
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Sulfamethoxazol	**R**
	Trimethoprim/Sulfamethoxazol	
Macrolide	**Tylosin**	**R**

4.2.5.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clone IV, which turned resistant to tetracycline. Clone II showed the same resistance profile as the parental strain MRS17/02046. Clones I, III, IV and V became intermediate to streptomycin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clone V, which acquired resistance to imipenem. Clones II and III showed the same resistance profile as the parental strain MRS17/02046. Clones I, IV and V became intermediate to streptomycin.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. There was no increasing resistance detected. Clones I, II, IV and V showed the same resistance profile as the parental strain MRS17/02046. Clone III became intermediate to streptomycin.

In total there were two increasing resistances detected.

4.2.5.3 Exposure to calgonit sterizid Ecokok

30 minutes after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. There was no increasing resistance detected. Clones I, II, III and V showed the same resistance profile as the parental strain MRS17/02046. Clone IV became intermediate to streptomycin.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. Increasing resistance was detected in all clones, except clone V. Clones I, II, III and IV acquired resistance to tetracycline. Clones III and V became intermediate to streptomycin.

5h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. Increasing resistance was detected in clones I, IV and V. These clones acquired resistance to tetracycline. Clones II, III and IV became intermediate to streptomycin.

In total there were seven increasing resistances detected, which belong to one antimicrobial class (Table 18).

Table 18: Number of single increased resistances and number of antimicrobial classes detected for MRS17/02046 after exposure to calgonit sterizid Ecokok

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
MRS17/02046	CS Ecokok	0,0625	30min	I	0	0
MRS17/02046	CS Ecokok	0,0625	30min	II	0	0
MRS17/02046	CS Ecokok	0,0625	30min	III	0	0
MRS17/02046	CS Ecokok	0,0625	30min	IV	0	0
MRS17/02046	CS Ecokok	0,0625	30min	V	0	0
MRS17/02046	CS Ecokok	0,0625	3h	I	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	3h	II	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	3h	III	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	3h	IV	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	3h	V	0	0
MRS17/02046	CS Ecokok	0,0625	5h	I	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	5h	II	0	0
MRS17/02046	CS Ecokok	0,0625	5h	III	0	0
MRS17/02046	CS Ecokok	0,0625	5h	IV	1	(1) tetracycline
MRS17/02046	CS Ecokok	0,0625	5h	V	1	(1) tetracycline
					7	1 (tetraycycline)

4.2.5.4 Exposure to calgonit sterizid P12 DES

No bacterial growth was present 30 minutes, 3h and 5h after exposure to calgonit sterizid P12 DES.

4.2.6 *Salmonella* Infantis PA19/26029 yellow

4.2.6.1 Field strain

The field strain PA19/26029 yellow is naturally resistant to oxacillin and tylosin, and intermediate to streptomycin. These are representing three antimicrobial classes, namely penicilline, macrolide and aminoglycoside (Figure 11, Table 19).

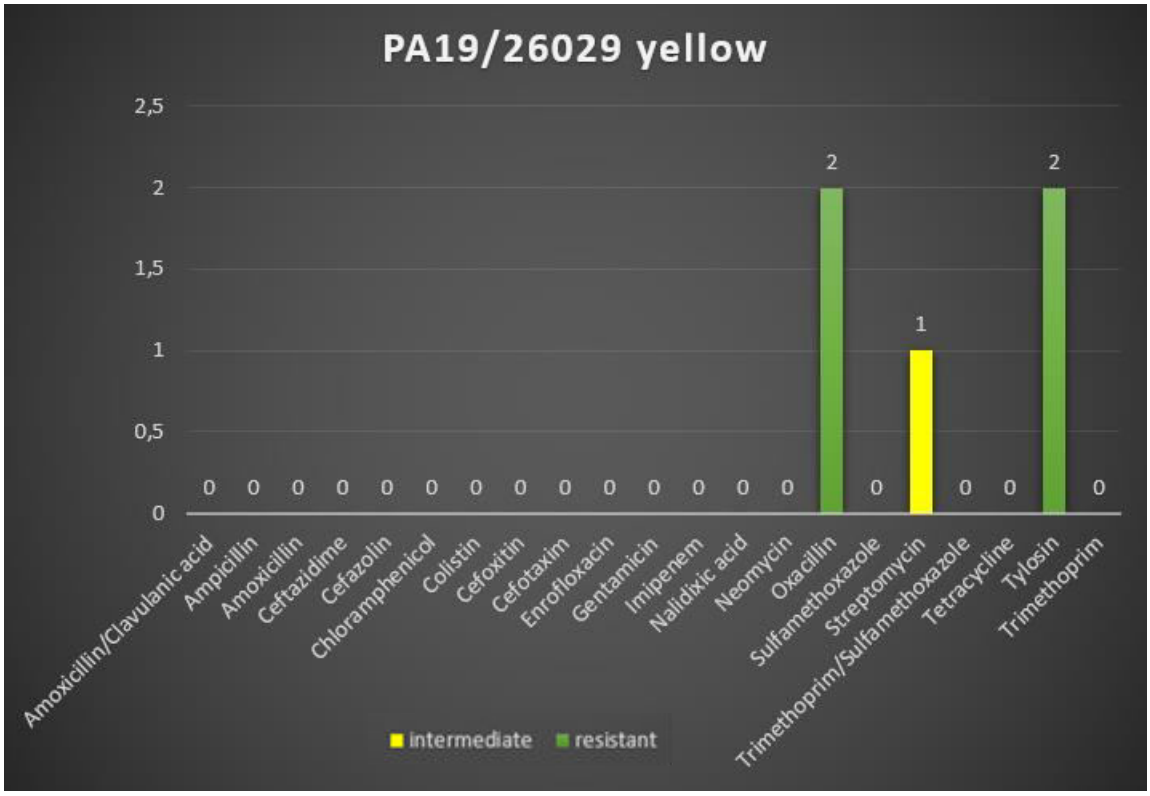

Figure 11: Resistance profile of PA19/26029 yellow

Table 19: Antimicrobial classes of PA19/26029 yellow

antimicrobial class	*antimicrobial substance*	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanic acid	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	**I**
Carbapenem	Imipenem	
Tetracycline	Tetracycline	
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Sulfamethoxazole	
	Trimethoprim/Sulfamethoxazole	
Macrolide	**Tylosin**	**R**

4.2.6.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.5%. Increasing resistance was detected in all clones. Each clone acquired resistance to cefoxitin. Clones II, III, IV and V additionally acquired resistance to sulfamethoxazole. Clone I additionally became intermediate to cefazolin and surprisingly sensitive to streptomycin.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clones I, III and IV. Clones I and IV acquired resistance to cefoxitin. Clone I additionally became resistant to sulfamethoxazole. Clone III acquired resistance to streptomycin. Clone V surprisingly turned sensitive to streptomycin. Clone II showed the same resistance profile as the parental strain PA19/26029 yellow.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in clones II and III, both acquiring resistance to cefoxitin. Clone III additionally turned intermediate to cefazolin. Clones I and IV showed the same resistance profile as the parental strain PA19/26029 yellow. Clone V surprisingly became sensitive to streptomycin.

In total there were seventeen increasing resistances detected, which belong to three different antimicrobial classes (Table 20).

Table 20: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 yellow after exposure to Virkon™ S

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 yellow	Virkon™ S	0,5	30min	I	2	(1) cephalosporine
PA19/26029 yellow	Virkon™ S	0,5	30min	II	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	Virkon™ S	0,5	30min	III	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	Virkon™ S	0,5	30min	IV	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	Virkon™ S	0,5	30min	V	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	Virkon™ S	0,25	3h	I	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	Virkon™ S	0,25	3h	II	0	
PA19/26029 yellow	Virkon™ S	0,25	3h	III	1	(1) aminoglycoside
PA19/26029 yellow	Virkon™ S	0,25	3h	IV	1	(1) cephalosporine
PA19/26029 yellow	Virkon™ S	0,25	3h	V	0	0
PA19/26029 yellow	Virkon™ S	0,25	5h	I	0	0
PA19/26029 yellow	Virkon™ S	0,25	5h	II	1	(1) cephalosporine
PA19/26029 yellow	Virkon™ S	0,25	5h	III	2	(1) cephalosporine
PA19/26029 yellow	Virkon™ S	0,25	5h	IV	0	0
PA19/26029 yellow	Virkon™ S	0,25	5h	V	0	0
					17	**3 (cephalosporine, aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations)**

4.2.6.3 Exposure to calgonit sterizid Ecokok

30 min after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected in all clones, except clone V. Clones I, III and IV acquired resistance to sulfamethoxazole. Clones II and IV became resistant to streptomycin. Clone IV additionally turned resistant to cefoxitin. Clone V showed the same resistance profile as the parental strain PA19/26029 yellow.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. Increasing resistance was detected in all clones. Clones I, II, III and IV acquired resistance to sulfamethoxazole. Clones II and V became resistant to streptomycin. Clone IV additionally turned resistant to cefoxitin and clone I additionally acquired resistance to imipenem and turned intermediate to cefazolin.

5h after exposure to calgonit sterizid Ecokok two clones were picked at a MIC of 0.0625%. Increasing resistances were detected in both clones. They both acquired resistance to sulfamethoxazole.

In total there were seventeen increasing resistances detected, which belong to four different antimicrobial classes (Table 21).

Table 21: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 yellow after exposure to calgonit sterizid Ecokok

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 yellow	CS Ecokok	0,125	30min	I	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,125	30min	II	1	(1) aminoglycoside
PA19/26029 yellow	CS Ecokok	0,125	30min	III	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,125	30min	IV	3	(3) cephalosporine, aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,125	30min	V	0	0
PA19/26029 yellow	CS Ecokok	0,0625	3h	I	3	(3) cephalosporine, carbapenem and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,0625	3h	II	2	(2) aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,0625	3h	III	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,0625	3h	IV	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,0625	3h	V	1	(1) aminoglycoside
PA19/26029 yellow	CS Ecokok	0,0625	5h	I	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	CS Ecokok	0,0625	5h	II	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
					17	**4 (cephalosporine, aminoglycoside, carbapenem and diaminopyrimidine, sulfamethoxazole and combinations)**

4.2.6.4 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.0625%. Increasing resistance was detected in clones I, II and III. Clones IV and V showed the same resistance profile as the parental strain PA19/26029 yellow. Clones I, II and III acquired resistance to sulfamethoxazole. Clones I and II additionally became resistant to streptomycin.

3h after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in clones II and V. Clones I, III and IV showed the same resistance profile as the parental strain PA19/26029 yellow. Clone II acquired resistance to sulfamethoxazole and turned intermediate to cefazolin. Clone V became resistant to cefoxitin and streptomycin.

5h after exposure to calgonit sterizid P12 DES three clones were picked at a MIC of 0.03125%. Increasing resistance was detected in all three clones. Clones I and III acquired resistance to streptomycin. Clones II and III additionally acquired resistance to cefoxitin. Clone II additionally became resistant to sulfamethoxazole and intermediate to cefazolin.

In total there were fifteen increasing resistances detected, which belong to three different antimicrobial classes (Table 22).

Table 22: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 yellow after exposure to calgonit sterizid P12 DES

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 yellow	calg. ster. P12 DES	0,0625	30min	I	2	(2) aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	calg. ster. P12 DES	0,0625	30min	II	2	(2) aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	calg. ster. P12 DES	0,0625	30min	III	1	(1) diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	calg. ster. P12 DES	0,0625	30min	IV	0	0
PA19/26029 yellow	calg. ster. P12 DES	0,0625	30min	V	0	0
PA19/26029 yellow	calg. ster. P12 DES	0,03125	3h	I	0	0
PA19/26029 yellow	calg. ster. P12 DES	0,03125	3h	II	2	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	calg. ster. P12 DES	0,03125	3h	III	0	0
PA19/26029 yellow	calg. ster. P12 DES	0,03125	3h	IV	0	0
PA19/26029 yellow	calg. ster. P12 DES	0,03125	3h	V	2	(2) cephalosporine and aminoglycoside
PA19/26029 yellow	calg. ster. P12 DES	0,03125	5h	I	1	(1) aminoglycoside
PA19/26029 yellow	calg. ster. P12 DES	0,03125	5h	II	3	(2) cephalosporine and diaminopyrimidine, sulfamethoxazole and combinations
PA19/26029 yellow	calg. ster. P12 DES	0,03125	5h	III	2	(2) cephalosporine and aminoglycoside
					15	**(3) cephalosporine, aminoglycoside and diaminopyrimidine, sulfamethoxazole and combinations**

4.2.7 *Salmonella* Infantis PA19/26029 black

4.2.7.1 Field strain

The field strain PA19/26029 black is naturally resistant to oxacillin, sulfamethoxazole and tylosin. These represent three antimicrobial classes, namely penicilline, diaminopyrimidine + sulfamethoxazole and combinations and macrolide (Figure 12, Table 23).

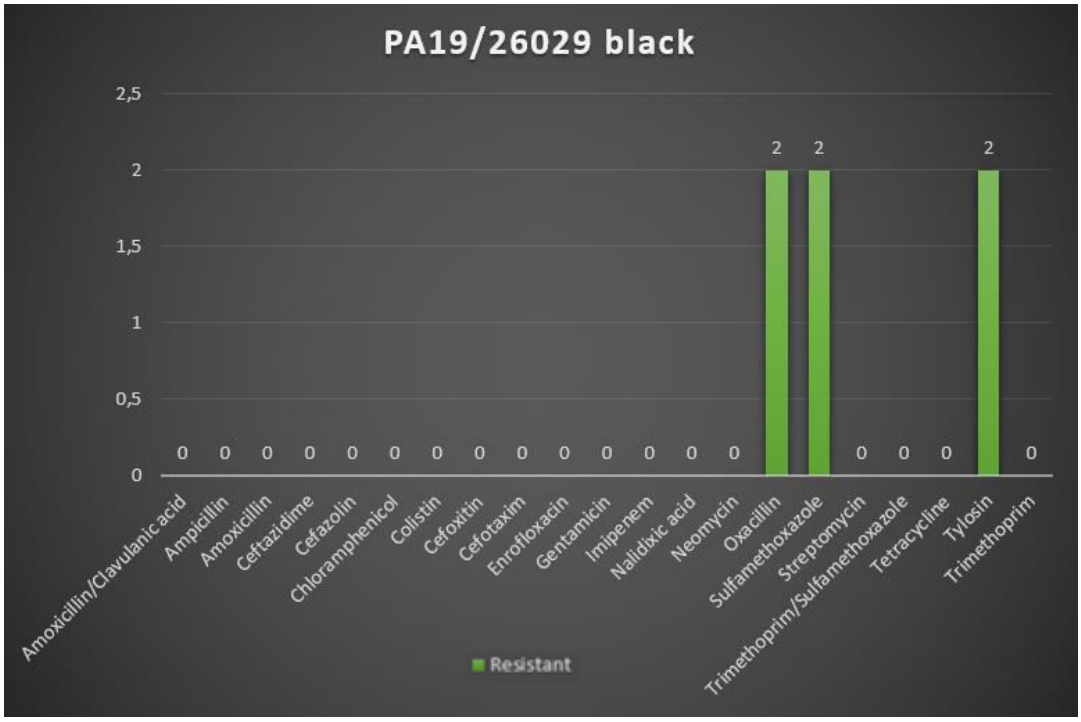

Figure 12: Resistance profile of PA19/26029 black

Table 23: Antimicrobial classes of PA19/26029 black

antimicrobial class	*antimicrobial substance*	
Penicilline and penicilline combination	Amoxicillin	
	Amoxicillin/Clavulanic acid	
	Ampicillin	
	Oxacillin	**R**
Cephalosporine	Cefazolin (first generation)	
	Cefoxitin (second generation)	
	Cefotaxim (third generation)	
	Ceftazidime (third generation)	
Chloramphenicol	Chloramphenicol	
Polypeptide	Colistin	
Quinolone	Enrofloxacin	
	Nalidixic acid	
Aminoglycoside	Gentamycin	
	Neomycin	
	Streptomycin	
Carbapenem	Imipenem	
Tetracycline	Tetracycline	
Diaminopyrimidine, sulfamethoxazole, and combinations	Trimethoprim	
	Sulfamethoxazole	**R**
	Trimethoprim/Sulfamethoxazole	
Macrolide	**Tylosin**	**R**

4.2.7.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S five clones were picked at a MIC of 0.5%. Increasing resistance was detected in clones I, II and V. Each clone acquired resistance to streptomycin. Surprisingly clones III and IV turned sensitive to sulfamethoxazole.

3h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in all clones. Clone I acquired resistance to streptomycin. Clones II, III, IV and V became intermediate to streptomycin. Clones IV and V additionally became resistant to cefoxitin. Surprisingly all clones turned sensitive to sulfamethoxazole.

5h after exposure to Virkon™ S five clones were picked at a MIC of 0.25%. Increasing resistance was detected in all clones, except clone III. Clone V acquired resistance to streptomycin. Clones I, II and IV turned intermediate to streptomycin. Surprisingly all clones became sensitive to sulfamethoxazole.

In total there were fourteen increasing resistances detected, which belong to two different antimicrobial classes (Table 24).

Table 24: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 black after exposure to Virkon™ S

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 black	Virkon™ S	0,5	30min	I	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,5	30min	II	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,5	30min	III	0	0
PA19/26029 black	Virkon™ S	0,5	30min	IV	0	0
PA19/26029 black	Virkon™ S	0,5	30min	V	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	3h	I	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	3h	II	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	3h	III	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	3h	IV	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	Virkon™ S	0,25	3h	V	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	Virkon™ S	0,25	5h	I	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	5h	II	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	5h	III	0	0
PA19/26029 black	Virkon™ S	0,25	5h	IV	1	(1) aminoglycoside
PA19/26029 black	Virkon™ S	0,25	5h	V	1	(1) aminoglycoside
					14	**2 (cephalosporine and aminoglycoside)**

4.2.7.3 Exposure to calgonit sterizid Ecokok

30 min after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.125%. Increasing resistance was detected in all clones. Clone III acquired resistance to streptomycin. Clones I, II, IV and V became intermediate to streptomycin. Clones I, II, III and V additionally turned resistant to cefoxitin. Clone III additionally turned intermediate to cefazolin. All clones, except clone III turned sensitive to sulfamethoxazole.

3h after exposure to calgonit sterizid Ecokok five clones were picked at a MIC of 0.0625%. Increasing resistance was detected in all clones. Clones I and V acquired resistance to streptomycin. Clones II, III and IV became intermediate to streptomycin. Clones IV and V additionally turned resistant to cefoxitin and clones III and V additionally turned intermediate to cefazolin.

5h after exposure to calgonit sterizid Ecokok two clones were picked at a MIC of 0.0625%. Increasing resistance was detected in all clones. Clones II, IV and V acquired resistance to cefoxitin. All five clones became intermediate to streptomycin. Clones III and IV additionally turned intermediate to cefazolin. All clones, except clone I turned surprisingly sensitive to sulfamethoxazole.

In total there were twenty-nine increasing resistances detected, belonging to two different antimicrobial classes (Table 25).

Table 25: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 black after exposure to calgonit sterizid Ecokok

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 black	calg. ster. Ecokok	0,125	30min	I	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	30min	II	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	30min	III	3	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	30min	IV	1	(1) aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	30min	V	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	3h	I	1	(1) aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	3h	II	1	(1) aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	3h	III	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	3h	IV	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,125	3h	V	3	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,0625	5h	I	1	(1) aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,0625	5h	II	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,0625	5h	III	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,0625	5h	IV	3	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. Ecokok	0,0625	5h	V	2	(2) cephalosporine and aminoglycoside
					29	**2 (cephalosporine and aminoglycoside)**

4.2.7.4 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 DES five clones were picked at a MIC of 0.03125%. Increasing resistance was detected in all clones. Clone III became resistant to streptomycin. Clones I, II, IV and V turned intermediate to streptomycin. Clones I, II and V acquired resistance to cefoxitin. Clone I additionally became intermediate to cefazolin. Surprisingly clone IV turned sensitive to sulfamethoxazole.

3h after exposure to calgonit sterizid P12 DES one clone was picked at a MIC of 0.03125%. Increasing resistance was present. Clone I acquired resistance to streptomycin.

No bacterial growth was present 5h after exposure to calgonit sterizid P12 DES.

In total there were ten increasing resistances detected, belonging to two different antimicrobial classes (Table 26).

Table 26: Number of single increased resistances and number of antimicrobial classes detected for PA19/26029 black after exposure to calgonit sterizid P12 DES

strain	disinfectant	%	time	clone	number of increasing resistances	number of antimicrobial classes
PA19/26029 black	calg. ster. P12 DES	0,03125	30min	I	3	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. P12 DES	0,03125	30min	II	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. P12 DES	0,03125	30min	III	1	(1) aminoglycoside
PA19/26029 black	calg. ster. P12 DES	0,03125	30min	IV	1	(1) aminoglycoside
PA19/26029 black	calg. ster. P12 DES	0,03125	30min	V	2	(2) cephalosporine and aminoglycoside
PA19/26029 black	calg. ster. P12 DES	0,03125	3h	I	1	(1) aminoglycoside
					10	2 (cephalosporine and aminoglycoside)

4.3 Agar diffusion test

4.3.1 *Salmonella* Infantis MRS16/01939

4.3.1.1 Field strain

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 16 mm (intermediate)
- Chloramphenicol: 20 mm (sensitive)
- Enrofloxacin: 18 mm (intermediate)
- Tetracycline: 0 mm (resistant)

4.3.1.2 Exposure to Virkon™ S

30 min after exposure to Virkon™ S there were no changes in susceptibility detected in all clones.

3h after exposure to Virkon™ S there were no changes in susceptibility observed in all clones for chloramphenicol, enrofloxacin and tetracycline as well as for clone II in ampicillin. Interestingly, clones I, III, IV and V became sensitive to ampicillin.

5h after exposure to Virkon™ S there were no changes in susceptibility detected in all clones to chloramphenicol, enrofloxacin and tetracycline. Interestingly, all five clones became sensitive to ampicillin.

4.3.1.3 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for chloramphenicol, enrofloxacin and tetracycline in all clones after all exposure times. Whereas, all clones became sensitive to ampicillin after all three exposure times, respectively.

4.3.1.4 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 DES there were no changes in susceptibility observed in all clones for chloramphenicol and tetracycline as well as for

clones I, II, III and V in ampicillin. Interestingly, all five clones turned sensitive towards ampicillin. Whereas Clone IV acquired resistance to enrofloxacin.

3h and 5h after exposure to calgonit sterizid P12 DES there was no bacterial growth detected.

4.3.2 *Salmonella* Infantis MRS17/00712

4.3.2.1 Field strain

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 24 mm (sensitive)
- Chloramphenicol: 30 mm (sensitive)
- Enrofloxacin: 36 mm (sensitive)
- Tetracycline: 28 mm (sensitive)

4.3.2.2 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times to chloramphenicol. Whereas after 30 min clones I, II and IV became intermediate to ampicillin and enrofloxacin and resistant to tetracycline. Clone V turned intermediate to enrofloxacin and resistant to tetracycline. After 3h clones I and II became intermediate to ampicillin and enrofloxacin and resistant to tetracycline. After 5h clone I acquired

resistance to ampicillin, enrofloxacin and tetracycline. Clones III and V became intermediate to ampicillin and enrofloxacin and resistant to tetracycline (Table 27, Figure 13).

Table 27: Presentation of the inhibition zone diameters (in mm) of the Agar diffusion test of *Salmonella* Infantis MRS17/00712 after exposure to Virkon™ S (MIC = 0.25%) and resistance profiling and development of each clone according to the CLSI reference level for antibiotics (Table 4) by color coding compared to the parental strain MRS17/00712 (field strain)

	sensitive intermediate resistant			
	Ampicillin	**Chloramphenicol**	**Enrofloxacin**	**Tetracycline**
MRS17/00712 (field strain)	24	30	36	28
MRS17/00712 Virkon™ S 0,25% 30min clone I	16	22	18	0
MRS17/00712 Virkon™ S 0,25% 30min clone II	16	22	18	0
MRS17/00712 Virkon™ S 0,25% 30min clone III	24	32	34	28
MRS17/00712 Virkon™ S 0,25% 30min clone IV	14	22	18	0
MRS17/00712 Virkon™ S 0,25% 30min clone V	18	24	22	0
MRS17/00712 Virkon™ S 0,25% 3h clone I	16	20	18	0
MRS17/00712 Virkon™ S 0,25% 3h clone II	14	24	18	0
MRS17/00712 Virkon™ S 0,25% 3h clone III	24	38	34	28
MRS17/00712 Virkon™ S 0,25% 3h clone IV	24	30	34	28
MRS17/00712 Virkon™ S 0,25% 3h clone V	24	28	34	24
MRS17/00712 Virkon™ S 0,25% 5h clone I	12	18	16	0
MRS17/00712 Virkon™ S 0,25% 5h clone II	24	30	32	28
MRS17/00712 Virkon™ S 0,25% 5h clone III	14	22	20	0
MRS17/00712 Virkon™ S 0,25% 5h clone IV	24	34	38	26
MRS17/00712 Virkon™ S 0,25% 5h clone V	16	22	18	0

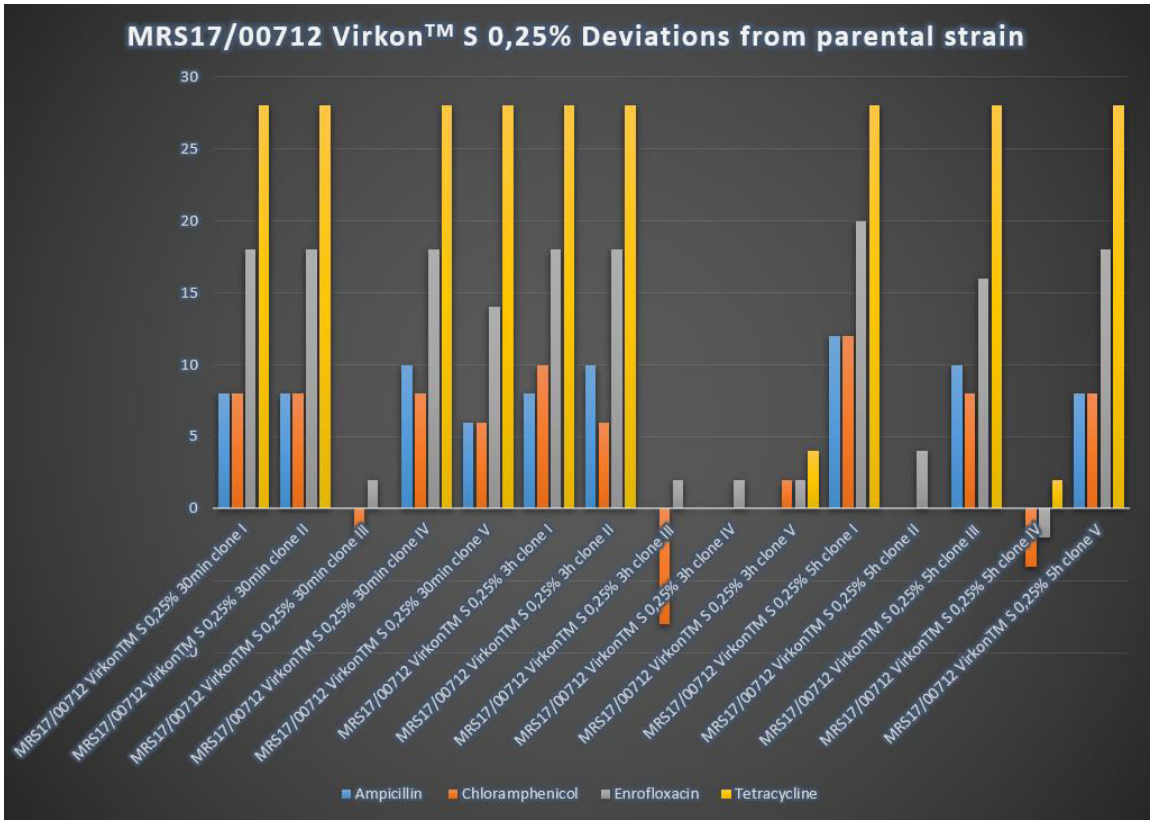

Figure 13: Deviations from the parental strain MRS17/00712 in the inhibition zone diameters after exposure to Virkon™ S. Bars above the base line represent increasing resistance as the inhibition zone diameter was decreased. Bars under the base line show an increased inhibition zone diameter

4.3.2.3 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.2.4 Exposure to calgonit sterizid P12 DES

30 min, 3h and 5h after exposure to calgonit sterizid P12 DES no bacterial growth was found.

4.3.3 Variants of *Salmonella* Infantis MRS17/00712

4.3.3.1 MRS17/00712 small colony

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 30 mm (sensitive)
- Chloramphenicol: 32 mm (sensitive)
- Enrofloxacin: 38 mm (sensitive)
- Tetracycline: 30 mm (sensitive)

4.3.3.1.1 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.3.1.2 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.3.1.3 Exposure to calgonit sterizid P12 DES

30 min, 3h and 5h after exposure to calgonit sterizid P12 DES no bacterial growth was found.

4.3.3.2 MRS17/00712 medium colony

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 22 mm (sensitive)
- Chloramphenicol: 22 mm (sensitive)
- Enrofloxacin: 20 mm (intermediate)
- Tetracycline: 0 mm (resistant)

4.3.3.2.1 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.3.2.2 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards chloramphenicol, enrofloxacin and tetracycline, respectively. An intermediate state was acquired for ampicillin in all clones after 30 min, in clone V after 3h and in clones I, II and III after 5h (Table 28, Figure 14).

Table 28: Presentation of the inhibition zone diameters (in mm) of the Agar diffusion test of *Salmonella* Infantis MRS17/00712 medium colony after exposure to calgonit sterizid Ecokok (MIC = 0.25%) and resistance profiling and development of each clone according to the CLSI reference level for antibiotics (Table 4) by color coding compared to the parental strain MRS17/00712 medium colony

	sensitive intermediate resistant			
	Ampicillin	**Chloramphenicol**	**Enrofloxacin**	**Tetracycline**
MRS17/00712 medium colony	22	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 30min clone I	16	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 30min clone II	16	24	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 30min clone III	16	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 30min clone IV	16	24	22	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 30min clone V	16	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 3h clone I	18	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 3h clone II	18	22	22	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 3h clone III	18	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 3h clone IV	18	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 3h clone V	16	22	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 5h clone I	16	24	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 5h clone II	16	22	22	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 5h clone III	16	24	20	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 5h clone IV	22	22	18	0
MRS17/00712 medium colony calg.ster.Ecokok 0,125% 5h clone V	22	22	18	0

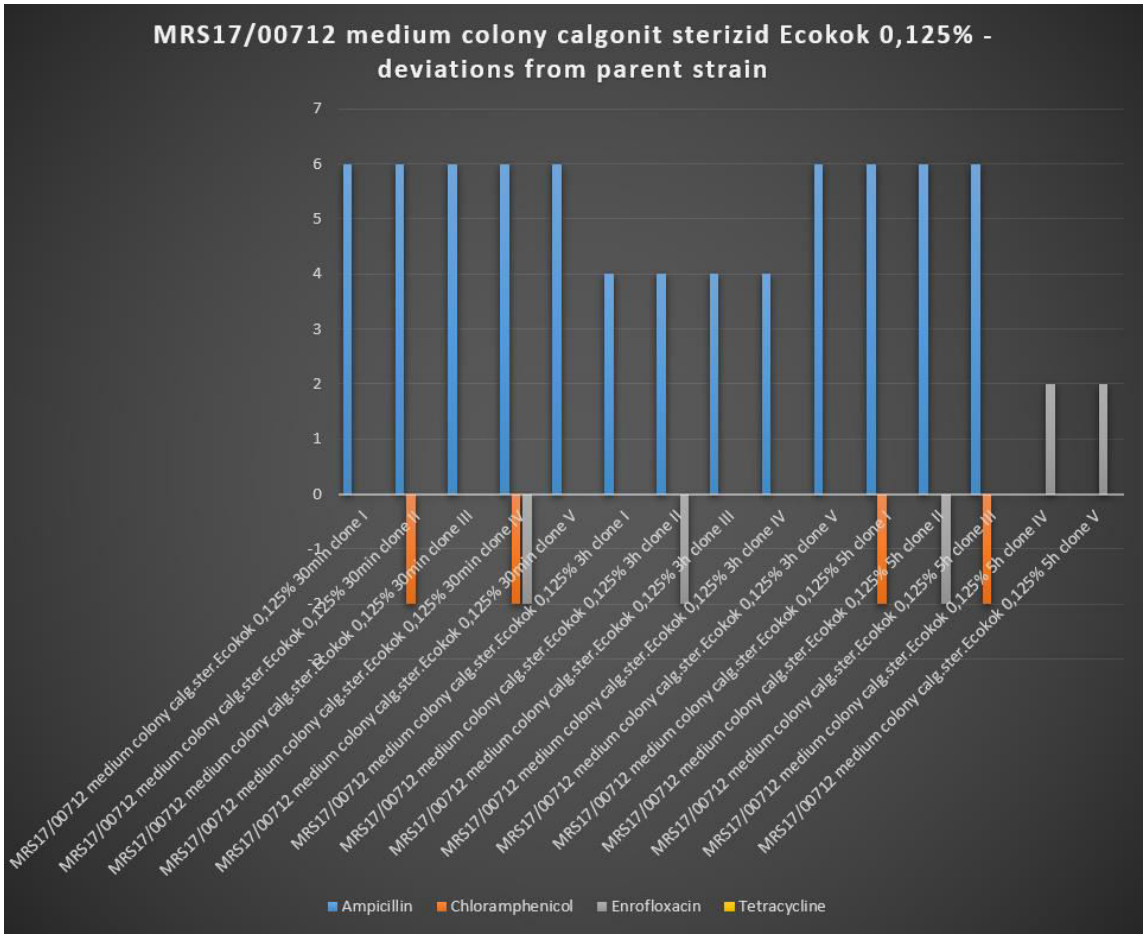

Figure 14: Deviations from the parental strain MRS17/00712 medium colony in the inhibition zone diameters after exposure to calgonit sterizid Ecokok. Bars above the base line represent increasing resistance as the inhibition zone diameter was decreased. Bars under the base line show an increased inhibition zone diameter

4.3.3.2.3 Exposure to calgonit sterizid P12 DES

30 min after exposure to calgonit sterizid P12 there were no changes in susceptibility detected for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively. Whereas an intermediate state was acquired for ampicillin in clone II after 3h. 5h after exposure to calgonit sterizid P12 DES no bacterial growth was found.

4.3.3.3 MRS17/00712 large colony

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 22 mm (sensitive)
- Chloramphenicol: 22 mm (sensitive)
- Enrofloxacin: 18 mm (intermediate)
- Tetracycline: 0 mm (resistant)

4.3.3.3.1 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.3.3.2 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards chloramphenicol, enrofloxacin and tetracycline, respectively. An intermediate state was acquired for ampicillin in clones III and IV after 30 min, and in all clones after 3h and after 5h (Table 29, Figure 15).

Table 29: Presentation of the inhibition zone diameters (in mm) of the Agar diffusion test of *Salmonella* Infantis MRS17/00712 large colony after exposure to calgonit sterizid Ecokok (MIC = 0.125%) and resistance profiling and development of each clone according to the CLSI reference level for antibiotics (Table 4) by color coding compared to the parental strain MRS17/00712 large colony

	sensitive intermediate resistant			
	Ampicillin	**Chloramphenicol**	**Enrofloxacin**	**Tetracycline**
MRS17/00712 large colony	22	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 30min clone I	22	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 30min clone II	22	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 30min clone III	16	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 30min clone IV	16	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 30min clone V	22	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 3h clone I	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 3h clone II	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 3h clone III	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 3h clone IV	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 3h clone V	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 5h clone I	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 5h clone II	16	24	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 5h clone III	16	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 5h clone IV	14	22	18	0
MRS17/00712 large colony calg.ster.Ecokok 0,125% 5h clone V	14	22	18	0

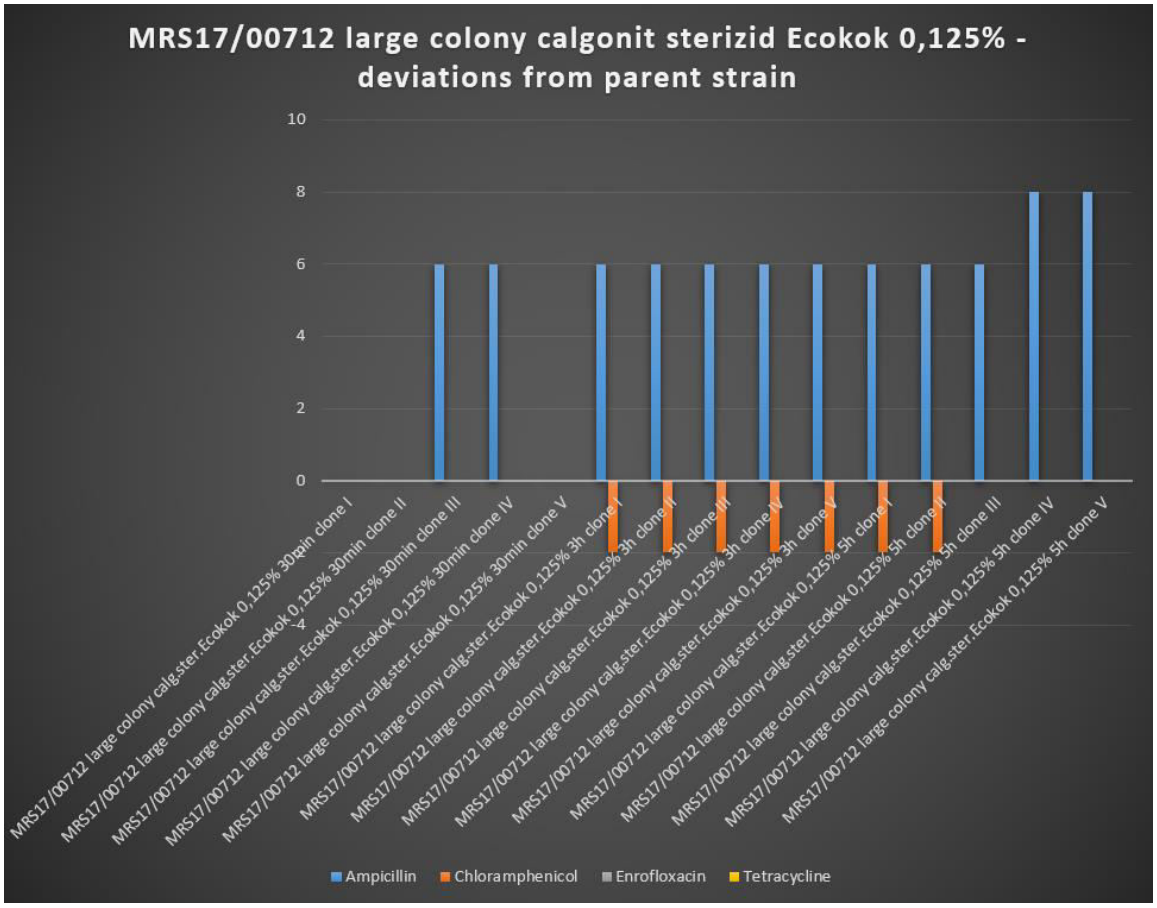

Figure 15: Deviations from the parental strain MRS17/00712 large colony in the inhibition zone diameters after exposure to calgonit sterizid Ecokok. Bars above the base line represent increasing resistance as the inhibition zone diameter was decreased. Bars under the base line show an increased inhibition zone diameter

4.3.3.3.3 Exposure to calgonit sterizid P12 DES

An intermediate state was acquired for ampicillin in clone I after an exposure time of 30 min. For all other clones and exposure times there were no changes in susceptibility observed.

4.3.4 *Salmonella* Infantis MRS17/02046

4.3.4.1 Field strain

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 26 mm (sensitive)
- Chloramphenicol: 30 mm (sensitive)
- Enrofloxacin: 28 mm (sensitive)
- Tetracycline: 10 mm (resistant)

4.3.4.2 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.4.3 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol and enrofloxacin, respectively. Interestingly, clone V after 3h and clone I after 5h became intermediate to tetracycline. All other clones after all exposure times remained stable for tetracycline.

4.3.4.4 Exposure to calgonit sterizid P12 DES

30 min, 3h and 5h after exposure to calgonit sterizid P12 DES no bacterial growth was found.

4.3.5 *Salmonella* Infantis PA19/26029 yellow

4.3.5.1 Field strain

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 20 mm (sensitive)
- Chloramphenicol: 18 mm (sensitive)
- Enrofloxacin: 24 mm (sensitive)
- Tetracycline: 18 mm (sensitive)

4.3.5.2 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.5.3 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.5.4 Exposure to calgonit sterizid P12

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.6 *Salmonella* Infantis PA19/26029 black

4.3.6.1 Field strain

After 24h incubation the following inhibition zone diameters could be measured:

- Ampicillin: 20 mm (sensitive)
- Chloramphenicol: 18 mm (sensitive)
- Enrofloxacin: 24 mm (sensitive)
- Tetracycline: 16 mm (sensitive)

4.3.6.2 Exposure to Virkon™ S

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.6.3 Exposure to calgonit sterizid Ecokok

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

4.3.6.4 Exposure to calgonit sterizid P12

There were no changes in susceptibility observed for all clones after all exposure times towards ampicillin, chloramphenicol, enrofloxacin and tetracycline, respectively.

5 Discussion

Possible influences of usage of disinfectants for bacteria and mechanisms of resistance have already been investigated to some extent in previous studies. It is commonly known that the industrial usage of disinfectants and the environmental biocide challenge can lead to the induction of bacteria with improved fitness and less susceptibility towards biocides and antimicrobial compounds (Chuanchuen et al. 2008, Condell et al. 2012, Gantzhorn et al. 2014, Karatzas et al. 2007, Soumet et al. 2016, Webber et al. 2015, Whitehead et al. 2011). So it is important to know the mechanisms and modes of action as well as to detect and to avoid possible application faults of disinfectants and antimicrobial compounds. To fight bacteria like *Salmonella* Infantis only strict cleaning and disinfection measures lead to a certain improvement of the current situation. But, recurrent infections of birds and persistent contamination of stables are still an important issue.

The objective of the present diploma thesis was to investigate if the development of antibiotic resistance based on the application of disinfectants might stick to parameters like exposure time to disinfectants, specific disinfectants or certain *Salmonella* Infantis strains. Interestingly *Salmonella* Infantis isolates with already higher initial resistances showed a lower increase in new resistances as isolates with less initial resistances. It was also observed that new resistances developed mainly in specific antimicrobial classes, which differed from strain to strain but stayed commonly constant within the same strain and therefore were independent from disinfectant or exposure time.

With a look at the *Salmonella* Infantis isolates included in this study, MRS16/01939 showed the more resistant initial susceptibility profile, whereas MRS17/00712 was initially more sensitive. After exposure to disinfectants deviations from parental strains were more often present in MRS17/00712 than in MRS16/01939.

Interestingly MRS17/00712 variant small colony showed the same initial resistance profile in microdilution as the parental strain MRS17/00712 and the same MIC in clones but mostly no increased or decreased resistances after exposure to disinfectants. So it proofed to be very stable instead.

Whereas MRS17/00712 variants medium and large colony initally proofed to be more resistant, developed more resistances after exposure to disinfectants. So these variants tend to be concurrently more reactive and more virulent than the small colony variant.

Salmonella Infantis strain MRS17/02046 was more resistant initally but did not develop crucial differences in its resistance profile in clones after exposure to disinfectants despite the presence of a pESI plasmid. But, a resistant state instead of an intermediate state in tetracycline was found in few clones.

Salmonella Infantis isolates MRS19/26029 black and yellow showed both a more sensitive initial resistance profile and hereafter proofed to be more reactive and with increased resistances after exposure to disinfectants despite the absence of a pESI plasmid.

In summary the different reaction patterns of the different *Salmonella* Infantis isolates indicate the fact that strains with more sensitive initial resistance profiles more often develop new additional resistances possibly due to a higher evolutionary selective pressure (Darwin 1860, Lambert and Kussell 2015) or better opportunities in interference with internal cellular processes like the presence of a plasmid or other virulence associated mechanisms (Alba et al. 2020, Condell et al. 2012, Hensel 2004, Suez et al. 2013).

Exposure time, together with recommended commercial concentration as well as the minimum inhibitory concentration, respectively, are important parameters concerning resistance acquirement. There was a tendency that clones developed more easily additional resistances after a shorter exposure time, like after 30 minutes more than after 3 hours and after 3 hours more than after 5 hours. This could even be a problem in broiler farms or slaughterhouses when cleaning and disinfection are not performed correctly, for example when exposure times are kept too short, when dust and dirt is not removed sufficiently or when drying of surfaces was done incorrectly and puddles emerge (Ahaduzzaman et al. 2021, Iwabuchi et al. 2010, Sommer et al. 2012). In this case the disinfectant is diluted and this can lead to the survival of resistant bacteria by undergoing MICs.

Another observation was that *Salmonella* Infantis isolates included in this study were in general mostly reactive with Virkon^{TM} S, then calgonit sterizid Ecokok and least with calgonit sterizid P12 DES. No growth was observed with calgonit sterizid DS 680. This

fact was explained due to a high recommended commercial concentration of 2.0 %, a short effective recommended exposure time of 30min and its composition from aldehyde and quarternary ammonium compounds, which proved higher efficacy in *Salmonella* elimination than cresols and least peroxygen compounds (Drauch et al. 2020).

Another challenge in disinfection represents the ability of *Salmonella* spp. to form biofilms under specific conditions (Agostinho Davanzo et al. 2021, Yin et al. 2018). The occurrence of biofilms was observed and investigated for other bacterial species, and may be a considerable future issue in fighting *Salmonella* as well.

Resistance mechanisms which are in focus for discussion also include internal bacterial cell components, like efflux pumps or the pESI plasmids. Treatment with sublethal concentrations of chemical antimicrobial agents can foster the expression of MDR efflux pumps in bacterial cells (Gilbert et al. 2002). There is a link proven between the expression and presence of efflux systems, the occurrence of so called quorum sensing which means cell-to-cell signaling as a response to extracellular chemical signals and the ability to biofilm formation and differentiation (Chan and Chua 2005, Soto 2013). It can be concluded that the exposure to biocides in sublethal concentations may provoke intrinsic signal ways and the expression of efflux pumps for both disinfectants and antibiotics in surviving bacteria, as well as the occurrence of alterations in phenotype and therefore different resistance profiles in this study.

Differences in results between microdilution assay and disk diffusion test can be traced back to lower specifity and sensitivity in the latter due to measuring ranges dependent on the examiner and less comparability due to only four measured antibiotic substances in this test setting (Balke et al. 2004, Bauer et al. 1959, Bauer et al. 1966, Häussler et al. 2003).

In this study antimicrobial classes involved in increased resistances were predominantly represented by cephalosporines (first generation and second generation) and quinolones. Beside these there were aminoglycoside, tetracycline and sulfamethoxazole involved. MDR efflux pumps are assumed as important resistance features in these classes (Blair et al. 2014, Nishino et al. 2021). To prevent a global health issue concerning MDR in bacteria like *Salmonella* spp. it is indispensable to deal with efflux pump mechanisms by further investigations. Combating efflux-based resistance mechanisms in bacteria is of public concern and is only possible by either the development of an improved new

generation of antimicrobial agents or by the use of molecules blocking efflux systems, so called efflux pump inhibitors (EPIs) (Amaral et al. 2011, Lomovskaya and Bostian 2006, Nikaido and Pagès 2012, Pagès and Amaral 2009).

The plasmid of Emerging *Salmonella enterica* serovar Infantis (pESI plasmid) is already known to be present in some isolates provoking MDR. This plasmid is known as carrier for multiple resistance genes (Alba et al. 2020, Bogomazova et al. 2020, Carattoli 2003, Lee et al. 2021). Especially so called extended-spectrum β-lactamase (ESBL) genes were found to play a crucial role in resistance development (Pietsch et al. 2021). The presence of the pESI plasmid in some of the isolates which were investigated in this study proved to be very important to draw conclusions concerning new-acquired resistances. Isolates harbouring this plasmid showed higher intrinsic initial resistances than strains without. Although these strains tend to remain more stable, nevertheless they have shown the ability to acquire new additional resistances. As pESI-like megaplasmids have shown the capability of introducing resistance against third generation cephalosporine it represents a major threat and implicites to be of emerging concern in further studies (Pietsch et al. 2021).

6 Summary

Salmonella spp. is a worldwide appearing bacterial pathogen. There are host-specific and non-host-specific serotypes occurring within more than 2600 known serotypes. *Salmonella* Infantis belonging to non-host-specific serovars is mainly found in broilers and broiler meat and is able to cause severe disease in humans. The zoonotic potential and the more and more frequent appearance of multiresistant *Salmonella* Infantis strains contemplates a significant global health issue. There are different bacterial clusters/strains in different geographical regions found, which also proved to show differences in persistence. So far, intensive cleaning and disinfection programs in poultry houses as well as slaughter houses are used to eliminate *Salmonella* Infantis. Recently, reports of increasing prevalence of antibiotic multiresistant isolates started the discussion on possible influences of the use of disinfectants. Therefore, the present study was performed to assess whether a possible link between the application of disinfectants and

the acquirement of resistance to antimicrobial compounds in *Salmonella* Infantis exists. Our results show that under specific circumstances the development of resistances in certain clones of *Salmonella* Infantis increases. This fact is mainly lead back to the ability of bacterial cells to develop intrinsic resistance mechanisms, such as being capable of forming biofilms, the acquirement of active efflux pumps or plasmids, which foster the development of new resistance genes and therefore makes it more and more complicated to combat mutants with improved fitness and persistence. Improper cleaning and disinfection in farms and slaughterhouses, e.g. application to disinfectants in sublethal concentrations or puddle emergence due to improper drying, can implicate higher resistance in surviving clones against biocides as well as antimicrobial compounds by intrinsic cell signal ways and possible horizontal gene transfer. There are many possible conditions which foster resistance development. In the present investigation we found the following ones: a) field isolates with initially lower resistances, b) an improper dilution of disinfectants, c) too short exposure time, d) disinfectants with peroxygen compounds or cresols, and e) the presence of the pESI plasmid. To prevent a new arising global health problem through bacterial mutants with MDR, permanent surveillance programmes and further studies and investigations on *Salmonella* Infantis are indispensable, beside concurrent genetic analysis of persisting strains and combating these with the use of adapted technologies like bacteriophages, efflux pump inhibitors, or vaccines.

7 Zusammenfassung

Salmonella spp. ist ein weltweit verbreiteter bakterieller Infektionserreger. Unter den mehr als 2600 bekannten Serovaren befinden sich wirtsspezifische und nicht-wirtsspezifische Serovare. *Salmonella* Infantis zählt zu den nicht-wirtsspezifischen Serovaren, kommt hauptsächlich im Masthuhn vor und kann beim Menschen ernsthafte Erkrankungen hervorrufen. Das zoonotische Potential und das immer häufigere Auftreten von multiresistenten *Salmonella* Infantis Stämmen stellen ein ernsthaftes globales Gesundheitsrisiko dar. *Salmonella* Infantis umfasst eine heterogene Bakteriengruppe, charakterisiert durch unterschiedliche Cluster in unterschiedlichen geographischen Regionen. Konzertante Reinigungs- und Desinfektionsmaßnahmen sind aktuell die einzigen gezielten Vorgehensweisen, um *Salmonella* Infantis aus kontaminierten

Geflügelstallungen und Schlachthöfen zu eliminieren; allerdings mit unterschiedlichen Erfolgen. Ein weiteres Augenmerk wird auf das vermehrte Auftreten von Antibiotika-resistenten *Salmonella* Infantis Stämmen gelegt. Deshalb wurde diese Studie durchgeführt, um einen möglichen Zusammenhang zwischen der Anwendung von Desinfektionsmitteln und dem Erwerb von Antibiotika-Resistenzen bei *Salmonella* Infantis zu untersuchen. Unsere Ergebnisse zeigen, dass unter bestimmten Umständen die Resistenzentwicklung von *Salmonella* Infantis Stämmen zunimmt. Dieser Umstand wird hauptsächlich zurückgeführt auf die Fähigkeit der Bakterien, intrinsische Resistenzmechanismen zu entwickeln, wie zum Beispiel die Fähigkeit Biofilme aus zu bilden, damit verbunden der Erwerb von aktiven Efflux-Pumpen, die sowohl Antibiotika als auch Desinfektionsmittel aus der Zelle schleusen können, oder Plasmide, wodurch die Bildung neuer Resistenzgene gefördert wird. Dies macht es wiederum schwieriger neue Mutanten mit verbesserter Fitness und Tenazität zu bekämpfen. Unsachgemäße Reinigung und Desinfektion in landwirtschaftlichen Betrieben und Schlachthöfen, wie zum Beispiel die Anwendung von Desinfektionsmitteln in subletalen Konzentrationen oder Pfützenbildung aufgrund unsachgemäßer Trocknung, können höhere Resistenzen in überlebenden Klonen sowohl gegen Biozide als auch gegen antimikrobielle Wirkstoffe bewirken. Es gibt viele verschiedene Bedingungen, welche Resistenzen fördern können. In der aktuellen Studie waren diese: a) Feldstämme mit einem sensitiveren Ausgangsresistenzprofil, b) verdünnte Desinfektionsmittel (minimale Hemmkonzentration wird unterschritten), c) zu kurze Einwirkzeit, d) Desinfektionsmittel auf Basis von Peroxidverbindungen und Kresolen, und e) das Vorhandensein des pESI Plasmids. Um einem neu aufkommenden globalen Gesundheitsproblem durch multiresistente bakterielle Mutanten entgegen zu wirken, sind ständige Überwachung und weitere Studien und Untersuchungen an *Salmonella* Infantis unabdingbar, neben fortlaufenden Analysen des Genoms von hartnäckigen Stämmen und der Bekämpfung letzterer mithilfe angepasster Technologien wie Bakteriophagen, Efflux-Pumpen-Inhibitoren oder Impfungen.

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9 List of Figures and Tables