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In-vitro testing of bacteriostatic and bactericidal efficacy of commercial disinfectants against *Salmonella* Infantis reveals substantial differences between products and bacterial strains

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ABSTRACT

Salmonella (*S.*) Infantis is currently the most common serovar in broilers and boiler meat in the European Union. In the field, eradication of *S.* Infantis in affected poultry flocks is considered extremely difficult. Despite stringent cleaning and disinfection measures between the placement of flocks, recurrent infections are often reported. So far, the efficacy of disinfectants on *S.* Infantis has rarely been studied. Therefore, in the present *in-vitro* study the bacteriostatic and bactericidal efficacy of ten commercial disinfectants were tested against seven *S.* Infantis field isolates. Combinations of aldehyde and quarternary ammonium were the active compounds of five, peroxygen of three, cresol and alkylamines of one disinfectant, respectively. Investigations were performed according to standard protocols and regulations. Different concentrations of disinfectants were used to test the bacteriostatic efficacy. Different temperatures and low and high protein exposures were applied as variables to investigate the bactericidal efficacy. Following neutralization of the disinfectants an additional incubation step was introduced to investigate the revitalisation potential of *S.* Infantis. The bacteriostatic efficacy could be assessed for seven disinfectants. For three disinfectants a bacteriostatic effect was observed when the recommended concentration was used, whereas with four disinfectants only increased concentrations led to this effect. The bactericidal efficacy was not influenced by temperature, whereas high protein exposure decreased the efficacy of nine disinfectants. Furthermore, reactivation of *S.* Infantis was revealed after application of disinfectants for the majority of products. Interestingly, the strain of *S.* Infantis influenced the efficacy of the disinfectants. Overall, products based on aldehydes and quarternary ammonium compounds proved most efficient, followed by peroxgen, cresol and alkylamines.

1. Introduction

Broilers are an important reservoir for various *Salmonella* (*S.*) serovars, which are known as agents of human foodborne diseases. The latest report of the European Food Safety Authority revealed *S.* Infantis as the most common serovar in fowl (*Gallus gallus*) with a continuing increase of isolates in broiler flocks and broiler meat over the last years (EFSA, 2019). Actually, *S.* Infantis accounts for 36.5% and 55.7% of all serotyped *Salmonella* isolates from broiler flocks and broiler meat, respectively. Furthermore, recent data also showed that *S.* Infantis had become the fourth most frequent serovar in human salmonellosis in the European Union (EFSA, 2019).

Different to *S.* Enteritidis and *S.* Typhimurium where multiple ways of introduction into a broiler house are known, the sources of *S.* Infantis still remain difficult to trace (Gradel and Rattenborg, 2003; Liljebjelke et al., 2005; Marin et al., 2011). In general, *Salmonella* can persist and even multiply in remaining organic matter, a certain risk for succeeding flocks (Gosling et al., 2016). Therefore, significant resources are spent on cleaning and disinfection of poultry houses. However, in contrast to serovars like *S.* Enteritidis and *S.* Typhimurium which can usually be eliminated within a single or a few flock cycles, farms persistently infected with *S.* Infantis are constantly reported (Gradel and Rattenborg, 2003; Ortali, 2019; Pate et al., 2019; Pless et al., 2019). These findings indicate that *S.* Infantis might be more resistant to commonly used disinfectants based on an increased biofilm-forming ability, a higher tolerance to thermal, acid and osmotic stress (Moraes et al., 2018). Interestingly, previous studies have already described variations in susceptibility to disinfectants between *Salmonella* strains and even

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within strains of the same serovar (Sander et al., 2002; Thomson et al., 2007).

So far, only few studies are available on *in-vitro* efficacy testings of commercial disinfectants against *Salmonella* serovars (Gosling et al., 2016; Gradel et al., 2004, 2005; Jang et al., 2017; McLaren et al., 2011; Sander et al., 2002). In these studies, different contamination methods were used, *e.g.* various faecal suspension, spiked organic matter or surface contamination models, claiming their ability to more closely mimic real-life conditions compared to official suspension methods. However, such tests are known to be difficult to standardize compromising reproducibility and comparison among laboratories (Reybrouck, 1999).

It has been shown previously that effectiveness of a disinfectant is mainly dependent on the active compound chosen, its concentration and the cleanliness of the surfaces to which it is applied (Featherstone et al., 2010). Therefore, the aim of the present study was to investigate the bacteriostatic and bactericidal efficacy of commonly used disinfectants in poultry houses against *S*. Infantis based on standard protocols, which are applied to license such products. Hence, the concentration of the different disinfectants necessary to elicit a bacteriostatic effect against *S*. Infantis was determined. Furthermore, the bactericidal efficacy of the different disinfectants against *S*. Infantis was evaluated by applying different incubation temperatures and protein exposures mimicking a clean and a dirty environment *in-vitro*. By introducing an additional incubation step after neutralizing the disinfectants the revitalisation potential of *S*. Infantis was investigated for the first time. Finally, the influence of the *S*. Infantis strain itself on the efficacy of the disinfectants was determined.

2. Material and methods

2.1. Salmonella Infantis strains

Seven *S*. Infantis field strains were used in the present *in-vitro* study. Six strains originated from Austria, and one from Russia (Table 1). All strains were derived from broiler flocks and were stored at −80 °C by adding 2 ml of 40% glycerol/10 ml brain heart infusion broth (Oxoid, ThermoFisher Scientific, Vienna, Austria). Before testing, the strains were thawed and cultivated on blood agar (37 °C, aerobically, 24 h). For all bacteriostatic and bactericidal *in-vitro* testing a liquid culture of each strain was made in 10 ml tryptone soya broth (TSB, Oxoid, Basingstoke, UK) incubated at 37 °C, aerobically for 24 h at 150 rpm of shaking. By applying serial dilutions (1:10) in phosphate buffered substrate (PBS, GIBCO, Paisley, UK) on tryptone soya agar (TSA Oxoid, Basingstoke, UK) the CFU count was determined as 10^9 CFU/ml which was used as standard concentration for all test settings.

2.2. Disinfectants

Ten commercially available disinfectants based on four different active compounds were used. The active ingredients of five disinfectants were combinations of aldehydes and quarternary ammonium (QUATS), three disinfectants contained peroxygen compounds and one disinfectant contained cresol and alkylamines, respectively (Table 2).

Table 1
Origin of *S*. Infantis strains.

Strain name	Serovar	Origin	Persistent on farm
MRS17/00712	*S*. Infantis	Austria, Upper Austria	No
PA18/18672	(6,7:r:1,5)	Austria, Upper Austria	No
MRS16/01939		Austria, Carinthia	Yes
PA18/19764		Austria, Styria	Yes
PA19/00011		Austria, Styria	Yes
PA19/00130		Austria, Styria	Yes
PA13/02890		Russia	No data available

The concentrations were prepared in purified water according to the protocol of the manufacturer.

2.3. Bacteriostatic efficacy

The bacteriostatic efficacy was tested according to the guidelines of the German Association of Veterinary Medicine (DVG, chapter IV, 2017). For this, 1 ml of each *S*. Infantis liquid culture (containing 10^9 CFU/ml) was transferred into 9 ml tryptone-natrium chloride solution (1 g tryptone (Merck, Darmstadt, Germany) and 8.5 g of NaCl (Carl Roth, Karlsruhe, Germany)/l purified water). An aliquot of 0.1 ml was transferred in 5 ml double concentrated TSB containing 5 ml of disinfectant with the recommended concentration, the twofold concentration and the fourfold concentration. This resulted in three test settings for each *S*. Infantis field strain and each disinfectant, which were incubated aerobically at 37 °C, for 72 h. Bacteriostatic effect was assessed visually by evaluating the translucence of the liquid suspension: a clear suspension indicated no bacterial growth (bacteriostatic effect), a turbidity of the suspension confirmed bacterial growth (no bacteriostatic effect).

2.4. Bactericidal efficacy

The bactericidal efficacy was tested according to the guidelines of the German Association of Veterinary Medicine (DVG, chapter V, 2017). Briefly, two equal suspensions were made consisting of 1 ml *S*. Infantis liquid culture mixed with 1 ml purified water containing a low protein exposure (3 g bovine serum albumin/l, BSA, Sigma Aldrich, St. Louis, USA). In the same way, suspensions mixed with 1 ml purified water containing a high protein exposure (10 g BSA/l and 10 g yeast extract/l, Sigma Aldrich, St. Louis, United States) were made. Afterwards, 8 ml of the disinfectant in the recommended concentration was added to each of these suspensions. Incubation was performed at 10 °C or 20 °C for the recommended residence time according to manufacturers' guidelines. Then, the disinfectant was neutralized by adding 8 ml of Dey-Engley Neutralizing Broth (Sigma-Aldrich, St. Louis, United States). After a neutralizing time of 5 min serial dilutions (1:10) in PBS were performed, and 100 µl were plated on TSA in duplicate. The plates were incubated aerobically for 24 h after which CFU were counted, and mean values were calculated. The remaining suspension was further incubated at 37 °C, aerobically for 72 h. Again, from this suspension serial dilutions (1:10) in PBS were performed and 100 µl were plated on TSA in duplicate. The plates were incubated aerobically for 24 h, CFU were counted and the mean value of bacterial growth was calculated. A 5-log reduction of CFU counts was considered a bactericidal effect.

2.5. Statistical analysis

A linear regression model was implemented in R (R Core Team (2019), version 3.6.1. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). To account for multiple testing, Tukey's Honestly Significant Difference (HSD) test was performed and p-values ≤ 0.05 were considered significant.

3. Results

3.1. Bacteriostatic efficacy

For three disinfectants containing aldehydes and quarternary ammonium compounds, namely ALDEKOL DES® 04, calgonit DS 680, ROTIE-CID F1 the bacteriostatic efficacy could not be assessed. These products produced turbidity in the suspension immediately after preparation.

A bacteriostatic effect against all *S*. Infantis field strains tested was observed when applying the recommended concentration of the

Table 2
Disinfectants used in the present study classified according to their active compounds with recommended concentrations and residence times.

Active compound	Trade name	Concentration	Residence time
Aldehyde and quarternary ammonium compounds	ALDEKOL DES® 04	2.0%	120 min
	calgonit DS 680	2.0%	30 min
	calgonit sterizid P12 DES	0.5%	15 min
	DESINTEC® FL-des GA forte	2.5%	60 min
	ROTIE-CID F1	1.0%	120 min
Peroxygen compounds	DESINTEC® Peroxx Liquid	0.5%	60 min
	ROTIE-PER Spezial	1.0%	30 min
	Virkon™ S	1.0%	30 min
Cresol	calgonit sterizid ECOKOK	0.5%	30 min
Alkylamines	Profex 99	1.5%	15 min

disinfectants calgonit sterizid P12 DES, DESINTEC® FL-des GA forte and calgonit sterizid ECOKOK.

A significant increase of bacteriostatic efficacy could be achieved for two products based on peroxygen compounds, DESINTEC® Peroxx Liquid and ROTIE-PER Spezial, by a twofold increase of the recommended concentration ($p < 0.05$). Here, the efficacy raised from 85.7% to 100% and from 42.9% to 85.7% for DESINTEC® Peroxx Liquid and ROTIE-PER Spezial, respectively. The disinfectant Virkon™ S did not show any bacteriostatic effect until a concentration fourfold higher than the recommended was applied resulting in 100% efficacy ($p < 0.001$). Interestingly, Profex 99 showed the least bacteriostatic effect. When applying the recommended and the twofold higher concentration of this product no bacteriostatic effect was found. Only when a fourfold higher concentration was used a bacteriostatic effect was noticed in two out of seven (28.6%) *S*. Infantis strains ($p < 0.001$) (Table 3). The different *S*. Infantis strains did not influence the bacteriostatic efficacy of the disinfectants tested.

3.2. Bactericidal efficacy

The bactericidal efficacy did not differ between 10 °C and 20 °C in any of the disinfectants tested. Fig. 1 illustrates the results for the low and the high protein exposure, after neutralization of disinfectants and further 72 h incubation of the suspensions. Data is presented in mean values with a standard error of ± 0.94.

In the presence of a low protein exposure bacterial growth after neutralization was only found in case of one product, namely calgonit sterizid P12 DES (1.18 CFU/ml). When these neutralized suspensions were further incubated for 72 h a significant ($p < 0.05$) multiplication of *S*. Infantis was observed for calgonit sterizid P12 DES (10.9 CFU/ml), ROTIE-CID F1 (3.7 CFU/ml) and Virkon™ S (4.2×10^1 CFU/ml).

In the presence of a high protein exposure bacterial growth after neutralization was found for the products ALDEKOL DES® 04 (1.8 CFU/ml), calgonit sterizid P12 DES (3.0×10^1 CFU/ml), ROTIE-CID F1 (9.3 CFU/ml), DESINTEC® Peroxx Liquid (1.2 CFU/ml), ROTIE-PER Spezial (2.6 CFU/ml), Virkon™ S (3.5×10^2 CFU/ml), calgonit sterizid ECOKOK (7.7×10^1 CFU/ml) and Profex 99 (3.7×10^2 CFU/ml). Following further incubation after neutralization *S*. Infantis could be reisolated from all disinfectant suspensions, except in case of calgonit DS 680. Surprisingly, this procedure resulted in a general increase of 6× to 7× $\log_{10}$ in *S*. Infantis CFU counts. The mean CFU counts were as follows: ALDEKOL DES® 04 1.2×10^8 CFU/ml, calgonit sterizid P12 DES 1.36×10^8 CFU/ml, DESINTEC® FL-des GA forte 4.0×10^7 CFU/ml, ROTIE-CID F1 2.1×10^7 CFU/ml, DESINTEC® Peroxx Liquid 5.75×10^7 CFU/ml, ROTIE-PER Spezial 9×10^7 CFU/ml, Virkon™ S 3.0×10^8 CFU/ml, calgonit sterizid ECOKOK 1.21×10^8 CFU/ml and Profex 99 2.4×10^7 CFU/ml. This finding was significant ($p < 0.05$) for the products calgonit sterizid P12 DES, ROTIE-CID F1, DESINTEC® Peroxx Liquid, ROTIE-PER Spezial, Virkon™ S, calgonit sterizid ECOKOK and Profex 99.

Fig. 2 presents all results from bactericidal efficacy tests grouped according to the active compounds of the disinfectants with a logarithmic transformation of the CFU/ml. Aldehydes and quarternary ammonium compounds proved to be most effective with the lowest reisolation of *S*. Infantis which corresponds to an efficacy of 84.29%. Next to this, products containing peroxygen, cresol and alkylamines with a higher CFU counts resulting in lower efficacy: 76.29% efficacy for peroxygen, 58.93% efficacy for cresol, and 50.0% efficacy for alkylamines.

Summarizing all bactericidal testings, a trend towards an influence of the *S*. Infantis strain itself on the efficacy of the disinfectants was observed. A significantly higher bacterial growth ($p < 0.05$) was observed in case of *S*. Infantis strains PA13/02890 and PA19/00011 compared to the other strains as shown in Fig. 3.

4. Discussion

In the EU the number of *S*. Infantis isolates from broiler flocks and meat has steadily increased over the past years (EFSA, 2019). This indicates that this *Salmonella* serovar has the ability to persist on farms once established and to spread along the entire broiler production chain (Pate et al., 2019). It is well known that the elimination of *S*. Infantis from poultry houses as well as slaughterhouses by applying disinfectant and cleaning intensively is difficult. Therefore, for the first time an *in vitro* study was performed focusing on the investigation of the bacteriostatic and bactericidal efficacy of commercially available disinfectants against different *S*. Infantis field strains. For this purpose, standardized suspension test methods were applied. These standard protocols are usually employed for licencing of disinfectants by obligatory use of certain bacteria namely *Staphylococcus aureus*,

Table 3
Bacteriostatic efficacy (in %) of seven disinfectants against *S*. Infantis with the recommended, twofold or fourfold higher concentrations.

Disinfectant	Recommended concentration	Twofold concentration	Fourfold concentration
calgonit sterizid P12 DES	100%	100%	100%
DESINTEC® FL-des GA forte	100%	100%	100%
DESINTEC® Peroxx Liquid	85.7%	100%	100%
ROTIE-PER Spezial	42.9%	85.7%	100%
Virkon™ S	0%	0%	100%
calgonit sterizid ECOKOK	100%	100%	100%
Profex 99	0%	0%	28.6%

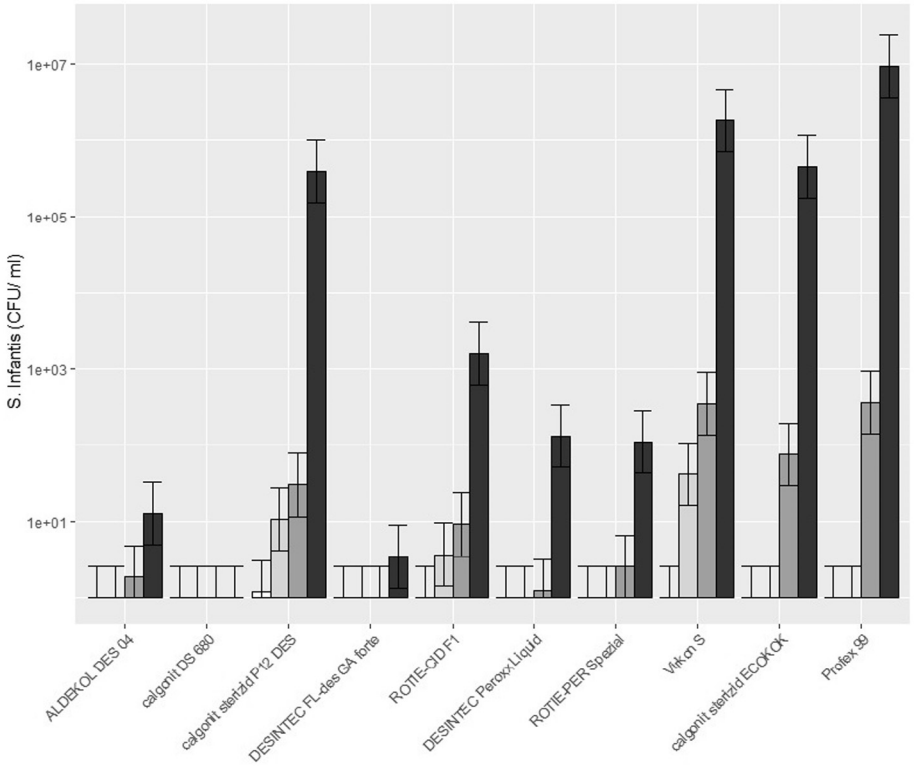

Fig. 1. Bacterial growth (mean CFU/ml, ± standard error) of *S*. Infantis determined after neutralization of the disinfectants and after further 72 h incubation applying low and high protein exposure. Bars in white colour = CFU count after neutralization low protein exposure applied; bars in light grey colour = CFU count after further 72 h incubation low protein exposure applied; bars in dark grey colour = CFU count after neutralization high protein exposure applied; bars in black colour = CFU count after further 72 h incubation high protein exposure applied.

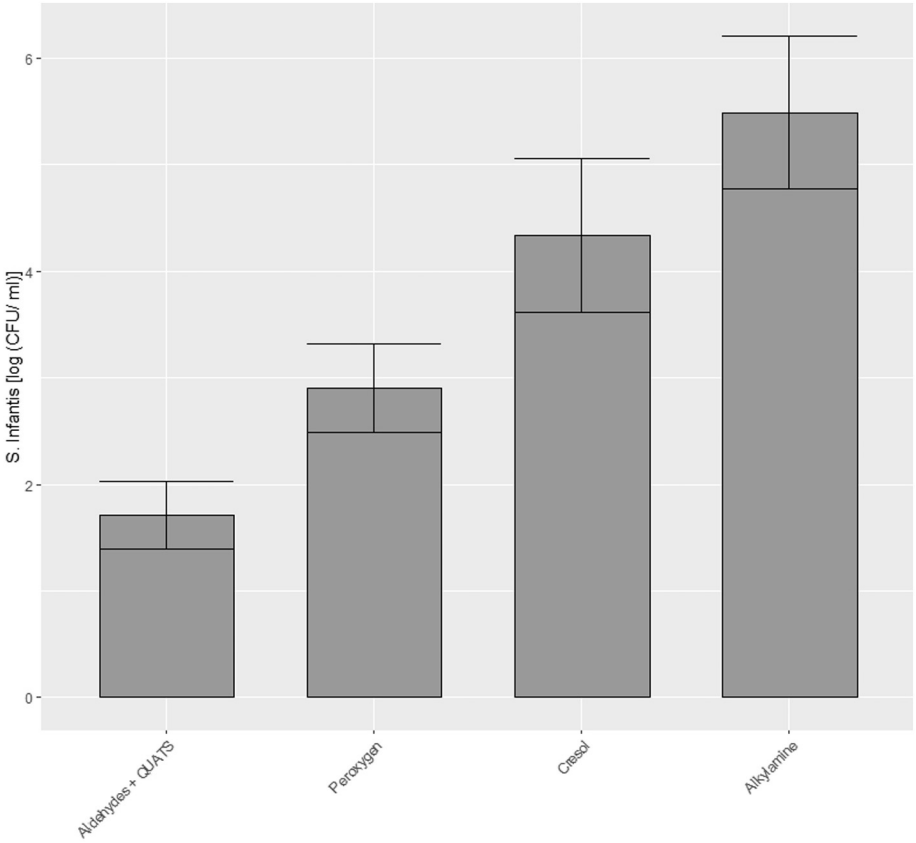

Fig. 2. Comparison of the bacterial growth (mean log CFU/ml, ± standard error) of *S*. Infantis depending on the active compounds of disinfectants comprising the results of all bactericidal test settings.

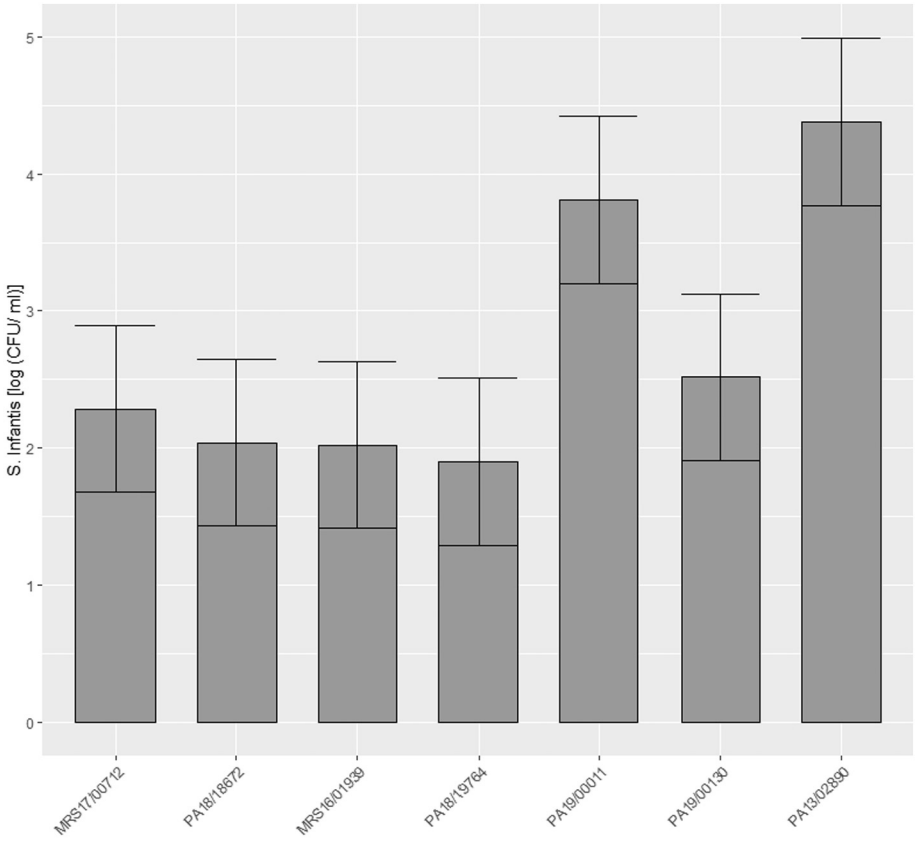

Fig. 3. Variety in bacterial growth behavior (mean log CFU/ml, ± standard error) of *S.* Infantis strains comprising the results of all bactericidal test settings.

Enterococcus hirae, *Pseudomonas aeruginosa* and *Proteus vulgaris* (*hauseri*) as reference strains. All disinfectants used in the present study are registered for the use in animal husbandry. For the first time these test settings were also applied with *S.* Infantis. In previous studies suspension methods were often criticized not being able to mimic the on-farm situation which led to the use of diverse materials in combination of varying spiked organic matter unable to be standardized (Gosling et al., 2016; Gradel et al., 2004; McLaren et al., 2011). With this, the greatest disadvantages of such methods are now scientifically accepted, namely difficulties in control and reproducibility of results due to the influence of many variables and the impossibility to compare results (Gradel et al., 2004; McLaren et al., 2011). To overcome this drawback in the present study, parameters like temperature and organic matter were mimicked under standardized *in-vitro* conditions, altogether factors known to influence the efficacy of disinfectants in the field.

When applying the bacteriostatic test setting it was recognized that this method was not applicable for three out of five disinfectants containing a combination of aldehydes and quarternary ammonium compounds, namely ALDEKOL DES® 04, calgonit DS 680 and ROTIE-CID F1. In these cases, the visual assessment of a bacteriostatic effect was not possible as these disinfectants caused turbidity in the test suspensions. Whereas the products calgonit sterizid P12 DES, DESINTEC® FL-des GA forte and calgonit sterizid ECOKOK proved bacteriostatic at the recommended concentration, an increase of the concentrations was needed for the remaining products. This finding was evident for all three products based on peroxygen compounds, a finding previously reported for other *Salmonella* species as well (Gradel et al., 2004, 2005; McLaren et al., 2011). Surprisingly, with Profex 99, containing alkylamines as active compounds, the concentration needed to achieve a bacteriostatic effect on at least 29% of *S.* Infantis strains was fourfold higher than recommended by the manufacturers. However, in the field application of higher concentrations of disinfectants may not be feasible as this might cause adverse health effects on humans, and also might increase the corrosive properties of a product resulting in severe damages to poultry house interiors.

Based on field reports of non-satisfying disinfectant efficiency against *S.* Infantis it was speculated that the *S.* Infantis strain itself might have an influence on the bacteriostatic effect of a product. But, similar to previous results from other *Salmonella* serovars no influence was found in the present bacteriostatic test setting (Chylkova et al., 2017)

It is known that the temperature might have an influence on the outcome of disinfection by negatively affecting the bactericidal efficacy when low. But, similar to previous studies no significant loss of efficacy was found for any of the disinfectants tested at low (10 °C) compared to room (20 °C) temperature (Berchieri and Barrow, 1996; Gradel et al., 2004). Temperatures below 10 °C might have a negative impact on the efficacy of disinfectants as shown by Jang et al. (2017). Such investigations were not included in the present study as the applied standard procedures have to be performed at 10 °C. However, for future studies it might be of interest to evaluate these methods at temperatures below 10 °C.

Remaining organic material in poultry or slaughterhouses is known to decrease the efficacy of disinfectants (Carrique-Mas et al., 2009; De Quadros et al., 2020; Wales et al., 2006). To mimic a low and a high amount of organic matter *in-vitro* two different concentrations of protein exposure were applied. The efficacy of the majority of disinfectants was not influenced in the presence of a low protein concentration. In contrast, applying a high protein concentration led to a reduction in the effectiveness of all disinfectants tested, except calgonit DS 680. This negative effect was also reported from the field and illustrates once more the importance of proper cleaning procedures before applying disinfectants (Carrique-Mas et al., 2009; Mueller-Doblies et al., 2010). Beside proteins, fats which can be mainly found as feed residues in troughs are also considered to protect bacteria during disinfection procedures. Interestingly, previous surface tests revealed that fat

(rapeseed oil) was generally more protective than conventional layer feed (Gradel et al., 2004). Unfortunately, suspension methods are not suitable to investigate possible protective effects of fats as they will not dissolve in the liquid media used for these studies.

Bacteria surviving the cleaning and disinfection process in low amounts might revitalise during the service period between the setting of broiler flocks and consequences on early infections. Gradel et al. (2004) already criticized that most published disinfection tests do not incorporate a time span between disinfection and possible recovery procedures resulting in misleading efficacy results. Therefore, for the first time, the possible revitalisation potential of *S*. Infantis was investigated by introducing an additional incubation period of the test suspensions after neutralizing the disinfectants. Except for calgonit DS 680, *S*. Infantis revitalised during the experiment. This finding is important for the field as more cleaning and disinfectant steps might be needed to successfully eliminate *S*. Infantis from a premise, which has already been shown for *S*. Java (Kloska et al., 2017).

A variation in the efficacy against *S*. Typhimurium and *S*. Enteritidis of disinfectants having the same active ingredients has been observed previously (McLaren et al., 2011). This agrees with present findings for products based on combinations of aldehydes and quarternary ammonium and on peroxygen. Independent from exposure to high or low levels of protein differences might be due to the formulation process of products, indicating that the compound *per se* does not determine efficacy alone. Also *S*. Infantis strains may differ in their susceptibility to detergents, a feature already known for other *Salmonella* strains and serovars (Sander et al., 2002; Thomson et al., 2007). In the present study, a tendency towards higher resistance was found for two *S*. Infantis strains, both isolated on farms reporting recurrent infections. Future studies may resolve the genetic and/or metabolic mechanisms responsible for such differences (Kornschober et al., 2019).

Overall, products containing a combination of aldehydes and quarternary ammonium compounds were superior in combating *S*. Infantis, in agreement with previous studies for other *Salmonella* serovars (Martelli et al., 2017; Mueller-Doblies et al., 2010). In contrast, De Quadros et al. (2020) found that none of the *S*. Infantis strains isolated from swine slaughterhouses were susceptible to this group of disinfectants. This discrepancy may be due to genetic variations between *S*. Infantis strains derived from different hosts or different geographic areas, as shown previously for *S*. Heidelberg (Antony et al., 2018). Previous data showed good efficacy of peroxygens on different *Salmonella* serovars including *S*. Infantis (De Quadros et al., 2020; Gradel et al., 2004). This agrees with the present findings as products containing this active compound achieved the second-best efficacy results. A variability in efficacy against *Salmonella* between products within the chemical group of cresols is known (Gosling et al., 2016). Calgonit sterizid ECOKOK is mainly recommended as disinfectant against endoparasites, but an effect against bacteria is also claimed. In our hands, this product showed limited efficacy against *S*. Infantis. Profex 99, containing alkylamines as active compounds, was least effective. Profex 99 is widely used as disinfectant in poultry slaughterhouses in Austria. Based on the presented data its application in the recommended form needs to be critically reconsidered.

The present *in vitro* investigations provide important data to improve performances of cleaning and disinfection procedures of *S*. Infantis contaminated premises. However, differences in susceptibility of individual *S*. Infantis isolates urge for further investigations and support the need to apply standardized test protocols.

Authors contributions

CH and MH prepared the concept and design of the work. Experiments and data collection were performed by VD, CI and CH. Preparation of data and their statistical analysis was done by CV and VD. The manuscript was prepared by VD and CH. MH and CV contributed to the finalization of the manuscript.

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Declaration of competing interest

The authors declare that the present work was undertaken in the absence of any commercial or financial relationships that could be construed as conflict of interest.

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