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ORIGINAL ARTICLE

Synergistic antibacterial effect of disinfectants and microbubble water to *Salmonella* Typhimurium

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Salmonella is a pathogenic bacterium that has long been important industrially because it has a wide host range and can be transmitted to humans through direct contact as well as indirect contact such as food contaminated with animal waste. Understanding how to reduce *Salmonella* contamination in pig farms is important for public health and the livestock industry from an economic perspective. In the swine industry, high concentrations of disinfectants have been applied because it is difficult to effectively control *Salmonella* in environments contaminated with organic substances. In order to evaluate the synergetic effect of disinfectants, the efficacy of two commercial disinfectants diluted in hard water and microbubble water (MBW) were compared under the laboratory condition. Different concentrations of both disinfectants combined with 1% detergent diluted in the two diluents were evaluated for their antibacterial effect. In the case of monopersulfate-based disinfectant groups, the growth of *Salmonella* was not observed at 1:200 dilution with both the hard water and MBW combined with 1% detergent. In the case of citric acid-based disinfectant, the bacterial growth was not observed at 1:800 dilution with MBW combined with 1% detergent. Our results show that the use of MBW as a diluent might improve the biological activities of acid-based disinfectant.

Key Words: Disinfectant, Detergent, Microbubble water, *Salmonella* Typhimurium

INTRODUCTION

Salmonella is gram-negative intracellular pathogenic bacteria that has long been important industrially because it has a wide host range and can be transmitted to humans through direct contact as well as indirect contact such as food contaminated with animal waste (Eng et al, 2015; Heier et al, 2016; Rönnqvist et al, 2018; Ostanello and De Lucia 2020). Animal-derived bacterial infections are a serious public health risk factor as well as an occupational risk for farm workers who come into regular contact with livestock (Fosse et al, 2009; Wales et al, 2011; Snary et al, 2016). Several studies on human *Salmonella* infections showed that 22% of Dutch cases and 14% of Danish cases were attributed to pork or pork products (Authority 2006; Bonardi 2017). In order to reduce *Salmonella* contamination in livestock, farm hygiene management through cleaning and disinfection is one of the important factors (Moretro et al, 2009; Andres and Davies 2015). Many studies have emphasized the usefulness of cleaning and disinfection in farms and livestock-related facilities in reducing the level of *Salmonella* infection in pigs (Andres and Davies, 2015; Martelli et al, 2017; Walia et al 2017). However, the antibacterial efficacy of disinfectants used in farms and livestock facilities is often reduced owing to organic substances such as feces left on surfaces and biofilms produced by bacteria (da Costa Luciano et al, 2016; Skowron et al, 2019). In a swine industry, there is a tendency to use high concentrations of disinfectants because it is difficult to effectively control *Salmonella* in environments contaminated with organic substances. Such increased use of disinfectant chemicals and their subsequent discharge into wastewater may cause adverse impacts on aquatic ecosystems, accumulation on vegetables, and contamination of the food chain via wastewater irrigation and sludge application (Dewey et al, 2021; Subpiramaniyam 2021; Marteinson et al, 2022). Therefore, it is necessary to develop a method that can effectively control pathogens while reducing the amount of disinfectant used.

Microbubble water (MBW) refers to water with gaseous structures comprising either a single gas or mixed gases, with diameter ranging from microns to nanometers, which have extensive uses in waste water purification, drug delivery system, aquaculture, cleaning, and some early industrial applications (Patel et al, 2021; Zhang et al, 2022). It finds applications in several fields owing to its unique properties such as longer stability, free radical formation, scouring, surface attraction, and oxidation, which gives benefits such as controlling the pathogen growth and biofilm formation as well as improving the solid/oil/liquid separation processes (Patel et al, 2021). The removal of microbial pathogens such as *Escherichia coli* and *Salmonella*, foodborne pathogens, as well as pesticides, was reported during washing with MBW with and without additives/oxidizing agents (Zhang et al, 2022). Additionally, a previous study showed that carbon dioxide MBW could significantly improve the antimicrobial efficacy of chlorine and peracetic acid against *Escherichia coli* and *Listeria* respectively (Patel et al, 2021). To the best of our knowledge, no studies have evaluated whether MBW as a diluent solution is associated with improving the effectiveness of disinfectants. The purpose of this study is to evaluate the synergy effect of microbubbles, in order to reduce the amount of disinfectant used and to control *Salmonella* more effectively. The present study was performed: (1) to evaluate the effect of two types of commercial disinfectants mixed with alkaline based detergent; (2) to test the effect of MBW dilutions on the efficacy of disinfectants to inactivate *Salmonella* Typhimurium.

MATERIALS AND METHODS

Preparation of the *Salmonella*

All potency tests were conducted in accordance with the disinfectant potency test guidelines (Ministry of the Agriculture, Forestry and Livestock Quarantine Headquarters notice in Republic of Korea). *Salmonella* Typhimurium (ATCC 14028) was used in this study. The test strains were distributed in a sterilized nutrient medium and inoculated at 37℃ for 24±2 h before being used. *Salmonella* suspensions in sterile saline solution were prepared to an optical density equal to 0.5 McFarland standard (approximately 10^8 CFU/mL).

Preparation of the Disinfectants and Detergent

For this study, we chose two different types of disinfectants: monopersulfate-based disinfectant (V; Virkon-S., Bayer Korea, Seoul, Korea) and citric acid-based disinfectant (F; FARMCARE liquid., CTC Bio Inc., Seoul, Korea). All disinfectants were approved by the Animal and Plant Quarantine Agency (QIA), Korea. Table 1 shows the main components of the disinfectants and detergents used in this study. The detergent used in this study was a foaming alkaline Kenosan farm cleaner (Grifoam, Animed, Gyeonggi., Korea) based on 2-(2-butoxyethoxy) ethanol and sodium hydroxide. For the

Table 1. Chemical compound of the monopersulfate-based disinfectant (V) and citric acid-based disinfectant (F)

	Ingredient name	Content (g/kg)
Disinfectant (V)	Monopersulfate compound	500 g
	Sodium chloride	15 g
	Malic acid	100 g
	Sulfamic acid	50 g
	Sodium hexametaphosphate	181 g
	Sodium dodecyl benzene sulphonic acid additives	150 g
Disinfectant (F)	Quaternary ammonium chloride	100 g
	Anhydrous citric acid	200 g
	Phosphoric acid	100 g
	Excipient (purified water)	

study, the detergent was diluted to 1% concentration according to the manufacturer's instructions.

Preparation of the organic dilution solution

MBW was produced through a microbubble generator and pre-operated at room temperature 1 h before use. MBW was filtered using a 0.2 μm sterile PES membrane filter and immediately diluted with a test solution. Hard water was prepared by dissolving 0.305 g of anhydrous calcium chloride and 0.139 g of magnesium chloride hexahydrate in 1 L distilled water. It was sterilized at high pressure (121℃, 15 min) and stored at 4℃ before use. To prepare the organic matter/solution, yeast extract was dissolved in hard water to a concentration of 20%. The prepared organic solution was sterilized at high pressure (121℃, 15 min) and stored at 4℃. It was diluted with hard water to form a solution with organic material content of 5%, and its pH was adjusted to 7.0 with 1 N sodium hydroxide. Basic proliferative medium containing 5% fetal bovine serum (FBS) was used as the bacterial neutralization solution. The disinfectant was diluted to 1:100 to 1:1,600 using a sterile organic solution. This was the chosen concentration as the majority of the disinfectants performed well at this ratio, and a weaker concentration was required to determine any synergistic or antagonistic effects when the detergent and disinfectant were combined. After mixing 4 mL of each *Salmonella* culture in 96 mL of hard water and 5% organic diluent, 2.5 mL of this was extracted and mixed with 2.5 mL of the same amount of disinfectant to react at 4℃ for exactly 30 min and mixed every 10 min. To neutralize the effectiveness of the disinfectant, 1.0 mL of the mixture was immediately taken out and mixed in 9.0 mL of a 37℃ medium (5% organic diluent) and then 0.1 mL was mixed in a test tube containing 2 mL medium. Each solution was tested thrice consecutively and incubated at 37℃ for 48 h.

Estimation of *Salmonella* growth

The growth of bacteria was confirmed by McFarland Equivalence Turbidity Standards (McFarland, 1907). 0.5 McFarland standard was prepared by adding 85 mL of 1% (w/v) H_2SO_4 to 0.5 mL of 1.175% (w/v) barium chloride dihydrate ($BaCl_2.2H_2O$), made up to 100 mL with deionized water and mixed well. Optical density (OD) was measured at a wavelength of 600 nm using a spectrophotometer. The disinfectant was determined to be effective against *Salmonella* if no growth was recognized in the three nutritional media based on turbidity. The control group was tested under hard water conditions without a disinfectant, and the pathogen titer was confirmed to be 2×10^5 CFU/mL or higher in the neutralization reaction stage.

Statistical analysis

All results obtained in this study were statistically analyzed using Student's t-test and expressed as mean ±standard deviation using SPSS ver. 21.0. The different mean values of the diluents, hard water and MBW, were compared, and $P<0.05$ was considered significant.

RESULTS

Table 2 and 3 show that effects of V and F on *Salmonella* inactivation in each treatment groups. In case of the hard water, V-disinfectant inactivated *Salmonella* at a 1:200 dilution ratio, and F inactivated *Salmonella* at a 1:100 dilution ratio. In this conditions, adding 1% detergent to the disinfectant had no effect on the increase in *Salmonella* control efficacy. In case of MBW conditions, *Salmonella* was inactivated 1:400 dilution ratio in the F compared V had no significant effect. The disinfectant is mixed with 1% cleaning agent and diluted in microbubbles. In the V disinfectant, there was no significant difference from the diluted in hard water. In contrast, in the F disinfectant, *Salmonella* was not detected even at 1:800 dilution ratio, which was the most effective com-

Table 2. Effects of monopersulfate-based disinfectant (V) on *Salmonella* inactivation in each treatment groups

Disinfectant	Diluent	Group	×1	×200	×400	×800	×1,600
Control			0.72±0.02				
Detergent	HW	HW+1% Det	0.8±0.06				
	MBW	MBW+1% Det	0.8±0.02				
Disinfectant	HW	HW+V		0.06±0.02*†	0.71±0.01*	0.73±0.01*	0.74±0.01*
	MBW	MBW+V		0.82±0.05*	0.83±0.03*	0.82±0.04*	0.89±0.03*
Detergent+ Disinfectant	HW	HW+V+1% Det		0.06±0.03*†	0.81±0.06	0.73±0.01	0.74±0.01
	MBW	MBW+V+1% Det		0.01±0.01*†	0.93±0.04	0.88±0.08	0.72±0.02

*P<0.05; The different mean values of the diluents, hard water and MBW, were compared.

†No growth.

HW, hard water; MBW, microbubble water; Det, detergent.

Table 3. Effects of citric acid-based disinfectant (F) on *Salmonella* inactivation in each treatment groups

Disinfectant	Diluent	Group	×1	×100	×200	×400	×800	×1,600
Control			0.79±0.07					
Detergent	HW	HW+1% Det	0.8±0.06					
	MBW	MBW+1% Det	0.8±0.02					
Disinfectant	HW	HW+F		0.01±0.01*†	0.62±0.02*	0.62±0.06*	0.72±0.03	0.73±0.02*
	MBW	MBW+F		0.02±0.01*†	0.02±0.01*†	0.03±0.02*†	0.71±0.05	0.8±0.02*
Detergent+ Disinfectant	HW	HW+F+1% Det		0.04±0.34*†	0.64±0.02*	0.66±0.04*	0.7±0.02*	0.76±0.08
	MBW	MBW+F+1% Det		0.02±0.01*†	0.02±0.01*†	0.01±0.01*†	0.01±0.01*†	0.76±0.04

*P<0.05; The different mean values of the diluents, hard water and MBW, were compared.

†No growth.

HW, hard water; MBW, microbubble water; Det, detergent.

pared to all other conditions in this study.

DISCUSSION

Disinfection is a commonly used crucial aspect of pathogens control in environment. However, products and chemical groups of disinfectant agents vary in their activity against different pathogens (Gosling et al, 2017). Not all disinfectants or disinfectant product formulations are equally effective, and some disinfectants are less effective than others (Cabrera et al, 2017; Gosling et al, 2017). A variety of researchers have extensively reviewed the chemical characteristics and modes of action of disinfectants that are commonly used in livestock units (Cabrera et al, 2017; Jang et al, 2017; Aksoy et al, 2020; Gómez-García et al, 2022). Various efforts have been made to control *Salmonella*, but it is still challenging to completely eradicate infections. The acid and peroxymonosulfate components are widely used as chemical agents for disinfection and known to have good bactericidal effects, while complying with the regulatory standards of food, livestock industry, and public health (Dibner and Buttin 2002; Mroz 2005; Seo et al, 2013; Bai et al, 2022). The acid-based components could decrease the extension of the lag phase and inhibit the physiological state values of *S.* Typhimurium when the pH was lower than 4.5. Potassium peroxymonosulfate or potassium monopersulfate is a product widely used disinfectant in a variety of industrial applications (Kunanusont et al, 2020; Moraes et al, 2022).

Many studies highlight the usefulness of cleaning and disinfection in the swine industry to reduce the level of *Salmonella* in environment. Additionally, several approaches have been investigated however, difficulties in eliminating *Salmonella* remain. During normal cleaning and disinfection, the recommended protocol for farmers

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is to allow drying time between the application of a detergent and disinfectant. However, in practice, there is not always sufficient time before the next batch of pigs is due to arrive, to fully adhere to the process. Washing alone had no effect on *Salmonella* prevalence, with 87.2% (157/180) of swabs having tested as *Salmonella*-positive; using only detergent after power washing resulted in a reduction in the percentage of *Salmonella*-positive swabs to 54% (58/108), but results still showed the presence of *Salmonella* in the facility (Walia et al, 2017).

In this study, we evaluated the effect of a mixture of disinfectant and detergent on *Salmonella* inactivation and the synergistic effect of microbubbles. Because no study to date has investigated the various combinations with detergent and disinfectants and using MBW as a diluent to eliminate *Salmonella*. As a predictable result, the concentration of the disinfectant and the type of the mixed solution have a significant impact on the inactivation efficiency of *Salmonella*. In present study, when only disinfectant was used, the monopersulfate-based disinfectant was evaluated to have a better *Salmonella* inactivation effect than the citric acid-based one. This These results need to be interpreted carefully. Although both disinfectants showed an effect on *Salmonella* even at higher dilution rates than the manufacturer's recommended concentration, it may be different from actual fecal contamination conditions. In spite of previous study (Gosling et al, 2017), the combination of disinfectants and cleaning agents did not increase the effect of *Salmonella* inactivation compared to that with the use of disinfectants alone in this study. These results differ from previous experimental results, which are thought to be due to differences in the experimental conditions. In previous studies, the test was conducted using the fecal floating model, whereas the organic matter model was used in our study, which may have resulted in lower efficacy of the cleaning agent. In previous studies conducted under fecal contamination conditions, the *Salmonella* inactivation effect was better when the detergent alone was applied (unpublished). However, when MBW was mixed with disinfectants and a cleaning agent, the *Salmonella* inactivation effect was improved. MBW rapidly shrinks and collapses upon receiving physical stimuli. The OH free radicals produced by the microbubble collapse are considered to have a bactericidal effect, although the precise mechanism involved remains unknown (Agarwal et al, 2011; Tsuge et al, 2009; Tamaki et al, 2018). MBW produces active oxygen that decomposes toxic compounds, disinfects water, and cleans solid surfaces including membranes. Since the rate of increase in the internal pressure of a microbubble is inversely proportional to its size, a high-pressure spot is eventually created at the final stage of the microbubble collapse (Agarwal et al, 2011). However, *Salmonella* could not be inactivated when microbubbles were applied alone in this study. On the other hand, when an acidic disinfectant was diluted with MBW, it was much more effective in inactivating *Salmonella*. when the acidic disinfectant was diluted in MBW, the same antibacterial effect was observed even for higher dilutions of the disinfectant. However, there was no difference in the efficacy of peroxymonosulfate-based disinfectants between hard water conditions and MBW conditions. As a result, for *Salmonella* control, it may be more effective to use diluted MBW with a low concentration of acid-based disinfectant and a cleaning agent.

CONCLUSION

Although many studies have been conducted on bacterial inhibition using microbubbles in various industrial fields, there were no studies have evaluated whether MBW is associated with improving the effectiveness of disinfectants.

As our results, when a mixture of a citric acid-based disinfectant and detergent diluted in MBW is used, efficient disinfection of *Salmonella* can be achieved and the amount of disinfectant used can also be reduced. When the disinfectant alone or in combination with a detergent diluted in hard water is applied, the antibacterial effect achieved is not as optimal. Further studies may be need to evaluate the efficacy of disinfectants according to different level of the organic contamination conditions.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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