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Bactericidal Efficacy of a Disinfectant Spray Containing a Grapefruit-seed Extract, Citric acid, Malic acid and Benzalkonium Chloride against *Salmonella* Typhimurium and *Brucella ovis*

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ABSTRACT - *Salmonella* spp. and *Brucella* spp. can cause considerable diseases on both humans and animals. In addition, these microorganisms cause the economic loss in animal farming and food industry. In this study, the disinfection efficacy of a disinfectant spray, composed to grapefruit seed extract, citric acid, malic acid and benzalkonium chloride, was evaluated against *S.* Typhimurium and *B. ovis*. A bactericidal efficacy test by broth dilution method was used to determine the lowest effective dilution of the disinfectant following exposure to test bacteria for 30 min at 4°C. The disinfectant and test bacteria were diluted with hard water (HW) or organic matter suspension (OM) according to treatment condition. On HW condition, the bactericidal activity of the disinfectant spray against *S.* Typhimurium and *B. ovis* was 5 and 4 fold dilutions, respectively. On OM condition, the bactericidal activity of the disinfectant spray was 2 and 1 fold dilutions against *S.* Typhimurium and *B. ovis*, respectively. As the disinfectant spray possesses bactericidal efficacy against foodborne pathogens such as *S.* Typhimurium and *B. ovis*, the disinfectant spray can be used to control the spread of bacterial diseases.

Key words : Disinfectant spray, *Salmonella* Typhimurium, *Brucella ovis*, Disinfectant efficacy

All over the world, *Salmonella* Typhimurium (*S.* Typhimurium) is responsible for approximate a third of all cases of foodborne diseases[1]. Salmonellosis is an increasingly important health concern and is usually associated with the consumption of *Salmonella*-contaminated foods, mainly of animal origin, including beef, pork, poultry and turkeys[2]. The disease in human is characterized by self-limiting gastroenteritis that occasionally can cause fever, systemic infection, and severe inflammation of the intestinal mucosal epithelium[3]. In food animals, *Salmonella* infections play a significant role in public health and particularly in food safety, as food products originated from animals are believed to be the major source of salmonellosis in human[4].

Brucellosis is a zoonosis that is transmitted to humans from infected animals' meat, milk, urine, body fluids, and pregnancy material, as well as from milk products prepared by infected milk[5]. The disease is generally transmitted to humans by eating cheese, made from raw milk, and milk products such as cream and butter[6]. In addition, the disease may be transmitted by eating animals' reticulo-endothelial organs, such as the spleen and liver, without proper cooking[7]. *Brucella* organisms are non-motile gram-negative coccobacilli or short small rods measuring about 0.6-1.5 × 0.5-0.7 μm without bipolar staining, not encapsulated bacteria[8]. There are six recognized species of the genus which include *Brucella abortus* (*B. abortus*), *Brucella melitensis* (*B. melitensis*), *Brucella ovis* (*B. ovis*), *Brucella canis* (*B. canis*), *Brucella neotome* (*B. neotome*) and *Brucella suis* (*B. suis*)[9]. In six recognized *Brucella* species, *B. abortus*, *B. melitensis, B. ovis* and *B. suis* are responsible for bovine, ovine and caprine, and swine brucellosis, respectively[10,11].

Due to abuse and overuse of antibiotics in animal agriculture and medicine, antibiotic-resistant strains of *Salmonella* and *Brucella* are increasing[12,13]. Therefore, the effective cleaning and disinfection are essential measures for the

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prevention of infections and outbreaks. The choice of proper biocides is very important for the cleaning and disinfectant regimes. In food production, hospital settings, and the home, the increase of biocide use is contributing to the emergence of biocide resistant strains[14]. Thereby, biocides are often composed of a mixture of ingredients that act upon a wide range of cellular mechanisms and targets, which makes it difficult for bacteria to become resistant to biocides[12].

In the world, salmonellosis and brucellosis cause significant economic loss in agriculture and the food industry[15,16]. The use of disinfectant is very effective for successful control of diseases from bacteria, fungi and parasites[17,18]. In the food industry, regular cleaning and disinfection are the most efficient way to control pathogenic bacteria that are prevalent in food and the environment[19,20]. There are many kinds of disinfectants for food utensils and premises, such as alcoholic solutions and hypochloric solutions, including organic acid, sodium hypochlorite, alcohol-based products and quaternary ammonium compounds[20,21]. However, there is not the efficacy test for the disinfectant spray containing the grapefruit-seed extract, citric acid, malic acid and benzalkonium chloride (BAC) against foodborne pathogenic bacteria. Therefore, this study was carried out to examine bactericidal efficacy of a disinfectant spray against *S.* Typhimurium and *B. ovis*.

Materials and Methods

Bacteria and culture

The test bacteria, *S.* Typhimurium (G-B-14-21-62) and *B. ovis* (ATCC 25840) were obtained from the Korean Veterinary Culture Collection (KVCC, Seoul, Korea). The strains were maintained as frozen glycerol stock. *S.* Typhimurium were cultured in Luria-Bertani (LB) broth containing 1.5% agar. *B. ovis* were spread in Brucella broth containing 5% fetal bovine serum and incubated at 37°C under 5% CO_2 condition.

Disinfectant spray

A disinfectant spray (grapefruit-seed extract 750 mg, citric acid 600 mg, malic acid 187.5 mg and BAC 75 mg per 300 mL) was provided by Korea Thumb Vet Co. Ltd. (Iksan, Korea). The disinfectant was stored in the dark in room temperature and prepared for dilution on the day of evaluation. Determination of the bactericidal efficacy of the disinfectant was based on Animal and Plant Quarantine Agency (Anyang, Korea), Regulation No. 2013-34[22].

Diluents and treatment condition

Testing was based on bactericidal effects of the disinfectant diluents in two treatment conditions (hard water (HW) and organic matter (OM) condition) and pathogen control (sanitizer negative control) in Table 1. HW, an ingredient of HW treatment condition, was made by adding anhydrous $CaCl_2$ 0.305 g and $MgCl_2 \cdot 6H_2O$ 0.139 g into one liter distilled water. Organic suspension, an ingredient of OM treatment condition, is a solution of 5% (w/v) yeast extract in HW. The test organisms were prepared by titration of each cultural broth into at least 10^8 colony-forming units (CFU)/mL viable organisms with the same kind of diluents of treatment condition.

Table 1. Experimental design for the determination of the bactericidal efficacy of the disinfectant spray

Treatment condition[1)]	Contents according to treatment condition[2)]			
	HW	OM	Disinfectant	Bacteria
HW condition	+	-	+	+
OM condition	+	+	+	+
Bacteria control	+	-	-	+

[1)]HW, standard hard water; OM, organic matter.
[2)]+, presence; -, absence

Experimental procedures

For the efficacy test against *S.* Typhimurium, the disinfectant was diluted 3, 4, 5, 6, 7 and 8 times with HW, and diluted 1, 2, 3, 4, 5 and 6 times with OM, respectively. For the efficacy test against *B. ovis*, the disinfectant was also diluted 2, 3, 4, 5, 6 and 7 times with HW, and diluted 1, 2, 3, 4, 5 and 6 times with OM, correspondingly.

To verify the lowest effective dilution of the disinfectant, five serial dilutions of the disinfectant were prepared and placed at 4°C prior to test reaction. 2.5 mL of each the disinfectant diluent was mixed with the same amount of test organism followed by contact time of 30 min at 4°C. During this period, the mixture was shaken at 10 min interval. At the end of 30 min contact time, one mL of the mixture was neutralized with 9 mL of nutrient broth containing 5% inactivated horse serum (BD Korea Co., Ltd., Incheon, Korea) at 37°C. 0.1 mL of the neutralized reaction mixture was subcultured into 10 mL of each recovery cultural broth at 37°C for 48 h in incubator. For the test of each bacteria control, 2.5 mL of HW was mixed with the same amount of each test organism followed by contact time of 30 min at 4°C, and then all procedure were undertaken in parallel for the disinfection test.

The valid dilution of the disinfectant was determined that the greatest dilution showing no growth in four or more in the five replicates was confirmed. The final dilution time was statistically determined by a median value among three valid dilution of the triplicate test, but each value of which should be within 20% experimental error. In each bacteria control, the number of bacterial growth in the five replicates was counted.

Table 2. Final valid dilution of the disinfectant spray against *S.* Typhimurium and *B. ovis*

Bacterial strains	Treatment condition[1]										
	HW				OM				BC		
	DT	1[2]	2	3	DT	1	2	3	1	2	3
S. Typhimurium	3	○[3]	○	○	1	○	○	○	+[5]	+	+
	4	○	○	○	2	○	×	○	+	+	+
	5	○	×[4]	○	3	×	×	×	+	+	+
	6	×	×	×	4	×	×	×	+	+	+
	7	×	×	×	5	×	×	×	+	+	+
	8	×	×	×	6	×	×	×	+	+	+
	VD		5.0		VD		2.0			+	
B. ovis	2	○	○	○	1	○	○	○	+	+	+
	3	○	○	○	2	×	○	×	+	+	+
	4	○	○	○	3	×	×	×	+	+	+
	5	○	×	×	4	×	×	×	+	+	+
	6	×	×	×	5	×	×	×	+	+	+
	7	×	×	×	6	×	×	×	+	+	+
	VD		4.0		VD		1.0			+	

[1] HW, standard hard water; OM, organic matter; BC, bacterial control; DT, dilution time; VD, valid dilution.
[2] The number of examinations.
[3] ○, growth inhibition, no growth of more than four in the five replicates.
[4] ×, no growth inhibition, growth of more than two in the five replicates
[5] +, all growth in each replicate.

Results

Table 2 shows the final valid dilution of the disinfectant spray. When the bactericidal effect on HW condition was evaluated, the antibacterial activity of the disinfectant showed on 5 and 2 fold dilutions against *S.* Typhimurium and *B. ovis*, respectively. With the investigation of the bactericidal effect of the disinfectant on OM condition, *S.* Typhimurium and *B. ovis* were inactivated on 4 and 1 fold dilutions, individually. Because the organic material interferes with the efficacy by either inactivating the disinfectant or blocking it from surface contact, the bactericidal activity of the disinfectant on the OM condition was lowered against pathogenic bacteria compared with HW condition.

Comparing the results of the disinfectant against two pathogenic bacteria in the present study, the bactericidal effect against *S.* Typhimurium was higher than that against *B. ovis* on the HW and OM condition. In each bacterial control, the growth of *S.* Typhimurium and *B. ovis* was verified in all replicates.

Discussion

In food-processing environments, the ability to persist and multiply makes contamination by foodborne pathogens difficult to control and eradicate. Therefore, food-processing facilities must be designed carefully, with an emphasis on effective cleaning and disinfection operations in the production line[23]. The use of efficient cleaning agents and disinfectants on surface-attached microbes minimizes contamination of the product, enhances shelf life and reduces the risks of foodborne illness[24].

Various methods are used during cleaning and disinfection processes, to sanitize food-processing environments through the use of alkaline and acid detergents, and to control the organism residing in the food and the processing devices through the use of a wide range of chemical disinfectants[25].

In a previous study[26], a grapefruit-seed extract had antimicrobial properties against a wide range of gram-negative and gram-positive organisms at dilutions found to be safe. In addition, organic acids, including citric acid and malic acid have been used to control foodborne pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli* and *S.* Typhimurium[27,28]. Furthermore, BAC is a quaternary ammonium compound that is widely used as disinfectant and cationic surface active agent for sanitation in food-processing lines and surfaces in the food industry[29], as clinical disinfectant and antiseptic in health care facilities and domestic households[30].

A previous study reported that the log-reduction was 6.72 after 99 mL of an antiseptic containing 85% ethanol was interacted with 1 mL of *S.* Typhimurium suspension for 15 sec[31]. In another previous study using a disinfectant containing 10% (w/v) povidone-iodine[11], the bactericidal activity against *S.* Typhimurium and *B. ovis* on HW condition was 400 and 150 fold dilutions, respectively and 5 and 20 fold dilutions on OM condition, respectively. In addition, a disinfectant (chlorine dioxide 0.01% (v/v), betaine HCl 0.5% (v/v) and propylene glycol 0.30% (v/v)) had a bactericidal activity against *S.* Typhimurium and *B. ovis* with 2.5 and 1.1 fold dilutions on HW and OM condition, respectively[32].

Considering the ingredient content and components of above disinfectants, bactericidal activity of the disinfectant spray used for this study was greater than that of the antiseptic composed of ethanol and the disinfectant composed of chlorine dioxide, betaine HCl and propylene glycol. On the other hand, compared with a disinfectant containing 10% povidone-iodine, the disinfectant spray had a low bactericidal activity on both HW and OM condition.

With the results from this study, the disinfectant spray as an effective and safe disinfectant can be applied to control foodborne pathogens in food-processing environments. In the present study, the disinfectant efficacy of the disinfectant spray has a limitation that the results are based on *in vitro* test. Organic material in suspension (OM condition) could not represent all possible parameters of *Salmonella* and *Brucella* contaminated food-industry environment.

Conclusions

In the food industry, salmonellosis and brucellosis were very important diseases because of high mortality for farmed animals, zoonoses and economic loss. In the study of the bactericide efficacy test of the disinfectant spray, the results suggest that the disinfectant has a safe and potential bactericidal activity against *S.* Typhimurium and *B. ovis*. So, the disinfectant spray can be used to control the spread of foodborne pathogens.

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국문요약

살모넬라증과 부루셀라증은 가축 및 사람에 심각한 피해를 유발하는 질병으로, 축산업과 식품산업에 많은 경제적 손실을 초래하고 있다. 본 연구에서는, 자몽종자추출물, 구연산, 사과산 그리고 염화벤잘코늄을 주성분으로 하는 스프레이형 소독제의 *Salmonella* Typhimurium과 *Brucella ovis*에 대한 효력시험을 수행하였다.

살균효력시험은 배지희석법에 따라 수행하였으며, 스프레이형 소독제와 시험 세균들을 처리조건에 따라 경수와 유기물로 희석하여 반응을 시켰다. 유기물 조건에서, *Salmonella* Typhimurium과 *Brucella ovis*에 대한 스프레이형 소독제의 살균력은 경수조건에서의 살균력과 비교하여 낮게 나타났는데, 이는 유기물들에 의한 소독제의 살균 유효성분에 대한 저해작용에 따른 것으로 사료된다.

스프레이형 소독제는 *Salmonella* Typhimurium과 *Brucella ovis*와 같은 인수공통전염병 유발 병원균들에 대해 살균효과를 나타내어, 살모넬라증과 브루셀라증과 같은 세균성 질병의 확산을 제어하는데 효과적으로 이용될 수 있을 것으로 사료된다.

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