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EVALUATING THE EFFECT OF VARIOUS TYPES OF DISINFECTANTS ON BACTERIAL BIOFILMS

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Abstract
Biofilm formation on equipment surfaces is a potential food safety hazard, providing increased resistance and persistence of pathogens and spoilage microorganisms in food production environments. The issue of preventing the biofilm formation is extremely important, since a wide range of disinfectants does not always provide the proper effect. The article discusses the antimicrobial effectiveness of disinfectants with various active ingredients (based on active chlorine, peracetic acid and quaternary ammonium compounds (QAC) with enzymatic substances) on binary biofilms. The objects of the study were the strains of pathogenic and opportunistic microorganisms isolated from abiotic surfaces of food production environments and food products. Different effects of disinfectants on biofilms formed by bacteria have been established. Disinfectant based on peracetic acid and chlorine had the greatest effect on binary biofilms of Brochothrix thermosphacta/Salmonella spp. and Staphylococcus equorum/Salmonella spp. The greatest antimicrobial effect on biofilm of Listeria monocytogenes 12/Pseudomonas azotoformans 6 was shown by a chlorine-based disinfectant. Disinfectants based on chlorine and QAC with enzymatic substances were most effective against the binary biofilm of L. monocytogenes 12/Salmonella spp. 14. However, none of the disinfectants had absolute antimicrobial effectiveness against the studied binary biofilms. Biofilm-forming microorganisms have shown resistance to the recommended concentrations of disinfectants. Therefore, currently, it is extremely important to revise approaches to hygiene at enterprises by finding working concentrations of new antimicrobial agents and new procedure that are effective for destroying biofilms.

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Introduction

The most dangerous phenomenon in the food industry is the ability of microorganisms to form biofilms on abiotic surfaces. The main and auxiliary equipment at food enterprises have an abiotic surface characterized by roughness, porosity, presence of joints, seams and other hardly accessible areas [1–3]. Such structural features of the equipment are a favorable environment for the development and attachment of biofilms.

The phenomenon of biofilm formation was discovered in the mid-1980s [4,5]. Over the next years, biofilm research proved that biofilm formation is inherent in a large number of pathogenic and opportunistic microorganisms [6, 7]. Some researchers consider biofilm formation as a pathogenicity factor [8].

Biofilm is a population of surface-associated microbial cells enclosed in polymeric extracellular matrix. According to literature, many pathogenic bacteria are associated with biofilms and in some cases actually grow in them, including *Legionella pneumophila* [9], *Staphylococcus aureus* [10], *Listeria monocytogenes* [11], *Campylobacter* spp. [12], *Escherichia coli* O157: H7 [13], *Salmonella typhimurium* [14], *Vibrio cholera* [15] и *Helicobacter pylori* [16].

Compared to planktonic cells, biofilm-associated cells are much more resistant to antimicrobials, including disinfectants. This increased resistance has a significant impact on the quality of hygienic measures at food enterprises.

Effective disinfection is necessary at food enterprises, since wet surfaces of objects in the production environment create favorable conditions for the growth of microorganisms [17,18,19]. Modern disinfectants used in the food industry include oxidizing agents such as hypochlorite, hydrogen peroxide, and peracetic acid; denaturing agents, for example alcohol-based products; non-oxidizing agents and agents that reduce interfacial tension; and enzyme-based compounds [20,21]. Disinfectants must be effective, safe, rinseable, and easy-to-use [20].

The resistance of biofilm-associated cells to disinfectants is explained by many factors, often acting simultaneously, which include the presence of extracellular polymers that interfere with diffusion/reaction and differences in physiological status depending on the biofilm layer [22,23].

There is also growing evidence that interspecies interactions within the biofilm matrix further enhance resistance to disinfectants compared to single-strain biofilms [24–27].

The permeability of the matrix may be reduced by various factors such as changes in the microenvironment, cell density and biofilm age. The last two factors are highly correlated and difficult to separate as the biofilm matrix becomes thicker and denser with age and the number of colony-forming units (CFU) increases. Despite this, biofilm age has been shown to play a more important role than cell density [28] in relation to increased tolerance to disinfectants.

The limited time for penetration of disinfectants into biofilm during hygienic procedures at food enterprises may result in low levels of antimicrobial agent exposure in the deeper layers of the biofilm. Consequently, microorganisms in the biofilm will develop adaptive responses to sublethal concentrations of disinfectants. Surface-associated bacteria are more difficult to kill than planktonic cells, so biofilm contamination of production environments increases microbial load and potentially reduces food safety and quality.

The complex nature of biofilm structure and the ability of biofilm-associated cells to be firmly attached to hardly accessible surfaces make the antimicrobial activity of currently used disinfectants less effective [29,30]. For all these reasons, it is necessary to consider whether the modern cleaning and disinfection procedures currently used in the food industry are effective, or whether new methodologies and strategies are needed to solve the problem.

The aim of this study was to determine the effectiveness of disinfectants used in the food industry for destructing the binary biofilms of pathogenic and opportunistic microorganisms.

Objects and methods

To determine the antimicrobial effectiveness of various disinfectants against formed biofilms, the following microorganisms were selected: *Pseudomonas azotoformans 6, Salmonella* spp. *14, Listeria monocytogenes 12* isolated from the environment of food enterprise, *Brochothrix thermosphacta 2726* and *Staphylococcus equorum 2736* isolated from pig carcass wipe samples, *Salmonella* spp. *38* isolated from a food product. Binary biofilms were formed from microorganisms: *Brochothrix thermosphacta 2726/Salmonella 38, Staphylococcus equorum 2736/Salmonella* spp**.** *38, L. monocytogenes 12/P. azotoformans 6, L. monocytogenes 12/Salmonella* spp. *14*.

The following substances were used as disinfectants:

— Disinfectant No. 1 for decontamination of equipment and premises at meat industry enterprises. Ingredients: tertiary amines (N, N-bis(3-aminopropyl) dodecyl amine 3 ± 0.5%), enzymes (carbohydrase 4 ± 1%, enzyme complex 4 ± 1%), quaternary ammonium compounds (QAC) (benzalkonium chloride 8 ± 0.6%, dodecyl dimethylammonium chloride 6 ± 0.4%). For the study, a working solution of the agent with a concentration of 0.085% was prepared.
— Disinfectant No. 2 for decontamination of process equipment and production facilities at meat industry enterprises. Ingredients: sodium salt of dichloroisocyanuric acid, as well as functional components that contribute to better dissolution of the agent. When dissolved in water, 2.7 grams of the product releases 1.5 grams of active chlorine. For the study, a working solution of the agent with an active chlorine concentration of 0.015% was prepared.
— Disinfectant No. 3 for decontamination of process equipment and production facilities at meat industry enterprises. Ingredients: peracetic acid (15.5–17.0%), hydrogen peroxide (15.8–18.0%), acetic acid, functional additives. For the study, a working solution of the agent with peracetic acid concentration of 0.05% was prepared.

Biofilm formation

Biofilms were formed at the solid surface/air interface. Biofilms of this type were obtained using glass fiber filters as substrates, which are an easily dispersed material, according to the method described earlier (Plakunov et al., 2016) [31]. Glass fiber filters (Whatman GF/F, UK) were cut into 15x15 mm squares and sterilized by autoclaving (20 min, 120 °C), then laid out on the surface of LB agar medium (Becton Dickinson, USA) in plates.

Bacterial cultures were separately grown in LB broth until stationary phase. Turbidity was prepared in pure 0.5 LB broth according to McFarland using DEN-1B McFarland Densitometer (Biosan, Latvia). Next, 40 µL of the obtained binary bacterial culture were applied in triplicate onto prepared sterile glass fiber filters in sterile plates with PCA agar medium. Cultures were grown in a thermostat for 48 h at 30 °C.

Effect of disinfectants on biofilms

After 48 hours of biofilm growth, they were treated with disinfectants. Solutions of disinfectants in sterile water were prepared immediately before being applied to the filters. Biofilms were removed from the surface of the growth medium, transferred to sterile plates, each was treated with disinfectant solutions in the amount of 100 µL, until the filter was completely wetted. The exposure time of disinfectants was 10 minutes. As a positive control, instead of disinfectant, sterile water in the amount of 100 µL was added to the surface of the medium with a formed biofilm.

The glass fiber filter was then placed in a flask with sterile saline. A sterile glass mortar and beads were used to homogenize the glass fiber filter. The resulting content of the flask was considered a first dilution. Aliquots of the obtained homogenates (100 µL) were diluted in 900 µL of sterile saline and a series of decimal dilutions was prepared. Then, homogenates were incubated in a thermostat at 30 °C for 24 h, followed by counting the colonies on the

plates. In each dilution, a number of viable cells (CFU/cm³) was determined by the microscopy method, after which the CFU titer in the primary filter homogenate was calculated. The experiments were performed in three independent biological replicates.

Statistical analysis

Statistical data processing was carried out using the Statactical software ver. 10.0.1011 (StatSoft). The results were calculated as "mean ± standard error". Differences with p-values of ≤ 0.05 were considered statistically significant.

Results and discussion

In their natural environment, biofilms are complex populations of different types of microorganisms, rather than single-species biostructures. Multispecies biofilms in their structure are more resistant to environmental conditions, including the action of disinfectants. A quantitative assessment of the antimicrobial effect of modern disinfectants on binary (multispecies) biofilms was carried out.

Based on information about the frequent detection of *Brochothrix thermosphacta* in various food products and objects at the production environment [32,33] and its genomic heterogeneity in terms of the presence of a gene potentially involved in the formation of a biofilm matrix [34], the effect of various disinfectants on binary biofilm formed by *Brochothrix thermosphacta* and pathogenic *Salmonella* spp. was studied. The antimicrobial effectiveness of disinfectant working solutions on biofilm is shown in Figure 1. Analysis of the obtained data shows the different effects of disinfectants on the biofilm formed by two types of microorganisms.

At the concentrations studied, disinfectant No. 2 showed an antimicrobial effect in contrast with the other two disinfectants. Disinfectant No. 1 based on QAC with enzymatic substances was the most ineffective against biofilm. Absolute antimicrobial effectiveness against biofilm was not observed with any of the disinfectants.

A different pattern regarding the effectiveness of disinfectants was observed with biofilm of *Staphylococcus equorum/Salmonella* spp. (Figure 2). Disinfectant No. 3 showed

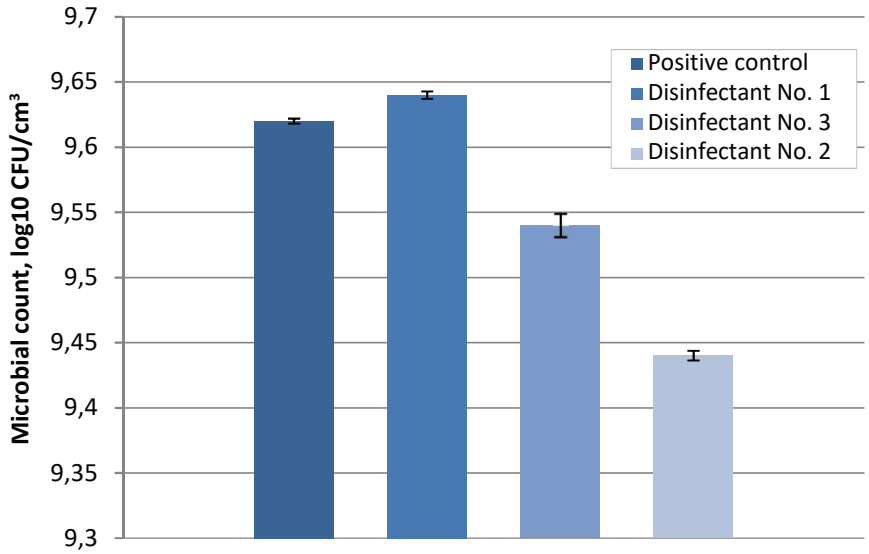

Figure 1. Results of disinfectants' effect on the binary biofilm of *Brochothrix thermosphacta/Salmonella* spp.

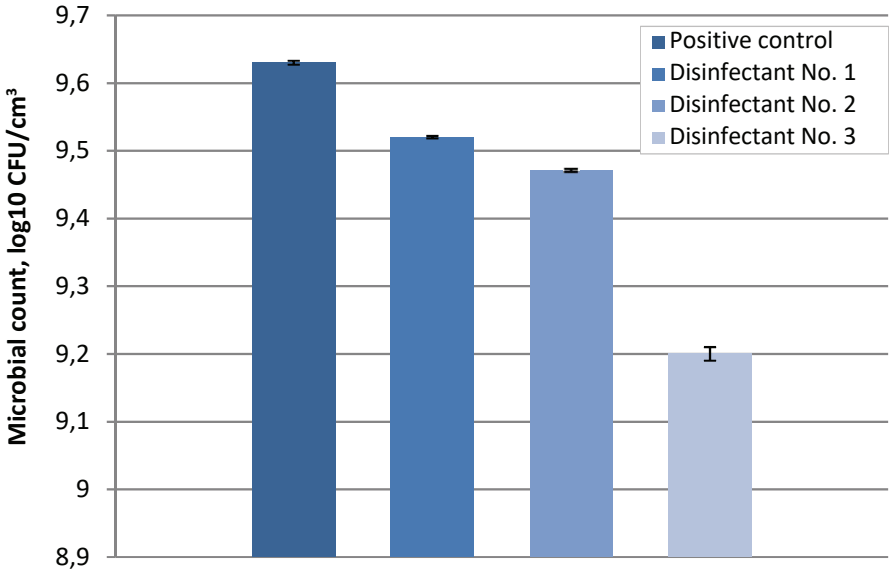

Figure 2. Results of disinfectants' effect on the binary biofilm of *Staphylococcus equorum/Salmonella* spp.

the highest antimicrobial activity against it. No significant differences in the effects of disinfectants No. 1 and No. 2 on the binary biofilm of *Staphylococcus equorum/Salmonella* spp. were established.

At the concentrations studied, disinfectants have shown to be ineffective against binary biofilms. According to the literature, biofilms composed of many types of bacteria may be more resistant to antibacterial agents, including antibiotics and disinfectants [35,36].

The persistence of *Salmonella* spp. in biofilm raises food safety concerns. *Salmonella* spp. are one of the main causes of foodborne infectious diseases [37]. Bacteria may enter food enterprises and spread through raw ingredients, dirty packaging, equipment, workers' hands and clothes. The ability to form biofilms only increases the rate and area of pathogen spread. The control and prevention of *Salmonella* spread in food production facilities depends on the correct implementation of comprehensive hygienic measures. However, the results obtained may indicate the need to revise the approaches to decontamination at enterprises.

Multispecies biofilms may include in their consortium not only pathogens, but also spoilage microorganisms such as *Brochothrix thermosphacta*. *Brochothrix thermosphacta* is one of the main spoilage microorganisms in meat products. *B. thermosphacta* has recently been identified in 80% of biofilms sampled at a meat processing facility, including both food contacting and non-contacting surfaces [32].

An equally significant pathogen is *L. monocytogenes*. The control of this pathogen has become one of the main goals in the food industry [38]. Biofilms of *L. monocytogenes* on food contacting surfaces have been identified as an important pathway for pathogen persistence and subsequent product contamination [39–41]. The highest antimicrobial effect on the biofilm of *L. monocytogenes 12/P. azotoformans 6* was showed by disinfectants No. 1 and No. 2, where microbial count after exposure decreased by 3.45 log and 3.15 log, respectively (Table 1).

Disinfectants No. 1 and No. 2 showed the best antimicrobial effect on cell combination in the biofilm of *L. monocytogenes 12*/*Salmonella* spp. *14*, where microbial count after exposure decreased by 2.14 log and 2.33 log, respectively. Disinfectant No. 3 based on peracetic acid showed the worst antimicrobial properties against the studied binary biofilms of *L. monocytogenes 12/P. azotoformans 6* and *L. monocytogenes 12*/*Salmonella* spp. *14*.

The results obtained showed that microbial counts in multispecies biofilms reduced when exposed to disinfectants, but these values were not significant, and absolute antimicrobial effectiveness was not observed. The results show that the use of disinfectants studied in this work is not always effective in elimination of the bacterial biofilms from the surfaces of food production environments. Definitely, after treatment with disinfectants in the studied concentrations, microorganisms remain on the surface.

The resistance of the formed biofilms to disinfectants based on active chlorine and peracetic acid was directly reflected in this study. This finding is of concern because the concentrations used in the experiment are commonly used to disinfect food equipment, especially in meat industry.

The ability to form biofilms is inherent not only in opportunistic microorganisms, but also in pathogenic ones. Recent data obtained by other scientists have shown that the formed biofilm of foodborne pathogens is highly resistant to sodium hypochlorite and peracetic acid [42]. In this study, binary biofilms of pathogenic *Salmonella* spp. and *L. monocytogenes*, as well as opportunistic bacteria, showed resistance to the working concentrations of solutions recommended for decontamination and used for disinfection at food enterprises. In the work by Byun et al. [43], chlorine-based disinfectants (NaOCl and ClO_2) were used to reduce the counts of planktonic cells and biofilms of *S. enteritidis*. As a result, it was shown that both preparations are effective as disinfectants when applied against planktonic cells at a dose of more than 100 μg/mL for 1 min, while biofilms were destroyed only when ClO_2 was applied at the same concentration for 5 minutes. In general, ClO_2 effectively reduced the counts of planktonic cells and biofilms of *S. enteritidis* compared to NaOCl under the same conditions. However, the presence of organic substances significantly reduced the effectiveness of the studied disinfectants [43]. The results obtained indicate that various disinfectants based on active chlorine, but with different active substances, may have different antimicrobial effects.

Disinfectant No. 1 based on QAC and containing additional enzymes for the destruction of biofilm matrix was effective against biofilms of *L. monocytogenes 12/P. azotoformans 6* and *L. monocytogenes 12/Salmonella* spp. *14*. As an environmentally friendly alternative for industrial surface cleaning, disinfectants with the addition of enzymatic agents have proven to be an effective tool against biofilms in the food industry [44]. It becomes apparent that biofilms must be destroyed by one of the accepted methods before decontamination.

The need to revise the approaches to decontamination at food enterprises arises. The development and evaluation of approaches to biofilm destruction on various objects at food enterprises are carried out all over the world.

Table 1. Results of disinfectants' effect on binary biofilms

Microbial composition of the biofilm	Disinfectant No. 1	Disinfectant No. 2	Disinfectant No. 3	Control
	Microbial count, log10 CFU/cm^3			
L. monocytogenes 12/P. azotoformans 6	**6.32 ± 0.08**	**6.62 ± 0.12**	**8.88 ± 0.08**	**9.77 ± 0.09**
L. monocytogenes 12/Salmonella* spp. *14	**6.48 ± 0.08**	**6.29 ± 0.11**	**7.71 ± 0.07**	**8.62 ± 0.08**

Conclusion

The present study examined the antimicrobial effectiveness of three disinfectants with different active ingredients used for decontamination at food enterprises against binary biofilms of pathogenic bacteria and spoilage microorganisms. The results of the study showed that the biofilm-associated microorganisms were resistant to the recommended concentrations of disinfectants used at food enterprises. It is evident that the objects in the production environment may act as containers for disinfectant-resistant bacteria. The search for fundamentally new methods of resistant bacteria elimination, including their biofilms, and the revision of approaches to decontamination at food enterprises are becoming increasingly important. The results of this study confirm the need to change approaches to ensuring microbiological safety at food industry enterprises.

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All authors bear responsibility for the work and presented data.

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