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EVALUATION OF APPROACHES TO INCREASE THE EFFECTIVENESS OF VARIOUS DISINFECTANTS AGAINST BIOFILM COMMUNITIES OF DIFFERENT AGES

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Abstract
Disinfectants are used as the main agents against microorganisms circulating on the surfaces of food enterprises. However, the adaptive ability of microorganisms to form biofilms complicates the process of surface cleaning and reduces the effectiveness of disinfectants. Modern disinfectants act against freely circulating microflora, but it is known that they are not always effective against biofilms. The purpose of this study was to investigate effective disinfectant compositions with bactericidal effect on binary bacterial biofilms of different ages. The article describes the effects of disinfectants based on chlorine, peracetic acid and quaternary ammonium compounds with enzymes in concentrations recommended by the manufacturer and increased several times on Salmonella 38, Brochothrix thermosphacta 2726 and Staphylococcus equorum 2736 planktonic cultures and binary biofilms. Binary biofilms of different ages (2 and 9 days old) were exposed to disinfectants with various active ingredients in combination with adjuvants, i. e. hydrogen peroxide 6% and various concentrations of isopropyl alcohol (30%). All products in concentrations recommended by the manufacturer did not have a disinfectant effect against the studied biofilm cultures. As a result of the work, it was found that the most effective disinfectants against multispecies biofilms were quaternary ammonium compounds in combination with enzymes and chlorine in combination with isopropyl alcohol (30%). The results obtained allow to expand knowledge about effective methods for controlling biofilms.

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Introduction

Food contamination causes great economic losses to society. Hygiene in the food industry is therefore of great importance and requires consideration of all types of microbiological hazards that may arise in the facilities (conveyor belts or slicing and packaging machines) and in the processing environment, as well as in raw materials throughout the process [1].

In food processing plants, failure to follow sanitation and disinfection procedures may result in the formation of bacterial niches that are not properly disinfected, so the bacteria are exposed to subinhibitory levels of disinfectants [2].

At the same time, dried organic matter and biofilms, if they are not removed during the washing process, may prevent the penetration of the disinfectant and reduce the effectiveness of surface cleaning [3].

In the food industry, pathogenic and spoilage microorganisms form biofilms on food contacting surfaces [4]. This results in equipment contamination, microorganism growth in drinking water systems, and post-processing contamination, which contributes to food spoilage and outbreaks of foodborne infections [5,6].

A number of studies have shown that multispecies biofilms commonly found in meat processing plants increase the resistance of bacteria to antibacterial treatment [7,8]. Non-lethal concentrations of antibiotics or disinfectants may stimulate the formation of bacterial biofilms [9,10], which are tolerant to aggressive external factors, including antimicrobial substances [11,12]. Over the years, this process has become better understood through researches; however, its potential risk and impact on food safety have not yet been fully established [13,14]. At the same time, complete inactivation and removal of mature biofilms formed on food contacting surfaces is difficult. Meat processing equipment with scratches, cracks or dents, and other hard-to-reach areas such as the underside of conveyor belts are potential niches for biofilm development. *Escherichia coli* O157: H7 and *Salmonella enterica* may form biofilms in meat processing plants [15,16], and in previous studies [17,18], many common disinfectants failed to

completely kill these biofilms due to the three-dimensional (3D) structure of the biofilm and the presence of bacterial extracellular polymeric substances (EPS) [19].

Biofilm formation provides significant benefits to bacterial cells by providing protection from physical and chemical stress. Multiple layers of surface-colonizing biofilms may prevent the penetration and diffusion of disinfectant solutions into the inner layers of biofilms. Thus, bacteria in these layers may be better protected and survive treatment with disinfectants. Surviving bacteria may multiply and contaminate food. Bacterial resistance to disinfectants also involves a nonspecific change in cell wall or membrane structure that affects cell permeability or increases efflux pump activity that helps biofilm cells to block and remove disinfectants outside the matrix. To counteract such resistance mechanisms, the ability of a disinfectant to dissolve the biofilm matrix, penetrate multiple layers of the biofilm, and disrupt the integrity of the cell membrane is critical to inactivate and remove the biofilms [20].

Given the growing interest in studying the resistance of biofilms to chlorine, quaternary ammonium compounds (QAC) and peracetic acid (PAA), many studies have been conducted [21]. Bacteria in mature biofilms are 10 to 1000 times more resistant to antibiotics than planktonic forms of bacteria [22], and resistance to biocides also exists. However, the etiology of this natural resistance is poorly understood and likely depends on many factors, mainly biofilm structural barriers and genetic adaptation factors. To explain this resistance, several authors [23] have proposed three possible causes under three hypotheses. The first is based on slow or incomplete diffusion of antibiotics into the inner layers of the biofilm. The second is the changes that occur in the biofilm microenvironment, as some biofilm bacteria enter a slow growth phase due to lack of nutrients or accumulation of harmful metabolites and therefore survive [24]. Finally, the third hypothesis indicates the presence in the biofilm of a subpopulation of cells, whose differentiation resembles the process of sporulation. They have a unique and highly resistant phenotype that protects them from the effects of antibiotics [25].

Therefore, the meat industry requires effective disinfectants for biofilm removal with an easy-to-implement protocol that can be applied to the production environment and various equipment, including hard-to-reach areas [26].

Improper disinfection of food contacting surfaces leads to the formation of biofilms, which puts food safety and public health at risk [27].

To overcome the problems posed by potent biofilm-forming bacteria with various complex resistance mechanisms, a comprehensive approach using multiple disinfectants or combining disinfectants with other cleaning methods has been proposed [28].

The purpose of this study was to investigate effective disinfectant compositions with bactericidal effect on binary bacterial biofilms of pathogenic and opportunistic bacteria of different ages.

Objects and methods

The objects of the study were the microorganisms *Brochothrix thermosphacta* 2726 and *Staphylococcus equorum* 2736 isolated from pork carcass swabs, and *Salmonella* sp. 38 isolated from a food product (pork steak). Strains were stored at –70 °C in Lennox broth (LB; Acumedia, Baltimore, MD) containing 15% glycerol. Before use, each strain was inoculated from the original glycerol solution into liquid LB medium and grown during the night time at 30 °C.

In the experiments, we used disinfectants approved for use in food production for treating working and production surfaces:

(1) Dimax Chlorine (INTERSEN-plus LLC, Russia). Dimax Chlorine is based on the sodium salt of dichloroisocyanuric acid. The product is in form of round white tablets with a characteristic odor of chlorine. The active ingredient is active chlorine, which is formed in water when the tablets are dissolved. The recommended concentration of active substances for sterilization of working surfaces is 0.015% in the working solution, exposure time is at least 5 minutes.

(2) BFR Biocid Enzym (BFR Labs LLC, Russia). BFR Biocid Enzym contains the following active ingredients: didecyldimethylammonium chloride (6.0%), NN-bis(3-aminopropyl) dodecylamine (3.0%), benzalkonium chloride (8.0%), as well as Enzumix multiple enzyme preparation containing a mixture of carbohydrases 3% to 5%, anticorrosion additives, technological and functional components. The recommended concentration of active substances for sterilization of working surfaces is 0.5% in the working solution, exposure time is at least 5 minutes.

(3) Peracetic acid as part of P3-Oxonia Active 150 product (Ecolab Production France SAS, France). Composition of the disinfectant: peracetic acid — PAA (15.5% to 17.0%), hydrogen peroxide (15.8% to 18.0%), acetic acid, functional additives. The recommended concentration of active substances for sterilization of working surfaces is 0.05% in the working solution, exposure time is at least 5 minutes.

Substances with different mechanisms of action were selected as *adjuvants* enhancing the biocidal effect of the main disinfectant:

— Hydrogen peroxide (6%) (Lega LLC, Russia) is a strong oxidizing agent; when interacting with catalase-positive microorganisms it forms gaseous oxygen.

— Isopropanol (30%) (Chemical line, Russia) has a protein-denaturing and coagulating effect on polymer solutions.

All these substances are approved for use in food production as disinfectants.

Preparation of microorganisms

The selection of strains for the formation of binary biofilms was based on the similarity of growth dynamics. Growth dynamics were assessed and reproduced on a CLARIO star device (BMG Labtech, Germany) for 36 hours (Figure 1).

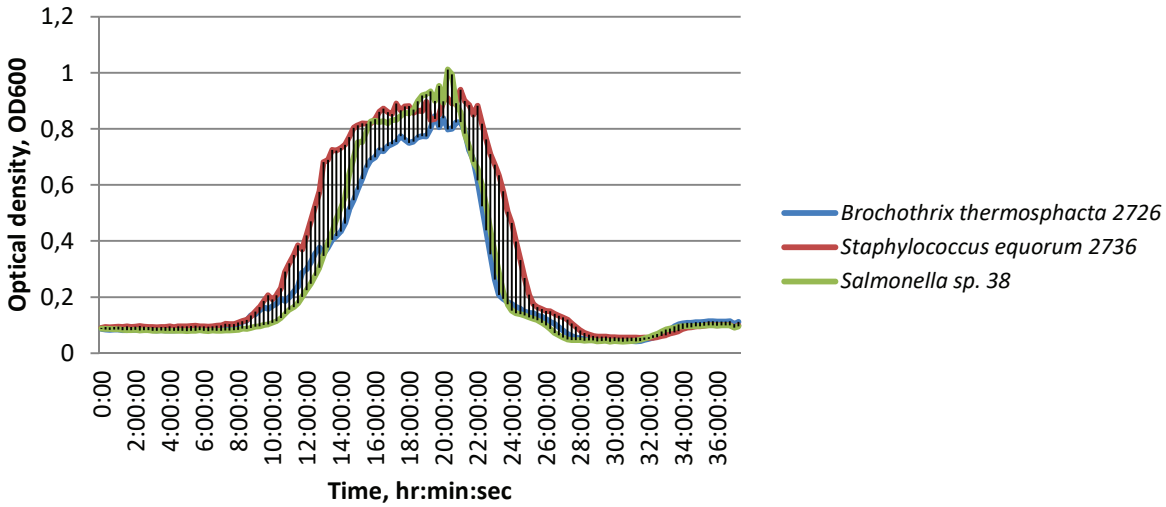

Figure 1. Growth dynamics for planktonic cultures of the studied microorganisms

Inoculation on a solid medium revealed the absence of an antagonistic effect on bacterial growth in binary cultures.

Effects of disinfectants on planktonic bacterial cultures

To assess the effectiveness of disinfectants on planktonic cultures of *Brochothrix thermosphacta* 2726, *Staphylococcus equorum* 2736, *Salmonella* sp. 38 using the suspension method in laboratory environment, the technique described in Guideline R4.2.3676–20 was used [29]. Working concentrations of disinfectant were selected in accordance with the manufacturers' recommendations, a well as increased concentrations (P3-Oxonia Active 150: 1%, 0.5%, and 0.05%; Dimax Chlorine: 0.30%, 0.15%, and 0.015%; BFR Biocid Enzym: 0.5%, 5.0%, and 10%).

Obtaining models of biofilms at the solid surface/air interface

Biofilms of this type were obtained using easily dispersible fiberglass materials as substrates according to previously described method [30]. Fiberglass filters (Whatman GF/F) were cut into 15x15 mm squares and sterilized by autoclaving (for 20 minutes, at a temperature of 120 °C), then laid out on the surface of LB agar medium in plates.

Bacterial cultures were grown separately in LB broth until the stationary growth phase. Turbidity was reproduced in pure LB0.5 broth according to McFarland method using DEN-1B Densitometer (Biosan, Latvia). To form binary biofilms, *Brochothrix thermosphacta 2726/Salmonella* sp. 38 and *Staphylococcus equorum* 2736/*Salmonella* sp. 38 cultures were mixed 1:1 in separate tubes. Next, the resulting bacterial suspensions were applied in amount of 40 µl onto previously prepared sterile fiberglass filters in sterile plates with PCA agar medium (bioMérieux, France) in duplicate. Then they were grown in a thermostat for nine days at a temperature of 30 °C.

Effects of disinfectants on biofilms

On days 2 and 9 of biofilm growth, they were treated with disinfectants with and without adjuvants. Disinfectant solutions in sterile water were prepared immediately before application to the filters. Biofilms were removed from the surface of the growth medium, transferred to sterile plates, and disinfectant solutions in amount of 100 µl were applied to each plate until the filter was completely wetted. The disinfectant exposure time was 10 minutes.

Then the fiberglass filter was placed in a Falcon tubes with sterile saline solution (10 µl). A sterile glass mortar and beads were used to homogenize the fiberglass filter. The resulting contents of the Falcon tubes were considered as the first dilution. Aliquots of the resulting homogenates (100 µl) were diluted in 900 µl of sterile saline solution and a series of decimal dilutions was prepared. In each dilution, the number of viable cells (CFU/ml) was determined by the cultural method and then the CFU titer in the primary filter homogenate was calculated.

The plates were incubated in a thermostat at 30 °C for 24 hours followed by counting the colonies on the plates. Experiments were performed in two independent biological replicates.

Statistical analysis

All studies were carried out in duplicate; each replication included two parallel experiments. When calculating CFU titers, mean values and experimental errors were determined using the average deviation of experimental values from the mean function of 5–7 independent samples using Microsoft Office Excel 2010. Differences between values were considered significant if they exceeded the level of experimental error (typically 20% or less) according to Student's t-test for $p = 0.05$. In the figures, data from typical experiments are presented as means ± experimental errors.

Results and discussion

Effects of disinfectants on planktonic bacterial cultures

In order to correct the concentrations of disinfectants recommended by the manufacturer, at the first stage of work, their biocidal effect was tested on planktonic bacterial cultures. Microorganisms in the planktonic state were not resistant to the recommended concentrations of the disinfectants. Antimicrobial activity suppressed the

viability of microorganisms, reducing growth by 7 orders of magnitude (Table 1).

Table 1. Cell viability of planktonic cultures (lg CFU/ml) after exposure (10 minutes) to disinfectants in concentrations recommended by the manufacturer

Planktonic cultures	Disinfectants			
	Control (no treatment)	Dimax Chlorine (0.015%)	P3-Oxonia Active 150 (0.05%)	BFR Biocid Enzym (0.5%)
	Cell count, lg CFU/ml			
Brochothrix thermosphacta 2726	9.04 ± 0.08	< 2	< 2	< 2
Staphylococcus equorum 2736	9.53 ± 0.10	< 2	< 2	< 2
Salmonella sp. 38	9.45 ± 0.06	< 2	< 2	< 2

A change in antimicrobial effect occurs when microorganisms form mono and binary biofilms, thereby increasing their resistance to disinfectants. Results for the antimicrobial effect of different concentrations of disinfectant working solution on binary biofilm of *Brochothrix thermosphacta* 2726/*Salmonella* sp. 38 are presented in Figure 2.

BFR Biocid Enzym at a concentration of 0.5% recommended by the manufacturer did not have disinfectant activity at either 2-day-old or 9-day-old biofilm. When the concentration of the solution was increased by 10 times, the antimicrobial effect was observed only on 9-day-old biofilm, where there was a decrease by log 5.44 compared to the control. The greatest antimicrobial effect was observed when the concentration of BFR Biocid Enzym was increased by 20 times. However, differences in the effects of the disinfectant depending on biofilm age were not observed in this case. A similar pattern was observed when exposed to P3-Oxonia Active 150 and Dimax Chlorine, where the greatest antimicrobial effect was noted for a concentration increased by 20 times. When exposed to the above-mentioned agents in the studied concentrations, a complete antimicrobial effect was not detected. At the same time, a 20-fold increase in concentrations did not show significant differences in the antimicrobial effect depending on the age of the biofilm.

Results for resistance of binary biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 to disinfectants are presented in Figure 3.

The concentrations recommended by the manufacturer for disinfection (BFR Biocid Enzym, 0.5%; P3-Oxonia Active 150, 0.05%; and Dimax Chlorine, 0.015%) had no antimicrobial effect on the biofilms studied. The concentration of P3-Oxonia Active 150 increased by 10 times (0.5%) had a greater antimicrobial effect against 9-day-old biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 compared to 2-day-old biofilm. Increasing the concentration by 20 times did not lead to a complete antimicrobial effect, but reduced the number of CFU by $\log_{10}$ 6.64 compared to the control.

Dimax Chlorine with 10-fold increased concentration (0.15%) showed a decrease in CFU for 2-day-old and in 9-day-old biofilms of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 by $\log_{10}$ 3.08 and $\log_{10}$ 6.64 compared to the control respectively. Exposure to a concentration increased by 20 times contributed to a decrease in CFU for 2-day-old and in 9-day-old biofilms by up to $\log_{10}$ 6.64 compared to the control.

In order not to go beyond the recommended concentrations of antimicrobial agents, but at the same time, to maintaining their effectiveness, a technique was used to increase the activity of the active substance due to the synergistic effect of an additional compound, an adjuvant [30,31].

Results for sensitivity of binary biofilms to enzymatic disinfectant based on QAC (BFR Biocid Enzym) in combination with adjuvants (isopropyl alcohol and peroxide) are presented in Figures 4 and 5.

The concentration recommended by the manufacturer for BFR Biocid Enzym of 0.5% was enhanced by hydrogen peroxide adjuvant at a concentration of 6%, but the combination had no antimicrobial effect on either 2-day-old or 9-day-old biofilms and did not significantly reduce CFU.

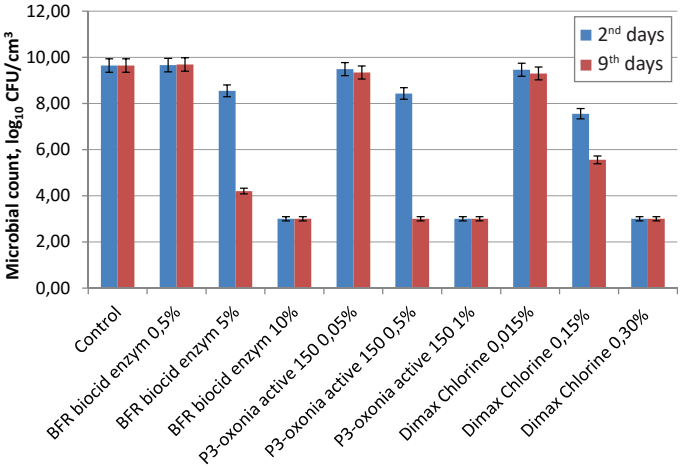

Figure 2. Sensitivity of binary biofilms of *Brochothrix thermosphacta 2726/Salmonella* sp. 38 of different ages to different concentrations of disinfectants

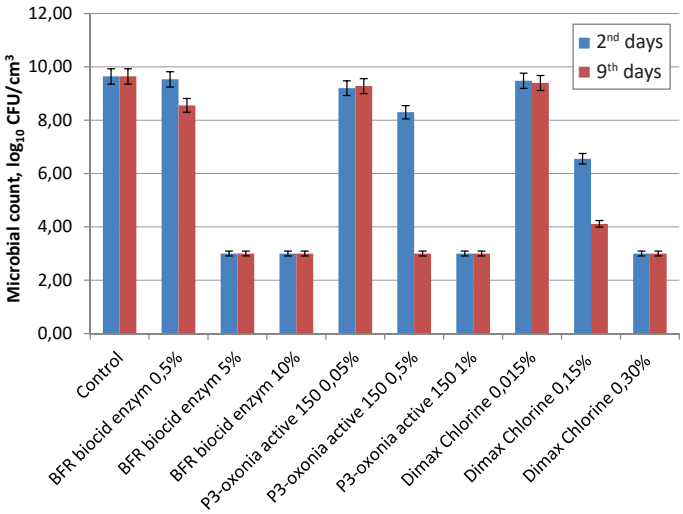

Figure 3. Sensitivity of binary biofilms of *Staphylococcus equorum 2736/Salmonella* sp. 38 of different ages to different concentrations of disinfectants

The addition of isopropyl alcohol adjuvant in varying concentrations had an antimicrobial effect depending on the concentration of isopropyl alcohol. Adding isopropyl alcohol 10% to the disinfectant did not have a significant antimicrobial effect on 2-day-old biofilm, but reduced CFU in 9-day-old biofilm by $\log_{10}$ 1.98 compared to the control. The combination of isopropyl alcohol 20% and BFR Biocid Enzym 0.5% showed a similar effect and reduced CFU in 9-day-old biofilm by $\log_{10}$ 1.9 compared to the control. A decrease in CFU of 2-day-old and 9-day-old biofilms by $\log_{10}$ 6.64 compared to the control was noted with the addition of isopropyl alcohol adjuvant at a concentration of 30%. At the same time, the combination of BFR Biocid Enzym with isopropyl alcohol 30% had the same effect on both 2-day-old and 9-day-old biofilms.

Results for sensitivity of binary biofilms of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 of different ages to enzymatic disinfectant based on QAC with different adjuvant concentrations are presented in Figure 5.

Hydrogen peroxide adjuvant at a concentration of 6%, added to the working concentration of enzymatic disinfectant based on QAC, did not have a strong antimicrobial effect on binary biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38. The addition of isopropyl alcohol at concentrations of 10% and 20% led to a decrease in CFU only in older 9-day-old biofilm by $\log_{10}$ 2.01 and $\log_{10}$ 1.89 compared to the control respectively. Exposure to a working concentration of disinfectant with the addition of isopropyl alcohol 30% contributed to CFU decrease in 2-day-old biofilm by $\log_{10}$ 6.64, and in 9-day-old biofilm by $\log_{10}$ 6.04 compared to the control.

The results for the antimicrobial effect of disinfectant based on peracetic acid in combination with various adjuvants on binary biofilms are presented in Figures 6 and 7.

Disinfectant based on PAA with a working concentration of 0.05% did not have a pronounced biocidal effect on either 2-day-old or 9-day-old biofilms; a decrease in CFU was only by $\log_{10}$ 0.15 and $\log_{10}$ 0.3 respectively. Addition of isopropyl alcohol adjuvant at concentrations of 10% and 20% did not reduce CFU in 2-day-old biofilm of *Brochothrix thermosphacta* 2726/*Salmonella* sp. 38 compared to the control, but reduced cell count in 9-day-old binary biofilm by $\log_{10}$ 1.34 and $\log_{10}$ 0.74 compared to the control respectively. The addition of isopropyl alcohol 30% as an adjuvant had no complete antimicrobial effect on 2-day-old biofilm, but contributed to CFU decrease in 9-day-old biofilm by $\log_{10}$ 4.64.

Results for sensitivity of binary biofilms of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 of different ages to disinfectant based on PAA with different adjuvant concentrations are presented in Figure 7.

The effect of disinfectant based on PAA with a working concentration of 0.05% on biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 had no significant antimicrobial effect; there was a decrease in the number of microorganisms by only $\log_{10}$ 0.44 and $\log_{10}$ 0.36 in 2-day-old

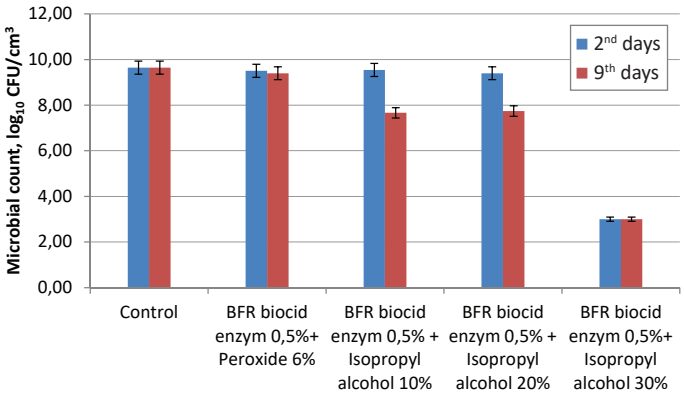

Figure 4. Sensitivity of binary biofilms of *Brochothrix thermosphacta 2726/Salmonella* sp. 38 of different ages to enzymatic disinfectant based on QAC with different adjuvant concentrations

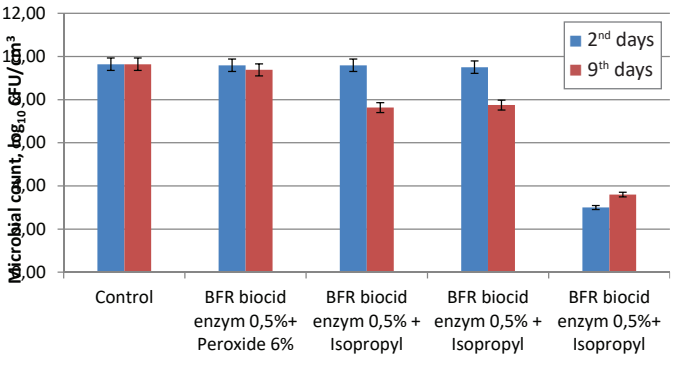

Figure 5. Sensitivity of binary biofilms of *Staphylococcus equorum 2736/Salmonella* sp. 38 of different ages to enzymatic disinfectant with different adjuvant concentrations

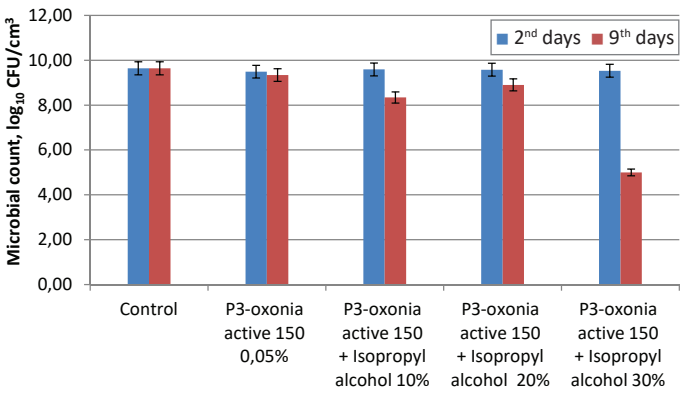

Figure 6. Sensitivity of binary biofilms of *Brochothrix thermosphacta/Salmonella* of different ages to disinfectant based on PAA with different adjuvant concentrations

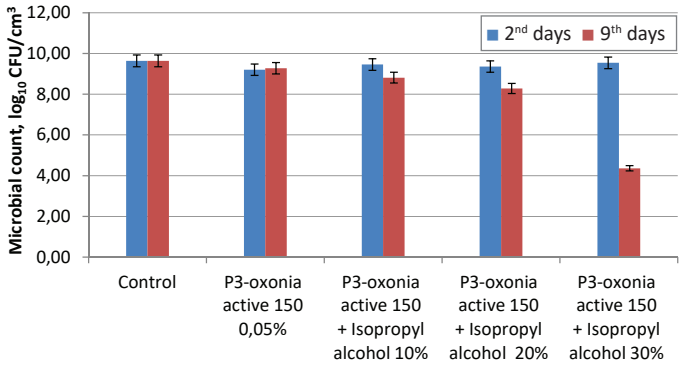

Figure 7. Sensitivity of binary biofilms of *Staphylococcus equorum 2736/Salmonella* sp. 38 of different ages to disinfectant with different adjuvant concentrations

and 9-day-old biofilms respectively. The combination of exposure to the recommended concentration of disinfectant based on PAA with an adjuvant in the form of isopropyl alcohol at concentrations of 10%, 20% and 30% slightly reduced CFU in 2-day-old biofilm by $\log_{10}$ 0.1 to $\log_{10}$ 0.28, and in 9-day-old biofilm by $\log_{10}$ 0.83 to $\log_{10}$ 1.36. The addition of isopropyl alcohol 30% as an adjuvant had an antimicrobial effect exclusively on 9-day-old biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 with a decrease in viable cell count by $\log_{10}$ 5.28 compared to the control.

The results for the effect of chlorine-based disinfectant, Dimax Chlorine, with adjuvants on binary biofilms are presented in Figures 8 and 9.

Exposure to disinfectant based on active chlorine with a working concentration of 0.015% had no antimicrobial effect on binary biofilm of *Brochothrix thermosphacta* 2726/*Salmonella* sp. 38. The combined effect of isopropyl alcohol adjuvant at concentrations of 10% and 20% did not significantly reduce CFU in biofilm of *Brochothrix thermosphacta* 2726/*Salmonella* sp. 38 of two ages studied. Addition of isopropyl alcohol 30% to the concentration of active chlorine disinfectant recommended by the manufacturer led to a significant cell count reduction by $\log_{10}$ 5.72 exclusively in 9-day-old biofilm.

Results for sensitivity of binary biofilms of *Staphylococcus equorum* 2736/*Salmonella* sp. 38 of different ages to chlorine-based disinfectant with different adjuvant concentrations are presented in Figure 9.

The working concentration of disinfectant based on active chlorine recommended by the manufacturer had no antimicrobial effect on binary biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38. The combined effect of disinfectant and isopropyl alcohol adjuvant at concentrations of 10%, 20% and 30% showed no antimicrobial effect on 2-day-old binary biofilm of *Staphylococcus equorum* 2736/*Salmonella* sp. 38. However, a clear decrease in CFU was observed in 9-day-old biofilm when exposed to disinfectant with the addition of isopropyl alcohol 30%, where the decrease in cell count was by $\log_{10}$ 6.64 compared to the control, while with the addition of 10% and 20%, cell count decreased by $\log_{10}$ 0.76 and $\log_{10}$ 1.26 respectively.

The main theoretically important and practically focused result of the research is the expansion of our knowledge about the biofilm development and methods for their effective control. The work includes a comparative analysis of binary biofilm resistance to disinfecting agents depending on the biofilm age and the type of disinfectants used individually or together with agents that enhance the antimicrobial effect (adjuvants).

The results obtained on the pronounced synergistic effect of adjuvants and traditional disinfectants significantly complement the knowledge about methods for combating biofilms. To control biofilms, several dozens of special substances with different types of action on biofilms have been proposed (inhibiting the synthesis or destroying matrix components and cellular structures of the biofilm phenotype, hydrolases disrupting signal transmission, inhibitors of cellular metabolism, etc.) [32,33]. A possible problem for the practical use of new substances and approaches is the lack of data on their use in real production environment or in disease treatment, as well as the lack of safety reports and approvals.

An important approach to control biofilms is the creation of complex disinfectants from those known and used, which, in our opinion, is more effective, because it relies on the use of substances with known mechanisms of action that are already approved for practical use. Many examples of such combinations are known [34,35,36]. Disinfectants with physical [37,38] and biological factors [39] have been successfully used. The effect of antimicrobial agents on biofilms has been demonstrated to be enhanced in the presence of ultrasound [37], rotating magnetic field [38], or antagonistic bacteria (*Pseudomonas aeruginosa*) [39].

In addition to obvious effectiveness of the simultaneous use of two or more antimicrobial factors [40], there is an approach that includes the use of additional effects, not necessarily biocidal, but enhancing the effectiveness of the biocide used. Such additional substances are called adju-

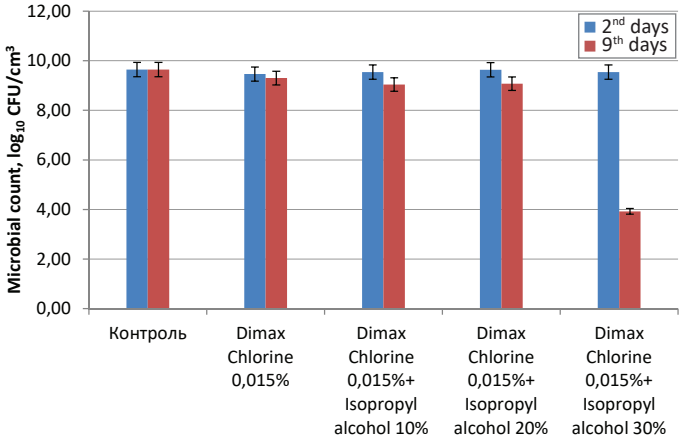

Figure 8. Sensitivity of binary biofilms of *Brochothrix thermosphacta 2726/Salmonella 38* to disinfectant with different adjuvant concentrations

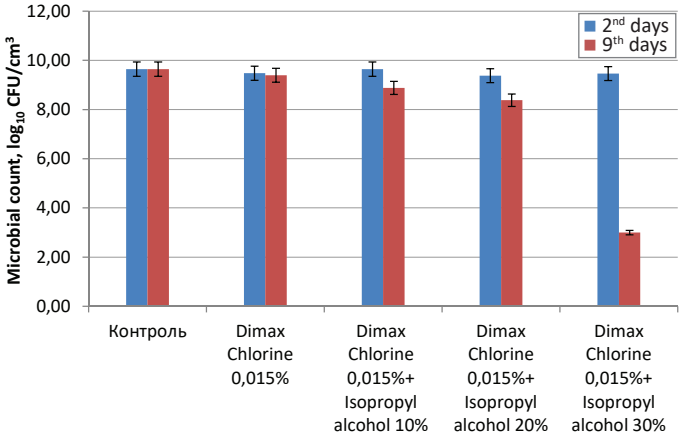

Figure 9. Sensitivity of binary biofilms of *Staphylococcus equorum 2736/Salmonella* sp. 38 to disinfectant with different adjuvant concentrations

vants (enhancers). This term was originally used in medicine for enhancers of the immune response [41]. Some substances that enhance the effect of a disinfectant on biofilms are also adjuvants (hydrolases).

In [42], the authors showed a sterilizing effect on binary biofilms of non-pathogenic bacteria when exposed to a disinfectant with the addition of adjuvants. The biocidal activity of BFR Biocid Enzym was increased to the greatest extent by adjuvants that additionally disrupt the structure of the matrix, i. e. H_2O_2 (due to rupture of the matrix by the resulting oxygen) and alcohols, which cause coagulation of matrix biopolymers [42]. Whereas in this work, on binary biofilms of pathogenic and opportunistic bacteria, it was shown that the most effective enhancement of BFR Biocid Enzym and Dimax Chlorine based on active chlorine occurred when they were combined with isopropyl alcohol (30%) disrupting the structure of the matrix, as it was noted above.

Resistance of microorganisms to disinfectants in multispecies biofilms is also related to biofilm age. Mature biofilms are generally more resistant to stress conditions than newly formed biofilms due to the robust three-dimensional structure of bacterial cell layers forming a physical barrier that restricts and prevents the penetration of disinfectants or other chemicals [43]. In our study, 9-day-old binary biofilms demonstrated greater sensitivity to the studied antimicrobial compositions than 2-day-old ones. This may be due to the fact that by the 9th day of cultivation, the biofilms were already at the stage of disintegration and release of free microorganisms.

Conclusion

Biocidal concentrations of disinfectants used in production environment (Dimax Chlorine, PAA and BFR Biocid Enzym) were established in relation to binary biofilms of pathogenic and opportunistic strains formed on fiberglass carriers. For all products, the concentrations recommended by the manufacturer had no disinfectant effect against the studied biofilm cultures. An increase in concentration by 20 times (with the same exposure time of 10 minutes) had a complete biocidal effect (6 orders of magnitude) on the studied binary biofilms. Chlorine-containing disinfectant at recommended concentrations and in combination with isopropyl alcohol was less effective than QAC-containing disinfectant (BFR Biocid Enzym). It may be assumed that agents whose action is based on oxidative reactions (Dimax Chlorine, P3-Oxonia Active 150) form multiple concentration resistance of biofilm strains due to constant surface treatment in production environment. Even with the addition of a non-oxidizing agent (isopropyl alcohol), a synergistic disinfectant effect was not established. We observed a biocidal effect when combining Dimax Chlorine and isopropyl alcohol 30% only in old biofilms. Whereas, when biofilms were exposed to QAC-containing biocide (BFR Biocid Enzym) with the addition of protein coagulating agent (isopropyl alcohol 30%), a decrease in the number of viable cells by 6 orders of magnitude was observed. At the same time, the combination of BFR Biocid Enzym with oxidizing agent (peroxide 6%) had no biocidal effect.

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