Evaluation of new antimicrobial products based on potassium monopersulfate for disinfection of poultry farms

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Abstract

Bird drinkers used on poultry farms are highly susceptible to the formation of bacterial biofilms which can cause several diseases for animals and humans. This study evaluated the antimicrobial activity of new potassium monopersulfate-based products against bacterial biofilms. Four monopersulfate-based products named as P.A, P.B, P.C, and P.D. developed by the Hidrodomi do Brasil industry were evaluated. The products were tested against avian pathogenic *Escherichia coli*, *Salmonella enteritidis*, and *Listeria monocytogenes* mature biofilms. To verify the antimicrobial activity of the products, tests were initially performed against planktonic bacterial cells to determine their minimum inhibitory and bactericidal concentration. In the next step, the antimicrobial activity of the products was further evaluated in biofilms grown on acrylic specimens by counting colony-forming units (CFU) and also by employing both scanning electron microscopy (SEM) and confocal fluorescence microscopy. All products showed effectiveness against the bacterial species tested. It has been observed that P.A with concentrations lower than 0.13% presented the most effective action against planktonic cells and biofilms showing efficiency in inhibiting bacterial growth. P.A was able to completely eliminate both *S. enteritidis* and *L. monocytogenes* mature biofilms. The evaluated products, notably P. A., showed potential to be used as sanitizers and disinfectants on poultry farms.

Introduction

In 2020, the world chicken meat market had a production of 100,413 thousand tons of chicken, of which 13,845 thousand tons were produced in the Brazilian market. Brazil is one of the largest producers of chicken meat, exporting 4,231 thousand tons in 2020 (ABPA, 2021). On poultry farms, chickens are placed in chicken beds where they sleep, eat, drink water and defecate, creating a favorable environment for contamination and infections of these birds (GEWEHR, 2003). Most of these infections are caused by bacteria that form biofilms on floor fodder (traditionally composed of straw, hay or wood shavings) and in drinking fountains.

The term biofilm is used to describe bacterial populations enclosed in a matrix, adherent to each other and/or to surfaces, and it may be formed by one or several species, reflecting complex communities of microcolonies separated by water-filled channels (JAY, 2005). Environments with greater water availability are conducive to greater biofilm formation, including bird drinkers on poultry farms (WEBSTER, TRAN, 2013; AZEREDO; OLIVEIRA, 2000).

Potassium peroxymonosulfate ($2KHSO_5.KHSO_4.K_2SO_4$), or also potassium monopersulfate, is a product widely used in a variety of industrial applications as an oxidizing agent. Monopersulfate alone is not an effective disinfectant as active chlorine compounds, but when activated catalytically, thermally or photolytically, the radicals formed are powerful oxidants with potent disinfecting action (ANIPSITAKIS et al., 2008).

Bacterial biofilms are among the main concerns in commercial poultry, as they can be sources of infections in birds and, consequently, in humans, and also generate economic losses to producers due to sanitary embargoes (STOODLEY et al., 2002). It is essential to remove these pathogens from the poultry farm environment so that there is no economic damage and risks to human health. Therefore, this study evaluates the antimicrobial activity of four products based on potassium peroxymonosulfate against biofilms of different bacterial species frequently associated with both human and avian infections.

Material And Methods

Reagents

The products based on potassium peroxymonosulfate was supplied by Centro de Pesquisa, Desenvolvimento e Inovação Hidrodomi do Brasil, from Ribeirão Preto - SP. The products evaluated were: P.A, which is a mixture of sodium dichloroisocyanurate and potassium monopersulfate; P.B, containing potassium monopersulfate, citric acid, sodium hexametaphosphate, sodium lauryl sulfate; P.C, composed of potassium monopersulfate, citric acid, sodium dichloroisocyanurate. Additionally, the product P.D, composed of sodium percarbonate, citric acid, sodium lauryl sulfate and tetra-acetyl ethylene diamine, was used to be compared with those with monopersulfate in terms of antimicrobial activity.

All products were prepared at a concentration of 0.05% (m/v), aiming to standardize the concentrations for comparative purposes in the different tests carried out. Sodium dichloroisocyanurate (D.C) was used as a control, prepared at a concentration of 8 ppm, mimicking chlorinated water which contains 5 ppm of active chlorine (PFUNTNER, 2011).

Bacterial strains

The antimicrobial potential of the products was evaluated against three bacterial strains: avian pathogenic *Escherichia coli* (APEC), *Salmonella enteritidis* and *Listeria monocytogenes*. The strains were cryopreserved at -80 °C in cryotubes containing BHI (Brain Heart Infusion) liquid culture medium (Oxoid, UK) with 15% glycerol.

Evaluation of the minimum inhibitory and bactericidal concentration

The microdilution method employing 96-well plates was used to evaluate the Minimum Inhibitory Concentration (MIC), according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2020). This protocol was adopted with adaptations by using resazurin to reveal bacterial growth, as described by Pitondo-Silva *et al*. (2016).

A bacterial inoculum suspension was prepared in sterilized saline solution from recent bacterial cultures, aiming to obtain a turbidity corresponding to the 0.5 McFarland scale ($1.5x10^8$ CFU mL^{-1}). From this initial suspension, 500 µL were pipetted and transferred to a test tube containing 4.5 mL of sterilized saline solution ($1,5x10^7$ CFU mL^{-1}).

In the 96-well round-bottom plate, the first column served as a negative control and 100 µL of Müeller-Hinton broth (Oxoid, UK) was added to verify the medium sterility. Column 12, on the other hand, served as a positive control, adding 90 µL of the same culture medium plus 10 µL of the inoculum, allowing for evaluating the bacterial growth viability. From the second to the eleventh column, two-fold serial dilutions were carried out with the solutions of the products. The initial concentration was 2% (m/v), covering a concentration range from 2% to 0.004% (m/v). Finally, 10 µL of the bacterial inoculum was added to all tested solutions reaching a final volume of 100 µL.

After an incubation time interval off 18 h at 37°C, the plates were inserted into the instrument to analyze the solutions. Two qualitative readings were visually performed. In the first one, without chromogenic indicators, observing only the turbidity or not of the culture medium. In the other one, reading was performed by applying 30 µL of resazurin after 3 h of incubation at 37°C. Resazurin has an original blue color due to the redox reaction, indicating the absence of bacterial growth and, in the reduced form, it has a pink or purple color, indicating bacterial growth.

All tests were performed in triplicate, which was compared, and the MIC was determined as the concentration equivalent to the well with the lowest concentration of the product that did not allow bacterial growth.

For the determination of the minimum bactericidal concentration (MBC), the wells that did not show visible bacterial growth and also the wells of the negative and positive controls were selected. A volume of 10 µL of each one was transferred to Petri dishes containing Müller Hinton Agar (Oxoid, UK). The plates were incubated for 24 h at 37°C and afterwards, a visual evaluation was performed to determine the MBC by verifying the presence or absence of bacterial colonies. The MBC was considered the lowest concentration in which there was no bacterial growth.

Formation of bacterial biofilms on acrylic specimens

Biofilms were formed on 1 cm^2 acrylic specimens previously sterilized. The strains *E. coli* APEC, *S. enteritidis* and *L. monocytogenes* were previously reactivated in BHI broth, incubated at 37°C for 24 h, and subsequently inoculated onto plates with BHI Agar under the same conditions. With the bacteria grown, 1 mL of inoculum corresponding to 1 McFarland scale ($3x10^8$ CFU mL^{-1}) in BHI broth was transferred to sterilized 5 mL tubes containing the acrylic specimens. The tubes were incubated for 21 consecutive days at 37 °C with daily changing of inoculum, aiming to obtain dense and mature biofilms.

The formation of biofilms on the surface of the specimens was performed in octuplicate, allowing the statistical analysis of the results considering the action of each product against the different bacterial species tested. Negative control was also performed in which no bacteria were added.

Evaluation of products in biofilms

The evaluation of cell viability after microbial adhesion on the surface of the specimens was performed by quantifying the CFU. After 21 days of incubation, the specimens containing the adhered biofilms were washed with 2 mL of phosphate buffer solution (PBS) to remove non-adhered planktonic cells. Subsequently, the biofilms were exposed to the action of the products by adding 2 mL of P.A, P.B, P.C and P.D at a concentration of 0.05% m/v and also 2 mL of D.C at 8 ppm. The solutions containing the products were kept in contact with the specimens for five minutes. For the positive control, 2 mL of PBS was added to the specimen, mimicking the protocol performed with the products. For microscopic analyses, an additional specimen was prepared for each product tested, including controls, from which biofilms were not collected.

After five minutes of action, the products were removed and the specimens were placed in clean 5 mL tubes containing 2 mL of PBS for the collection of biofilms. The tubes containing the specimens were vortexed (Leucotron, Brazil) under maximum speed for 30 s. Then, they were placed in an ultrasonic vat (Kondortech, Brazil) for 480 s. In the next step, the collected aliquots of the detached biofilms were submitted to six serial dilutions e to determine the number of CFU/mL of each bacterial strain. After serial dilution, 100 µL of each aliquot was inoculated by spreading method on BHI Agar plates and incubated under the same conditions previously described.

After the incubation period, the images of the plates have been documented and the CFUs were counted using the ImageJ program (Schneider, 2012). Plates with visible colonies were selected from the serial dilutions obtained from the collected biofilms. These colonies were counted and the values of the CFU/mL were converted to a logarithmic scale for better statistical analysis of the results.

Morphological analysis of biofilms

The morphological analysis of the biofilms adhered to the acrylic specimens was performed by scanning electron microscopy (SEM). After treatment with the products, the specimens selected were fixed with 2.5% glutaraldehyde for 24 hours at 4 °C. Then, they were washed with PBS to remove the glutaraldehyde solution excess as well as

planktonic cells deposited on the surface of the specimens. The samples were sequentially dehydrated in ethanol solutions with ascending concentrations (30%, 50%, 70%, 80%, 85% and 100%) for five minutes in each one. After dehydration, the samples were placed in a desiccator for 72 h (HUANG *et al*., 2017; LI *et al*., 2018).

To evaluated bacterial adhesion, the acrylic specimens from each experimental group were placed on an aluminum plate, fixed with double-sided carbon adhesive tape and metalized with gold-palladium alloy (Balt-Tec SCD-050, Florida, USA) for 120 s. After metallization, the samples were analyzed using SEM (JSM-6610LV Scanning Electron Microscope, JEOL, Tokyo, Japan). The surfaces were observed in a secondary electron regime at a voltage of 10-15 kV with the magnification of 500x, 2000x and 5000x to generate the photomicrographs. Each sample was observed at five different equidistant points.

Confocal fluorescence microscopy

Confocal fluorescence microscopy (Leica SP5 Confocal Microscope, Wetzlar, Germany) analysis was performed to verify the live and dead bacteria adhered to the specimens after the action of the products and also the ability of the products to remove biofilms. After treatment with the products, the specimens were stained with the bacterial viability kit (Live/Dead Bac Light Bacterial Viability and Counting Kit, Molecular Probes, Eugene, OR, USA) for 15 min, following the protocol described by JOSHI, *et al*. (2010). The kit features fluorescence markers to detect live and dead bacteria, with the SYTO 9 reagent staining viable cells in green (penetrating cells with unaltered membranes) and isopropidium iodide staining dead cells in red (penetrating cells with harmed membranes).

Statistical analysis

After obtaining all the results, statistical analyzes were performed to compare the action of the products against the bacterial species tested. Firstly, the Kolmogorov-Smirnov test was performed to verify if the samples had a normal distribution. Then, the Levene test was applied to verify if the variants were homogenous. After confirming the normality and homogeneity of the data, the single-factor analysis of variance test (ANOVA) was applied, comparing whether groups showed statistically significant differences. In addition to the ANOVA test, the Tukey-Kramer post-test was also performed to compare all groups, showing which ones presented statistical differences. The analyzes were performed using the software Graph Pad Prism, version 5 and Microsoft Excel 2016.

Results And Discussion

Evaluation of the MIC and MBC

The evaluation of MIC and MBC was initially carried out to define the lowest effective concentrations of the products studied against the bacterial species in the planktonic form. Subsequently, these results were used to establish the concentrations to be tested against the same bacterial species in the form of mature biofilms adhered to acrylic specimens.

The product control D.C lead to bacterial inhibition at concentrations lower than 0.03% (Table 1). These results showed that the product was more efficient than the others to inhibit bacterial growth against the three species studied. These results corroborate the data presented by Zabot (2016), which demonstrated that sodium dichloroisocyanurate has an effective inhibitory activity for bacterial species, especially *S. enteritidis* at a concentration of 60 ppm. Among the products tested, P.A obtained the best results in the MIC test, at a concentration of 0.03% m/v for APEC and *S. enteritidis* and 0.13% m/v for *L. monocytogenes* (Table 1). P.A is a mixture of sodium dichloroisocyanurate with potassium monopersulfate. Dichloroisocyanurate has a stable chemical structure that

slowly reacts with organic matter present in water. This reaction leads to a slower release of hypochlorous acid, which penetrates the bacterial cell destroying it, and increasing the effectiveness of the disinfection process. This compound is highly water-soluble, it does not significantly change the pH of its solution and presents a very low contents of insoluble solids (ZABOT, 2016). Potassium monopersulfate is a salt that has broad-spectrum antibacterial and antiviral properties, it is active even in the presence of organic matter, does not cause corrosion in metals and its oxidizing properties compromise the main physical and chemical components of microorganisms (SILVA; MAYRINK; LISARDO, 2021).

The antimicrobial activity of sodium dichloroisocyanurate associated with potassium monopersulfate was previously described by Almeida (2020) who demonstrated the bactericidal concentration against *Enterococcus faecalis* was 75% lower when compared sodium dichloroisocyanurate and 50% lower when compared to potassium monopersulfate alone. Such results indicate the synergism of the association of these salts, enhancing their antimicrobial action when it is considered the effect individually obtained.

P.B exhibited better results than P.C for APEC, *S. enteritidis* and *L. monocytogenes* (Table 1). Both products are based on monopersulfate. The difference between them is related to their chemical composition. P.B contains citric acid, and sodium hexametaphosphate and sodium lauryl sulfate, while P.C has potassium monopersulfate, citric acid, sodium dichloroisocyanurate in its formulation. Citric acid is water-soluble and the acid itself and its formed salts have the antimicrobial activity to control pathogens in fresh and processed chicken meat, but its use is potentially limited by the requirement to keep the pH low for optimal antimicrobial activity (ZABOT, 2016). Sodium hexametaphosphate is a cyclophosphate that has bactericidal and bacteriostatic properties (MAGALHÃES, 2019). Sodium lauryl sulfate is a surfactant, and also it has antimicrobial activity and can inhibit bacterial enzymes associated with fluoride release (JARDIM JÚNIOR *et al.*, 1998).

P.D was used as a comparative product, as it has some of the components the tested products have, but with no monopersulfate. This product presented a less effective antimicrobial activity, which has been demonstrated by its values of MIC with a concentration of 0.25% m/v for APEC and *L. monocytogenes*, and 0.13% m/v for *S. enteritidis*. These values are low when compared to the values reached by the others. P.D contains sodium percarbonate, citric acid, sodium lauryl sulfate and tetraacetyl ethylene diamine (TAED). Sodium percarbonate, when dissolved in an aqueous medium, forms carbonate ions (CO_3^{2-}) and hydrogen peroxide (H_2O_2). The release of these compounds exerts a mechanical cleaning, in addition to having an antimicrobial action (SILVA, 2021; SESMA; MORIMOTO, 2011). On the other hand, TEAD is an oxygen release potentiator that acts as a catalyst in the release of active oxygen when combined with oxidizing compounds, such as sodium percarbonate, which has activity against biofilms and contaminants in water (MONTEIRO, 2018).

MBC values demonstrated that the tested products have similar efficiencies, with concentrations ranging from 0.25% m/v to 0.5% m/v (Table 1).

All tested products, as well as the comparative product, are innovative and present formulations that are not reported in the literature. Therefore, comparative analyzes are restricted to products already described in previous studies.

Table 1

Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) results obtained for the different products against the bacterial species tested.

Bacterial species	Products									
	P.A		P.B		P.C		P.D		D.C	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Escherichia coli APEC	0.03%	0.50%	0.06%	0.50%	0.13%	0.50%	0.25%	0.25%	0.02%	0.50%
Listeria monocytogenes	0.13%	0.25%	0.13%	0.25%	0.13%	0.25%	0.025%	0.25%	0.03%	0.50%
Salmonella enteretidis	0.03%	0.50%	0.06%	0.50%	0.25%	0.50%	0.13%	0.25%	0.02%	0.25%

Evaluation of products in biofilms

Biofilms show more tolerance to antimicrobial agents than do planktonic cells. In this sense, it has been postulated that the antibiotic concentrations required to inhibit or kill bacteria in biofilms may be from 100-fold to 1000-fold greater than those required to inhibit or kill planktonically grown strains (SEDLACEK and WALKER, 2007; AIRES et al., 2017). Based on this theory, we initially tested the products at concentrations of 5%, 1% and 0.1%. However, all these concentrations completely eliminated bacterial biofilms for all species. The lowest concentration that allowed the visualization of biofilms and counting of CFUs was 0.005% for all products. Therefore, this was the standardized concentration for biofilm tests.

It is important to carry out tests on mature biofilms to mimic what happens in the water troughs of poultry farms. It is common for bacterial bile to form on the inside of the polyvinyl chloride (PVC) tubes that distribute water to the birds, which can cause infections throughout the farm (GEWEHR, 2003). Initially, we tried to standardize the specimens for the formation of biofilms in PVC material. However, the autoclave sterilization process deformed the specimens, interfering with the standardization of sizes for statistical validation. Therefore, the tests were performed on polyethylene specimens that withstand the sterilization process without deforming.

At a concentration of 0.005% for 5 min., the specimens exposed to the products presented plaques with countable CFU numbers for all bacterial species. P.A, in its turn, was the only product that presented a count of 0 CFU for *S. enterica* and *L. monocytogenes* while for APEC, the mean was 2.52 CFU/mL. As expected, the plates of the positive controls, which were exposed to PBS, showed uncountable CFUs (Table 2).

Table 2

Counts (log_{10}) of the average colony forming units (CFU) obtained for the three bacterial species after exposure to the tested products.

Bacterial species	Products				
	P.A	P.B	P.C	P.D	D.C
Escherichia coli APEC	2.52	7.58	5.36	5.14	6.02
Salmonella enteritidis	0	5.53	5.68	6.16	6.04
Listeria monocytogenes	0	3.93	4.59	5.12	5.08

Analysis of microbial morphology and adhesion

SEM analyzes were used to verify the action of the tested products on biofilms. Furthermore, with the positive controls, it was possible to evaluate the adhesion and morphology of biofilms in the specimens. All bacterial species evaluated were able to form biofilms on the specimens. Therefore, it was possible to evaluate the action of the products on the biofilm structures (Figure 1). It was also possible to observe the product P.A. was the most effective to disrupt the structure of biofilms considering all three bacterial species studied, including the comparative product D.C. (Figure 1).

The fluorescence microscopy results including the live/dead assays demonstrated the presence of dead bacteria (red) over the live ones (green) From the data obtained in the assay, it was possible to observe that the treatments with the products had a lower incidence of live bacteria in comparison to the positive controls and also to D.C.

The methodology is useful to show whether the biofilms adhered to the specimens are viable or not, *i. e.,* capable of causing infection in chickens or not. It is not possible to get these data from SEM, as the biofilm may have remained adhered to the specimen suffering the product action but with no removal.

Data provided by using a live/dead kit allowed us to observe that all products were able to kill bacterial cells adhered to the specimens. However, the P.A. presented greater effectiveness in disrupting biofilms when compared to the other products, the positive control and D.CAs can be seen in Figure 1, proportionally and comparatively visualized, there are more dead cells than live cells on the specimens (Figure 2).

Statistical analysis

According to the Kolmogorov-Smirnov test, all groups presented values with normal distribution, which means that the values are symmetrical about the mean. With the Levene test, it was found that the groups were homogeneous among themselves, so it was possible to proceed with the ANOVA.

The ANOVA test is based on the *p*-value, it indicates the error probability when stating that the samples are not different, therefore, the smaller the p-value, the greater certainty of the difference among compared data. The highest *p*-value accepted under the conditions that the tests were performed was 0.05. Above this value, the samples no longer present statistical confirmation of their difference. The test was performed for each group of a bacterial species, with exposition to the products. According to the results, for APEC, a *p*-value equal to $1.7x10^{-33}$ was obtained, for *S. enteritidis* equal to $2.02x10^{-37}$ and for the *L. monocytogenes,* the value was $1.42x10^{-39}$.

After ANOVA analysis, the Tukey-Kramer post-test was performed, comparing two groups to each other to verify whether or not they could have a statistical difference. Firstly, the *p* value was sought in the comparison among the groups of products for a specific bacterium (Table 3).

Table 3

Tukey-Kramer post-test comparing the action of the products among themselves, for the bacterial strains tested

p value				
Group 1	**Group 2**	**APEC**	***S. enteritidis***	***L. monocytogenes***
P.B	P.A	1.77636×10^{-14}	1.77636×10^{-14}	1.77636×10^{-14}
P.B	P.D	1.77636×10^{-14}	2.91401×10^{-06}	1.77636×10^{-14}
P.B	P.C	1.77636×10^{-14}	0.269625497	5.17364×10^{-09}
P.B	D.C	1.82077×10^{-14}	0.000107857	1.83187×10^{-14}
P.A	P.D	1.77636×10^{-14}	1.77636×10^{-14}	1.77636×10^{-14}
P.A	P.C	1.77636×10^{-14}	$1.77636X10^{-14}$	1.77636×10^{-14}
P.A	D.C	1.77636×10^{-14}	$1.77636X10^{-14}$	1.77636×10^{-14}
P.D	P.C	0.090362978	0.001260975	2.30661×10^{-08}
P.D	D.C	3.08317×10^{-11}	0.750711406	0.708232303
P.C	D.C	3.82861×10^{-08}	0.031527246	9.65686×10^{-07}

For APEC, the post-test showed that the product with the best result was P.A, with an average of 2.52 CFU/mL, being statistically different from all other products. P.C and P.D exhibited a mean of 5.36 and 5.14 CFU/mL respectively. However, they did not present a statistically significant difference between them, with a *p*-value of 0.09036. A better antimicrobial activity was observed for the three evaluated products in comparison to the control D.C, which had an average count equal to 6.02 CFU/mL. P.B had an average of 7.57 CFU/mL, being less efficient than the other products.

For *S. enteritidis*, P.A was the most effective, as it obtained a count of 0 CFU/mL, *i. e.,* there was no bacterial growth on the plates, eliminating the bacterial biofilm. P.B and P.C presented averages of 5.53 and 5.68 CFU/mL, respectively. The *p*-value in the statistical comparison was 0.2696255, demonstrating that they are not statistically different from each other. P.D presented an average of 6.19 CFU/mL while D.C reached an average equal to 6.04 CFU/mL. The *p*-value resulting from this comparison was 0.7507114, therefore they did not present a statistical difference between them. For *S. enterica,* the products P.A, P.B and P.C demonstrated an antimicrobial activity more efficient than the control D.C.

For *L. monocytogenes*, P.A was also more effective with an average of 0 CFU/mL. The second-best antimicrobial activity was gained by P.B with an average of 3.93 CFU/mL, followed by P.C with an average of 4.56 CFU/mL. P.D showed no statistical difference related to the control product again, with a *p*-value equal to 0.7082323, and their averages were 5.12 and 5.08 CFU/mL respectively. Hence, P.A, P.B and P.C revealed an antimicrobial action more efficient to cause biofilm disruption than the D.C control.

In all comparisons with the studied products, P.A presented a p-value equal to $1.776x10^{-14}$, for the three bacterial species evaluated. Thus, it has been proved there is a difference statistically significant the between the P.A and the other products when it comes to antimicrobial activity. In addition, the P.A presented the lowest averages in terms of CFU count, indicating its greater effectiveness.

After analyzing the products concerning bacterial species, the values of each bacterium were crossed for a specific product. This analysis demonstrated that P.D had no statistically significant difference in treatment for APEC and *L. monocytogenes*, with a *p*-value of 0.6522139. D.C showed no statistical difference for APEC and *S. enterica*, with a *p*-value of 0.7137928.

Conclusion

After analyzing the results, it was possible to conclude that all the tested products, as well as the comparative and the control products, have antimicrobial activity by inhibiting and also killing, at different levels, the bacterial strains evaluated in both forms of planktonic cells and mature biofilms.

The antimicrobial action effectiveness of the products was confirmed in the bacterial cell viability assessment test. P.A proved to be the most efficient product for the three bacterial species tested. The other tested products hit varying efficiency for the bacteria tested.

Confocal microscopy showed that all products generated a greater number of dead bacteria when compared to the positive control, concluding that all of them have bactericidal activity.

In general, P.A showed the highest efficiency to eliminate mature biofilms of all bacterial species tested. However, all products tested had the potential to be used as agricultural sanitizers, as they showed antimicrobial activity at low concentrations.

Declarations

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Author contributions André Pitondo-Silva conceived and designed the experiments. Miguel Augusto de Moraes conducted all experiments. Mariana Oliveira-Silva and Rafael da Silva Goulart supported in the antimicrobial susceptibility experiments. Carlos Eduardo Saraiva Miranda provided support in chemical analysis and solution preparation. Manoel Henrique Cintra Gabarra assisted in statistical analysis. Paulo Garcia de Almeida formulated and supplied the products. All authors reviewed and approved the final manuscript.

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Figures

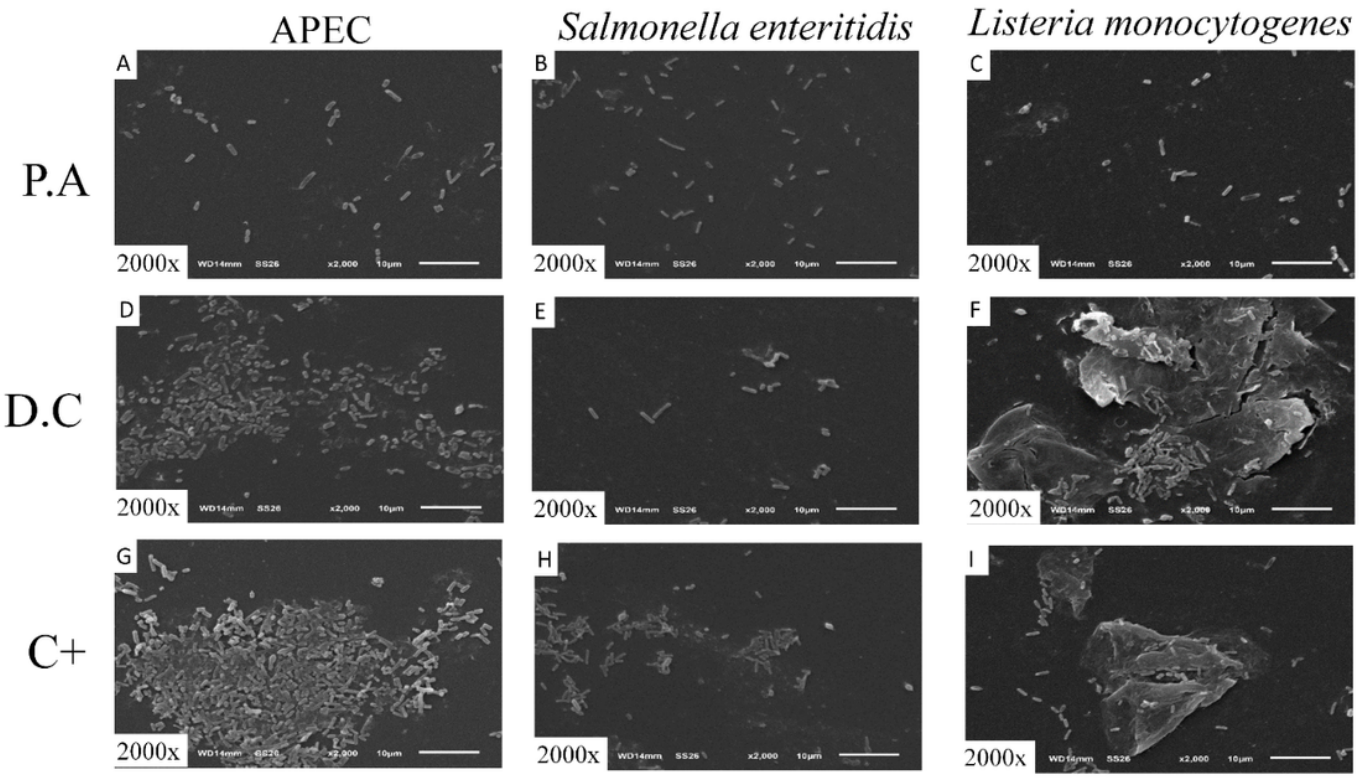

Figure 1

Scanning electron microscopy of P.A, D.C and positive control, at 2000x magnification, for the three bacterial species

A - P.A microscopy for avian pathogenic *Escherichia coli*; B - P.A microscopy for *Salmonella enteritidis*; C - P.A microscopy for *Listeria monocytogenes*; D - D.C microscopy for avian pathogenic *Escherichia coli*; E - D.C microscopy for *Salmonella enteritidis*; F - D.C microscopy for *Listeria monocytogenes*; G - Microscopy of the positive control for avian pathogenic *Escherichia coli*; H - Microscopy of the positive control for *Salmonella enteritidis*; I - Microscopy of the positive control for *Listeria monocytogenes*.

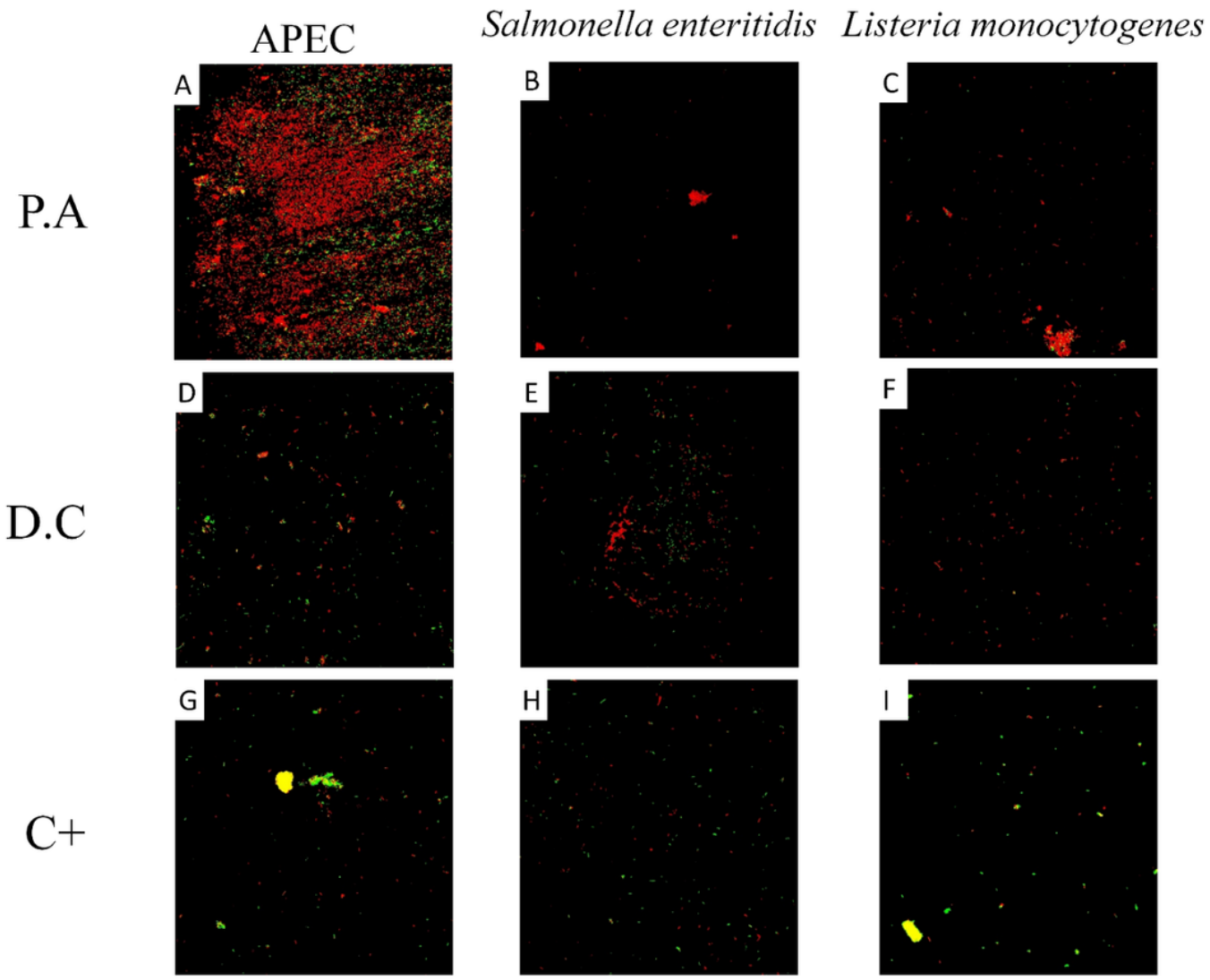

Figure 2

Confocal microscopy showing viable and non-viable cells, by treatment with P.A, D.C and positive control, for the three bacterial strains.

Green color - Viable cells (live); Red color - Non-viable (dead) cells; Yellow color - overlapping of viable and non-viable cells; A - Confocal microscopy of P.A treatment for avian pathogenic *Escherichia coli*; B - Confocal microscopy of P.A treatment for *Salmonella enteritidis*; C - Confocal microscopy of P.A treatment for *Listeria monocytogenes*; D - Confocal microscopy of the D.C treatment for avian pathogenic *Escherichia coli*; E - Confocal microscopy of D.C treatment for *Salmonella enteritidis*; F - Confocal microscopy of P.A treatment for *Listeria monocytogenes*; G - Confocal microscopy of the positive control treatment for avian pathogenic *Escherichia coli*; H - Confocal

microscopy of the positive control treatment for *Salmonella enteritidis*; I - Confocal microscopy of the treatment of the positive control for *Listeria monocytogenes*.