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ARTICLE

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Inactivation of *Pseudomonas aeruginosa* in mineral water by DP1 bacteriophage immobilized on ethylene-vinyl acetate copolymer used as seal caps of plastic bottles

Júnia Novello[1,2] | **Sanna Sillankorva**[3] | **Priscila Pires**[4] | **Joana Azeredo**[4] | **César Henrique Wanke**[1] | **Eduardo César Tondo**[2] | **Otávio Bianchi**[1,5]

[1]Exact Sciences and Engineering Knowledge Area, University of Caxias do Sul, Caxias do Sul, Brazil

[2]Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

[3]INL - International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga, Braga, Portugal

[4]Center of Biological Engineering, University of Minho, Braga, Portugal

[5]Department of Materials Engineering, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

Correspondence
Júnia Novello and Otávio Bianchi, Exact Sciences and Engineering Knowledge Area, University of Caxias do Sul, Francisco Getúlio Vargas Road, 1130, 95070-560, Caxias do Sul, Rio Grande do Sul, Brazil.
Email: jclnovello@ucs.br (J.N.) and otavio.bianch@gmail.com (O.B.)

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Abstract

Pseudomonas aeruginosa has been found in bottled natural mineral water, even though its presence is not allowed in this product by different food regulations. This study aimed to investigate the inactivation of *P. aeruginosa* present in mineral water by vB_PaeM_CEB_DP1 (short name DP1) bacteriophage immobilized on ethylene-vinyl acetate (EVA) copolymer used as seal caps of plastic bottles. EVA was chemically modified using microwave-assisted alcoholysis, improving polymer-phage binding. After that, DP1 phage was attached to EVA and EVA-OH copolymers and both surfaces were tested for plaque formation using *P. aeruginosa*. Then, both materials containing immobilized phages were used as seal caps of plastic bottles and its antimicrobial capacity was tested against *P. aeruginosa* contaminating mineral water. The EVA-OH resulted in higher hydrogen bond density that contributed significantly to the phage immobilization on the polymer surface. The polymers containing immobilized phages were able to reduce 0.53 log of *P. aeruginosa* population present inside mineral water bottles after 14 days.

KEYWORDS

applications, functionalization of polymers, packaging

1 | INTRODUCTION

During the last years, the consumption of natural mineral water has increased worldwide, mainly because consumers are more concerned about the increasing water contamination, the successful marketing strategies of the bottling companies, and the frequent chlorine taste and odor of tap waters supplied by municipal bodies.[1] Beyond that, chlorine and chloramine used to the treatment of public water may react with organic matter dissolved in the water, forming cytotoxic products, such as halamines, haloacetamides, halonitriles, and heterocyclic amines.[2,3]

Natural mineral waters are not sterile and complex bacterial ecosystems with high phenotypic and genotypic diversity may be present.[4] The bacterial load of bottled mineral water is dependent on the quality of natural

spring-water and on the good hygienic practices carried out inside industrial facilities. Microorganisms can be present on the surfaces of pumps, pipes, caps, and bottles,[3] resulting in the contamination of end products. Bacterial counts of bottled mineral water can reach values ranging from 10^4 to 10^5 colony forming units (CFU) per ml after some days of storage[5]; however, pathogens are not expected to be present.

According to the Brazilian regulation and international directives,[6,7] mineral water for human consumption has to be free of *Pseudomonas aeruginosa*.[4] This microorganism can be found in different natural and aqueous environments, being possible to contaminate mineral water during bottling process.[8] *Pseudomonas aeruginosa* is an opportunistic human pathogen that has been associated with some waterborne disease cases and frequent nosocomial-acquired outbreaks.[9] This bacterium can cause chronic persistent infections and form biofilms on different surfaces, becoming resistant to antimicrobial therapy, host immune response,[10] and sanitizers.

Pseudomonas aeruginosa can contaminate water and other fluids, such as milk in feeding bottles, and cause outbreaks.[4] According to Rosenberg and Hemandez-Duquino,[8] *Pseudomonas* adhesion on plastic bottle surfaces generally reaches higher numbers than on glass bottle surfaces. One hypothesis for this finding is the permeability of the external plastic vapors, for example, oxygen, which consequently enhances survival and microbial growth. Beyond that, the rapid microbial growth after filling may also be related to oxygenation of mineral water, temperature rise during the storage, and the small number of nutrients present on package surfaces.[11]

Bacteriophages (phages) were successfully used for the first time to treat bacterial infections a decade before the discovery of penicillin. They are not pathogenic for man, and they are particularly efficient for their target bacteria. Initially, the phage injects its nucleic acid into the bacteria and then these do DNA copies. After that, they make and package more of phages into new structures. Lastly, the phages produce toxic chemicals that rupture the bacterial host, releasing its new virus to the outside.[12]

Recently, phages have emerged as a new approach to control bacterial contamination in food industries,[13] particularly in animal husbandry, horticulture, and agriculture.[14] As examples, the products ListShield and EcoShield are made up of bacteriophages and are sold to control *Listeria* and *Escherichia coli* O157, respectively, while LISTEXTM P100 is a GRAS (generally recognized as safe) product available for use in food.[13,15]

Several companies worldwide are using bacteriophages to control bacterial contamination. Pearson et al.[16] anchored *E. coli* and *Staphylococcus aureus* phages on polyethylene (PE) and polytetrafluoroethylene (PTFE) surfaces and were able to reduce bacterial counts and biofilms. Wang et al. immobilized phage T4 on the polyhydroxyalkanoate (PHA) surfaces and the infective capability of attached phages was tested exposing the phage-immobilized surfaces to *E. coli*. In both studies,[16,17] the formation of specific chemical bonds between polymer-phage was essential for the increase of the phage binding on the surface of the polymers.

Several approaches have been employed in the immobilization of phages in polymers bulk or surfaces.[16,18,19] Encapsulation of phages in solvent-emulsion microspheres may result in loss of phage activity due to the organic solvent employed.[20] The creation of antimicrobial surfaces with covalent bonding or by electrostatic interactions between polymer-phage, taking advantage of the different charge distribution between head and tail, seems like a promising strategy.[16,18] A limitation of these approaches is to use systems that are already in production scale since they use chemical reagents that can contribute to reduced product integrity. In the case of drinking water, contamination with small amounts of organic solvents can render a valuable asset unusable.[21] Thus, cleaner approaches to products that are already in use can directly contribute to the advance of phage use.

These approaches cannot use systems that are already in production scale, as they use chemical reagents that can contribute to the reduction of product integrity. In the case of drinking water, contamination with small amounts of organic solvents can render a valuable commodity useless. Thus, cleaner approaches to products that are already in use can directly contribute to the advancement of phage use.

The FDA regulates the ethylene-vinyl acetate (EVA)-vinyl alcohol (VOH) copolymer for direct contact with food. The partial or complete alcoholysis or hydrolysis of EVA copolymers complying complies with section 177.1350 of the FDA regulamentations.[22] EVA copolymers have low cytotoxicity[23,24] and have been used as a medical device construction proposal due to its chemical resistance, toughness, clarity, and color stability. Ethylene copolymers are derived from ethylene and another compound with a polar functional group such as VOH, vinyl acetate (VA) are the most frequently used food-packaging polymers. However, EVOH or EVA-OH has the most promising gas-barrier properties due to better chain packing aided by hydrogen bonds.[25,26]

The objective of the present study was to immobilize DP1 phages on the surfaces of EVA used as seal caps of

plastic bottles for mineral water. We also investigated the samples of EVA and EVA-OH produced by microwave-assisted alcoholysis, aiming to understand how chemical interaction occurs between the polymer groups and phages and its capacity to eliminate *P. aeruginosa* in mineral water. Therefore, polymer seal caps with immobilized DP1 phages can be a strategy to contribute with the control of *P. aeruginosa* contamination of bottled mineral water.

2 | EXPERIMENTAL

2.1 | EVA films: Preparation and characterizations

EVA copolymer (Braskem, HM 728, Triunfo, Brazil) with 28 wt% of VA content and melt flow index (190°C/2.16 kg) of 6.0 g/10 min, average molecular weight of M_n = 146,000 g/mol, and polydispersity index of M_w/M_n = 3.2 determined by thermogravimetry and gel permeation chromatography measurements were used in this study. EVA copolymer was chemically modified by alcoholysis reaction and was produced according to the procedure described by Paradinha et al.[26] in order to improve the polymer/phage compatibility.

EVA pellets (26 g) were solubilized in Tetrahydrofuran (THF, ACS grade—VETEC, Xerém, Brazil) (104 g at 45°C) and placed into a 250-ml three-necked flask equipped with a reflux condenser. Then, 60 ml of 0.5 M alcoholic sodium hydroxide (NaOH, ACS, 97% of purity, VETEC, Xerém, Brazil) were added into the EVA/THF solution and the medium was microwave refluxed for 30 min.

After the alcoholysis reactions, EVA was neutralized with 10 ml solution of 3 M hydrogen chloride (HCl, ACS Grade, VETEC, Xerém, Brazil). Then, the samples were washed using boiled ultrapure water (Milli-Q) to remove the alcoholysis products such as sodium acetate. The degree of chemical modification of EVA was determined using ^{1}H NMR (Bruker Fourier 300 Mhz in $CDCl_3$ at 25°C) and ATR-FTIR (PerkinElmer, Spectrum 400 equipped with an attenuated total reflectance ATR diamond crystal at 45°, resolution of 2 cm^{-1}, 32 scans from 4,000 to 500 cm^{-1}) (see Supporting Information). The immobilization of the phages and subsequent characterizations of the EVA and EVA-OH samples were done on films produced by compression molding at 160°C and 6 MPa (2 min). The contact angle measurements were made using sessile drop technique (DSA 30/Krüss, Germany). The contact angle values were computed using *Surftens 3.0TM* software (Frankfurt, Germany). This technique was performed to evaluate the polarity change after the chemical modification of the EVA by alcoholysis reaction. For this, 10 drops of deionized water and *n*-hexadecane were placed on different parts of EVA surface films (60 × 25 mm^2). The contact angle was measured on each side of the surface, totalizing 10 measurements for each liquid. The highest and lowest values were discarded. The arithmetic mean and the standard deviation were, respectively, the mean value and the error measurement. The contact angles of the surfaces of the films that characterized the wettability were analyzed according to the literature,[27] in which the surfaces were classified as hydrophilic (contact angle with water less than 90°) or hydrophobic (contact angle with water between 91° and 150°) and superhydrophobic (contact angle with water greater than 151°). Besides, the free energy of the surfaces was estimated according to Owens and Wendt.[28]

2.2 | Bacterial strains and culture preservation conditions

The microbiological assays were performed using *P. aeruginosa* strain PAO1 isolated from an endoscope (Rouen, France). Phage vB_PaeM_CEB_DP1 (short name DP1) used in this study was isolated from the hospital effluents of the Hospital de São João (Porto, Portugal).[29]

Pseudomonas aeruginosa strain PAO1 was cryopreserved at −80°C in Tryptone Soya Broth (TSB) with 30% (v/v) of glycerol (AppliChem, Germany), and DP1 Phage was kept at 4°C in buffer saline magnesium (SM buffer; 5.8 g/L NaCl, 2 g/L $MgSO_4.7H_2O$, 50 ml of 1 M Tris-HCl [pH 7.5]) (Applichem, Germany).

2.3 | Bacteriophage production

Phages were produced using the protocol described by Pires et al. [29] First, bacterial lawns were prepared, pouring 0.1 ml of overnight-grown *P. aeruginosa* suspension using the pour plate technique. After drying, plates were added with 3 ml of top-agar (TSB + 0.6% [w/v] agar). Then, sterile paper (80 g/m^2) strip rectangles (1 cm per 5 cm) were immersed in the phage stock solution and streaked on the bacterial lawns. The plates were incubated from 16 to 18 h at 25°C and then 3 ml of SM buffer was added. The plates were placed under agitation (120 rpm) at 4°C during 16–18 h and, afterward, the liquid phase and top-agar (TSB, 0.6% [w/v] agar) layer were collected, centrifuged, and the supernatant was filtered (0.2 μm MilliporeSigma, Germany).

2.4 | Phage attachment on EVA and EVA-OH surfaces

The DP1 phage immobilization on EVA and EVA-OH surfaces was performed according to the protocol described by Pearson et al., with some modifications.[16] The assays were performed on sterile 24-well plates (flat-bottomed uncoated polystyrene tissue culture plates, Orange Scientific, Belgium). EVA and EVA-OH films were placed into each well and 1,000 µl of phosphate-buffered saline (PBS) buffer (0.1 mol/l, pH 7.4, HiMedia, India), containing 2.5 mmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich, Germany) and 2.5 mmol *N*-hydroxysuccinimide (Sigma-Aldrich, Germany) were added for 2 h in order to create —COO— groups followed by washing with PBS. Then, the coupons were immediately immersed into 10 ml buffer solution containing 500 µl of concentrated phage for 16 h. After that, the coupons were washed seven times in 15 ml PBS buffer in order to remove all nonbonded phages. ATR-FTIR monitored the presence of phages on EVA surfaces.

2.5 | Plaque formation assay for the phage immobilized on surfaces

The films containing attached bacteriophages were used for the plaque formation assay as described in Reference [16] with some modifications. *Pseudomonas aeruginosa* was subcultured on Trytone Soya Agar (TSA; HiMedia, India) at 37°C for 24 h. After that, colonies were inoculated into 15 ml of TSB at 37°C for 18 h, at 90 rpm. An amount of 100 µl of cell suspension (8.0 log CFU/ml) was spread on Petri plates containing TSA using a swab and, after 10 min, the coupons with attached and nonattached-phage (1×1 cm^2) were stabbed on the agar into Petri plates. The plates were incubated at 37°C for 24 h, and the formation of clear plaques around the surfaces was observed and counted. Images were taken to record the plaques produced by phages.

2.6 | Antimicrobial activity in mineral water bottles

Commercial 500 ml (±20 ml) bottles of mineral water were purchased in a local market of Porto Alegre city, Southern Brazil. The composition of mineral water was as follows: pH 7.1, bicarbonate 122.64 mg/l, calcium 24.10 mg/l, chloride 1.22 mg/l, fluoride 0.19 mg/l, lithium 0.011 mg/l, magnesium 2.75 mg/l, potassium 1.93 mg/l, sodium 14.74 mg/l.

Neutral liquid soap (Kalykim, Brazil) was used to wash the external side of bottles, which were dried using paper towels and sanitized with 70% ethylic alcohol [v/v]. A volume of 5 ml of mineral water was removed from each bottle. Then, 5 ml of the sterile mineral water containing *P. aeruginosa* was added, reaching a final concentration of 10^4 CFU/ml into each of the three bottles used for each of the following treatments. EVA copolymer seals with and without attached DP1 phage were placed into three sets of three bottle caps (MOI=1). After that, the bottles with *P. aeruginosa* were closed and incubated at 25°C upside down without agitation. *Pseudomonas aeruginosa* and DP1 phages counts were carried out just after inoculation and then after 1, 7, and 14 days, using Cetrimide Agar Base plates (HiMedia, India), incubated at 35°C for 48 h. The difference between the initial and final bacteria or phage count was expressed in CFU/ml, and the results of plaque counts were expressed in PFU/ml, respectively.

Water bottles without the seal polymers and without *P. aeruginosa* were used as negative controls. All the counts were carried out in triplicates, and the experiment was independently repeated three times.

3 | RESULTS AND DISCUSSION

The NMR spectra (Supporting Information) showed at $\delta = 2.05$ and 4.88 ppm (α-H). The EVA spectra had a characteristic peak due to methyl and methine protons of the VA.[30,31] A broad peak at 1.4–1.7 ppm, corresponding to the β-H was observed (CH_2 in VA segment). The methyl and methine protons and other signals in the chemical modified sample (EVA-OH) were the same found in pure EVA. The regions between 1.4 and 1.7 ppm showed the signal for CH_2 in VA and OH segments (β-H). The peak at $\delta = 3.76$ ppm observed in EVA-OH was characteristic of the hydroxyls groups generated by alcoholysis reactions.[26]

FTIR spectrum of EVA and EVA-OH samples (showed in Supporting Information) demonstrated the characteristics bands of C═O stretching relative to ester carbonyl group (at 1738 cm^{-1}), C—O stretching at 1,240 cm^{-1}, and symmetric stretching vibration of C—O—C band at 1,016 cm^{-1} related to the VA group in copolymer.[26,32] The CH_2 stretching band at 2,920 cm^{-1}, CH_2 deformation band at 1,370 cm^{-1}, and CH_2 scissoring band at 14,65 cm^{-1} were observed. After hydrolysis, the appearance of two new bands was noted. One related to the VOH (C—O) at 1,280 cm^{-1} and other related to the —OH stretching at 3,080–3,700 cm^{-1}, from the intermolecular hydrogen bond, because of the VOH group. The hydroxyls and carboxylate groups were possibly

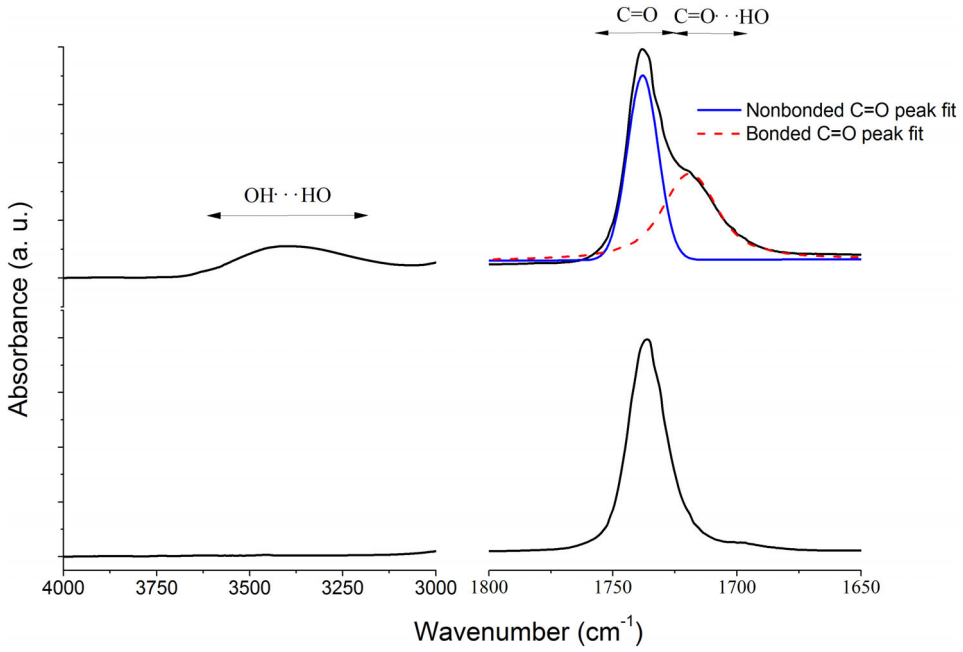

FIGURE 1 FTIR spectra OH (4,000–3,000 cm^{-1}) and C═O (1,800–1,650 cm^{-1}) regions. C═O region was fitted using Voigt functions ($R > 0.994$) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Surface properties of EVA and EVA-OH copolymers

	Contact angle (degree)		Surface energy (mJ/m^2)		
Sample	**Water**	***n*-hexadecane**	**Polar**	**Dispersive**	**Total**
EVA	86.4 ± 1.7	25.3 ± 1.6	25.0 ± 0.3	0.5 ± 0.4	25.5 ± 0.4
EVA-OH	81.0 ± 2.5	15.0 ± 1.8	26.7 ± 0.2	1.4 ± 0.7	27.2 ± 0.6

responsible for the immobilization of the phage on the polymer surface. Figure 1 shows FTIR spectra for the region of the OH and C═O groups for EVA and EVA-OH. In these regions, it is possible to identify the effect generated from the alcoholysis reaction. The NMR and FTIR results confirmed that part of the acetate groups was converted into hydroxyl groups after alcoholysis reaction. As a result of the partial alcoholysis reaction, there was a formation of HO···HO and C═O···HO. The alcoholysis degree estimated by NMR was ~42.6%.

It was observed that the surfaces of the polymers used are hydrophilic and have an angle of contact with water (θ_w) was less than 90°.[28] EVA-OH was the most hydrophilic surface ($\theta_w = 80 \pm 3°$) (Table 1). Thus, by the nature of DP1 phages, which has peptide bonds, it should increase the phage and EVA-OH surface interactions and thus remains more strongly linked when compared to the pure EVA. As well as the contact angle, there was also the variation of the surface energy components for EVA-OH. As expected, EVA-OH polarity was higher (26.7 mJ/m^2) than pure EVA. Thus, the increase in hydrophilicity favors the immobilization of the phage by specific interactions.[16,17]

Pearson et al. also verified the presence of carboxyl (—COOH) and hydroxyl (—OH) groups after the modification of PE and PTFE surfaces with maleic anhydride.[16] Moreover, these two groups are responsible for the immobilization of the phage T4 onto PHA surfaces.[17]

FTIR was used to verify the attachment of DP1 phage to the surface of the polymers (Figure 2a). A band at 1,640 cm^{-1}, relative to the primary amide, was observed for both polymers and phage systems.[33] However, for pure EVA, a lower intensity was observed, which is indicative of the smaller amount of the phage bonded, also noted in the region of 3,700–3,060 cm^{-1}. The difference in intensity was due to the formation of interactions —NH···OH— by hydrogen bonds. Through the mathematical deconvolution of the area in the C═O region (1,738 cm^{-1}), it was possible to estimate the relative amount of hydrogen bonding in the carbonyl group. EVA-OH without phages showed the relative index of 0.52, whereas for the EVA-OH with phages demonstrated 0.41. Therefore, it is possible to infer that the phage also associates through interactions NH···OH, which suggests that the association phage–EVA-OH also occurs through multiple hydrogen bonds. Concerning the EVA, the C═O group limits the formation of specific interactions when compared to EVA-OH. Consequently, the relative index of hydrogen bonding in the carbonyl group for pure

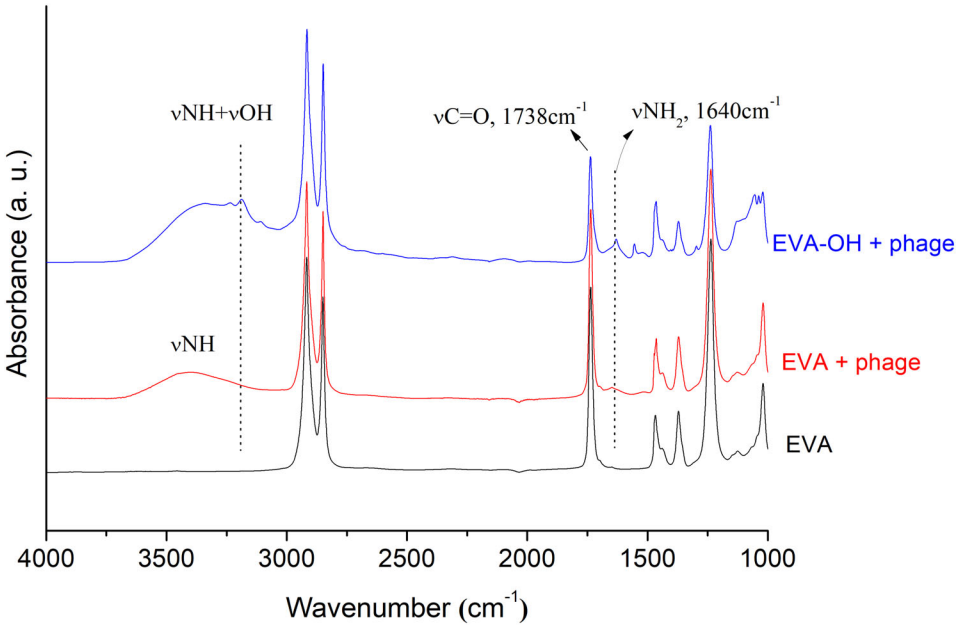

FIGURE 2 FTIR spectra of EVA and EVA-OH with attached DP1 phage. Absorbance intensity shows the increase of the peptide bond interactions between phage and polymers [Color figure can be viewed at wileyonlinelibrary.com]

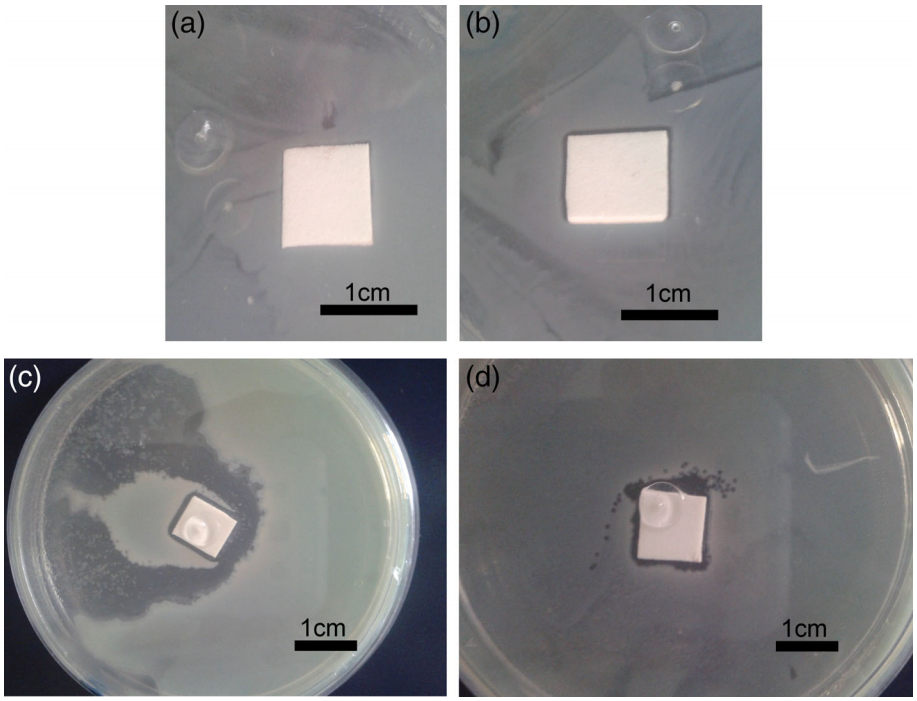

FIGURE 3 Transparent halos (plaque formation assays) on (a) positive and (b) negative controls, (c) EVA and (d) EVA-OH with DP1 phages against *P. aeruginosa* in water bottles [Color figure can be viewed at wileyonlinelibrary.com]

EVA/phase is lower than the EVA-OH, demonstrating the value of 0.14.

According to Figure 3a,b, it was possible to verify a transparent halo (lysis plaques) formed around the polymers with the immobilized phage. This result demonstrated that the EVA surfaces were able to inactivate *P. aeruginosa*, as a result of the phage activity.

As the incubation time increased, the size of plaques increased too. Moreover, the plaques did not occur in control coupons (positive and negative controls) (Figure 3a,b). Similar results were demonstrated by Pearson et al.,[16] who immobilized T1 bacteriophages on PE and PTFE surfaces which were able to inactivate *S. aureus* and *E. coli*. The use of EVA-OH for the phage attaching is interesting because it is less toxic than EVA and produces a more efficient barrier for gases than other olefinic polymers.[34] So, a similar approach could be used to immobilize specific bacteriophages on the surfaces of EVA used as bottle cap seals in order to control *P. aeruginosa* in natural mineral waters, and this was done in the present study.

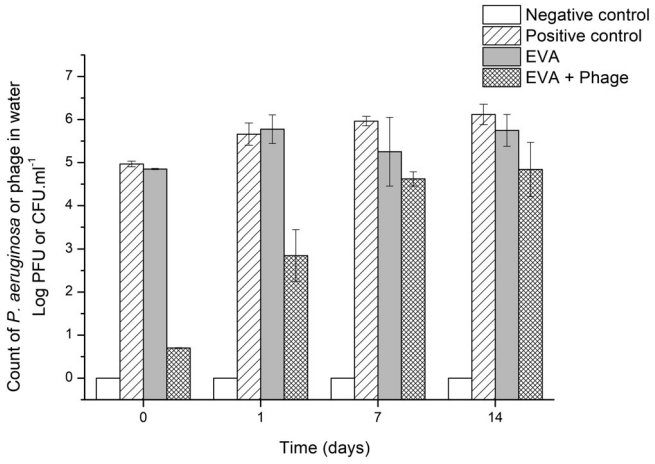

FIGURE 4 *Pseudomonas aeruginosa* (log CFU/ml) and DP1 bacteriophages (log PFU/ml) counts after 0, 1, 7, and 14 days of assay, carried out for testing the anti-*P. aeruginosa* activity of EVA polymers used as cap seal containing immobilized DP1 phages inside mineral water bottles. Containing the virus ("Bottle with EVA"), without the virus but with the bacteria ("Positive control bottle"), and without the virus and bacteria ("Negative control bottle")

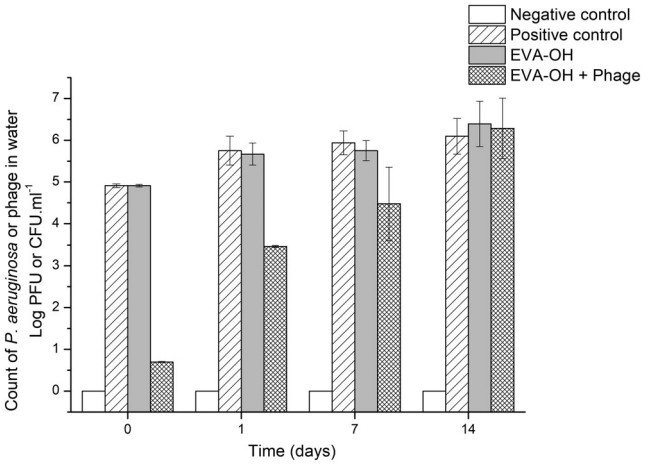

FIGURE 5 *Pseudomonas aeruginosa* (log CFU/ml) and DP1 bacteriophages (log PFU/ml) counts after 0, 1, 7, and 14 days of assay carried out for testing the anti-*P. aeruginosa* activity of EVA-OH copolymers used as cap seal containing immobilized DP1 phages inside mineral water bottles. Containing the virus ("Bottle with EVA-OH"), without the virus but with the bacteria ("Positive control bottle"), and without the virus and bacteria ("Negative control bottle")

It was observed that the numbers of DP1 phages increased inside mineral water bottles until 14 days (5 or 6 log PFU/ml). Furthermore, there was a less plaque-forming unit (4.8 log PFU/ml) inside bottles with EVA (Figures 4 and 5) when compared to bottles with EVA-OH, indicating that the higher concentration of polar groups resulted in greater immobilization of phages through peptide bonds.[16,17]

Nonetheless, it was observed that the most significant bacterial reduction (0.53 log reduction) occurred at 14 days in the mineral water bottles containing EVA polymer with DP1 phages (Figure 4). Despite the antimicrobial effectiveness of the DP1 phage against *P. aeruginosa* cells, the phages were not able to eliminate all microorganisms. It is possible that the agitation of bottles could result in better reduction levels, simulating what happens during mineral water bottle transport. We decided to carry out this test without agitation aiming to access the worst scenario.

Antibiotic treatment is currently the most widely used therapy for *P. aeruginosa* infections. However, over the past few years, this bacterium has grown impervious toward several antimicrobial agents like antibiotics and disinfectants. Antibiotic treatment is currently the most widely used therapy for *P. aeruginosa* infections. Consequently, antibiotic resistance has been observed in some *P. aeruginosa* strains. So strategies to address this have focused on the use of bacteriophage mixes with or without the antibiotics,[35,36] using the surface modification with copper[37] and silver[37,38] or graphene oxide,[39] to improve antibacterial effect. Copper and silver ions concentrations tested (0.1–0.8 mg/l) achieved more than 99.999% reduction of *P. aeruginosa*, in 6, 12, and 96 h, respectively.[37,38] But combination of copper and silver ions exhibited a synergistic effect against *P. aeruginosa*.[37]

Pseudomonas aeruginosa cells increased up to 1.75 log CFU/ml over 14-day incubation period inside bottles containing the polymer without DP1 phages ("positive control bottle"). Thus, EVA copolymers containing attached DP1 phages, mainly EVA-OH, seem to be a viable alternative to reduce *P. aeruginosa* into mineral water bottles. This is an approach that needs attention due to the effects of low toxicity.[36] However, others studies are necessary for validation of the use of this seal caps of water bottles, regarding the applications in the beverage industries.

4 | CONCLUSION

According to our results, it was possible to produce a nontoxic and more hydrophilic EVA copolymer with antimicrobial action against *P. aeruginosa*. Moreover, to the best of our knowledge, there are no reports in the literature which investigate EVA-OH copolymers with immobilized phages, which characterizes the innovative characteristic of this research.

Furthermore, the EVA-OH copolymers used as seal caps of plastic bottles for mineral water were able to reduce the counts of the *P. aeruginosa* present in mineral water bottles, indicating a possible control measure for controlling the eventual contamination of mineral

water by *P. aeruginosa*. However, other experiments are necessary in order to test new conditions able to reduce the time of action of the DP1 phage against *P. aeruginosa*, such as virus concentration and water temperature. The proposed approach is promising as it uses a polymer that is already used in the manufacturing of bottle seals.

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ORCID

Otávio Bianchi https://orcid.org/0000-0001-7493-8163

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