NATURAL COMPOUNDS AND NOVEL SOURCES OF ANTIMICROBIAL AGENTS FOR FOOD PRESERVATION AND BIOFILM CONTROL

EDITED BY: Lizziane Kretli Winkelströter, Eugenia Bezirtzoglou and Fabricio Luiz Tulini

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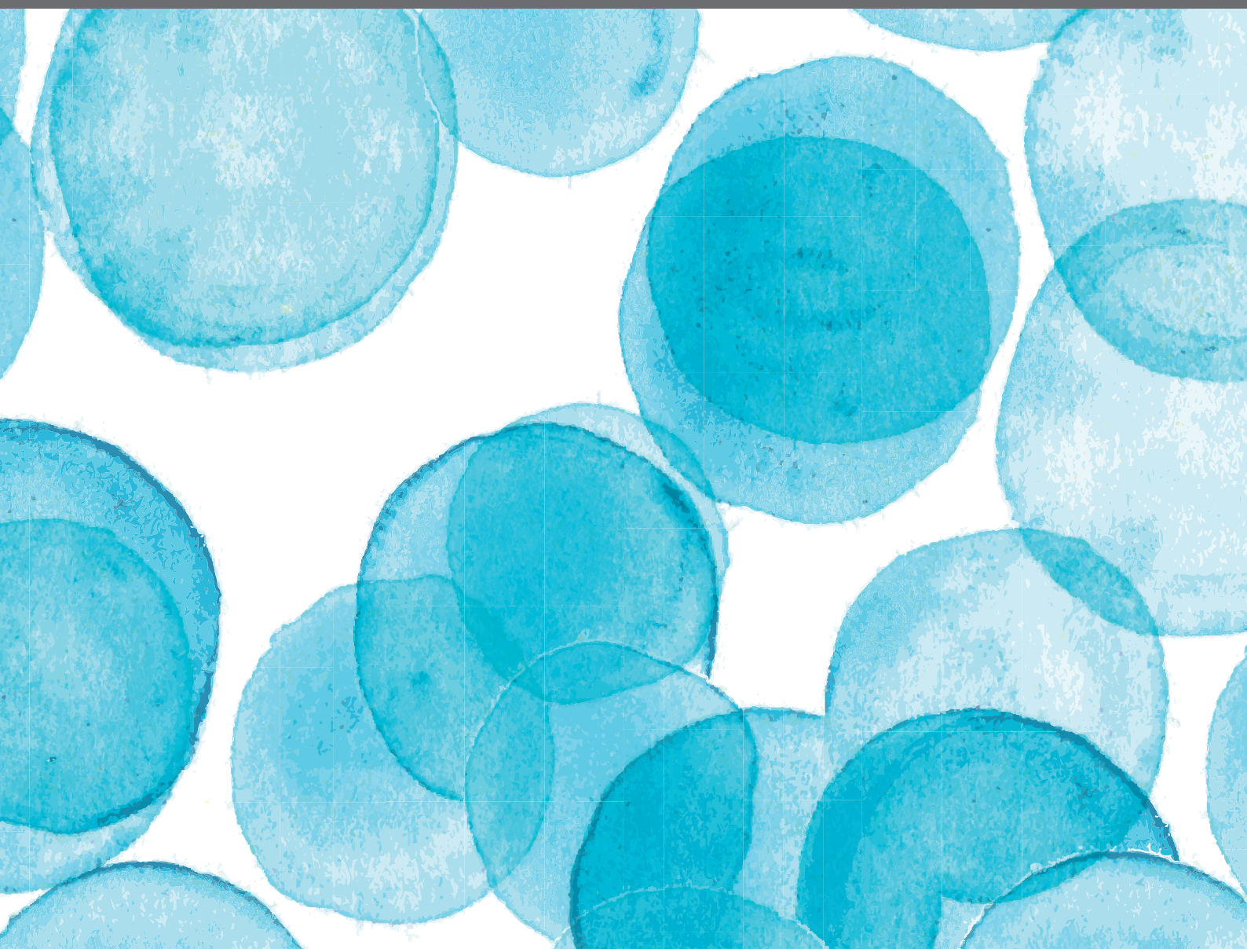

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NATURAL COMPOUNDS AND NOVEL SOURCES OF ANTIMICROBIAL AGENTS FOR FOOD PRESERVATION AND BIOFILM CONTROL

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EDITORIAL
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Editorial: Natural Compounds and Novel Sources of Antimicrobial Agents for Food Preservation and Biofilm Control

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Editorial on the Research Topic

Natural Compounds and Novel Sources of Antimicrobial Agents for Food Preservation and Biofilm Control

Biofilms can be defined as highly structured sessile microbial communities, embedded in an extracellular polymeric substance, which allows irreversible adherence to biotic and abiotic surfaces. In nature, biofilms can be formed by numerous species of bacteria, fungi, protozoa, and algae. Among the species of microorganisms commonly involved in the formation of biofilms are *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli*, and *Candida albicans* (Joshi et al., 2021; Srinivasan et al., 2021). The ability to form biofilms determines the pathogenicity of these microorganisms. Currently, it is believed that 80% of human bacterial infections are associated with biofilm formation, especially those involving the use of medical devices, such as catheters, heart valves, contact lenses among others (Algburi et al., 2017; Joshi et al., 2021; Srinivasan et al., 2021).

The development of microbial biofilms also occurs frequently in the food industries, since there is a large amount of nutrients available in equipment, utensils and contact surfaces. Biofilms have a higher resistance to sanitizers and can lead the equipment to corrosion, causing a negative impact on the quality of the final product. Its presence in food and processing can cause serious damage to public health due to problems associated with foodborne diseases and food deterioration (Mevo et al., 2021).

The production of safe and high-quality food has become a challenge for the food industry due to food deterioration, which is caused by the undesirable growth of pathogenic/deteriorating microorganisms. This deterioration can lead to food loss and waste. To reduce losses, the food industry uses synthetic preservatives to control microbial growth in order to extend shelf life, quality, and food safety. However, some chemical additives when ingested in large quantities can cause undesirable reactions to consumers. Nowadays, it has been observed an increased consumer preference for more natural food additives and concern about the safety of synthetic preservatives that have encouraged the food industry to look for environmentally friendly alternatives. Thus, the intention arose to replace the use of traditional synthetic preservatives with natural antimicrobials in food (Mei et al., 2019; Yu et al., 2021).

In addition, the indiscriminate use of antimicrobials, including antibiotics and biocides, has led to the development of resistance in food and hospital bacteria. These resistances pose a threat to public health, as they reduce the efficacy of the same compounds, thus increasing mortality and morbidity (Srinivasan et al., 2021). Microorganisms in the form of biofilms become substantially

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more resistant to the action of antibiotics than those living alone. This fact can be attributed to the weak penetration and diffusion of antimicrobial drugs through the extracellular polymer matrix, strong expression of efflux pumps and enzymes capable of degrading antimicrobial molecules (Algburi et al., 2017). These mechanisms have driven several studies in an attempt to develop materials resistant to the adhesion of microorganisms, in addition to possible treatments in the case of biofilms already formed.

Thus, it is essential to study new antimicrobial compounds in the control of growth and dissemination of pathogens. Compounds of natural origin have been presented as promising alternatives to antimicrobial agents currently used, not only as possible food preservatives, but also as adjuvants in the process of disinfection of surfaces and in the fight against infections.

This Research Topic aims to address several natural agents with antimicrobial action and/or antibiofilm focused on food preservation and biofilm control, i.e., phytochemicals, biosurfactants, antimicrobial peptides and microbial enzymes, together with their sources, mechanism of action by interference in quorum detection pathways, and also, interruption of the extracellular polymeric substance, and its inhibiting concentrations. We had successfully received 19 submitted manuscript that followed the revision process and finally, it was concluded in 13 approved articles.

Review article create a readable synthesis of the best resources available in the literature for an important current area of research. In the present Research Topic, four reviews were published and contributed to suggest new research directions, to strengthen support for existing theories and/or identify patterns among existing research studies. Oulahal and Degraeve focused on a review that provided an overview of current knowledge regarding the promises and the limits of phenolic-rich plant extracts for food preservation and biofilm control on food-contacting surfaces. Khorshidian et al. investigated the possible application of pediocin (Pediocins, which belong to subclass IIa of bacteriocin characterized as small unmodified peptides with a low molecular weight, are produced by some of the *Pediococcus* genus bacteria), in preservation of meat and meat products against *L. monocytogenes*. Maurya et al. developed a review that aimed to present the practical application of nanoemulsions (a) by addressing their direct and indirect (EO nanoemulsion coating leading to active packaging) consistent support in a real food system, (b) biochemical actions related to antimicrobial mechanisms, (c) effectiveness of nanoemulsion as bio-nanosensor with large scale practical applicability, (d) critical evaluation of toxicity, safety, and regulatory issues, and (e) market demand of nanoemulsion in pharmaceuticals and nutraceuticals along with the current challenges and future opportunities. Das et al. developed a review that deals with the advancement in nanoencapsulation-based edible coating of essential oil with efficient utilization as a novel safe green preservative and develops a green insight into sustainable protection of fruits against fungal- and mycotoxin-mediated quality deterioration.

Several studies (nine original research) have addressed this theme and proved the potential application of natural and alternative components in food preservation and biofilm control. Many plants have been known to exert antimicrobial properties due to their content of secondary metabolites. Over the past decade, much attention has been placed on the study of phytochemicals for their antimicrobial activity (Barbieri et al., 2017). In the present Research Topic Santos et al. developed an alcohol-free high-performance extractive approach to recover antibacterial and antioxidants phytochemicals from red propolis. The authors found inhibition halo on the growth of *Staphylococcus aureus* and *Salmonella* enteritidis bacteria. Khelissa et al. prepared ruthenium (II) cationic water-soluble complex by a reaction between dichloro (para-cymene) ruthenium (II) dimers and aminooxime ligands in a 1:2 molar ratio. Antibacterial and antibiofilm activities of the synthetized complex were assessed against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*. The results revealed that the ruthenium (II) complex has higher antibacterial and antibiofilm activities in comparison with free ligands or the enantiopure (R)-limonene.

Phenolic compounds are natural substances that can be obtained from plants and play an important antimicrobial effect. In the present Research Topic, Santos et al. aimed to evaluate the potential of phenolic compounds for QS inhibition in a QS biosensor strain (*Chromobacterium violaceum*) and three foodborne bacterial species (*Aeromonas hydrophila, Salmonella enterica* serovar Montevideo, and *Serratia marcescens*). Those authors found that curcumin, capsaicin, and resveratrol inhibited violacein production by *C. violaceum*. Biofilm formation was inhibited by resveratrol in *A. hydrophila*, by capsaicin and curcumin in *S.* Montevideo and by resveratrol and capsaicin in *S. marcescens*.

Microbial secondary metabolites are low molecular mass products, not essential for growth of the producing cultures, but very important for human health. They include antibiotics, antitumor agents, cholesterol-lowering drugs, and others (Allemailem, 2021). In the Research Topic, Kumari et al. aimed to assess potential of biosurfactants screened from a novel yeast and their inhibition against food spoilage fungi. Authors illustrated the antifungal activity of sophorolipid biosurfactant from Metschnikowia genus for the first time and suggested a novel antifungal compound against food spoilage and human fungal pathogens. Chen et al. hypothesized that deinococcal cellular constituents play a pivotal role in preventing *S. aureus* colonization by inhibiting biofilm formation. Theirs experiments proved that DeinoPol is a key molecule in the negative regulation of *S. aureus* biofilm formation by *D. radiodurans*. Therefore, DeinoPol could be applied to prevent and/or treat infections or inflammatory diseases associated with *S. aureus* biofilms.

In this scenario, Taggar et al. demonstrated that an antimicrobial peptides (named as peptide-Ba49) isolated from *Bacillus subtilis* subsp. *spizizenii* strain exhibited strong antibacterial efficacy against *S. aureus* ATCC 25923. The first article of this topic, Zhang et al. evaluated the efficacy of mixtures of natural antimicrobial compounds, namely reuterin, microcin J25, and lactic acid, for reducing the viability of *Salmonella enterica* and total aerobes on broiler chicken carcasses. They found that sprayed onto chilled chicken carcasses, this

reuterin + lactic acid mixture reduced *Salmonella* spp. counts. The synergy of reuterin with lactic acid or microcin J25 as inhibitors of bacterial growth was significant. Choyam et al. demonstrated that *Bacillus* antimicrobial peptide (BAMP) produced by *Bacillus paralicheniformis* exhibited a bacteriostatic effect on *Salmonella typhi* and controls the viability of *Listeria monocytogenes* in chicken meat efficiently.

Nanotechnology is increasingly used to target bacteria as an alternative for food safety and biofilm control (Vinci and Rapa, 2019). In this Research Topic Puranen et al. evaluated the efficacy of nanomaterials and blue light illumination for *L. monocytogenes* ATCC 7644 biofilm inactivation. The results found by the authors demonstrated that nanocoating with visible light illumination could be an effective and safe method for enhancing food safety in food processing facilities to control biofilm formation.

In summary, this Research Topic provides a better understanding of the main natural and alternative components that exhibit action in the food preservation and/or in the biofilm control of pathogenic species. The results obtained from the use of these compounds have aroused great interest. There is an expectation that further *in vitro* and *in vivo* studies will be conducted to better understand their metabolic pathways, mechanisms of action, to define details about its safety and to develop regulations for its use. In this way, we hope that this Research Topic can generate knowledge and open ways for the construction of new systems and strategies to combat biofilms and food contamination.

AUTHOR CONTRIBUTIONS

LW, EB, and FT edited the topic and wrote the manuscript. All authors listed have made a substantial contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Inhibitory Activity of Natural Synergetic Antimicrobial Consortia Against *Salmonella enterica* on Broiler Chicken Carcasses

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The currently most utilized antimicrobial agent in poultry processing facilities is peracetic acid, a chemical increasingly recognized as hazardous to human health. We evaluated the efficacy of mixtures of natural antimicrobial compounds, namely reuterin, microcin J25, and lactic acid, for reducing the viability of *Salmonella enterica* and total aerobes on broiler chicken carcasses. The compounds were compared singly and in combination with water and 0.1% peracetic acid. The minimum inhibitory concentrations of reuterin, lactic acid, and microcin J25 against *S. enterica* serovar Enteritidis were respectively 2 mM, 0.31%, and 0.03 μM. *In vitro*, the combinations of reuterin + lactic acid and reuterin + microcin J25 were synergic, making these compounds effective at four times lower concentrations than those used alone. *Salmonella* viable counts fell to zero within 10 min of contact with reuterin + lactic acid at 10 times the concentrations used in combination, compared to 18 h in the case of reuterin + microcin J25. Sprayed onto chilled chicken carcasses, this reuterin + lactic acid mixture reduced *Salmonella* spp. counts by 2.02 Log CFU/g, whereas reuterin + microcin J25 and peracetic acid reduced them by respectively 0.83 and 1.13 Log CFU/g. The synergy of reuterin with lactic acid or microcin J25 as inhibitors of bacterial growth was significant. Applied as post-chill spray, these mixtures could contribute to food safety by decreasing *Salmonella* counts on chicken carcasses.

Keywords: chicken carcass, natural antimicrobials, microcin J25, reuterin, lactic acid, *Salmonella*

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INTRODUCTION

Nontyphoidal serovars of *Salmonella enterica*, commonly associated with poultry, pose a well-known health risk. As foodborne pathogens, they cause much hospitalization, sometimes with fatal outcome. About 35% of foodborne illnesses traceable to poultry are due to *S. enterica* at a social cost of about $700 million annually (Morris et al., 2011). The prevalence of *Salmonella* on processed poultry meat has been estimated at 20–43% (Scheinberg et al., 2013; Trimble et al., 2013). According to the Canadian Food Inspection Agency (2018), all the holders of licenses to produce poultry for distribution are expected to have control strategies in place to eliminate microbial pathogens or prevent them from reaching dangerous levels. The Performance

Standard for *Salmonella* in young chicken carcasses tolerates up to five positive test results in a set of 51 samples (Annex U: USDA, 2017). Poultry producers and processors must therefore employ efficient preventive strategies of *Salmonella* control throughout the chain.

A variety of treatments have been reported to reduce the microbial load of chicken carcasses after slaughter. These include hot water, infrared radiation, gamma radiation, and spray/chill systems enhanced with chemicals such as chlorine, trisodium phosphate, hydrogen peroxide, ozonated water, and ethanol (El-Ziney et al., 1999). Furthermore, a wide range of organic acids have been considered and tested in poultry products. The lack of safety and the chemical nature of most of these compounds make their use in the food sector controversial. The increasing demand of the consumers for safer and natural compounds makes the development of new alternatives urgently needed. Previously, chlorine has been utilized as one of the primary antimicrobial agents in poultry processing plants for carcass decontamination; but replaced with peracetic acid during the past decade. Obtained by combining acetic acid and hydrogen peroxide, peracetic acid is effective at concentrations of 200–2000 ppm in aqueous solution. However, it is unstable, corrosive, and reportedly an irritant to the upper respiratory tract, eyes, and skin (Dittoe et al., 2019). When used for decontamination purposes, it can cause undesirable color, texture, and flavors to develop in chicken products. The search continues for the means of ensuring food safety while maintaining quality using milder alternative natural compounds.

Bio-preservation refers to the use of microorganisms and/or their metabolites to increase product shelf life and ensure food safety. Lactic acid bacteria are able to produce a variety of antimicrobial substances, including organic acids, bacteriocins, and low-molecular-mass compounds such as short-chain fatty acids and reuterin (Reis et al., 2012; Hernández-Aquino et al., 2019). Reuterin (3-hydroxypropionaldehyde) is a neutral broad-spectrum antimicrobial compound produced from glycerol by *Lactobacillus reuteri*. It is water-soluble, effective over a wide pH range and not inactivated by enzymes (El-Ziney et al., 1999). These advantages make it suitable as a preservative in a variety of foods including meat and poultry products. Bacteriocins are proteinaceous molecules exhibiting bacteriostatic or bactericidal activities covering relatively narrow spectra of bacterial taxa, generally related closely to the producing strain. They act by forming pores in cell membranes and/or inhibiting cell wall synthesis (Roces et al., 2012). Produced by *Escherichia coli*, microcin J25 is bactericidal to several Gram-negative foodborne pathogens including *E. coli* and *Salmonella*. Its peculiar lasso structure makes it highly resistant to thermal denaturation. Though attractive to the food industry, it is being adopted very slowly in large part because of its narrow spectrum of activity, sensitivity to food enzymes, and the possible development of resistant variants of pathogens.

In general, bacteriocins produced by Gram-positive bacteria have little or no impact on the viability of Gram-negative bacteria such as *Salmonella*. This resistance is due to the outer membrane, which acts as a barrier against the diffusion of large molecules such as proteins and hydrophobic substances including some antibiotics (Prudencio et al., 2016). The use of synergic combinations of compounds represents a promising strategy to overcome this obstacle. Counts of *S. enterica* in soybean sprouts have been reduced significantly using washing solutions containing enterocin AS-48 with lactic, polyphosphoric, peracetic or hydrocinnamic acids, or sodium hypochlorite (Molinos et al., 2008). Inhibition of planktonic and biofilm cultures of *E. coli* by colistin has been enhanced with nisin/enterocin (Al Atya et al., 2016). Bovicin HC5 appears to be effective against *Salmonella* when combined with EDTA (Prudencio et al., 2016). Nisin and high hydrostatic pressure appear to enhance each other as inactivators of total aerobic bacteria (Zhao et al., 2013). The use of antimicrobial combinations also makes the compounds effective at lower concentrations and the emergence of resistant variants much less likely (Bassetti and Righi, 2015; Gupta and Datta, 2019).

The objective of the present study was to evaluate reuterin, microcin J25, and lactic acid separately and in combination as natural-sourced inhibitors of *Salmonella* on broiler chicken carcasses.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Salmonella enterica serovar Enteritidis MNHN kindly provided by Prof. Sylvie Rebuffat (Muséum national d'Histoire naturelle, Paris, France), *Salmonella* Heidelberg CMBL4-8 (Université Laval METABIOLAC collection), and *Salmonella* Newport ATCC 6962 were used as test strains for antibacterial activity assays. *Lactobacillus reuteri* from broiler chicken intestine (isolate C1-14, unpublished) and *E. coli* MC4100 carrying the pTUC202 plasmid were used respectively for reuterin and microcin J25 production. *Salmonella* strains were maintained as glycerol stock at −80°C and cultured in nutrient broth (NB, Oxoid) at 37°C for 18 h prior to use. *Lactobacillus reuteri* was maintained in MRS broth (Nutri Bact, Terrebonne, Canada) and cultured at 37°C for 18 h under anaerobic conditions (Forma Scientific, United States). *Escherichia coli* was cultured at 37°C overnight under aerobic conditions in Luria-Bertani (LB) broth (Difco, Sparks, MD, United States) supplemented with 34 μg/ml chloramphenicol (MilliporeSigma, ON, Canada).

A mixture of *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Newport was used for the carcass trial. The three serovars were activated at 37°C in NB and sub-cultured by transferring 0.1 ml of 24 h suspension to 10 ml of fresh NB. They were then mixed together and centrifuged at 5000 × *g* for 15 min at 20°C (Multifuge 1S-R, Heraeus, Osterode, Germany) and washed twice with sterile buffered peptone water (Hardy Diagnostics, Santa Maria, CA, United States). The final suspension in peptone water was used as test inoculum, adjusted to a viable count of about 8 $\log_{10}$ CFU/ml. *Salmonella* spp. were enumerated on XLT-4 agar (Hardy Diagnostics).

Production and Quantification of Reuterin, Lactic Acid, and Microcin J25

A two-step fermentation process was used to produce reuterin as described previously (Vimont et al., 2019). *Lactobacillus reuteri* was cultured in 1 L of MRS medium supplemented with 20 mM glycerol and incubated overnight at 37°C.

The cells were then harvested by centrifugation at 1500 × *g* for 10 min at 20°C, washed with potassium phosphate buffer (0.1 M, pH 7.0), resuspended in 100 ml sterile aqueous solution of glycerol (300 mM). Reuterin was then collected after 2 h by centrifugation (10,000 × *g*, 10 min, and 4°C) and filtration through 0.2-μm pore size membrane filter. High-performance liquid chromatography (HPLC) was used to quantify the reuterin. The solution was analyzed by an HP1100 (Agilent Technologies, CA, United States) on a Coregel ION300 column (7.8 × 300 mm, Cobert Associates, Inc., Saint Louis, United States) with 10 mM H_2SO_4 as eluent at 40°C and a flow rate of 0.4 ml/min. Components were identified and quantified using a refractive index detector (Agilent Technologies). The compound was stored in solution at −20°C until use.

Microcin J25 was produced by *E. coli* MC4100 cultured in the minimal medium M63 following the method described and published by our laboratory (Hammami et al., 2015; Boubezari et al., 2018; Naimi et al., 2018; Ben Said et al., 2020). The bacteriocin was recovered from the supernatant of overnight culture using a Sep-Pak C18 35 cc vac cartridge (Waters, Milford, United States) at 4°C. Its concentration was calculated using an HPLC method previously described (Gomaa et al., 2017).

Lactic acid purchased from Laboratoire Mat Inc. (QC, Canada) was diluted in distilled water to achieve desired concentration before being used and sterilized by microfiltration (0.2 μm, MilliporeSigma).

The inhibitory activity of antimicrobial compound was verified visually using the agar well diffusion method (Naimi et al., 2018). About 25 ml of sterile medium containing 0.75% (w/v) agar was seeded with 1% (v/v) of an overnight culture of *Salmonella* Enteritidis and then poured into a sterile Petri dish. After solidification, wells were then cut and filled with 80 μl of the compound to be tested. Plate was incubated at 37°C for 18 h and the diameter of the inhibition zone was measured by a ruler.

Determination of Minimum Inhibitory Concentration and Synergism

A microdilution method described previously (Ben Said et al., 2020) was used with minor modifications. Two-fold serial dilutions in NB starting from 125 μl of tested antimicrobial compounds were prepared in assay plates (96 wells, Becton Dickinson Labware, Franklin Lakes, NJ, United States). Each well received 50 μl of overnight *Salmonella* Enteritidis culture diluted 1,000-fold in fresh medium. The plates were incubated for 18 h at 37°C and the absorbance at 595 nm was measured every 20 min using an Infinite® F200 PRO photometer (Tecan US inc., Durham, NC). The minimum inhibitory concentration (MIC) was the lowest concentration that prevented visible bacterial growth.

Synergic activity was evaluated using the checkerboard assay (Garcia, 2010; Laishram et al., 2017). Wells containing 50 μl of each antimicrobial agent received 100 μl of overnight *Salmonella* culture diluted to 5 × 10^5 CFU/ml. The plates were incubated at 35°C for 24 h under aerobic conditions (Hanchi et al., 2017). The fractional inhibitory concentration index (FICI) was calculated as follows:

$$FICI = \frac{MIC\ of\ \ compound\ A\ in\ combination}{MIC\ of\ \ compound\ A\ alone} + \frac{MIC\ of\ \ compound\ B\ in\ combination}{MIC\ of\ \ compound\ B\ alone}$$

In the remainder of this paper, MIC_c (MIC_c A or MIC_c B) refers to each MIC of each compound when used in the synergetic combination (A + B). The interaction is synergic if FICI is ≤0.5, additive or indifferent if FICI is in 0.5–4 range, and antagonistic if FICI is ≥4 (Hanchi et al., 2017). The minimum and fractional inhibitory concentrations were calculated using duplicate medians obtained in three independent experiments.

Measurement of *Salmonella* Growth Inhibition

Inhibition of *Salmonella* Enteritidis by reuterin, microcin J25, and lactic acid alone or in combination was evaluated in micro-assay plates. Bacteria were grown in LB, centrifuged (Multifuge 1S-R, Heraeus, Osterode, Germany) at 2500 × *g* for 15 min at 4°C, washed, and re-suspended in 0.85% saline. Plate wells were received 10^5 CFU/ml bacterial suspension, supplemented with antimicrobial agents, and incubated at 37°C. The concentration of each antimicrobial agent was MIC_c determined above. Growth was measured as absorbance at 595 nm (Tecan US inc., Durham, NC) every 45 min for 24 h based on three experimental repetitions. Viable counts were obtained in duplicate for 0, 3, 6, 12, and 24 h using the drop plate count method on LB agar (Difco, Sparks, United States).

Bacterial Inactivation Time-Course Curves

LB containing reuterin + lactic acid or reuterin + microcin J25 at 5 or 10 times the MIC_c was inoculated with *Salmonella* at 10^5 CFU/ml and held under ambient condition. Viable cells remaining after each exposure time were counted on LB agar. The exposure times were 10, 20, and 30 s; 1, 5, 10, 15, 20, and 30 min; and 3, 6, 12, 18, 20, 22, and 24 h.

Effectiveness of the Inhibitors on Broiler Carcasses

A total of 24 commercial broiler carcasses obtained immediately after processing without antimicrobial treatment were assigned randomly to the four treatment groups. A Health Canada pathogen challenge test protocol (Health Canada, 2012) was modified slightly for the mixed *Salmonella* test inoculum used in this study. The carcasses were inoculated on the medial and lateral sides with 1 ml (five times 200 μl) of bacterial suspension and then placed under a biohazard hood for 20 min. The load of attached cells was about 10^5 CFU per gram chicken carcass.

Carcasses were then treated by spray with 200 ml of the antimicrobial formulas including water, reuterin + lactic acid, reuterin + microcin J25, and peracetic acid. The procedure

was repeated for a total of six carcasses per treatment (3 × 2 replicates; Lemonakis et al., 2017). The peracetic acid was a commercial product (CHINOOK, Sani-Marc Inc., Victoriaville, QC, Canada) prepared according to the manufacturer's instructions. The natural inhibitors were applied at 10 times the MIC_c and the peracetic acid concentration was 0.1%. To reduce cross contamination, treated carcasses were placed in individual sterile poultry rinse bags (Nasco, Madison, United States) for holding at 4°C to mimic the refrigeration conditions used at industry level. They were tested for total aerobes and *Salmonella* after 24 h.

Each carcass was rinsed with 400 ml of buffered peptone water containing 0.1% sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ) to neutralize residual antimicrobial compound (Lemonakis et al., 2017) and then shaken vigorously for 60 s in a sterile chicken-rinse bag. Rinsing solution was collected in a conical tube and a 10 µl aliquot was diluted serially in 90 µl of phosphate-buffered saline in a flat-bottom 96-well micro-assay plate. Diluted aliquot was plated on tryptic soy agar for counting aerobic bacteria and on XLT-4 agar for *Salmonella*. These plates were incubated at 37°C for 24 h, and colonies were counted manually. On XLT-4 agar, only colonies with a black center were counted as *Salmonella*.

Statistical Analysis

Raw counts were log-transformed for statistical analysis. Reductions were determined as $\log_{10}$ (N_0/N), where N_0 was the average control count and N the experimental treatment count. Data were analyzed using SPSS software (version 22.0, IBM Corporation, Armonk, NY, United States) for ANOVA. Significant differences ($p < 0.05$) between treatment effects were declared on the basis of the Tukey's test.

RESULTS

Inhibitory Activity and Synergy of the Antimicrobial Agents

Reuterin and microcin J25 purity was evaluated by HPLC, and anti-*Salmonella* activity was confirmed by the agar diffusion test (**Figure 1**). The MIC values of the individual compounds and the two combinations against *Salmonella* Enteritidis are shown in **Table 1**. Reuterin alone had a MIC of 2 mM; lactic acid and microcin J25 inhibited *Salmonella* Enteritidis at 0.31% and 0.03 µM, respectively. When they were used in combinations, the MIC_c of the combinations were four times lower (0.5 mM, 0.078%, and 0.008 µM). According to the equation above, the combinations reuterin + lactic acid and reuterin + microcin J25 were both synergic (FICI = ¼ + ¼ = 0.5) at lower concentrations. Since no synergetic effect (FICI = 1) was observed for microcin J25 + lactic acid, this formula was not considered in this study.

Inhibition of *Salmonella* Growth by the Synergic Pairs

Figures 2A,B show the growth curves of *Salmonella* Enteritidis in LB broth containing reuterin, lactic acid, or microcin J25 alone or in combination at the MIC_c are listed in **Table 1**. Both combinations were entirely effective inhibitors of *Salmonella* growth for 24 h, whereas reuterin (0.5 mM) was the only effective single agent, stopping growth for 12 h. Neither lactic acid (0.078%) nor microcin J25 (0.008 µM) alone lowered viable counts, although optical density did appear to have been affected slightly.

In the absence of inhibitor, the viable count reached 11.5 $\log_{10}$ CFU/ml (**Figure 2B**). The combination of reuterin with lactic acid or microcin J25 inhibits the growth over time at these low concentrations. Although the bacteria were still present, slight drops of 0.27 and 0.54 $\log_{10}$ CFU/ml were observed for reuterin + lactic acid and reuterin + microcin J25 (**Figure 2B**). The agar diffusion result is shown in **Figure 2C** by using a high concentration to clearly distinguish the inhibition zone of the substance visually. There was no inhibition when reuterin or microcin J25 was used alone, but a 17-mm inhibition zone appeared when used in combination. And compared with lactic acid alone (16 mm), reuterin + lactic acid had a larger (25 mm) inhibition area.

Concentration Dependency of Anti-*Salmonella* Bactericidal Activity

The loss of viability of *Salmonella* Enteritidis in the presence of higher concentrations of the inhibitors is shown in **Figure 3**. Addition of five times the MIC_c of reuterin + lactic acid inhibited *Salmonella* within 30 min, and reuterin + microcin J25 (five times MIC_c) induced 2.58 Log_{10} CFU/ml decrease of *Salmonella* counts within 24 h. At 10 times the MIC_c of the reuterin + lactic acid pair, counts dropped to zero in 20 s (**Figure 3A**). However, more than 12 h were needed to get a similar drop at 10 times the reuterin + microcin J25 MIC_c (**Figure 3B**).

Validated by the agar diffusion inhibitory results of microcin J25 shown in **Figure 4**, *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Newport were selected to prepare a mixed inoculum, which was used for the following chicken carcass trial. The corresponding viable counts after treatment in broth culture at 10 times the MIC_c are shown in **Figure 5**. Reuterin + lactic acid significantly ($p < 0.05$) decreased the count of *Salmonella* immediately after treatment, and reuterin + microcin J25 had this significant effect after 6 h. These results confirmed that reuterin + lactic acid was the more potent of the two combinations, with an unequivocal bactericidal effect measurable within 10 min, while after 12 h for reuterin + microcin J25.

Antibacterial Activity of the Inhibitors on Chicken Carcasses

Total aerobes and *Salmonella* on broiler carcasses treated with the inhibitors in comparison with peracetic acid are shown in **Figure 6**. The two antimicrobial combinations showed a significant effect ($p < 0.05$), and the reuterin + lactic acid appeared to be even better than the industrial product used as prescribed, reducing total aerobes (**Figure 6A**) by 1.99 Log_{10} CFU/g relative to the negative control treatment (water).

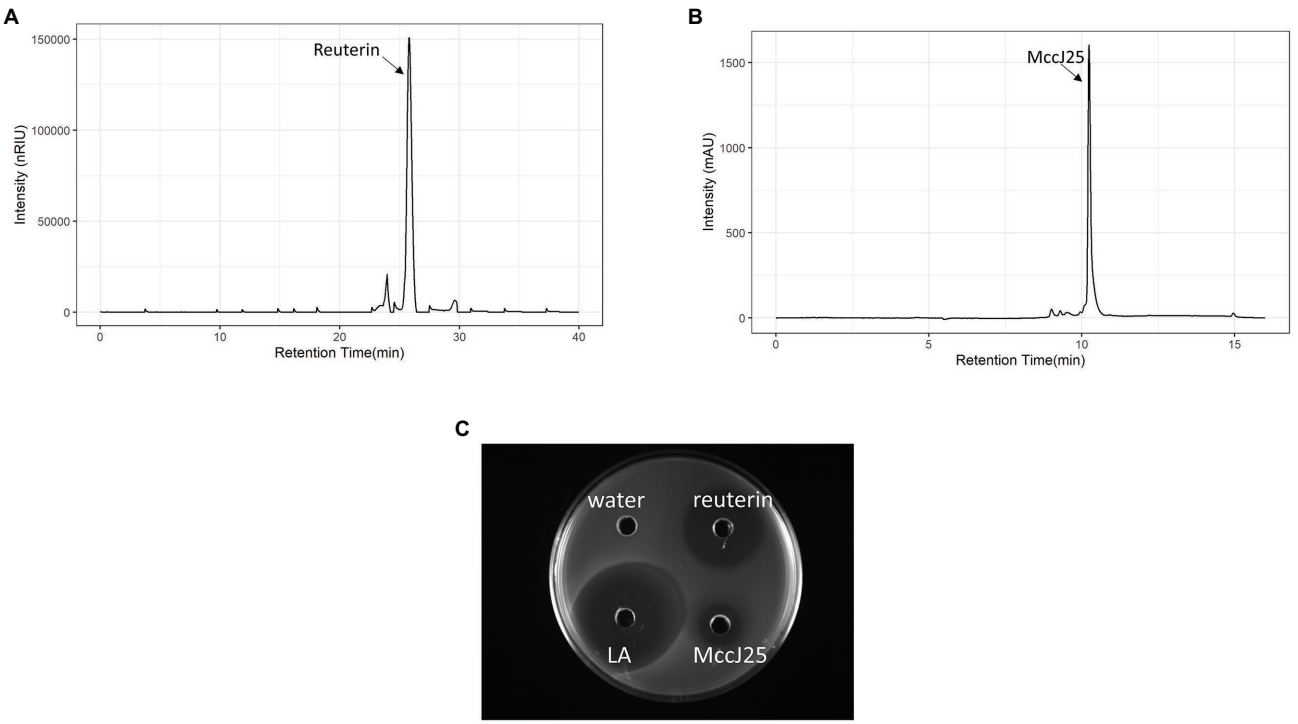

FIGURE 1 | HPLC chromatograms of reuterin **(A)** and microcin J25 (MccJ25; **B)**; inhibition of *Salmonella* Enteritidis MNHN by reuterin, MccJ25, and lactic acid (LA) in nutrient agar **(C)**.

TABLE 1 | Inhibitory concentrations of the compounds against *Salmonella* Enteritidis.

	Antimicrobial combination	
	Reuterin (mM)-Lactic acid (v/v%)	**Reuterin (mM)-Microcin J25 (μM)**
MIC (A/B)	2/0.31	2/0.03
MIC_c (A + B)	0.5 + 0.078	0.5 + 0.008
FICI	0.5	0.5
Interaction type	Synergic	Synergic

MIC (A/B) = minimum inhibitory concentration of agent A or B alone;
MIC_c (A + B) = each MIC of each compound when used in combination (A + B);
FICI = fractional inhibitory concentration index.

The peracetic acid and reuterin + microcin J25 treatment achieved similar total aerobic bacterial counts ranging from 3.51 to 3.99 Log_{10} CFU/g on carcasses. Their difference of bacterial number reduction was not statistically significant ($p > 0.05$).

Much the same pattern was obtained for *Salmonella*, as shown in **Figure 6B**. The significant reduction was 2.02 Log_{10} CFU/g for reuterin + lactic acid relative to treatment with water, while reuterin + microcin J25 and peracetic acid had smaller and similar potencies with a reduction of 0.83 and 1.13 Log_{10} CFU/g ($p < 0.05$), respectively. The treatment of both reuterin + microcin J25 and peracetic acid did not differ significantly in recovered *Salmonella* (3.66 and 3.36 Log_{10} CFU/g of *Salmonella*).

DISCUSSION

The current shift in consumer preference for foods preserved using natural substances rather than chemicals is not likely to fade. Demand for more nutritious and safer food is expected to continue to grow, which will increase the need for more natural and affordable inhibitors of foodborne pathogens. Natural antimicrobial compounds offer several advantages over current treatments. One of these is that using them in combinations should lessen the likelihood of antimicrobial resistance developing among the targeted pathogens. The goal of this work was to study the effectiveness of two synergic combinations of natural antimicrobial compounds applicable by spraying to reduce the viability of *Salmonella* on chicken carcasses.

Decontamination of poultry carcasses involves primarily rinsing and chilling with water followed by spraying or dipping in solutions that may contain chlorine, organic acids, and in some cases bacteriocins. However, chlorine concentrations as high as 3,400 ppm have been found to fail to eliminate *Salmonella* Typhimurium on turkey even while causing unacceptable changes in the appearance of the meat (Teotia and Miller, 1975). Organic acids, especially lactic and acetic, have been widely used on chicken meat surfaces because of their availability and low cost, but their efficacy may depend on the type of surface and on the tenacity with which the bacteria attach (Burfoot et al., 2015). Bacteriocins, such as nisin, are active against *Clostridium* spp. and *Listeria* spp. (Özel et al., 2018) but do not inhibit Gram-negative bacteria attached to meat surfaces

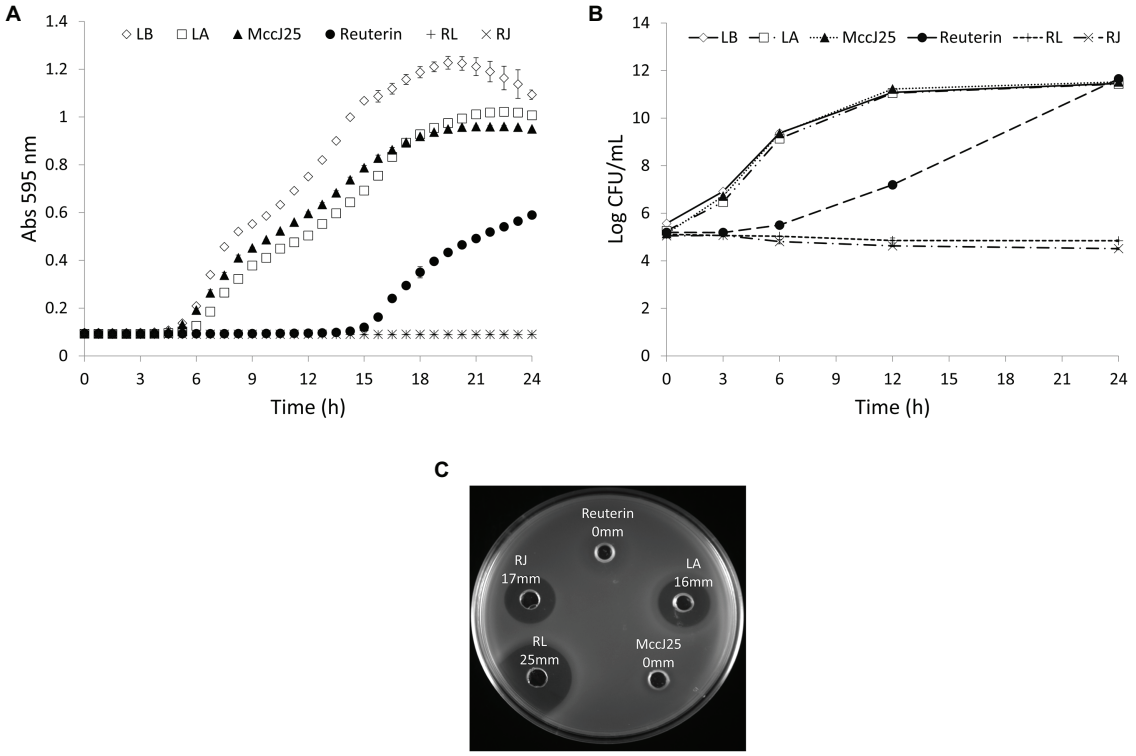

FIGURE 2 | Inhibition of *Salmonella* Enteritidis MNHN growth at 37°C in LB broth by reuterin, lactic acid (LA), microcin J25 (MccJ25), reuterin + LA (RL), and reuterin + MccJ25 (RJ). **(A)** Optical density measurement; **(B)** viable counts; and **(C)** agar diffusion test. Error bars indicate standard deviation.

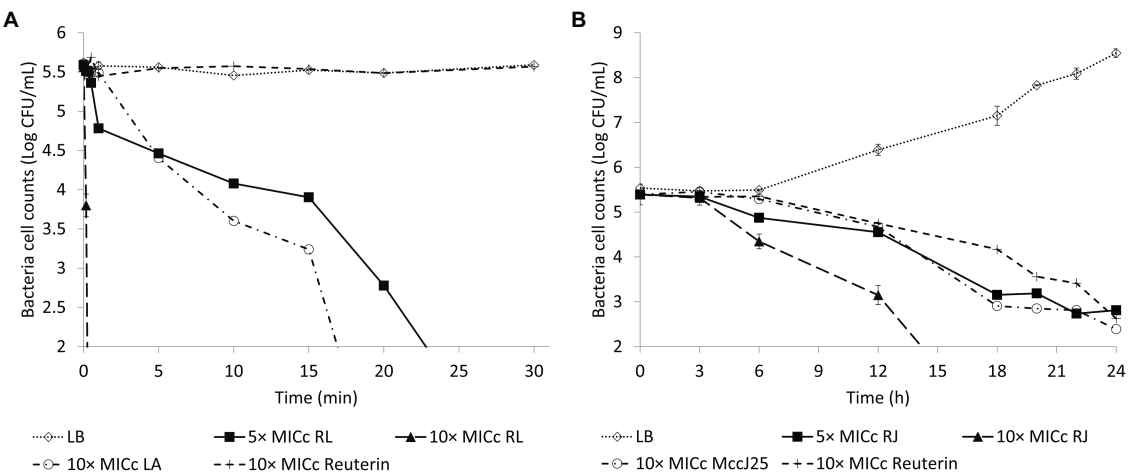

FIGURE 3 | Survival of *Salmonella* Enteritidis MNHN at room temperature after exposure to **(A)** reuterin, lactic acid (LA), and reuterin + LA (RL); or **(B)** reuterin, microcin J25 (MccJ25), and reuterin + MccJ25 (RJ) at 5 or 10 times minimum inhibitory concentrations in combination (MICc). Bars indicate standard deviation.

(He et al., 2016). Peracetic acid is an oxidizing agent that appears to denature proteins and enzymes and increase cell wall permeability by disrupting sulfhydryl and sulfur bonds (Rosario et al., 2021). However, it is corrosive, unstable, and an irritant in the upper respiratory tract even at low concentrations (Dittoe et al., 2019). The ideal treatment would be effective at low concentrations of agents having a broad antibacterial spectrum and low risk of being thwarted by the development of resistance strains.

In a previous study (Kuleasan and Cakmakci, 2002), reuterin alone was reported to inhibit the growth of *Listeria monocytogenes* on the surface of sausages. Lactic and acetic

acids in tandem have been found to reduce *Salmonella* cocktail (*Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Heidelberg ATCC 3347–1, and a wildtype *Salmonella*) on chicken parts (Ramirez-Hernandez et al., 2018). Microcin J25 has been shown to reduce *Salmonella* Typhimurium counts by approximate 3 Log_{10} CFU/ml in pork mincemeat extract (Yu et al., 2019). However, to our knowledge, no study has examined the efficacy of the combination of reuterin and bacteriocin. Inhibition of *E. coli* O157:H7 and *L. monocytogenes* by reuterin + lactic acid has been reported once, in the context of cooked pork surfaces (El-Ziney et al., 1999). Although 5% lactic acid added into 500 AU/ml of reuterin enhanced the antimicrobial activity by reduction of 1.88–2.9 Log_{10} CFU/cm^2 for *E. coli* O157:H7 and 0.64–0.7 Log_{10} CFU/cm^2 for *L. monocytogenes*, the concentration used for each compound was not determined by synergy test and considerably higher than the concentration of reuterin + lactic acid (5 mM + 0.78%) used in our work. In the present study, the use of a cocktail of three different *Salmonella* strains (*Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Newport) is based on recommendation of Health Canada challenge protocol. According to this recommendation, using a mixture of at least 3–5 different strains allows to take into consideration variation in growth and survival characteristics among strains and therefore provide more consistent and representative results. These three strains are sensitive at different extents to microcin J25 as shown by agar diffusion assay. Microcin J25 is known for its inhibition activity against Gram-negative bacteria including *Salmonella*, while reuterin is known for its large spectrum of inhibition activity against both Gram-positive and Gram-negative bacteria. Combining these two antimicrobials will allow a stronger inhibition activity and large spectrum of inhibition. We have shown that combinations of reuterin with lactic acid or microcin J25 are synergic as inhibitors of *S. enterica* serovars. At 10 times the MIC_c, both combinations reduced *Salmonella* counts to undetectable level within contact times that are applicable in the commercial context. Also, we can postulate that the mechanism of action of both combinations depends on the initial concentration. Indeed, at higher concentration (10 times MIC_c), the inhibition activity is bactericidal since we did not see growth over the first 24 h. However, at concentrations

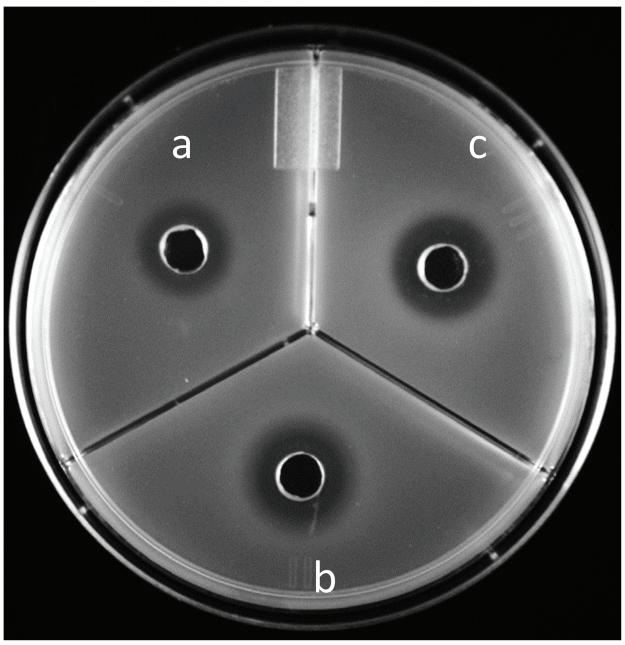

FIGURE 4 | Microcin J25 agar diffusion test using **(A)** *Salmonella* Enteritidis MNHN, **(B)** *Salmonella* Heidelberg CMBL4-8, and **(C)** *Salmonella* Newport ATCC 6962

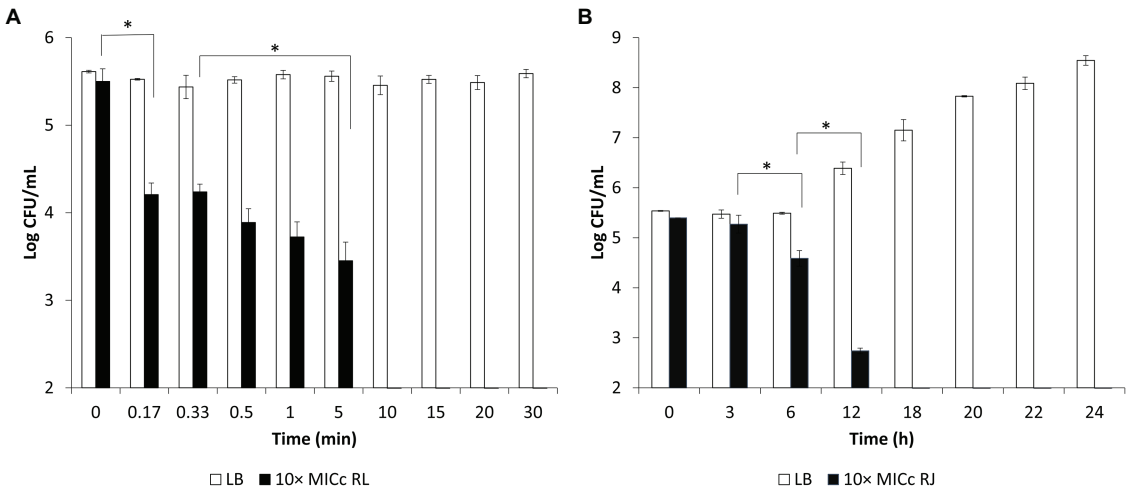

FIGURE 5 | Counts of viable *Salmonella* mixture (*Salmonella* Enteritidis MNHN, *Salmonella* Heidelberg CMBL4-8, and *Salmonella* Newport ATCC 6962) after exposure in 10 times minimum inhibitory concentrations in combination (10 × MIC_c) of reuterin + lactic acid (RL; **A)** and reuterin + microcin J25 (RJ; **B)**. LB broth as negative control. Bars indicate standard deviation. *$p < 0.05$.

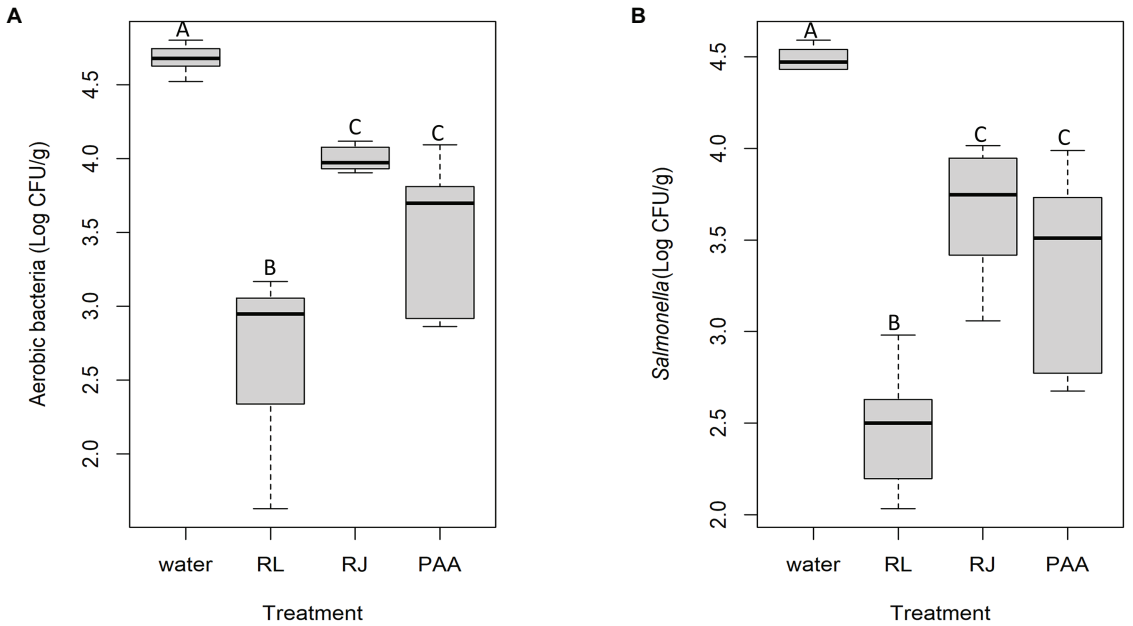

FIGURE 6 | Total aerobic **(A)** and *Salmonella* **(B)** viable counts on chicken carcasses sprayed with antimicrobials (RL = reuterin + lactic acid, RJ = reuterin + microcin J25, PAA = peracetic acid; $n = 6$ for each treatment). Different letters indicate significant differences ($p < 0.05$).

lower than 10 times MIC_c, the antimicrobial effect was bacteriostatic. These results were confirmed by *Salmonella* growth and concentration dependency assay.

The effectiveness of reuterin + lactic acid and reuterin + microcin J25 for reducing *Salmonella* counts on chicken carcasses suggests that these mixtures should be tested in poultry processing. Lactic acid is allowed at concentrations up to 5% to reduce *Salmonella* counts on animal carcasses (Lemonakis et al., 2017). Using sprayed 2% lactic acid, *Salmonella* Typhimurium has been reduced by 2 Log_{10} and total aerobes by 1.03 Log_{10} on chicken carcasses (Yang et al., 1998). In contrast, dipping in 5% lactic acid was found to reduce *Salmonella* by about 0.8–1.7 Log_{10} (Lemonakis et al., 2017). Microcin J25 used alone at concentrations of 8–16 μg/ml gave 3 Log_{10} reductions of *Salmonella* Typhimurium and *E. coli* O157:H7 (Yu et al., 2019). In our study, the concentration of lactic acid was less than 1%, which should have a smaller effect on sensory attributes, and both microcin J25 and reuterin were also used at lower concentrations in the combination. On the other hand, the antimicrobial combination at low concentration would consist ideally of compounds that work by fundamentally different mechanisms in order to lessen the likelihood of the development of resistance in bacterial species that pose serious threats to human health. In addition, in terms of cost, lower concentration and smaller volume are more cost-effective through the use of spray. Moreover, the raw materials used in the production of reuterin and microcin, such as glycerol, glucose, etc., are cheap and easy to obtain.

The method of application of the inhibitory product to processed poultry may have a measurable impact on the antimicrobial effect obtained. Immersion in acetic acid and in acidic electrolyzed oxidizing water was found to reduce *Salmonella* Typhimurium by respectively about 1.41 and 0.86 Log_{10}, whereas spray-washing with the same solutions had no effect (Fabrizio et al., 2002). In the present study, spraying peracetic acid reduced the *Salmonella* load by about 1.13 Log_{10} CFU/g compared to the control. On post-chilled ground chicken, 0.1% peracetic acid brought a nearly 1.5 Log_{10} reduction (Chen et al., 2014). These observations suggest that chilling might increase the efficacy of subsequent treatments intended to reduce *Salmonella* counts on poultry (Lemonakis et al., 2017) and that the application method may play an important role and needs to be compatible with chilling. Other factors, such as antimicrobial concentration and contact time, also seem to be involved in the variability of the results reported. In the case of chicken drumsticks dipped for 15 min in solution containing 220 ppm peracetic acid, *Salmonella* Enteritidis counts were reduced by 0.36 Log_{10} CFU/g on day 0 (del Rio et al., 2007). In contrast, post-chill immersion for 20 s in peracetic acid at 400 ppm and 1,000 ppm could reduce *Salmonella* Typhimurium and *Campylobacter jejuni* loads on chicken carcasses by up to 2 Log_{10} CFU/ml (Nagel et al., 2013). In our study, the antimicrobial effects differed not only in terms of numerical reduction but also the time course. The diversity of the mechanisms of action might be one reason for the time variance. The reuterin aldehyde group reacts with primary amines and thiol groups, which are present on many small molecules and proteins (Schaefer et al., 2010), which could explain why reuterin has a broad-spectrum effect on microorganisms. Microcin J25 appears to have two intracellular targets, namely RNA polymerase and the respiratory chain. However, uptake of microcin J25 by the target strain requires the outer membrane receptor FhuA and

the inner membrane proteins TonB, ExbD, ExbB, and SbmA (Ben Said et al., 2020), providing as many opportunities for resistance to develop as a result of structural mutations. In contrast, lactic acid is active in its undissociated form, which penetrates *via* the plasma membrane and reduces intracellular pH as well as disrupting the outer membrane of Gram-negative bacteria (Coroller et al., 2005). The antibacterial effect of the reuterin + lactic acid combination therefore occurs quickly (within 10 min), whereas reuterin in combination with microcin J25 needs somewhat more contact time in order to be effective against *Salmonella*.

Overall, this study shows that spraying a solution of natural inhibitors onto chicken carcasses results in significant but not vast reductions in viable counts of a pathogenic genus. By comparison, smaller than 1 Log_{10} reductions of total aerobic and *Salmonella* counts on broiler carcasses chilled for 45 min in aqueous ozone were observed decades ago (Sheldon and Brown, 1986) and modest reductions (0.53–0.69 Log_{10}) were obtained using acidic solutions relatively recently (Ramirez-Hernandez et al., 2018). It has been shown that bacteria reside not only on exposed skin or muscle surfaces, but also within holes left in the skin by feather removal (Zhang et al., 2013) and that these shelter bacteria from the effects of subsequent antimicrobial treatments. That bacterial loads are smaller in skin-off than skin-on products is therefore hardly surprising (Ramirez-Hernandez et al., 2018). Complete decontamination of poultry carcasses by antimicrobial agents is likely to remain elusive for the foreseeable future.

CONCLUSION

The results of the present study confirm that mixtures of reuterin with lactic acid or microcin J25 are synergic inhibitors of *Salmonella* and therefore warrant testing as candidates for improving the safety of poultry products. The combination of lactic acid with reuterin was more potent than peracetic acid. Synergic combinations of agents at low concentrations could contribute to slowing the development of resistance in pathogenic species and decrease the residual toxicity of food decontamination treatments. Thus, the two combinations, especially reuterin + lactic acid, appear to be suitable for application to broiler carcasses by spraying. Regulatory agencies should consider these results in developing the new strategic plan for *Salmonella* control in poultry meat products based on natural antimicrobials intended to improve occupational health and safety for poultry processing employees. Further study is needed to test these combinations against other pathogenic bacteria, such as *Campylobacter*, and to understand their impact on the shelf life of processed poultry and subsequent changes in the microbiome. Multiple antimicrobial combinations, sequential application, and different methods, such as dipping and immersion, may be needed in order to achieve the desired result.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the birds were raised in an off-campus commercial farm, and the current study was restricted to the microbiological evaluation of bird carcasses.

AUTHOR CONTRIBUTIONS

LZ, LBS, and IF designed the experiment. LZ performed the experiment and wrote the paper. LBS, MD, and IF edited the paper. All authors read and approved the final manuscript.

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Production of Sophorolipid Biosurfactant by Insect Derived Novel Yeast *Metschnikowia churdharensis* f.a., sp. nov., and Its Antifungal Activity Against Plant and Human Pathogens

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Biosurfactants are potential biomolecules that have extensive utilization in cosmetics, medicines, bioremediation and processed foods. Yeast produced biosurfactants offer thermal resistance, antioxidant activity, and no risk of pathogenicity, illustrating their promising use in food formulations. The present study is aimed to assess potential of biosurfactant screened from a novel yeast and their inhibition against food spoilage fungi. A novel asexual ascomycetes yeast strain CIG-6A^T producing biosurfactant, was isolated from the gut of stingless bee from Churdhar, HP, India. The phylogenetic analysis revealed that the strain CIG-6A^T was closely related to *Metschnikowia koreensis,* showing 94.38% sequence similarity in the D1D2 region for which the name *Metschnikowia churdharensis* f.a., sp. nov., is proposed. The strain CIG-6A^T was able to produce sophorolipid biosurfactant under optimum conditions. Sophorolipid biosurfactant from strain CIG-6A^T effectively reduced the surface tension from 72.8 to 35 mN/m. Sophorolipid biosurfactant was characterized using TLC, FTIR, GC-MS and LC-MS techniques and was a mixture of both acidic and lactonic forms. Sophorolipid assessed promising activity against pathogenic fungi viz. *Fusarium oxysporum* (MTCC 9913), *Fusarium solani* (MTCC 350), and *Colletotrichum gloeosporioides* (MTCC 2190). The inhibitory effect of biosurfactant CIG-6A^T against *F. solani* was studied and MIC was 49 μgm/ml, further confirmed through confocal laser scanning microscopy. We illustrated the antifungal activity of sophorolipid biosurfactant from *Metschnikowia* genus for the first time and suggested a novel antifungal compound against food spoilage and human fungal pathogen.

Keywords: ***Fusarium,*** **antifungal, sophorolipid, biosurfactant,** ***Metschnikowia***

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INTRODUCTION

Biosurfactants are amphiphilic molecules comprised of a hydrophobic moiety (hydrocarbon- fatty acid with variable chain length) and variable hydrophilic moieties (phospholipid- phosphate group; neutral lipid- alcohol or ester group; fatty acid or amino acid- carboxylate group) (Ibacache-Quiroga et al., 2013). The biosynthetic gene cluster responsible for the synthesis and assembly of

hydrophobic and hydrophilic moieties of biosurfactants varies depending on the microorganisms such as yeasts, bacteria, and fungi; they can produce a variety of biomolecules such as glycolipids, lipoproteins, lipopeptides, phospholipids, lipopolysaccharides (Ibacache-Quiroga et al., 2013; Van Bogaert et al., 2013). The glycolipids are the most studied biosurfactant and the best known are rhamnolipid, trehalolipid, mannosylerythritol lipids (MELs) and sophorolipids (SLs). SLs are mainly produced by non-pathogenic yeasts, e.g., *Candida batistae, C. apicola*, *Starmerella bombicola*, *Rhodotorula bogoriensis*, *Wickerhamiella domercqiae*, and *Rhodotorula babjevae* while MELs are produced by *Pseudozyma rugulosa*, *P. aphidis,* and *P. antacrtica* (Sen et al., 2017; Hipólito et al., 2020). SLs have wider commercialization value because of its higher production as compared to other glycolipid biosurfactants. They are comprised of a disaccharide sophorose associated to a terminal or sub-terminal fatty acid (chain length of usually 16–18 carbons) and are produced in lactonic (neutral) and acidic (anionic under alkaline conditions) forms (Hipólito et al., 2020; Twigg et al., 2020).

SLs have been recorded for a wide range of antimicrobial activities against various bacterial and fungal pathogens via., increased permeabilization, and membrane destabilization (Bluth et al., 2006). Earlier studies concerned with antifungal activities of SLs are somehow limited; therefore, there is necessity for more research to investigate the yeast species having high productiveness of biosurfactants and their applicability as an antifungal agent. The principal fungal pathogens such as *Fusarium*, *Colletotrichum*, *Aspergillus*, and *Botrytis* result in significant loss of wholesome stored fruits, grains and vegetables. The *Fusarium* genus causes the yellow and orange sporulation majorly in fruits and contaminates corn and roots (Hipólito et al., 2020). *Colletotrichum* is a major post-harvest pathogen which causes a significant loss of fruits such as mango, papaya, avocado, citrus and apple. It causes the brown rot on the infected areas initially, then the whole fruit may rot, leading to the development of fruiting bodies on the rotten surface (Tian et al., 2011). The continued use of agrochemicals has raised a serious concern for environment and food safety, so the plausible management strategies need to be investigated.

In this study, the yeast was isolated from insect gut because insect gut harbors an astonishing diversity of undescribed yeast species and is still grossly under-reported (Suh et al., 2005; De Vega et al., 2012). *Metschnikowia* clade is strongly associated with the insect-flower ecosystem (Gimenez jurado et al., 2003) and regularly encountered with the fruits, flowers, plant tissues, digestive tract, and frass of insects (Lachance, 2011). The yeast associated with the bees has a putative role as a producer of antimycotic substances which protect the bee hive from diseases (Hayden et al., 1997). So, analyzing the insect gut for novel yeast and their product might lead to more useful bioactive compounds. In the current study, a total of 3 strains were isolated from the gut of stingless bee, and they all represent one novel species. The biosurfactant of the strain CIG-6A^T was checked for the antifungal and antibacterial activity. To the best of our knowledge, this is the first report of antibacterial and antifungal activity of biosurfactant from *Metschnikowia* genus.

MATREIALS AND METHODS

Isolation, Characterization and Identification of the Strain CIG-6A^T

Isolation of Yeast Strains

The insect sample was collected from Churdhar, Himachal Pradesh, India, in 2016 and abbreviated as CIG[1]. The insects were placed in a sterile falcon without food for 2–3 days before dissection. The gut inhabiting yeasts were isolated using a previously described method (Suh and Blackwell, 2004). The homogenized sample was plated on yeast malt agar (YM), yeast peptone dextrose agar (YPD), and potato dextrose agar (PDA) supplemented with 100 mg/L of chloramphenicol to reduce the bacterial growth. The yeast colonies were purified, maintained, and preserved in 15% glycerol at −80°C and lyophilized for long-term storage (Saluja and Prasad, 2008).

Morphological and Physiological Characterization

The standard methods explained by Kurtzman were used to characterize the novel isolate (Kurtzman et al., 2011). Carbon assimilation tests were performed in Biolog YT microplate (Biolog, Inc., Hayward, CA) as per manufacturer instructions. Carbon fermentation and nitrogen assimilation tests were performed in a test tube using yeast nitrogen base (YNB), yeast carbon base (YCB) and starved inoculum. Sporulation was checked on different culture media such as YM agar, PDA, potato carrot agar, YCB with 0.01% ammonium sulfate, and cornmeal agar at 25°C for 21 days. The vegetative cell morphology and hyphae formation were observed using a confocal microscope (Nikon Instruments Inc., United States).

DNA Sequencing and Sequence Analysis

DNA isolation, PCR amplification, gel extraction, and sequencing of the ITS/D1D2 domain was performed using the method explained in Saluja et al. (Saluja and Prasad, 2008). The sequences obtained were submitted in the GenBank database and received the accession number MW244067 (ITS region) and MG821162 (D1/D2 region). The sequence of ITS/D1D2 region of the strain CIG-6A^T was compared with the GenBank database using the nBLAST[2] and the MycoBank database using pairwise sequence alignment. Sequences of the closely related species of strain CIG-6A^T were retrieved from the GenBank and aligned by using CLUSTAL W (Thanh et al., 2002). The tree was constructed using Kimura two-parameter correction with 1,000 bootstrap values using the neighbor-joining method in MEGA Version 7.0 (Thanh et al., 2002; De Vega et al., 2012).

Purification and Characterization of Biosurfactant CIG-6A^T

Extraction and Purification

For the biosurfactant extraction, the culture grown for 72 h at 180 rpm at 25°C, was centrifuged (8,000 rpm for 30 min).

[1]CIG, Churdhar Insect Gut.

[2]nBLAST, nucleotide Basic Local Alignment Sequencing Tool.

Extraction was performed using cell-free supernatant with an equal amount of ethyl acetate (1:1 ratio) in the separating funnel. The organic layer was then separated and vacuum dried in a rotary evaporator at 45°C (Fontes et al., 2012). Biosurfactant was purified by silica gel (60–120 mesh) column chromatography (Daverey and Pakshirajan, 2010). Glass column with dimensions 45 × 3.5 cm^2 was packed with silica gel in absolute methanol. The 5 ml of crude sample of biosurfactant (1 g), dissolved in methanol was loaded on the column and eluted by gradient system of methanol: chloroform (0–90% chloroform). The fractions were collected separately and vacuum dried at 45°C.

Biosurfactant Analysis by TLC (Thin Layer Chromatography)

The biosurfactant purified from silica gel (60–120 mesh) was dissolved in 100% methanol. The sample was spotted on the silica gel plate (Merck DC, Silica gel 60) and mobile phase used was chloroform: methanol: water (65: 25: 4, v/v). The silica plate was developed for lipid detection with fumes of iodine in a chamber, and later anthrone reagent was sprayed to detect the sugars. 1,4″-sophorolactone 6′,6″-diacetate (Sigma-Aldrich, United States) commercially available sophorolipid (SL-S) was used as a reference standard (Sen et al., 2017).

Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of biosurfactant were evaluated using infrared spectroscopy (FTIR system, Perkin Elmer, Branford, CT, United States). FTIR of standard SL-S along with the test biosurfactant in ATR (Attenuated total reflectance) was accomplished at a wavenumber and a resolution accuracy of 0.01 and 4 cm^{-1}, respectively, and 32 scans with the association for atmospheric CO_2 (Camargo et al., 2018). All of the data were corrected for the background spectrum.

Fatty Acid Analysis by GC-FID-MS (Gas Chromatography and Mass Spectra)

The esterified sample was used to determine the fatty acid composition of biosurfactant. The sample was prepared using the method explained in literature (Ribeiro et al., 2020). The sample was analyzed in gas chromatography with a flame ionization detector (GC/FID; Thermo-scientific TRACE 1300). The gas chromatograph equipped with DB-5 ms capillary column (30 m in length × 250 μm diameter × 0.25 μm) was used for analysis. The carrier gas used was helium at a flow rate of 1 ml/min. The detector and injector temperature were 320°C. The temperature of the oven was increased to 150°C at 10°C/min, and hold for 4 min, increased to 280°C at 4°C/min and held for 5 min. The fatty acids were identified using a standard external MIDI No. 1300-C mix $C_{9:0}$–$C_{20:0}$.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Biosurfactant separation and structural homologs identification was done by LC-MS (1260 Infinity HPLC, Agilent Technologies, United States) using a reverse-phase, C18 column (250 mm × 10 mm, 150 Å, Waters) (Nuñez et al., 2004) along with standard SL-S. The mobile phase contained solvent A (water supplemented with 1% TFA) and solvent B (acetonitrile having 1% TFA). The gradient elution, i.e., 5–20% solvent B in 5 min, 20–80% B in 25 min, and reverse 80–5% B in 3 min was used in HPLC. Mobile phase flow rate was maintained at 3.0 ml/min. ESI-MS (Electrospray ionization-mass spectroscopy) was accomplished in positive ion mode, and spectral range from 200 to 800 m/z was examined through Agilent Mass Hunter software.

Ionic Character Determination of Biosurfactant

Agar double diffusion method was used to determine the ionic charge of biosurfactant (Santos et al., 2013). Two regularly spaced rows of well were made on agar plate with low degree of hardness (1%). The one row of wells was filled with biosurfactant and one row of wells was filled with compound of known ionic charge. The 20 mM SDS (Sodium dodecyl sulfate) was used as anionic compound and 50 mM barium chloride was used as cationic compound. The biosurfactant from the strain CIG-6A^T was dissolved in methanol; therefore, control of methanol was maintained under similar conditions with SDS and barium chloride.

Physicochemical Properties of Biosurfactant CIG-6A^T

Surface Tension (ST) Measurement

ST of biosurfactant was determined using tensiometer through Wilhelmy plate method at 25°C upto 192 h. For precise measurements, ultrapure water having ST of 72.8 mN/m was used to calibrate the instrument.

Critical Micelle Concentration (CMC)

The CMC was estimated by measuring the ST of the biosurfactant until a constant value of ST was reached. The ST of column purified biosurfactant was measured from 1 to 12 mg/ml concentration up to a ST's constant value. The concentration at which ST value became constant was considered as CMC.

Emulsification Index (%E_{24})

The emulsification index (%E_{24}) was analyzed, as reported earlier (Elshafie et al., 2015). Equal concentration of biosurfactant (2 ml) was added with different hydrocarbons (olive oil, crude oil and mineral oil) and vortexed at maximum speed for 3 min and kept at room temperature for 192 h. The emulsions formed were differentiated with negative control, i.e., YM broth, and positive control, i.e., Tween-20.

Stability Studies

The biosurfactant stability was examined in reference to pH, salinity, and temperature. The temperature stability, pH and NaCl tolerance of crude biosurfactant were investigated by incubating at different temperatures (37, 40, 60, 80, 100, and 121°C for 15 min), different pH (2.0–12.0) and NaCl concentrations (2–15% w/v), respectively.

Antioxidant, Antimicrobial and Hemolytic Activity of Biosurfactant CIG-6A^T

Antioxidant Activity of Biosurfactant Strain CIG-6A^T

Free radical scavenging method was used to evaluate the antioxidant activity by using stable radical 2,2-Diphenyl-1-Picrylhyrdazyl (DPPH). The experiment was performed using the method according to Ribeiro et al. (2020). A stock solution of 200 μM DPPH was prepared in methanol. Different concentrations (2.5, 5.0, 10.0, and 20.0 mg/ml) of biosurfactant (40 μl) was mixed with 250 μl of DPPH to determine the antioxidant activity. L-ascorbic acid was used as standard and used in same concentration as biosurfactant from CIG-6A^T. After 30 min of incubation in dark, the absorbance was read at 517 nm. The experiment was carried out in triplicates and percentage of inhibition was calculated using the following equations $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$. Abs_0 is the control absorbance and Abs_1 is absorbance in the presence of biosurfactant.

Determination of Minimum Inhibitory Concentration (MIC)

Biosurfactant MICs were evaluated using the microtiter plate dilution assay. Bacterial test strains used are *Staphylococcus aureus* (MTCC 1430), *Klebsiella pneumoniae* (MTCC 618), *Vibrio cholerae* (MTCC 3904), *Listeria monocytogenes* (MTCC 839), *Pseudomonas aeruginosa* (MTCC 1934), *Bacillus subtilis* (MTCC 121), *Bacillus cereus* (MTCC 9490), *Salmonella enterica* (MTCC 3232), *Escherichia coli* (MTCC 1610), and *Micrococcus luteus* (MTCC 106). Whereas, biosurfactant antifungal activity was checked against plant and human pathogens viz. *F. solani* (MTCC 350), *F. oxysporum* (MTCC 9913), *Penicillium chrysogenum* (MTCC 160), *C. gloeosporioides* (MTCC 2190), *Alternaria alternata* (MTCC 10576), and *Botrytis cinerea* (MTCC 2349) using a technique of broth microdilution (Santos and Hamdan, 2005). After 48 h, absorbance of the plate was measured at 600 nm through plate reader (BMG Labtech, Germany), and MIC was recorded as the lowest concentration at which growth was not observed.

Hemolysis Assay

Hemolysis assay was performed using a blood sample collected from rabbit (New Zealand White) in a test tube containing EDTA. The blood sample was centrifuged for 5 min at 1,550 rpm and washed thrice with phosphate buffer saline (PBS) (Baindara et al., 2016). 1X-PBS was used to prepare erythrocyte suspension (10%) and incubated along with the increasing biosurfactant concentrations at 37°C for 24 h. The samples were then centrifuged, and cell-free supernatants were used to examine the erythrocyte lysis at a 405 nm wavelength. PBS and 1% Triton X-100 were taken as negative and positive controls, respectively.

Confocal Laser Scanning Microscopy

Cells of *F. solani* were grown in the liquid medium to late logarithmic phase and 1 ml culture was centrifuged for 3 min at 1,000 g, washed and incubated with biosurfactant for two different time intervals (48 and 72 h) at 25°C. After each time point, cells were washed and resuspended in PBS containing PI (10 μmol^{-1}) (Sen et al., 2020). Samples were incubated for 15 min and washed with PBS. Cells were suspended in PBS, and treated cells were observed microscopically (excitation wavelength 460–490 nm, emission wavelength > 520 nm). Untreated cells served as control.

Statistical and Structural Analysis

Data was represented as arithmetic average of minimum of three replicates and error bars defined standard deviations. The evaluation was executed using ANOVA, followed through the Tukey test with 95% of confidence level. For the present study, the biosurfactant's chemical structures were drawn by using MarvinSketch version 20.19[3]. The MarvinSketch is a Java-based software used for chemical drawing and editing of molecules in various file formats.

RESULTS

Isolation, Identification and Characterization of Strain CIG-6A^T

CIG-6A^T Delineation and Identification

On pairwise sequence alignment of ITS region, strain CIG-6A^T showed 5.3% sequence divergence from *M. koreensis* (12 gaps and 3 substitutions) and 6.03% from *M. reukaufii* (7 gaps and 10 substitutions). The sequence of D1D2 region of the strain CIG-6A^T differed from *M. koreensis* by 5 to 5.5% (4 gaps and 24 substitutions) and 5.73% from *M. reukaufii* (8 gaps and 21 substitutions). The phylogenetic analysis based on the sequence of the D1D2 region of 26S rRNA gene placed the strain CIG-6A^T near to *M. koreensis* with 71% bootstrap support (**Figure 1**). Strain CIG-6A^T differs in nutritional requirements from the closely related species by the pattern of fermentation of cellobiose, maltose, sucrose, D-gluconic acid, D-galactose, and palatinose (**Supplementary Table 1**).

Description of *Metschnikowia churdharensis* f.a., sp. nov.

Metschnikowia churdharensis f.a., sp. nov., (chur.dhar.en'sis. N.L. fem. adj. *churdharensis*, related to the place from where it was isolated).

Colonies of the strain CIG-6A^T are raised, dull, smooth, cream colored, entire, and butyrous after 5 days of growth on YMA plate at 25°C (**Supplementary Figure 1A**). The cells are ovoid and occurred in short-chains or singly; budding is polar and measured 8–9 by 5–6 μm (**Supplementary Figure 1B**). On the cornmeal agar plate, after the incubation of 21 days at 25°C, pseudohyphae are produced.

D-glucose, D-cellobiose, maltose, sucrose, and D-mannitol are fermented. D-cellobiose, gentiobiose, maltose, maltotriose, β-methyl-D-glucoside, arbutin (weak), D-sorbitol, sucrose, melezitose (weak), D-glucose, N-acetyl-D-glucosamine (weak), D-glucosamine (weak), salicin (weak), and D-mannitol are assimilated. L-malic acid, L-glutamic acid, D-trehalose, α-keto-glutaric acid, L-arabinose, 2-keto-D-gluconic acid, inulin, D-galactose, xylitol, L-sorbose, melibiose, D-ribose, D-xylose,

[3]http://www.chemaxon.com/products/marvin/marvinsketch/

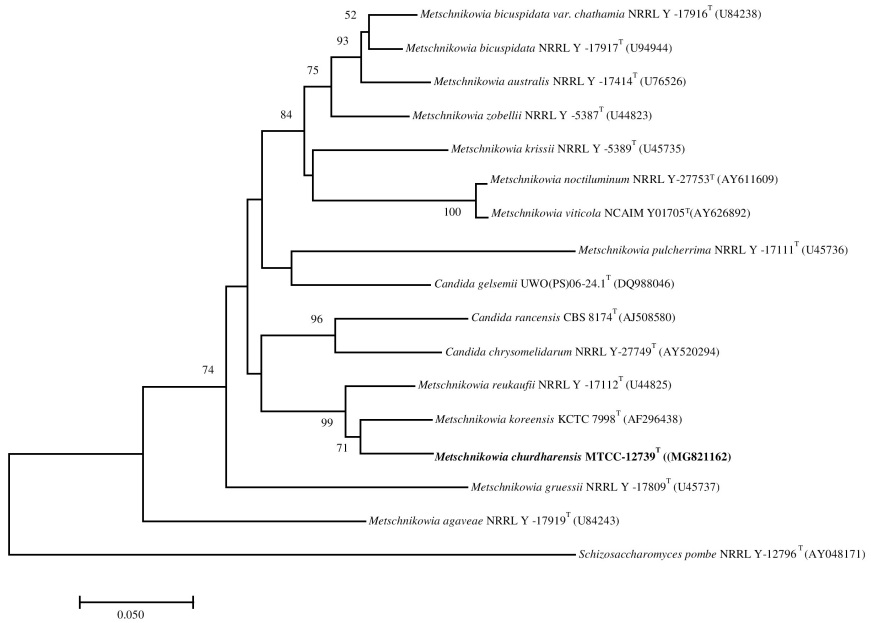

FIGURE 1 | The phylogenetic tree was drawn using a neighbor-joining method based on sequences of D1/D2 domain of 26S rRNA gene, showing the relationship between *Metschnikowia churdharensis* f.a., sp. nov., strain CIG-6A^T and related members of the genus *Metschnikowia*. *Saccharomyces pombe* (T) **(AY041781)** was used as an out-group. Substitution per nucleotide position is bar 0.050. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at nodes.

D-arabinose, erythritol, L-rhamnose, D-raffinose, and glycerol are not assimilated. Ethylamine, ammonium sulfate, cadaverine, nitrate (weak), lysine, nitrite (weak), creatinine (weak), and creatine (weak) are assimilated, and D-glucosamine and imidazole are not assimilated. In a vitamin free base medium, the growth is positive. Growth at 4, 12, 25, and 30°C is positive; at 37°C is negative. The acid production from glucose on custer's chalk medium is negative (no clearing of the medium around the streak). On 10% NaCl agar and 16% NaCl agar, no growth is observed, and on 10% NaCl/5% glucose, the growth is positive or weak; with 16% NaCl/5% glucose, no growth is observed. The growth on 50% glucose is positive; with 60% glucose is positive or weak or delayed. Growth in 1% acetic acid medium is negative. Starch like compound formation is negative, and gelatin liquefaction is positive. Growth in 0.01 and 0.1% cycloheximide is negative. Diazonium blue B (DBB) reaction and urea hydrolysis are negative.

CBS 15318 is the holotype of *Metschnikowia churdharensis* f.a., sp. nov., and isotype is MTCC 12739. This strain was deposited in an inactive metabolic state in Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India and Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. The MycoBank number of the species is MB 824669.

Physicochemical Properties of Biosurfactant CIG-6A^T

Tensioactive properties of the biosurfactant mainly relate to their potential to lower ST and CMC value. Biosurfactant from strain CIG-6A^T effectively reduced the ST from 72.8 to 35 mN/m. Growth kinetics demonstrated surface tension, biomass, and yield of biosurfactant of strain CIG-6A^T in **Figure 2**. As shown in **Figure 3**, CMC value was observed at 5 mg/ml, i.e., ST reduction was not observed any further on increasing the concentration of biosurfactant. Emulsification assay and emulsion stability after 24, 72, and 192 h were studied, as shown in **Table 1**. Biosurfactant was successfully stabilized and emulsified the formed emulsion with olive oil, mineral oil, and crude oil. E_{24} was highest against crude oil with 79% emulsion stability, whereas with mineral oil and olive oil, it was 60% emulsion stability. E_{24} was 0% with the negative control,

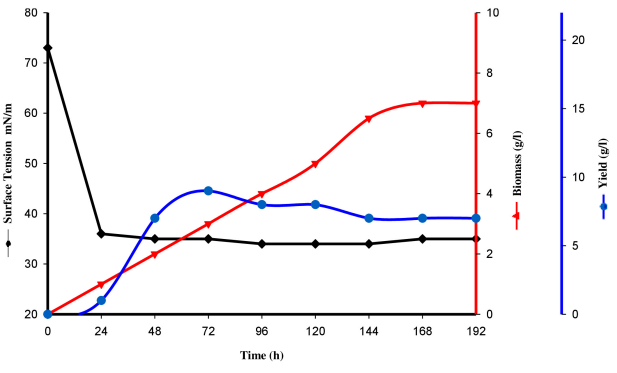

FIGURE 2 | Surface tension, growth kinetics, biomass and yield of biosurfactant from the strain CIG-6A^T grown at 25°C, 180 rpm, 2% inoculum (v/v) plotted as a time function.

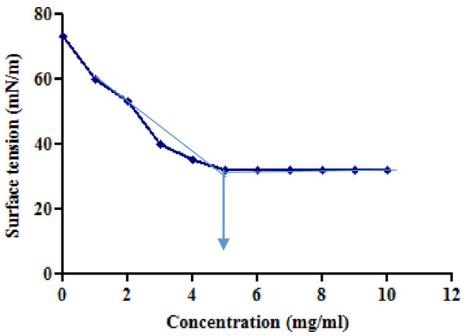

FIGURE 3 | Minimum surface tension and CMC of the biosurfactant was evaluated. Arrow illustrate CMC value of crude biosurfactant produced by the strain CIG-6A^T.

TABLE 1 | Emulsification index (EI) evaluated using biosurfactant produced form *Metschnikowia churdharensis* f.a., sp. nov., CIG-6A^T grown at 25°C, 180 rpm, 2% inoculum (v/v), 1% glucose (w/v), 1%NaCl (w/v) after 24, 72, and 192 h.

S.no	Hydrophobic substrates	Emulsification index (%)		
		24 h	72 h	192 h
1	Mineral oil	63 ± 0.11	63 ± 0.3	60 ± 0.12
2	Olive oil	65 ± 0.32	65 ± 0.2	63 ± 0.13
3	Crude oil	79 ± 0.21	77 ± 0.32	74 ± 0.22

i.e., media, and 100% with the positive control, i.e., Tween-20. The biosurfactant stability in terms of surface tension was evaluated. Thermal stability results revealed that biosurfactant surface tension was maintained up to 121°C (**Supplementary Figure 2A**). No change in surface tension was observed at pH 2.0, 4.0, 12.0, and 14.0 (**Supplementary Figure 2B**) and decreased surface tension was observed at pH 6.0, 8.0 and 10.0. No change in surface tension was observed till 10% of NaCl concentration (**Supplementary Figure 2C**).

Characterization of Biosurfactant CIG-6A^T

Thin-Layer Chromatography (TLC)

TLC was used as a prior methodology for biosurfactant compositional analysis. Lipids and sugars were detected by using iodine vapors and anthrone reagent, respectively. TLC chromatogram revealed the biosurfactant chemical form (**Supplementary Figure 3**) when compared with sophorolipid standard 1,4″-sophorolactone 6′,6″-diacetate (SL-S). In SL-S, two spots with R_f values 0.657 and 0.710 were observed. In CIG-6A^T sample, spots were observed with R_f values of 0.751 and 0.789, indicating lactonic SL's presence, whereas spots appeared with R_f value 0.105 and 0.167 indicating acidic SL in biosurfactant.

FTIR

FTIR spectra of SL-S (**Figure 4A**) and CIG-6A^T (**Figure 4B**). The absorption at 2,970 and 2931.65 cm^{-1} corresponds to symmetrical stretching (ν_s CH_2) and asymmetrical stretching (ν_{as} CH_2) of the methylene group, respectively. The absorption

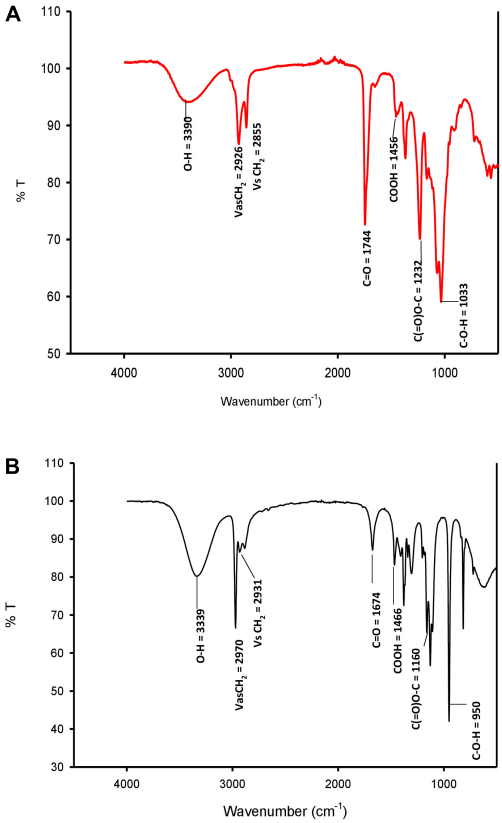

FIGURE 4 | FTIR spectra of standard sophorolipid, 1,4″-sophorolactone 6′,6″-diacetate **(A)** and biosurfactant produced by the strain CIG-6A^T **(B)**.

observed at 1674.35 cm^{-1} appears to be C = O lactone group. O-H stretch was represented by strong absorption at 3339.7 cm^{-1} as compared to the standard SL-S. C(= O)-O-C = stretch in lactone was observed by absorption at 1,160 cm^{-1}. The absorption at 1466.8 cm^{-1} and 3339.7 reflect C-O-H stretch in-plane bending of carboxylic acid, might be typical acidic sophorolipid of CIG-6A^T. Biosurfactant from CIG-6A^T was different from the standard in absorptions at 1674.35 and 950.66 cm^{-1} (C = O and C-O-H), respectively. FTIR data confirms that the biosurfactant from CIG-6A^T is both mixtures of acidic and lactonic SLs.

GC-FID-MS

GC-MS spectra revealed the composition of fatty acids in biosurfactant (**Figure 5**). Total of eight fatty acids were found in CIG-6A^T biosurfactant. The palmitic acid $C_{16:0}$ and linolenic acid $C_{18:3}$ were the predominant fatty acids in biosurfactant, whereas $C_{10:1}$, $C_{10:2}$, $C_{13:0}$, $C_{15:1}$, $C_{15:3}$, and $C_{18:2}$ fatty acids were present in lesser proportion.

LC-MS

In sample CIG-6A^T as well as SL-S, LC-ESI-MS were acquired in positive mode. It revealed the presence of protonated and sodiated adduct ions in both sample and the standard. MS of CIG-6A^T biosurfactant was analyzed at different retention

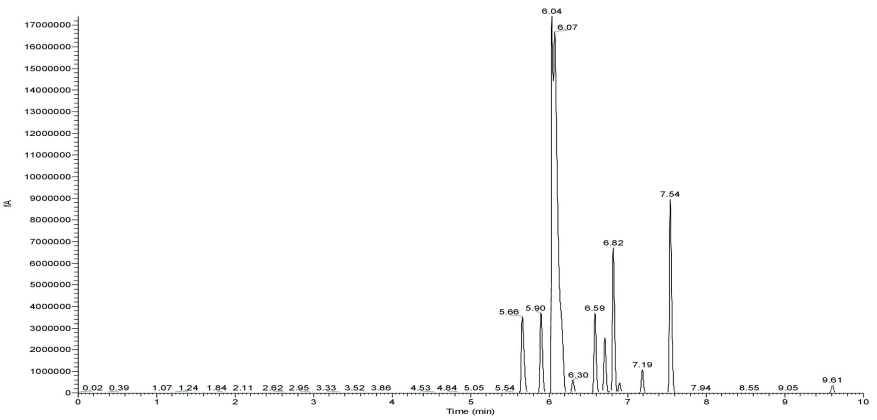

FIGURE 5 | GC-FID-MS spectra of the biosurfactant produced by the strain CIG-6A^T.

time points (**Figure 6**). Purified biosurfactant was a mixture of nine components. The sample contained seven lactonic and two acidic sophorolipid having a lipid chain of variable unsaturation and lengths. At the retention time (7.51 min), a diacetylated sodiated form of acidic sophorolipid was observed. Peak at m/z 678 and m/z 700 appears to $[M + H]^+$ ion of diacetylated acidic sophorolipid $Ac_2AS\ C_{16:0} + H^+$ and $[M + Na]^+$ ion of $Ac_2AS\ C_{16:0} + Na^+$, respectively as shown in **Figure 6A**. At the same retention time, peaks at m/z 210, 272, and 312 correspond to tridecanoic, octadecanoic and eicosanoic fatty acid chain fragments, respectively. Additionally, diacetylated lactonic form of hexadecanoic acid ($C_{16:0}$) lipid chain was detected at m/z 660 (**Figure 6B**). The peak at retention time 12.7 min corresponds to adducts of lactonic sophorolipids with variable length of unsaturated fatty acids viz., m/z 448.2, 492.29, 536.32, 580.34, 624.37, and 668.39 are equivalent to Ac_2 sophorose $+ Na^+$, LS $C_{10:1}$, LS $C_{13:0}$, LS $C_{15:3} + Na^+$, LS $C_{18:2} + Na^+$, and Ac_2 LS $C_{15:1} + Na^+$, respectively (**Figure 6C**). Fatty acids with m/z 300.13 correspond to $C_{18:3} + Na^+$ and m/z at 278.14 equivalent to $C_{18:3}$ detected at the same retention time. Retention time 12.2 min represents the lactonic and acidic form of CIG-6A^T sophorolipid with chain length LS-$C_{10:2}$ and AS $C_{10:2}$ with 18 Dalton difference at m/z 512 and 530, respectively (**Figure 6D**). Sophorolipids from SL-S were detected under the same conditions as showed in **Figure 6E**. The peaks at m/z 710.4 and 688 with RT 13.1 min correspond to $[M + Na]^+$ and $[M + H]^+$ ions of Ac_2 sophorolactone with C_{18} saturated fatty acid moiety. The finalized sophorolipid homologs list detected in CIG-6A^T with their least energy structures are shown in **Figure 7** and **Table 2**.

Ionic Character of Biosurfactant CIG-6A^T

Double diffusion agar test revealed the appearance of precipitation line between selected anionic compound (SDS) and the biosurfactant produced by the strain CIG-6A^T. Simultaneously, no line was observed between the cationic compound (barium chloride) and the biosurfactant from strain CIG-6A^T.

Antioxidant, Antimicrobial and Hemolytic Activity of Biosurfactant CIG-6A^T

Antioxidant Activity

DPPH method was used to evaluate the antioxidant property of biosurfactant using L-ascorbic acid as a standard and DPPH as control (**Figure 8**). The biosurfactant from CIG-6A^T showed maximum antioxidant activity of 62.98% (10 mg/ml) after 30 min of incubation, while the standard showed 93% of activity.

Antimicrobial and Hemolytic Activity of Strain CIG-6A^T

Biosurfactant from strain CIG-6A^T displayed broad-range activity against Gram-negative and Gram-positive bacteria. The MIC analysis of biosurfactant showed that it is more effective against *S. aureus* and *B. subtilis* with complete inhibition at 5 and 1 μg/ml concentration, respectively, whereas 12 and 18 μg/ml concentration of biosurfactant were required to inhibit *K. pneumonia* and *P. aeruginosa,* respectively (**Figure 9A**). Biosurfactant of CIG-6A^T showed activity against human and plant fungal pathogens, evaluated on MIC basis (50–1,000 μg/ml). CIG-6A^T biosurfactant revealed optimistic antifungal activity against *F. oxysporum* and *F. solani* as observed from low MIC value (**Figure 9B**). Hemolysis assay results did not appear to have any lysis of RBCs (Red blood cells) as shown in **Figure 9C** even at high MICs values.

Confocal Laser Scanning Microscopy

CLSM images (**Figure 10**) exhibited permeation of PI that increased the red fluorescence on the treatment with biosurfactant from strain CIG-6A^T, after 48 and 72 h of incubation, indicating the death of the cells. Whereas, untreated samples revealed no red fluorescence, suggesting healthy cells.

DISCUSSION

The phylogenetic analysis and biochemical characteristics comparison revealed that the strain CIG-6A^T, isolated from the gut of stingless bee, represents a novel yeast species. The

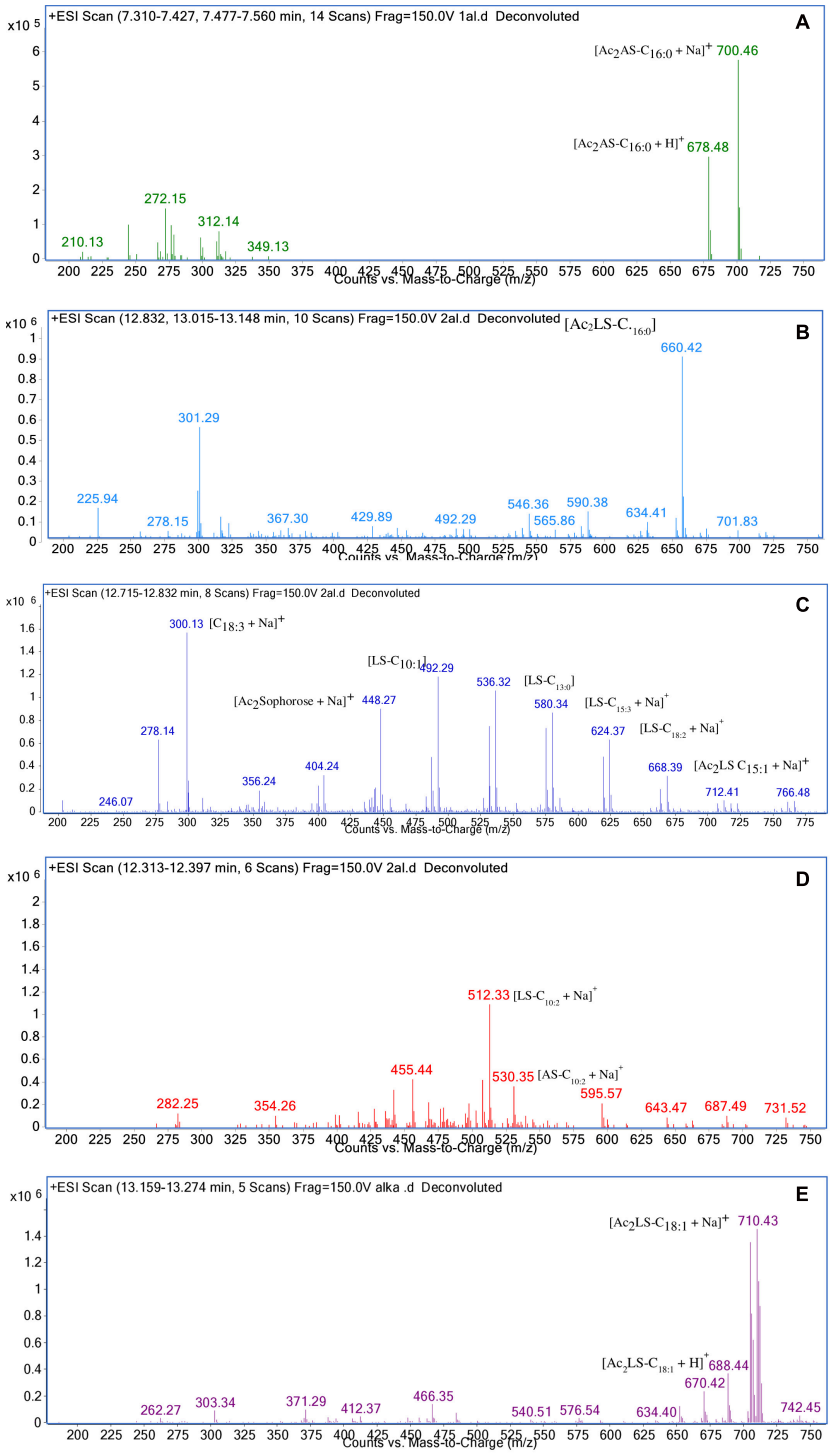

FIGURE 6 | Characterization of the biosurfactant produced by the strain CIG-6A^T using LC-MS in positive electrospray ionization mode (+ ESI). **(A)** MS showing the sodiated adducts of diacetylated acidic sophorolipids (AS) with hexadecanoic acid ($C_{16:0}$) lipid side chain at m/z values 678 and 700. **(B)** Diacetylated lactonic form of hexadecanoic acid ($C_{16:0}$) chain was detected at m/z 660. **(C)** The ion at m/z 492 and 536 corresponds to lactonic sophorolipid with decanoic acid and tridecanoic acid, respectively with lipid chain (LS-$C_{10:1}$ and LS-$C_{13:0}$). Two sodiated adducts of lactonic sophorolipid with pentadecanoic acid and octadecanoic acid, respectively with lipid chain (LS-$C_{15:3}$ and LS-$C_{18:2}$) were detected. Diacetylated sodiated adduct of sophorose moiety was observed at m/z 448 **(D)** Sodiated sophorolipid with the acidic and lactonic form with a decanoic fatty acid side chain (AS-$C_{10:2}$) was observed at m/z 530 and 512, respectively. **(E)** LC-MS spectra of the standard sophorolactone (SL-S), 1,4″-sophorolactone 6′,6″-diacetate in positive electrospray ionization mode (+ ESI). MS showing the protonated ion and sodiated adducts of di-acetylated lactonic sophorolipids (Ac_2LS) with octadecenoic ($C_{18:1}$) lipid side chains at m/z values 688 and 710, respectively.

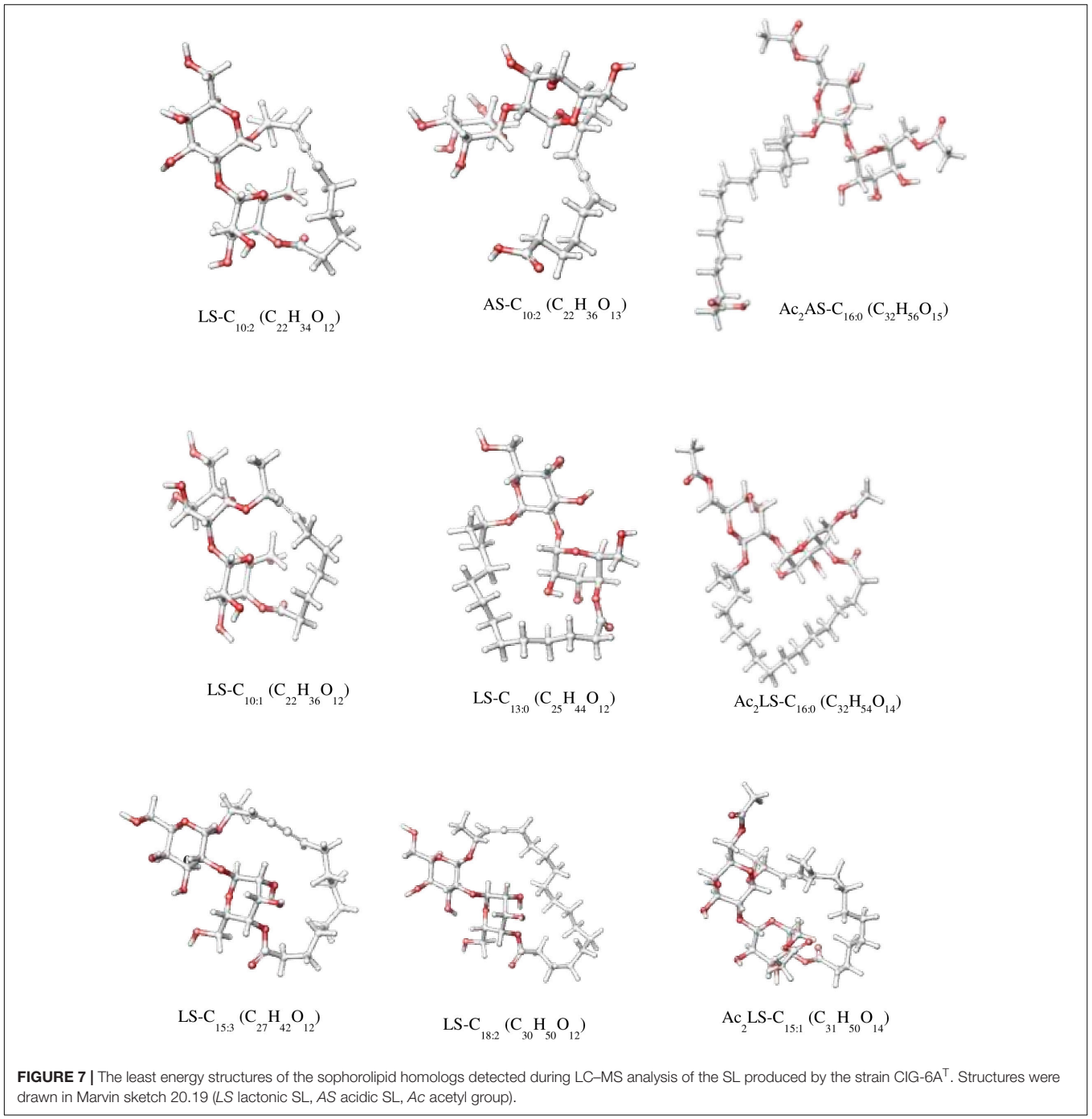

FIGURE 7 | The least energy structures of the sophorolipid homologs detected during LC–MS analysis of the SL produced by the strain CIG-6A^T. Structures were drawn in Marvin sketch 20.19 (*LS* lactonic SL, *AS* acidic SL, *Ac* acetyl group).

insect samples were collected during the summer season from Churdhar (30° 52′ 34.68″ N, 77° 24′ 4.68″ E) Himachal Pradesh, India, belonging to a very cold and high-altitude place. The *Metschnikowia* clade is diverse in morphological, physiological characteristics, and rRNA gene sequence. This diversification of *Metschnikowia* clade is mainly due to the expansion of flowering plants and insects associated with them (Sipiczki, 2014; Álvarez-Pérez et al., 2015). Different *Metschnikowia* clade members, i.e., *M. pulcherrima*, *M. gruessii*, *M. reukaufii*, and *C. rancensis* were most commonly present in the gastrointestinal tract of bumblebees. Yeasts use this symbiosis to survive during winters when flowers and active insects are absent (Brysch-Herzberg, 2004). *Metschnikowia* species provide nutritional factors to the insects and suppress the growth of opportunistic microorganisms that may hamper their symbiosis (Brysch-Herzberg, 2004). There are 26 *Candida* and 39 *Metschnikowia* species in *Metschnikowia* clade (Lachance, 2011; De Vega et al., 2012). The estimated global number of insects in Hymenoptera order is around 1,15,000, and estimated species are approximately more than 3,00,000 in numbers (Chapman, 2009). The total number of bees species

TABLE 2 | Type of sophorolipid homologs, Molecular mass and chemical structure of sophorolipid produced by the strain CIG-6A^T, along with sophorolipid standard, 1,4″-sophorolactone 6′,6″-diacetate (SL-S) as determined by LC-MS.

Homolog	Formula	Molecular mass	Type of SL	Source
AS-$C_{10:2}$	$C_{22}H_{36}O_{13}$	508.52	Acidic	SL-CIG-6A
LS-$C_{10:2}$	$C_{22}H_{34}O_{12}$	490.5	Lactonic	SL-CIG-6A
Ac_2LS-$C_{16:0}$	$C_{32}H_{54}O_{14}$	660.8	Lactonic	SL-CIG-6A
Ac_2AS-$C_{16:0}$	$C_{32}H_{56}O_{15}$	678.8	Acidic	SL-CIG-6A
LS-$C_{10:1}$	$C_{22}H_{36}O_{12}$	492.52	Lactonic	SL-CIG-6A
LS-$C_{13:0}$	$C_{25}H_{44}O_{12}$	536.62	Lactonic	SL-CIG-6A
LS-$C_{15:3}$	$C_{27}H_{42}O_{12}$	558.62	Lactonic	SL-CIG-6A
LS-$C_{18:2}$	$C_{30}H_{50}O_{12}$	602.72	Lactonic	SL-CIG-6A
Ac_2LS-$C_{15:1}$	$C_{31}H_{50}O_{14}$	646.73	Lactonic	SL-CIG-6A
Ac_2LS-$C_{18:1}$	$C_{34}H_{56}O_{14}$	688.8	Lactonic	SL-S

LS lactonic sophorolipid, AS acidic sophorolipid, Ac acetyl group. SL-CIG-6A, sophorolipid produced by Metschnikowia churdharensis f.a., sp. nov., SL-S, sophorolipid standard.

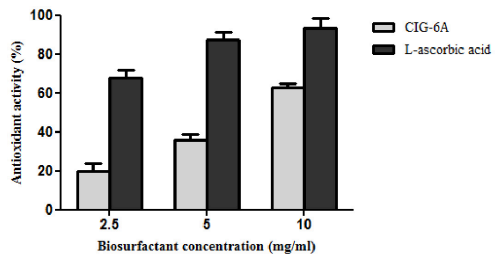

FIGURE 8 | Antioxidant activity of biosurfactant sophorolipid from strain CIG-6A^T in comparison with L-ascorbic acid using DPPH assay.

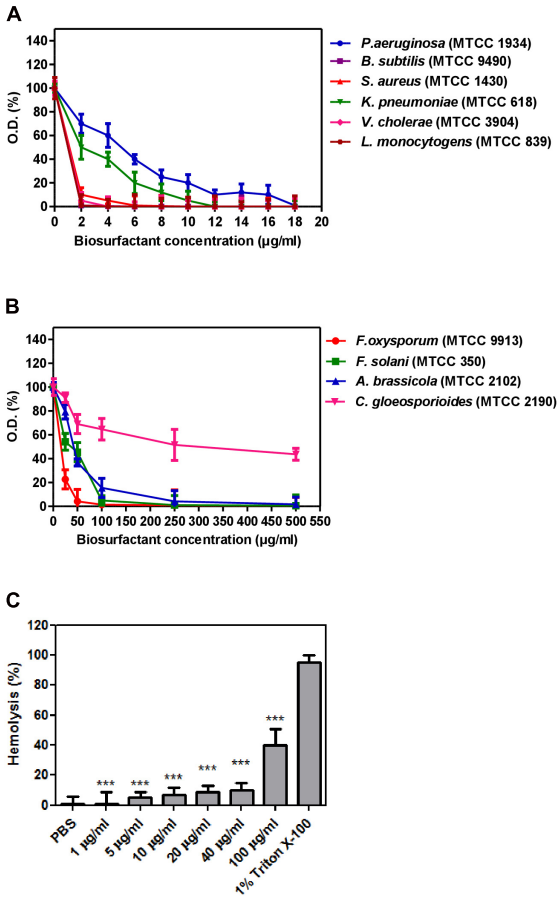

FIGURE 9 | Determination of minimum inhibitory concentrations (MICs) and hemolysis activity of biosurfactant produced from the strain CIG-6A^T in a microtiter plate assay performed in triplicate. **(A)** MICs against Gram-negative and Gram-positive bacteria. **(B)** MICs against pathogenic fungal strains. **(C)** Hemolysis assay of biosurfactant using RBCs of a rabbit. Purified biosurfactant and RBC samples were prepared in PBS. All the experiments are executed in triplicate. Bars show SD. *P*-values are showed above each bar (significance was at a P level of 0.05). ***$p \leq 0.001$.

accepted are about 20,000, and most of them were never studied for the yeast's presence (Rosa et al., 2003). Continued research on the yeast communities associated with bees might discover new species and their exploitation for bioactive compounds.

This study reports the novel yeast species, *Metschnikowia churdharensis* f.a., sp. nov., of strain CIG-6A^T having the ability to produce biosurfactant for the first time, which eventually characterized as sophorolipid with significant antifungal activity and having stable tensioactive properties. *Metschnikowia* clade has not been explored and characterized for the biosurfactant production, its antimicrobial and antifungal activity.

We analyzed physicochemical properties of biosurfactant from strain CIG-6A^T based on surface tension reducing ability, CMC, and emulsification activity. The potential biosurfactant is anticipated to reduce ST to nearly 35 mN/m (Camargo et al., 2018). Consequently, strain CIG-6A^T reduced ST below 35 mN/m value recommended a successful biosurfactant production. CMC equivalent to minimum surfactant concentration where monomers of surfactant begin forming micelles, at this point, medium solution interface in which biosurfactant diffused gets saturated with molecules of surfactant (Rufino et al., 2014). After CMC, no crucial ST lowering is observed, and low CMC value specifies a highly effective surfactant (Santos et al., 2018). CMC value acquired in our study was similar to biosurfactant produced by *Pediococcus dextrinicus* (Ghasemi et al., 2019). Emulsification is one of the properties of biosurfactant, which creates an emulsion of two immiscible liquids and increases bioavailability. Biosurfactant from strain CIG-6A^T contributed high%E_{24} with crude oil and low%E_{24} with mineral oil. Similar results were observed with biosurfactant produced from *Candida bombicola* (Elshafie et al., 2015).

Biosurfactant stability was assessed against a set of variable conditions, such as temperature, pH, and NaCl. The autoclaved sample had a minor change in surface tension. At the same time, a heated sample at 100°C has slight change in surface tension. Surface tension was stable at some pH and was relatively stable at all NaCl concentrations (as described above). Therefore, sophorolipid manifested magnificent stability over the evaluated range of temperature, pH and NaCl, suggested that there is a possibility of biosurfactant usage in food formulation.

Chemical characterization of biosurfactant produced by strain CIG-6A^T was performed using TLC, FTIR, GC-FID-MS, and

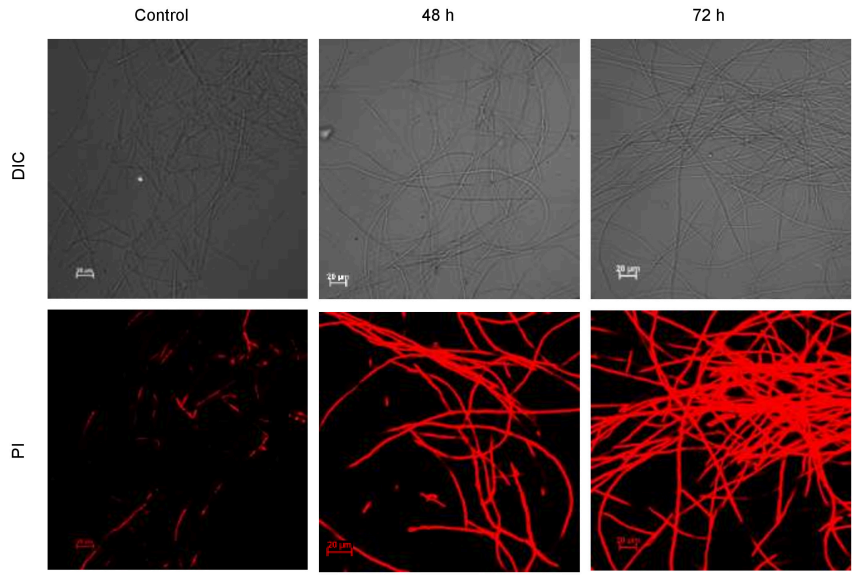

FIGURE 10 | Confocal laser scanning microscopic (CLSM) images of the inhibitory effect of biosurfactant from the strain CIG-6A^T against *Fusarium solani*. Scale bar = 20 μm.

LC-MS analysis regulated in contrast with SL-S. Sophorolipid produced by CIG-6A^T was a combination of lactonic and acidic sophorolipids analyzed through TLC. Sen et al. (2017) acquired R*f* values of acidic SL produced by *R. babjevae* in a similar range of 0.13–0.18, separated with the same mobile phase as used in this study.

The functional groups present in purified biosurfactant were estimated through FTIR and differentiated from the standard. Bands obtained in the sample and standard confirmed the lactonic SL presence. In contrast, acidic SL was confirmed through the presence of two bands at 3339.79 and 1466.8 cm^{-1}, often associated with the acidic SL in the literature (Daverey and Pakshirajan, 2009). Purified biosurfactant was analyzed by gas chromatography for the presence of different fatty acids and the gas chromatogram showed biosurfactant comprised of palmitic acid ($C_{16:0}$), capric acid ($C_{10:1}$), and linolic acid ($C_{18:2}$) and the predominant fatty acid present was palmitic acid ($C_{16:0}$). On ESI of small molecules, having a single functional group able to carrying electrical charge involves proton addition to the analyte ($M + H^+$) and cations adduction such as $M + Na^+$ due to salt presence (Nuñez et al., 2004). Similarly, our study observed both protonated and sodiated ions of lactonic and acidic SLs with different side chains of fatty acids (C_{10}–C_{18}). Similar sophorolipid ions with heterogenous composition were also observed for *R. babjevae* YS3 through LC-MS analysis (Sen et al., 2017). A mixture of sophorolipids was obtained in a production medium containing both lactonic and acidic sophorolipids, but acidic sophorolipid represent the most considerable fraction of the product. In our study, LC-MS analysis also confirms a similar composition for sophorolactone standards as previously reported (Sen et al., 2017). Biosurfactants can be non-ionic, anionic, or cationic in their hydrophilic portions, while their hydrophobic part composed of a branched or linear chain of hydrocarbons (C8–C18 carbon atoms). Anionic biosurfactant is very common in the genus *Candida* of yeast (Camargo et al., 2018) whereas cationic biosurfactants are rare in nature. The ionic character determination revealed that the biosurfactant from CIG-6A^T is cationic and reported for the first time from *Metschnikowia* clade. Regarding the results of DPPH sequestration, biosurfactant CIG-6A^T presented the capacity to donate the hydrogen, therefore, showed DPPH scavenging activity. As compared to the natural antioxidant such as L-ascorbic acid, biosurfactant seems to be less effective but it can be used as a good alternative for synthetic antioxidants.

Many microorganisms producing biosurfactants have been explored for their antimicrobial properties and are presently exploited to stem the incidences of antibiotic resistance afflicted today's world (Desai and Banat, 1997). Biosurfactants exhibited antagonistic activities that may be due to cellular membrane destabilization, which causes the extrusion of cytoplasm and, at last, results in rupturing of the cell (Hirata et al., 2009). Dengle-Pulate et al. (2014) reportedly used 1 and 6 μg/ml concentration to obtain complete *B. subtilis* and *S. aureus* inhibition, respectively and similar results were obtained in our study. There are very few studies on the antifungal activity of sophorolipids from yeast. We studied the antifungal activity against *F. oxysporum* and *F. solani*, which are the pathogens associated with post-harvest spoilage of vegetables like beans, potato and tomato (Kakde and Kakde, 2012). The promising antifungal activity was observed against plant and human pathogen *F. oxysporum* and *F. solani* at 49 and 98 μg/ml, respectively, indicates that it could have a possible application as a protective agent against post-harvest disease-causing fungal pathogens. The membrane integrity and viability can be determined by using PI, a membrane impermeable fluorescence dye, which binds to the nucleic acid, therefore

differentiate damaged cells from healthy cells. Upon treatment with biosurfactant from CIG-6A^T, live and dead cells of *F. solani*, stained with PI were visualized by CLSM, that confirm the irreversible damage to membrane integrity and structure damage. Previously, (Sen et al., 2020) demonstrated permeabilization of *Trichophyton mentagrophytes* membrane on treatment with sophorlipid produced by *R. babjevae*. Sophorolipid from CIG-6A^T has seven homologs of lactonic analog in contrast to two homologs of acidic analog, giving it more lipophilic character. It has been reported that lipophilic character of antifungal compound increased membrane permeability which hampers the ion transportation and latterly causing cell death (Di Pasqua et al., 2007). Therefore, biosurfactant sophorlipid from CIG-6A^T might exert its effect by changing the permeability of the fungal cells, thereby causing cell death.

This is the first study to demonstrate biosurfactant action against *F. oxysporum*, *F. solani*, *P. chrysogenum*, and *C. gloeosporioides* species capable of food spoilage and mycotoxin production. Speices of *fusarium* are capable of producing mycotoxins such as trichothecenes and fumonisins, which are often associated with toxicoses in humans and livestock. Trichothecenes causes toxicity to plants and animals by inhibiting ribosomal protein synthesis, RNA and DNA biosynthesis, and mitochondrial function. Fumonisins is a potential carcinogenic mycotoxin for human and causes lethal livestock diseases (Munkvold, 2017). The biosurfactant obtained from the present study appear to be more potent against food spoilage, plant and human fungal pathogens. Therefore, there is a new possibility for the application of biosurfactant as an alternative natural fungicide agent.

CONCLUSION

In this study, *Metschnikowia churdharensis* f.a., sp. nov., CIG-6A^T was isolated from the gut of stingless bee (Hymenoptera order). The CIG-6A^T showed the production of sophorolipid biosurfactant, which was described as thermostable, emulsion stabilizing, and antioxidant agent, thereby have potential use in food industry. Moreover, sophorolipid biosurfactant showed promising results against food spoilage fungal pathogens. Based on the promising results obtained in the study, this sophorolipid biosurfactant has biotechnological potential for the food industry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MG821162.

AUTHOR CONTRIBUTIONS

AK and SK conceived, designed, and performed the experiments. AP and GP supervised the project and provided the funding's. AP, GP, AK, and SK analyzed the data and wrote the manuscript. All authors approved the submitted version of research article.

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SUPPLEMENTARY MATERIAL

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Red Propolis as a Source of Antimicrobial Phytochemicals: Extraction Using High-Performance Alternative Solvents

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Propolis is a resinous material rich in flavonoids and involved in several biological activities such as antimicrobial, fungicide, and antiparasitic functions. Conventionally, ethanolic solutions are used to obtain propolis phytochemicals, which restrict their use in some cultures. Given this, we developed an alcohol-free high-performance extractive approach to recover antibacterial and antioxidants phytochemicals from red propolis. Thus, aqueous-solutions of ionic liquids (IL) and eutectic solvents were used and then tested for their total flavonoids, antioxidant, and antimicrobial activities. The surface-responsive technique was applied regarding some variables, namely, the time of extraction, the number of extractions, and cavitation power (W), to optimize the process (in terms of higher yields of flavonoids and better antioxidant activity). After that, four extractions with the same biomass (repetitions) using 1-hexyl-3-methylimidazolium chloride [C_6mim]Cl, under the operational conditions fixed at 3.3 min and 300 W, were able to recover 394.39 ± 36.30 mg RuE. g^{-1} of total flavonoids, with total antioxidant capacity evaluated up to 7595.77 ± 5.48 μmol TE. $g^{-1}_{\text{dried biomass}}$, besides inhibiting the growth of *Staphylococcus aureus* and *Salmonella enteritidis* bacteria (inhibition halo of 23.0 ± 1.0 and 15.7 ± 2.1, respectively). Aiming at the development of new technologies, the antimicrobial effect also presented by [C_6mim]Cl may be appealing, and future studies are required to understand possible synergistic actions with propolis phytochemicals. Thereby, we successfully applied a completely alcohol-free method to obtain antimicrobials phytochemicals and highly antioxidants from red propolis, representing an optimized process to replace the conventional extracts produced until now.

Keywords: Brazilian propolis, red propolis, flavonoids, ionic liquids, eutectic solvents

INTRODUCTION

Propolis is associated broad spectrum of activities, namely, fungicide (Marques das Neves et al., 2016), antioxidant, and antiparasitic (do Nascimento et al., 2016; Dantas Silva et al., 2017). Moreover, the impact generated by the pandemic COVID-19 has motivated the studies of propolis in action against infection by SARS-CoV-2 (Berretta et al., 2020; Refaat et al., 2021), whereas this natural product has anti-inflammatory properties (Lima et al., 2014; Washio et al., 2015; Kitamura et al., 2018) and action against some virus species (Hazem et al., 2017; Kwon et al., 2020). Recently, a clinical trial conducted in Brazil (National Clinical Trial Number: NCT04480593) has shown the efficacy of green propolis in supporting treatment in patients hospitalized with COVID-19 (Silveira et al., 2021).

Brazilian red propolis demonstrated strong cytotoxic potential *in vitro*, suggesting a potential therapeutic alternative for treatment against Chagas disease (Dantas Silva et al., 2017), schistosomiasis (Silva et al., 2021), and some types of cancer (da Silva Frozza et al., 2013; Dantas Silva et al., 2017; Banzato et al., 2020). These properties are related to propolis complex chemical composition, including formononetin, isoliquiritigenin, liquiritigenin, and biochanin A, as described by da Silva Frozza et al. (2013).

Traditionally, ethanolic solutions are the most conventional solvent used to obtain phytochemicals from red propolis. However, hydroalcoholic extracts' consumption has some restrictions in specific cultures (Muslim; Al-Ansari et al., 2019). Also, envisioning the purification of some specific molecules with different polarities, other volatile organic solvents (VOSs) are used such as hexane, methanol, and chloroform (Oldoni et al., 2011; Marques das Neves et al., 2016). These solvents are widely used in several industrial segments; however, beyond toxicity, there are risks for workers (high-volatility) and damage to the environment (environmental toxicity). Thus, alternative technologies have been required in order to replace these solvents with more sustainable alternatives (Choi and Verpoorte, 2019). In this scenario, the development of extraction methods using alternative solvents to replace VOS becomes a promising strategy for future commercial applications, which increases the number of applications in some industrial sectors, such as food, cosmetic, and pharmaceutic ones. Among the alternative solvents, ionic liquids (ILs) and eutectic solvents (ESs) stand out as promising candidates to replace VOS (Passos et al., 2014; Ventura et al., 2017). The non-volatile and flammability suggest that these compounds are an essential alternative in developing new extractive processes (Nam et al., 2015). Ionic liquid, for example, can assume several properties, it has been applied to obtain several molecules with different polarities, such as carotenoids (hydrophobic compounds; Xiao et al., 2018; de Souza Mesquita et al., 2019, 2020a,b; Murador et al., 2019a,b), as well as flavanones (hydrophilic compounds), depending on the ionic composition (Xiao et al., 2018). Another advantage of IL is the possibility to recycle and reuse, thus contributing to a low carbon footprint in the process (de Souza Mesquita et al., 2019, 2020a,b). The ES also has a high potential to extract bioactive compounds from natural products, including flavonoids and phenolic compounds, since they are considered as designer solvents and IL. In some cases, ES could improve the bioavailability and antioxidant activity of the extracts, which are considered as a promising characteristic for new applications, especially in the food sector (Meng et al., 2018; Funari et al., 2019; Liu et al., 2019; Murador et al., 2019a).

Using an alternative extraction strategy in terms of high-performance and transposing the limitations on the use of ethanol would make it possible to obtain a commercially differentiated red propolis extract obtained by alternative solvents. Thereby, this work aims to establish a new method of extracting antioxidant and antimicrobial phytochemicals from red propolis using alternative solvents, creating new possibilities for applying this biomass, which is considered as extremely important for different purposes.

MATERIAL AND METHODS

Materials

The following standards were used: rutin hydrate, formononetin, daidzein, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich (Darmstadt, Germany). The reagents 1-methylimidazolium, 1-chlorobutane, and potassium hexafluorophosphate; IL 1-n-butyl-3-methylimidazolium tetrafluoroborate ($[C_4mim][BF_4]$) and 1-hexyl-3-methylimidazolium chloride ($[C_6mim]Cl$); choline chloride, glycerol, 1,4-butanediol, and levulinic acid from Sigma-Aldrich (Darmstadt, Germany). Reagents sodium nitrite (Vetec®) and aluminum chloride (Proquímicos®); Fluorescein sodium salt and α, α′-Azodiisobutyramidine dihydrochloride (AAPH) from Sigma-Aldrich (Darmstadt, Germany). Culture mediums: Mueller Hinton Agar (Himedia®) and Nutrient broth (Himedia®). *Salmonella enteritidis* ATCC 13076 and *Staphylococcus aureus* ATCC 19095 were supplied by the Bacterial Culture Collection of the Oswaldo Cruz Institute – FIOCRUZ (Manguinhos, Rio de Janeiro, Brazil). Vancomycin (30 μg/disc) and Meropenem (10 μg/disc; DME®) antibiotic filter paper discs and inert filter paper discs (DME®).

Samples

The samples of red propolis were purchased from an apiary located in Porto de Pedras' municipality, in the state of Alagoas (Latitude: 9° 09' 11" S, Longitude: 35° 17'19" 17'19" W) in April 2018, by donation. The sample was composed of a single batch consisting of 350 g of sample and was lyophilized for 48 h, processed using an analytical mill (Ika®, model A11 Basic), vacuum-packed in sealer (TECMAQ®, model TM250), and maintained frozen at −40°C.

Extraction of Flavonoids From Red Propolis

Convectional Extraction

Ethanol was applied as a control to obtain red propolis extract. Thus, results using ethanolic solutions (70 and 95% v:v) were compared and evaluated with the developed method's extractive

performance based on alternative solvents. The homogenization was performed in an ultrasonic probe (500 W, 20 kHz, 4 mm in diameter; Unique, model DES500, Brazil) at 400 W for 5 min, followed by centrifugation at 4700 rpm for 15 min (process repeated twice). The solid-liquid ratio ($R_{S/L}$) was fixed at 1:3 (0.5 g of sample for 1.5 g of solvent). The procedure was performed in triplicate with three extractions for each sample. The supernatants were stored in a freezer at −40°C for further analysis.

Extraction of Flavonoids From Red Propolis With Ionic Liquids and Eutectic Solvents

Preliminary Tests

In total, four different IL were evaluated: 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4mim][BF_4]$) and 1-hexyl-3-methylimidazolium chloride ($[C_6mim]Cl$) – which were obtained commercially, 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$) and 1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4mim][PF_6]$) both synthesized in the laboratory, as described by Martins and de Rosso (2016). ($[C_4mim][BF_4]$) was synthesized in a round-bottom flask by mixing 0.10 mol of 1-methylimidazole and 0.10 mol of 1-chlorobutane. The mixture was stirred and refluxed (70–80°C) for 72 h. A viscous yellow liquid, which was washed twice with dichloromethane, was obtained. $[C_4mim]Cl$ was dried under vacuum at 100°C and crystallized at −40°C. $[C_4mim][PF_6]$ was synthesized by a mixture containing 0.01 mol of $[C_4mim]Cl$, and 0.01 mol of potassium hexafluorophosphate in distilled water was stirred vigorously for 45 min. The upper aqueous phase formed was separated and discarded; the remaining liquid was added to distilled water and stirred for 15 min. Then, 40 ml of chloroform was added. The solvent was evaporated under vacuum to yield a viscous liquid with a slightly yellow color. Besides, three ES were synthesized, associating choline chloride (CH) to three hydrogen donors: glycerol (CH–GLY), 1,4-butanediol (CH–BUT), and levulinic acid (CH–LEV), in a molar ratio of 1:2, as described by Liu et al. (2019). For each ES, 10% (g/g) water (co-solvent) was added and homogenized at 60°C for 10 min in a round-bottom flask.

The extractions were performed using a 1:3 solid-liquid ratio ($R_{S/L}$; g/ml), as in conventional extraction. The samples were maintained in an ice bath, and the homogenization was performed in a 500 W, 4 mm in diameter, and 20 kHz ultrasonic probe (Unique, model DES500, Brazil) at 400 W for 5 min. Then, the samples were centrifuged at 4700 rpm for 15 min. This procedure was performed in triplicate with three extractions for each sample (using the same biomass). The supernatants were stored in a freezer at −40°C for further analysis.

Optimization of Extraction Conditions Using Response Surface Methodology

The most effective solvent for the extraction of flavonoids was submitted to a Central Composite Design (CCD) to optimize the process. The effects of the variables number of extractions, time of extraction (T_{min}), and cavitation power (W) were assessed using the Central Composite Design Rotational (CCDR; 2^3), with six axial points (calculated by the interpolation considering the experimental values of factorial and central points) and three central points, totaling 17 trials. ILs, as well as ES, it has a high viscosity, and since propolis biomass is a very resinous substance, both factors difficult the extraction process; Thus, the variable ($R_{S/L}$) was fixed at 1:3, as it was the minimum amount of solvent that enabled a less viscous liquid for cavitation. The response was measured by total flavonoid yield and antioxidant activity using the oxygen radical absorbance capacity (ORAC) method.

Model Validation

An experiment contemplating the best extraction conditions was carried out, in triplicate, to validate the obtained model and verify the reproducibility of the method and compare the average of the extracts with the predicted model obtained in the experimental design.

Quantification of Total Flavonoids

The total content of flavonoids present in red propolis extract was quantified according to the method exposed by Zhishen et al., 1999 colorimetric method, based on the reaction with aluminum chloride. The absorbance of the solution was determined at 510 nm in a Varian® spectrophotometer. Total flavonoid contents were calculated as mg of rutin equivalent (RuE). g^{-1} using a calibration curve ranging between 200 and 700 ppm (R^2 = 0.998).

Analysis of Flavonoids in High Performance Liquid Chromatography

The flavonoids were separated and chromatographically identified by HPLC-DAD Shimadzu (Kyoto, Japan; Atlantis® C-18 column, size 4.6 mm × 250 mm) with quaternary pump (model LC-20AT), a DAD detector (Shimadzu, model SPD-M20A), a degassing unit (DGU-20A5), and a Rheodyne injection valve with a 20 µl loop. The extracts were filtered through a 0.22 µl filter (Millipore). The column was eluted using a linear gradient of acidified water (solvent A) and methanol (solvent B), starting with 80% solvent B up to 10 min, gradually reducing solvent B by 10% over time 15, 30, 45, 60, and 65 min, maintaining only solvent A, between 65 and 70 min, and increasing to 80% solvent B until 90 min (pump flow: 0.800 ml/min). The UV-visible spectra were obtained between 200 and 600 nm, and the chromatograms were processed at 280 and 355 nm. The red propolis flavonoids were tentatively identified based on the following information: elution order, retention time, and UV-visible spectral features compared to those of the standards analyzed under the same conditions and the available literature data. Co-chromatography was carried out with formononetin and daidzein analytical standards.

Evaluation to Deactivate Peroxyl Radicals by Hydrophilic Compounds in a Homogeneous System: Oxygen Radical Absorbance Capacity

The antioxidant activity against the peroxyl radical (ROO•) was measured by ORAC test, which is based on monitoring the effect of the hydrophilic or standard extract (Trolox) in

the analysis of fluorescence resulting from the oxidation of fluorescein induced by ROO• radical, generated by decomposition of the 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) at 37°C (Rodrigues et al., 2012).

The test was performed in 96-well microplates, where 150 µl of fluorescein (61 nm, prepared in 75 mm phosphate buffer pH 7.4) were transferred. Then, 25 µl of the propolis extracts were added in two different dilutions (1:1,000 and 1:10,000) in a phosphate buffer, or white (buffer solution only), or Trolox standard (control). The plate was incubated for 10 min at 37°C with intermittent shaking. After this time, 25 µl of AAPH solution (19 mm, prepared in phosphate buffer) was added to each well to generate radicals and microplate and inserted in the plate reader, where the fluorescence reading occurred at 538 nm after excitation at 485 nm, every minute, for 1 h and 30 min (Murador et al., 2016). The area under the fluorescence vs. time curve for the sample minus the area under the blank curve was compared with the standard Trolox curve. The results were expressed in µmol Trolox Equivalent (TE). g^{-1}.

Antimicrobial Activity Test

The disc diffusion technique was used to assess the propolis extracts' antimicrobial activity, in which the extracts were applied to filter paper discs, and these, placed on Mueller Hinton agar plates, previously sown with bacterial suspensions. The Gram-positive bacteria *St. aureus* ATCC 19095 and Gram-negative bacteria *Sa. enteritidis* ATCC 13076 were used for antimicrobial tests (CLSI/NCCLS, 2003; Winn, 2008). *Salmonella enteritidis* ATCC 13076 and *St. aureus* ATCC 19095 were supplied by the Collection of Bacterial Cultures of the Oswaldo Cruz Institute – FIOCRUZ (Manguinhos, Rio de Janeiro, Brazil), where they were grown on nutrient agar and incubated at 37°C for 24 h, then lyophilized and stored in vacuum sealed ampoules at a temperature between −10 and −20°C and transported to our laboratory in a thermic box. Analysis of purity, viability, morphology, and identity was carried out on the used batch.

Preparation of Discs

Inert, sterile Petri dishes were placed inert filter paper discs (DME®), and these were soaked with 10 µl of the optimized extracts in the dilutions 25 mg ml^{-1}, 75 mg ml^{-1} (in sterile saline), and pure extract. Inert discs were soaked with IL in the same conditions to characterize the negative control. For positive control, Vancomycin (30 µg/disc) and Meropenem (10 µg/disc; DME®) antibiotic filter paper discs were used for Gram-positive and Gram-negative bacteria, respectively.

Disc Diffusion Technique

The bacterial strains were reactivated, sowed in nutrient broth (tryptone soy broth), and incubated in a bacteriological oven at 36 ± 1°C for 24 h. Sample's inoculum was prepared according to document M2-A8 of the National Committee of Clinical and Laboratory Standardization/Clinical and Laboratory Standards Institute (NCCLS/CLSI) by carrying out a direct suspension, in sterile saline, from the growth in broth. The suspension's turbidity was adjusted by comparing it with the 0.5 tubes of the McFarland scale, that is, an inoculum containing approximately 1.0×10^8 CFU/ml (CLSI/NCCLS, 2003; Winn, 2008).

The suspension was applied to Mueller Hinton agar Petri dishes, aided by a sterile swab, moving it in eight directions so that the plate was fully sown. After about 15 min, the disks impregnated with extracts were applied with the aid of flamed forceps. Then, Petri dishes were inverted and incubated in an oven at 36 ± 1°C for a period of between 16 and 18 h (CLSI/NCCLS, 2003; Winn, 2008).

After the incubation period, the antimicrobial action was read and assessed by the formation of a growth inhibition halo, which was measured using a millimeter rule (CLSI/NCCLS, 2003).

Statistical Analysis

The tests were conducted in triplicate, and data expressed as mean ± standard deviation (SD). The results were submitted to analysis of variance (ANOVA), the comparison between averages established by Tukey's HSD *post hoc* test, adopting the 95% level of significance. All analyses were performed with StatSoft software STATISTICS® version 13.3 (Tulsa, United States).

RESULTS AND DISCUSSION

Efficiency in the Extraction of Flavonoids: Preliminary Tests

The 16 results obtained from the preliminary tests are presented in **Table 1** as well as conventional extraction and screening with IL and ES. Most of the tested solvents showed equivalent or superior efficacy, in terms of flavonoid concentration, compared to hydroalcoholic extracts used to relate the alternative solvents' extraction power with the conventional method. The extract containing [C_6mim]Cl/water in a 10:1 (g/g) ratio demonstrated twice the extractive potential of 95% ethanol. In contrast, ES CH-BUT and CH-LEV and IL [C_4mim]Cl with water in the proportion 10:3 (g/g) were those with the lowest efficiency in extract flavonoids from red propolis; however, changing the ratio to 1:1 (g/g) of IL/water, the extraction efficiency doubled, equivalent to the yield obtained with 70% ethanol. In the literature, no studies of propolis extracts with IL were found, but comparing [C_4mim]Cl extractor potential of flavonoids from heather, it showed superior performance compared to the 60% ethanol extract (Drozdz and Pyrzynska, 2019). The anion of IL acts in disrupting the structure of the natural products' matrix. At the same time, the imidazolium cation plays an essential role due to the aromatic π-cloud, which strongly interacts with polar and aromatic analytes (Ventura et al., 2017).

Generally, extracts presented different characteristics, according to the solvent and co-solvent used, especially regarding color. The yield of flavonoids of most extracts with the solvents tested in this study showed higher values than conventional extracts found in the literature (Santana Andrade et al., 2017; Devequi-Nunes et al., 2018). The substitution of VOS by

TABLE 1 | Determination of total flavonoids in red propolis extracts made with different ionic liquids and eutectic solvents, compared with the conventional solvent.

Extractor solvent	Co-solvent	Co-solvent/ IL or ES	Total flavonoids (mg RuE. g^{-1})*
Ethanol 70%	-	-	$112.93^{ab} \pm 9.20$
Ethanol 95%	-	-	$275.85^{fgh} \pm 26.91$
[C_4mim]Cl	Ethanol	1:4	$290.77^{gh} \pm 39.83$
[C_6mim]Cl	Ethanol	1:4	$249.48^{fgh} \pm 11.36$
[C_4mim][BF_4]	Ethanol	1:4	$229.57^{efg} \pm 13.15$
[C_4mim][PF_6]	Ethanol	1:4	$212.05^{def} \pm 24.09$
[C_4mim]Cl	Ethanol	1:1	$166.11^{bcde} \pm 14.56$
[C_6mim]Cl	Ethanol	1:1	$298.03^{h} \pm 36.17$
[C_4mim][BF_4]	Ethanol	1:1	$153.57^{bcd} \pm 7.49$
[C_4mim]Cl	Water	10:3	$69.46^{a} \pm 14.21$
[C_4mim]Cl	Water	1:1	$144.54^{bc} \pm 22.98$
[C_6mim]Cl	Water	1:1	$179.47^{cde} \pm 29.04$
[C_6mim]Cl	Water	10:1	$581.06^{i} \pm 20.97$
CH–BUT	Water	10:1	$66.58^{a} \pm 8.38$
CH–LEV	Water	10:1	$62.78^{a} \pm 7.95$
CH–GLY	Water	10:1	$152.97^{bcd} \pm 11.90$

**Values are equal to mean ± standard deviation (SD). Means followed by the same letter in column do not differ by Tukey's test at 5% significance. Results expressed in mg of rutin per gram of sample.*

alternative solvents (non-volatile solvents) is a promising strategy increasingly addressed. While these alternatives can positively contribute to the environment, there may also be an improvement in terms of the yield of bioactive compounds. Some studies indicate the effectiveness of the use of some ES and IL in the extraction of flavonoids from several natural products, considering conventional solvents. However, no reports compare these solvents in samples of Brazilian red propolis, which have stood out for their biological activities (Koutsoukos et al., 2019; Trusheva et al., 2019).

From the preliminary tests, variables were selected for the experimental design (CCDR 2^3), analytical viability was considered, associated with the efficiency to extract flavonoids. The IL [C_4mim][PF_6] possesses some properties, such as low water solubility, which can cause some difficulties to dissolve with the tested co-solvents. On the other hand, [C_4mim][BF_4] is more soluble with the co-solvent; however, this IL's extraction produced a heterogeneous extract.

Notably, propolis presents a natural resin with a complex composition. Thus, there is an inherent difficulty in standardizing the method for extract preparation with alternative solvents, which also have a high viscosity. In this study, all operational conditions were standardized and further optimized. However, some extracts presented different behaviors, forming multiphase systems, in addition to the formation of an emulsion, making them unfeasible for analysis, such as the case with extracts composed of [C_4mim][PF_6] and [C_4mim][BF_4], using water as a co-solvent. Those differences highlight the importance of evaluating different solvents and conditions to find the best manner to extract bioactive compounds. Those distinct characteristics may have occurred for fluoridated IL are unstable compounds in water since they hydrolyze, forming by-products that can produce specific toxicity (Plechkova and Seddon, 2008; Freire et al., 2010). For this reason, IL [C_4mim][PF_6] and [C_4mim][BF_4] were discarded from the screening of solvents. The extract with [C_6mim]Cl in its largest proportion obtained an increase in flavonoids, about three times concerning the ratio 1:1 (with water).

Among the ES tested, extraction with choline chloride and glycerol did not significantly different compared to the extract with 70% ethanol in flavonoid concentrations. Since alcohol is used as the most common extractor among propolis extracts by several authors (Alencar et al., 2007; da Silva Frozza et al., 2013; Santana Andrade et al., 2017), equivalence in the extractive power of ES is crucial to obtain the exact yield using an alternative solvent. Trusheva et al. (2019) tested the extract with choline chloride and glycerol, and it showed 8.4% of total flavones and flavonols compared to 70% ethanol (11.3%) in propolis samples of poplar type. Funari et al. (2019) tested the same ES, resulting in a lower performance of phenolic compounds than 70% ethanol in green propolis samples. Some authors highlight the influence of water on the ES's composition, showing that this may be the main factor that induces selectivity in extraction (Liu et al., 2018). The presence of water decreases the viscosity of ES, while it increases their polarity, interfering in the extractive power with low polarity (Funari et al., 2019). However, eutectic mixtures containing more than 30% water must be avoided, as this percentage results in the loss of existing hydrogen bonds, resulting in the de-characterization of the ES structure, which negatively influences its interaction with the flavonoid (Meng et al., 2018). Propolis is mainly composed of flavonoid aglycones and their derivatives, which are predominantly less polar than those glycosylated ones (Zhang et al., 2012; Huang et al., 2014). Thus, a higher percentage of water in ES would reduce the extractive potential of these compounds. On the other hand, ES presents high viscosity, reducing extraction efficiency by slow mass transfer (Fraige et al., 2019). This explains the fact that ES is not effective in extracting propolis flavonoids.

A global evaluation was executed to optimize the extraction methods using alternative solvents and, although the IL [C_4mim]Cl has formed a homogeneous extract, with a high capacity to extract flavonoids, [C_6mim]Cl performed better with water as a co-solvent, being this solvent chosen for the stage of extraction optimization, through the experimental design using response surface methodology (RSM).

Optimization of Ionic Liquid Extraction

Central Composition Design Rotational

As mentioned before, [C_6mim]Cl showed better extraction yield in total flavonoids based on the preliminary test, and it was selected for the experimental design using a CCDR 2^3 (**Table 2**). Two responses were considered as results from the assays performed using the conditions specified by the experimental design, flavonoid yield, and antioxidant activity. All results are presented in **Table 2**.

The flavonoid yield values obtained ranged between 260.85 and 470.96 mg RuE. g^{-1} of the sample. For the CCDR, three variables were considered: the number of extractions (X_1), time of extraction (min; X_2), and cavitation power (W; X_3); however, after data evaluation using 95% of confidence, the effect of

TABLE 2 | Design of experiment 2^3 using 1-butyl-3-methylimidazolium chloride [C_6mim][Cl] and 10% water as a co-solvent.

Test	Number of extractions (X_1)	Time of extraction (min; X_2)	Cavitation power (W; X_3)	Flavonoids (mg RuE. g^{-1})*	ORAC (μmol TE. g^{-1})	Predictive ORAC (μmol TE. g^{-1})	Deviation (%)
1	3 (−1)	5.0 (−1)	200 (−1)	398.72	4173.39	5163.56	−23.73
2	5 (+1)	5.0 (−1)	200 (−1)	386.55	6538.71	7398.45	−13.15
3	3 (−1)	10.0 (+1)	200 (−1)	260.85	4678.07	5163.56	−10.38
4	5 (+1)	10.0 (+1)	200 (−1)	392.82	7012.41	7398.45	−5.51
5	3 (−1)	5.0 (−1)	400 (+1)	376.65	5596.88	5163.56	7.74
6	5 (+1)	5.0 (−1	400 (+1)	323.09	8034.37	7398.45	7.92
7	3 (−1)	10.0 (+1)	400 (+1)	355.54	4244.95	5163.56	−21.64
8	5 (+1)	10.0 (+1)	400 (+1)	307.04	8033.95	7398.45	7.91
9	2 (−1.68)	7.5 (0)	300 (0)	303.48	3921.17	3453.96	11.91
10	6 (+1.68)	7.5 (0)	300 (0)	384.51	6493.30	7208.58	−11.02
11	4 (0)	3.3 (−1.68)	300 (0)	470.96	8689.50	8011.80	7.80
12	4 (0)	11.7 (+1.68)	300 (0)	383.30	8019.79	8011.80	0.10
13	4 (0)	7.5 (0)	130 (−1,68)	447.60	5290.34	5331.27	−0.77
14	4 (0)	7.5 (0)	470 (+1.68)	432.01	6480.77	5331.27	17.74
15	4 (0)	7.5 (0)	300 (0)	488.26	5794.64	5331.27	8.00
16	4 (0)	7.5 (0)	300 (0)	435.65	6621.68	5331.27	19.49
17	4 (0)	7.5 (0)	300 (0)	428.47	3966.62	5331.27	−34.40

*The numbers in parentheses correspond to the coded values in the CCDR. *The model generated for this response was not predictive.*

variables assessed was not statistically significant on the evaluated response. Also, no significant interaction between the variables used (R^2 < 0.7) was observed. Therefore, data could not be adjusted, and it was not possible to generate a predictive model as well as an equation representing the conditions evaluated. It is difficult to indicate a reason for the lack of adjusting among the experimental data and the predictive values. Often this occurs due to several factors acting together.

Regarding the antioxidant activity against peroxyl radicals, the CCDR assays provided values ranging between 3921.17 and 8689.50 μmol TE. g^{-1} of extract, and two variables were considered to obtain Equation 1: number of extractions (X_1) and time of extraction (X_2). The equation is constructed considering only terms representing the variables with significative effects over the response (antioxidant activity). Thus, not statistically significant terms were incorporated into the lack of fit to calculate the R^2 and F ratio.

$$Antioxidant\ activity\left(\mu\text{mol TE}.g^{-1}\right) = 5331.3 + 117.4\left(X_1\right) + 949.7\left(X_2\right)^2 \quad (1)$$

The conditions provided the best antioxidant activity (8689.50 μmol TE. g^{-1} of the sample) from experiment 11 (**Table 2**), whose conditions were: four extractions with the same biomass, time of extraction at 3.3 min, and 300 W of cavitation power. The dependent (antioxidant activity) and independent variables were fitted to a second-order model, and they were examined in terms of goodness of fit. Therefore, ANOVA was used to evaluate the adequacy of the fitted model.

In this study, R^2 was 0.70, and the estimated F value was approximately five times the F tabulated for antioxidant activity, showing that the proposed model was predictive, with a 95% confidence level. The obtained model for antioxidant activity was used to construct the response surface presented in **Figure 1** to understand the interactions between the number of extractions (X_1) and time of extraction (X_2) required to maximize the antioxidant activity provided by the red propolis extracts.

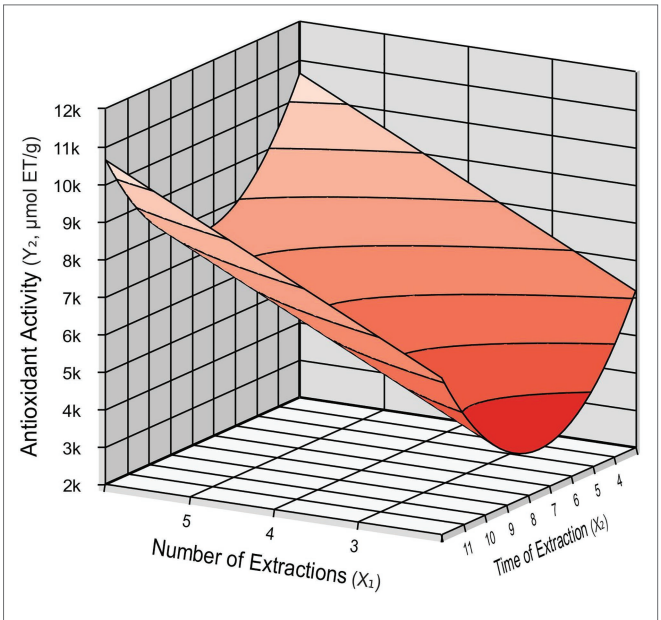

FIGURE 1 | Response surface for antioxidant activity as a function of the number of extractions (X_1) and time of extraction (X_2).

Analyzing the response surface and the contour diagram presented in **Figure 1**, two work regions provide the optimal conditions for antioxidant activity, evaluating the number of extractions and time of extractions. Both areas are comprising a higher number of extractions. The minimum and maximum levels can be considered for variable extraction time since both presented high antioxidant activity values in propolis extracts. The results show that [C_6mim]Cl has a strong extraction potential, intensified with each repetition of the process, even at the lowest extraction time tested, exhibiting affinity with

the bioactive compounds contained in propolis, which represents an advantage in the extraction process, confirmed by the better performance in antioxidant activity assay.

Despite no model being obtained for flavonoids concentrations, the highest value obtained, considering the assays based on the CCDR, for the flavonoid yield was provided by the same conditions presented in the predictive model for antioxidant activity, showing a possible relationship between the flavonoids contained in red propolis in their antioxidant action.

Experimental Validation

Based on the response surface analysis (**Figure 1**), we determined the extraction condition in which higher antioxidant activity is obtained. Such conditions are shown in **Table 3**, which indicates separately the values in which variables X_1, X_2, and X_3 provided the best values for antioxidant activity from the red propolis extracts.

The validation was carried out employing an experimental test under these conditions, presenting, by determining the extract antioxidant activity, 7595.77 μmol TE. g^{-1}, a deviation of 5.48% concerning the result predicted by the experimental design. The results obtained confirm that the obtained model is predictive and reproducible for antioxidant activity, considering the assessed conditions in this work. Results presented related to antioxidant activity were superior to those obtained by some authors, such as Santana Andrade et al. (2017, 6,665 μmol TE. g^{-1}). This optimized extract showed 394.39 ± 36.30 mg RuE. g^{-1} regarding total flavonoids, highlighting the close relationship between antioxidant activity and flavonoid concentration in the propolis extract, which has high levels of these polyphenols.

In the commercial context, the process of obtaining alcohol-free propolis extract is notably relevant. In countries, where most of the population is Muslim, less consumption of alcohol occurs, since it is forbidden to drink alcoholic beverages in the Islamic religion, requiring control in the production and import of alcohol in these countries (Al-ansari et al., 2015, 2019; Amin-Esmaeili et al., 2018). In addition to this group, abstainers and children are others who would be restricted from consuming alcoholic extracts. Although today various options for aqueous propolis extracts exist and studies related to them, hydroalcoholic extracts, in many cases, perform better in terms of biological activities (Hazem et al., 2017; Sayyadi et al., 2020). The proposal presented in this work represents an advantage concerning other processes in the yield of bioactive compounds.

TABLE 3 | Extraction condition for the antioxidant activity model validation.

Independent variables	Levels
Number of extractions (X_1)	0 (4)
Time of extraction (X_2)	−1.68 (3.3 min)
Cavitation power (X_3)	0 (300 W)

Red Propolis Extract Flavonoid Composition

The optimized and validated extracts were analyzed by high performance liquid chromatography (HPLC; **Figure 2**) and chromatogram, as shown in **Figure 3**. The characteristics of the flavonoids separated in red propolis extract are shown in **Table 4**. Medicarpin (peak 3) and isoliquiritigenin (peak 5) were the major constituents in an extract with [C_6mim]Cl, representing 24 and 18% of total flavonoids content, respectively, followed by hesperetin derivative (peak 4) and formononetin (peak 6), which contributed 17 and 12%, respectively. The extraction processes using hydrophobic IL [C_6mim]Cl proved to be selective for more hydrophobic flavonoids (medicarpin). Thus, the higher hydrophobicity of the IL implies the extraction of the most hydrophobic components of the biomass because of the π–π, n–π, and hydrophobic bonds (de Souza Mesquita et al., 2019; Murador et al., 2019b).

Red propolis can be found in tropical countries like Brazil, Cuba, Venezuela, and Mexico (Alencar et al., 2007; Cuesta-Rubio et al., 2007; Lotti et al., 2010; López et al., 2014). In research on the phytochemical markers of red propolis, daidzein was not found in all samples from Alagoas, the same geographical origin as the sample in this study. At the same time, formononetin and biochanin A were found in all studied samples (López et al., 2014).

This result demonstrates the variety in propolis chemical composition, although some authors have found these compounds in samples of red propolis. It is known that propolis chemical composition varies according to original geographic region, and propolis phytochemical profile can vary due to several factors

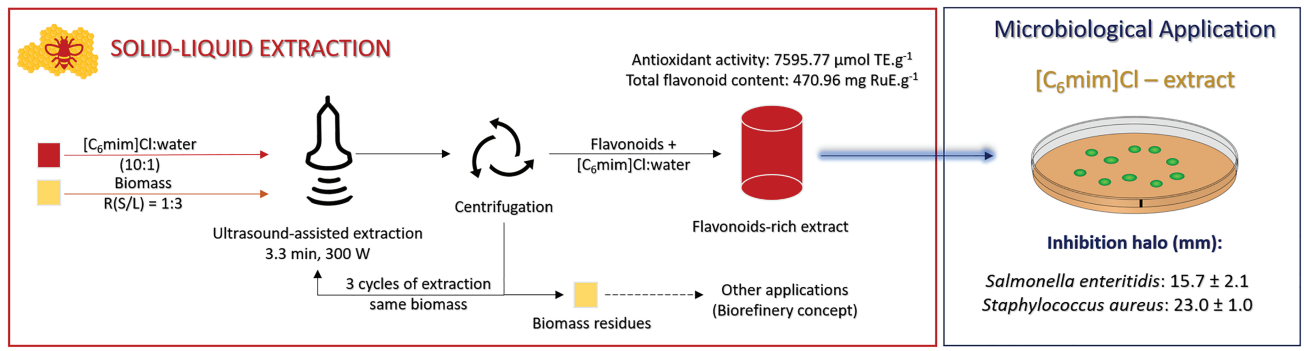

FIGURE 2 | Optimized process designed for high recovery of phytochemicals from red propolis.

such as local flora, season, and extraction methods, resulting in different extracts (Alencar et al., 2007; Cavalcanti de Pontes et al., 2018; do Nascimento et al., 2019). da Silva Frozza et al. (2017) identified some compounds of biological interest, such as isoflavones formononetin, and biochanin A besides isoliquiritigenin in samples of red propolis from Alagoas. These compounds were found in this study, but others flavonoids demonstrating once again the variability of this type of extract (Bankova, 2005; Escriche and Juan-Borrás, 2018; do Nascimento et al., 2019).

Antimicrobial Activity Test

The extract containing IL presented antimicrobial activity against the two bacteria tested, as described in **Table 5**. The optimized extract pure with IL (394.39 mg RuE. g^{-1}) and the negative control with IL [C_6mim]Cl presented no significant difference in the antimicrobial action against both tested bacteria, indicating that this solvent contributed to the antimicrobial effect. Some review articles report the antimicrobial activity of imidazolium-based IL (Reddy and Nancharaiah, 2020; Sivapragasam et al., 2020). The mechanism of IL antimicrobial activity is not known. Still, there is evidence that the long alkyl chain linear of the cation positively influences this activity, which may disrupt membrane proteins (Docherty and Kulpa., 2005; Duman et al., 2019; Florio et al., 2019).

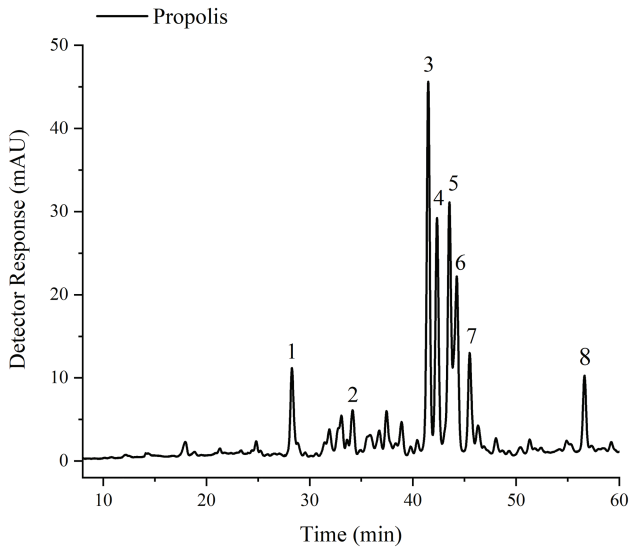

FIGURE 3 | Typical chromatogram of red propolis flavonoids extract obtained from [C_6mim]Cl. Chromatograms were processed at 280 nm.

The optimized process is presented in **Figure 2**. The disc with the extract in the concentration of 75 mg ml^{-1}, which contains 23.8 μg RuE/disc, showed antimicrobial action against *Sa. enteritidis*. The extract in the concentration of 25 mg ml^{-1} (7,9 μg RuE/disc) presented no antimicrobial activity for these bacteria. There were no comparative studies in the literature regarding the effect of the red propolis against this bacterium. Still, it is known that Gram-negative bacteria tend to better resist these compounds due to the intense lipid layer, making the cell wall impermeable for several macromolecules (Efenberger-Szmechtyk et al., 2021). Despite this, with approximately twice the extract concerning the amount of antibiotics, it was possible to verify antimicrobial activity against this Gram-negative bacterium.

The antimicrobial action against *St. aureus* from the extract with IL (394 mg RuE. g^{-1}) was statistically higher concerning the positive control for this bacterium. The extracts in all dilutions showed antimicrobial activity against this Gram-positive bacterium, including negative control. Despite this, the extract diluted to a concentration of 75 mg. 10^{-1} showed more significant inhibition compared to IL in the same conditions.

The antimicrobial action of the red propolis extract in Gram-positive bacteria has already been evidenced by some authors, who have also found promising results on the activity against the bacterium *St. aureus* (Souza Machado et al., 2016; Dantas Silva et al., 2017; Regueira-Neto et al., 2018; Silva et al., 2019).

The excellent performance of the red propolis extract with [C_6mim]Cl can be attributed to the increase in phytochemicals achieved by the extraction. At the same time, regarding negative control, the IL, as it is non-volatile, remained impregnated in the disc. Thus, tests involving other microbiological techniques are necessary to compare with the results obtained. Besides, methods for separating the IL from the extract – which is one of the advantages of using IL, as it can be recycled and reused – must

TABLE 4 | Chromatographic and UV-visible (UV-vis) characteristics of the flavonoids identified in the red propolis extract obtained from [C_6mim]Cl.

Peak	*tr* (min)[a]	Compound[b]	λ_{max} (nm)[c,d]	Standard Co-chromatography	References
1	27.5	Liquiritigenin	205, 246, 276, 312	No	Li et al., 2015
2	33.3	Daidzein	238, 249, 259 (sh), 303 (sh)	Yes	Marques das Neves et al., 2016
3	40.6	Medicarpin	237, 242, 281	No	da Silva Frozza et al., 2013
4	41.5	Hesperetin derivative	237, 242, 284	No	da Silva Frozza et al., 2013
5	42.6	Isoliquiritigenin	280, 370	No	Li et al., 2015
6	43.4	Formononetin	215, 244, 305	Yes	Srivastava et al., 2015; Marques das Neves et al., 2016
7	44.6	Biochanin A	260, 310 (sh)	No	do Nascimento et al., 2016
8	55.8	Pinocembrin	244, 280	No	do Nascimento et al., 2016

[a]*Retention time on the C18 column.*
[b]*Tentatively identified.*
[c]*Linear gradient of methanol/acidified water.*
[d]λ_{max}*: maximum absorption wavelength (nm).*

TABLET 5 | Antibiogram of red propolis extracts.

	Inhibition halo (mm)*	
Tested extract**	***Salmonella enteritidis***	***Staphylococcus aureus***
C(+)***	$34.7^{d} \pm 1.5$	$20.4^{b} \pm 0.5$
NC N.d.	$15.0^{b} \pm 0.0$	$22.0^{bc} \pm 0.0$
NC25	$0.0^{a} \pm 0.0$	$7.2^{a} \pm 0.3$
NC75	$0.0^{a} \pm 0.0$	$8.2^{a} \pm 1.0$
EXT N.d.	$15.7^{b} \pm 2.1$	$23.0^{c} \pm 1.0$
EXT25	$0.0^{a} \pm 0.0$	$8.0^{a} \pm 0.9$
EXT75	$6.8^{c} \pm 0.8$	$10.3^{d} \pm 0.6$

*Values are mean ± standard deviation (SD). Means followed by the same letter in column do not differ by Tukey's test at 5% significance. **C(+) = Positive control; NC N.d. = Negative control no dilution; NC25 = Negative control 25 mg ml^{-1}; NC75 = Negative control 75 mg ml^{-1}; EXT N.d. = Extract no dilution (394 mg RuE. g^{-1}); EXT25 = Extract 25 mg.ml^{-1} (7,9 μg RuE/disc); EXT75 = Extract 75 mg.ml^{-1} (23,8 μg RuE/disc). ***Meropenem (10 μg/disc) – Salmonella enteritidis and Vancomycin (30 μg/disc) – Staphylococcus aureus.*

be carried out to enhance the extract viability and to minimize possible toxic effects originated at the IL (do Nascimento et al., 2019).

The determination of toxicity and antimicrobial activity is crucial to release an IL to the industry. Although these compounds are considered sustainable due to their possibility of being recycled and reused, non-flammability and non-volatility are characteristics that associate them with "green" chemistry. Some articles have shown some IL based on imidazolium that are associated with toxicity. Therefore, it was impossible to consider IL as genuinely green compounds (Plechkova and Seddon, 2008). Cationic elements of IL play an essential role in microorganisms, depending on the length of the alkyl chain, contributing to toxicity (Fister et al., 2017) with significant antimicrobial activity against Gram-positive and Gram-negative bacteria (Docherty and Kulpa., 2005; Patil et al., 2020; Sivapragasam et al., 2020). This may explain the action of the IL against the tested bacteria. Thus, it is necessary to understand the behavior of both IL and red propolis individually to verify if, in fact, the sample showed antimicrobial activity or if the effect shown was overestimated by the action of the IL on bacteria.

On the other hand, the antimicrobial activity of [C_6mim]Cl can be an advantage to enable the use of the extract in several applications, such as food packaging incorporated into biodegradable films (de Souza Mesquita et al., 2020a,b). The optimized extracts supported the antimicrobial activity, inhibiting the growth of both *St. aureus* and *Sa. enteritidis*, the latter being a Gram-negative bacterium, a type known to obtain a more complex cell wall, rich in lipopolysaccharides. An interesting point was the antimicrobial action of pure [C_6mim]Cl, suggesting that this solvent interacts with the cellular components of the bacterial wall, generating the need to know these mechanisms, in addition to also evaluating the toxicity in human cells.

CONCLUSION

Propolis is known for thousands of years for its diverse biological actions. However, its use is limited in certain groups. The study of alternative solvents, moreover, to be an appealing commercial strategy from an economic point of view, enables expanded research on propolis chemical composition. [C_6mim]Cl was able to extract total flavonoid contents better than ethanol in the two concentrations tested in the screening tests. Furthermore, the optimization of the process determined the best conditions for preparing highly antioxidant extracts. Thus, it was possible to obtain a high-performance extract regarding flavonoids, with antioxidant and antimicrobial activity, as to expose in **Figure 3**, we developed a complete and optimized process designed for high recovery of phytochemicals from red propolis, compared to the methods previously developed in the literature mediated by VOS or ES.

In summary, we developed an ultrasound-assisted extraction under the operational conditions optimized at three cycles of extractions with the same biomass, up to 3.3 min at 300 W of cavitation, which was possible to recover 470.96 mg of phytochemicals with high antioxidant activity (7595.77 μmol TE. g^{-1}). Besides, the biomass residues can be used for other applications, applying the biorefinery concepts, which are extremely important in a sustainable society. In the end, the extracts were evaluated by microbiological assays, showing that the use of IL displays positive effects against *Sa. enteritidis* and *St. aureus*.

Nevertheless, some steps are still necessary in order to promote the characterization of this complex biomass and spur future investigations to minimize the effects inherent to the process, including the recycling and reuse of the [C_6mim]Cl, to make the method more sustainable. Besides, we suggest that future studies focus on understanding IL's interaction with the phytochemicals extracted from red propolis as a way to use it more effectively, better using these benefits from its rich composition in phytochemicals, also assuring the sustainable use of the raw material.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CS contributed to the execution and analysis of all experiments. VR contributed to the planning of experiments. AB contributed to the analysis and interpretation of the data included. All authors wrote and reviewed the results and approved the final version of the manuscript.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Antibiofilm Effects of Nanoparticles and Visible Light Illumination Against *Listeria monocytogenes*

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Listeria monocytogenes bacteria pose a particular risk to the food industry as the species is known to form biofilm and to survive in a wide range of challenging environmental conditions. *L. monocytogenes* can cause listeriosis, a serious food-borne disease, and effective and safe antibiofilm materials and sanitary methods for food processing environments are intensively sought. A variety of nanoparticle materials have been recognized as safe to use in food environments, which allows the application of nanomaterials also for food safety purposes. Nanoparticles together with light illumination generate reactive oxygen species which inactivate bacteria by breaking down cell membranes, proteins, and DNA. The main objective of this study was to evaluate the efficacy of nanomaterials and blue light illumination for *L. monocytogenes* ATCC 7644 biofilm inactivation. Biofilm was allowed to form for 72 h on nanocoated stainless steel and aluminum plates, after which the plates were illuminated. Non-coated control plates were used to evaluate the antibiofilm efficacy of nanocoating. Plate count method was used to evaluate bacteria counts after illumination. Nanocoating did not affect initial biofilm formation compared to the control plates. Biofilm was significantly ($p < 0.05$) reduced on stainless steel, aluminum, and TiO_2-coated aluminum plates after 72-h illumination by 1.9, 3.2, and 5.9 log, respectively. Nanocoating with visible light illumination could be an effective and safe method for enhancing food safety in food processing facilities to control biofilm formation. Evidence of antibiofilm properties of nanomaterials together with visible light illumination is limited; hence, future studies with variable light intensities and nanomaterials are needed.

Keywords: ***Listeria monocytogenes*, biofilm, nanoparticle, blue light, food safety**

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INTRODUCTION

Listeria monocytogenes bacteria are a serious risk for the food industry as they form biofilm on a variety of surfaces and can survive in a range of challenging environmental conditions (McKenzie et al., 2013; Li et al., 2018; Lee et al., 2019). The pathogen can grow at refrigeration temperatures and survive in a wide pH range and at high osmotic stress (Bucur et al., 2018), making it highly challenging to inactivate. Biofilm control is an important food safety and public health concern as *L. monocytogenes* can cause listeriosis, a serious food-borne disease, especially for pregnant women and the elderly (Farber and Peterkin, 1991). The European

Center for Disease Prevention and Control (ECDC) reports that in the EU/EEA, 30 countries reported 2,502 listeriosis cases in 2017 and cases show an increasing trend (ECDC, 2020), possibly due to the increasing size of the elderly population (European Food Safety Authority, 2018).

Biofilm allows bacteria to adhere to surfaces. It develops by irreversible attachment of bacteria, maturation, and dispersion, and is one of the major means by which bacteria spread and cause contamination (Petrova and Sauer, 2016). Biofilm can be formed on any surface, but by using nanomaterial coatings, individual bacterial cells attached to the surface can be destroyed before they start to form biofilm and thus reduce the risk of contamination and possible infections (Aponiene et al., 2017). Nanomaterials are 1–100 nm size particles, such as titanium or zinc oxides that can produce free radicals during illumination leading to the death of bacteria cells by damaging cellular components such as membrane, proteins, and DNA (Khezerlou et al., 2018). The general properties of nanomaterials, such as hardness, hydrophobicity, large surface area, and optical characteristics (Khan et al., 2019; Chan et al., 2021), make them excellent surface coatings, but they can also provide surfaces with highly beneficial self-cleaning and antimicrobial properties. TiO_2-coatings are used, for example, in public areas to ensure surface hygiene and to facilitate sanitation (Nanoksi, 2021).

Visible light illumination together with nanoparticle coating has been shown to be effective in controlling biofilm (Aponiene et al., 2017), although studies using visible light illumination and nanoparticles against *L. monocytogenes* biofilm are limited. A recent study by Aponiene et al. (2017) found that *L. monocytogenes* biofilm was reduced by 4 log from a plastic surface after zinc oxide nanoparticle coating and 405 nm light illumination (34.6 J/cm^2). Another study investigating biofilm resistance has shown that after 400 nm illumination together with zinc oxide nanoparticle coating, planktonic listeria cells underwent 7-log reduction after exposure of 17.3 J/cm^2 (Kairyte et al., 2013). In addition, titanium dioxide (TiO_2) nanoparticles have been found to enhance the antibiofilm properties of UV light (Chorianopoulos et al., 2011). Visible light and nanomaterials could be used in the food industry to ensure, among other cleaning measures, the hygiene of surfaces.

Surface contamination is the most likely route for listeria contamination of food products. A study evaluating listeria in fish factories found that up to 7.2% of process surfaces were contaminated, even though the surfaces were cleaned before taking the samples (Miettinen et al., 2001). In another study, listeria was found in a pork processing plant, especially the brining machine, with 35% of the finished products found positive for *L. monocytogenes* (Berzins et al., 2010). In the food industry, a variety of methods, such as sanitizers, enzymes, heat, and non-thermal treatments such as UV light, have been used to control biofilm formation (Galié et al., 2018), and new methods are constantly being developed and studied (Gray et al., 2018). The main objective of this study was to evaluate the photocatalytic activity of TiO_2-nanomaterial-coated surfaces against *L. monocytogenes* ATCC 7644 biofilm.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

Bacterial culture of *L. monocytogenes* ATCC 7644 was inoculated into 10 ml tryptone soy broth medium (TSB, Lab M) and grown overnight (16 h, +37°C). The bacterial culture was sedimented by centrifugation (2,900 × g, 5 min) after which the pellet was resuspended in 0.9% NaCl-peptone solution. Bacterial optical density was set to 0.1–0.15 (λ = 625 nm) using spectrophotometry to achieve a bacteria culture of approximately 10^8 cfu/ml. This suspension was further a 100-fold diluted in TSB. Aluminum and stainless steel plates (8 cm × 2 cm) with or without nanocoating were placed into petri dishes and submerged in TSB with *L. monocytogenes* at the desired dilution. Samples were incubated at +37°C for 72 h to allow biofilm formation. The incubation plates were then washed three times with 0.9% NaCl solution in an orbital shaker for 3 × 1 min at 200 rpm (Heidolph Unimax 2010) to remove unattached bacteria.

Materials

Aluminum and stainless steel plates with and without TiO_2 nanocoating were used as samples. Before each experiment, the plates were disinfected and washed with water and detergent, rinsed with distilled water, and wiped with 70% ethanol. The plates were then sterilized at +160°C for 180 min. Triplicates of the samples were used in each experiment.

LED Light Source and Illumination Conditions

A LED light source emitting 405 nm light was used for biofilm inactivation. The light intensity was measured using a thermal power sensor S314C (Thorlabs). Light intensity was 1.0–1.4 mW/cm^2 and power 20 W. The light source was attached to the wall of the refrigerator (Porkka Finland Oy) vertically, so the light hit the samples horizontally. Distance from light to samples was 30 cm. Temperature was set at +5 ± 2°C. The light dose was calculated as E = Pt, where E is the energy density (J/cm^2), P is the power density (W/cm^2), and t is time (seconds).

Detachment of Biofilm

After light exposure, the samples were transferred into sterile 50 ml falcon tubes and 50 ml of 0.9% NaCl-peptone was added. The samples were sonicated for 3 min at 35 kHz at 120% power (Elma, Transsonic Digital S) to detach the listeria cells from the plates (Bjerkan et al., 2009; Li et al., 2018). The sample tubes were vortexed (30 s, 5000 rpm), and the desired dilutions plated onto tryptone soy agar (TSA, VWR) plates. The TSA plates were incubated for 48–72 h at +37°C before colony counting.

Statistics

IBM SPSS Statistics 27 software was used for statistical analysis. Data were not normally distributed, so a nonparametric test with Bonferroni correction was performed. Values of p lower than 0.05 were considered statistically significant.

TABLE 1 | Number of *Listeria monocytogenes* on stainless steel plates after 0–72-h 405 nm light illumination at +5 ± 2°C.

Illumination 405 nm (h)	Light dose (J/cm²)	Stainless steel, light	Stainless steel, dark	TiO_2-coated stainless steel, light	TiO_2-coated stainless steel, dark
0	0	5.8 ± 0.119[a]	5.8 ± 0.119	5.9 ± 0.142	5.9 ± 0.142
2	8.6	4.2 ± 0.111[c]	5.1 ± 0.554	4.8 ± 0.286	5.6 ± 0.064
12	51.8	5.4 ± 0.251	5.2 ± 0.113	4.9 ± 0.147	5.3 ± 0.312[b]
24	13.7	4.4 ± 0.070[d]	5.4 ± 0.207	4.7 ± 0.043	5.7 ± 0.170
48	207.4	4.1 ± 0.150	5.7 ± 0.182	4.2 ± 0.582[e]	6.0 ± 0.026
72	311.0	3.9 ± 0.300	6.0 ± 0.187	3.8 ± 0.965	6.1 ± 0.047

Results are represented as logarithmic colony forming units in ml, with ± standard deviation of three replicates. Different letters indicate significant ($p < 0.05$) difference between samples.

[a]*Significant difference from stainless steel, 72 h.*

[b]*Significant difference from TiO_2-coated stainless steel, dark 72 h.*

[c]*Significant difference from TiO_2-coated stainless steel, dark 2 h.*

[d]*Significant difference from TiO_2-coated stainless steel, dark 24 h.*

[e]*Significant difference from TiO_2-coated stainless steel, dark 48 h.*

RESULTS

Effects of TiO_2 nanocoating and visible light illumination (405 nm, 0–311 J/cm²) on *L. monocytogenes* biofilm are presented in **Tables 1** and **2**, which show the cumulative light dose and the results from the dark controls. After primary biofilm attachment of approximately 6 log (CFU/ml), listeria cells were attached to every sample irrespective of the material or TiO_2 nanocoating (**Tables 1** and **2**).

Stainless Steel

On both illuminated stainless steel plates, biofilm was reduced approximately 2 log after 72 h compared to initial attachment before light illumination. There were no significant differences between stainless steel and TiO_2-coated stainless steel at any time point, but listeria biofilm was significantly ($p < 0.05$) reduced from stainless steel after 72-h illumination. No antibiofilm effects were observed in the dark controls (**Table 1**).

Aluminum

Listeria biofilm was significantly ($p < 0.01$, $p < 0.05$) reduced from illuminated TiO_2-coated aluminum and non-coated aluminum plates by 5.9 and 3.2 log, respectively. Also, in the dark control of TiO_2-coated aluminum, the number of listeria cells was significantly ($p < 0.01$) reduced after 72 h (**Table 2**).

DISCUSSION

Antibiofilm effect of TiO_2-nanomaterial coating together with visible light illumination against *L. monocytogenes* was studied on stainless steel and aluminum surfaces. 405 nm visible light illumination was used to activate the antimicrobial properties of the nanomaterial, i.e., reactive oxygen species production, which could be one of the mechanisms behind the antimicrobial results (Khezerlou et al., 2018).

Discussion of Results

Stainless Steel

Nanomaterial coatings have been reported to possess antibacterial effects in previous studies (Aponiene et al., 2017; Krumdieck et al., 2019). However, in this study, the nanomaterial did not have an additional antibacterial effect against *L. monocytogenes* compared to solely visible light illumination on stainless steel. With or without nanomaterial, the number of listeria cells decreased similarly after 72-h illumination (**Table 1**). Reduction of listeria from non-coated stainless steel plate is similar to the Li et al. (2018) study, where listeria biofilm was significantly reduced from stainless steel after 405 nm illumination. Although TiO_2 has been found to have antibacterial properties, a study by Chung et al. (2009) found that the growth of bacteria on stainless steel with TiO_2 thin film under visible light illumination did not differ from the control stainless steel surface without TiO_2. Those observations are similar to the findings of the present study, in which no antibiofilm differences were observed between TiO_2-stainless steel and the control. On stainless steel, listeria increased in the dark control at chilled temperatures, which shows the persistence of listeria, but also indicates that nanomaterial requires light to activate reactive oxygen species formation (Chorianopoulos et al., 2011; Shim et al., 2016; Aponiene et al., 2017).

The ineffectiveness of the nanomaterial on stainless steel could be partly explained by the horizontal light angle. Also, the finishing of surface material could be a challenge when applying nanomaterial. For example, the hydrophobicity of stainless steel may also explain the similarity of the results between TiO_2-coated and non-coated stainless steel. Stainless steel is a versatile surface material, but multiple findings indicate that it may not be the definitive choice when seeking self-cleaning properties for food contact surfaces.

Aluminum

TiO_2 nanomaterial coating had an additional antibiofilm effect compared to solely illumination of the aluminum surface. Approximately 3-log reduction was found between TiO_2-coated aluminum and aluminum samples after 72-h illumination (**Table 2**). The number of listeria cells decreased more from aluminum than

TABLE 2 | Number of *L. monocytogenes* on aluminum plates after 0–72-h 405 nm light illumination at +5 ± 2°C.

Illumination 405 nm (h)	Light dose (J/cm²)	Aluminum, light	Aluminum, dark	TiO_2-coated aluminum, light	TiO_2-coated aluminum, dark
0	0	6.1 ± 0.374[a]	6.1 ± 0.374[b]	5.9 ± 0.107[c]	5.9 ± 0.107[d]
2	8.6	4.6 ± 0.163	4.4 ± 0.399	4.8 ± 0.370	5.1 ± 0.109
12	51.8	5.1 ± 0.448	5.2 ± 0.272	5.0 ± 0.335	5.3 ± 0.168
24	13.7	4.3 ± 0.363	4.8 ± 0.414	3.7 ± 0.369	4.8 ± 0.178
48	207.4	3.1 ± 0.250	4.7 ± 0.391	1.7 ± 0.310	4.9 ± 0.374
72	311.0	2.9 ± 0.328	4.7 ± 0.226[e]	0.0 ± 0.000	1.8 ± 0.285

Results are represented as logarithmic colony forming units in ml, with ± standard deviation of three replicates. Different letters indicate significant ($p < 0.05$) difference between samples.

[a]*Significant difference from aluminum, 72 h.*

[b]*Significant difference from aluminum, dark 2 h.*

[c]*Significant difference from TiO_2-coated aluminum, 72 h ($p < 0.01$).*

[d]*Significant difference from TiO_2-coated aluminum, dark 72 h ($p < 0.01$).*

[e]*Significant difference from TiO_2-coated aluminum, 72 h.*

stainless steel surfaces, which could be explained by aluminum oxide (Al_2O_3) formation on the surface of aluminum. Al_2O_3 has been found to inhibit bacterial growth (Doskocz et al., 2017; Sikora et al., 2018), although in this study the antibacterial effect of Al_2O_3 is not visible at time point 0 h, since the initial attachment of listeria cells is similar between stainless steel and aluminum (**Tables 1** and **2**). The combined effect of Al_2O_3 and nanomaterial could be the reason for the complete inactivation of listeria from the TiO_2-coated aluminum plate after 72-h illumination. Interestingly, on the dark control plate of TiO_2-coated aluminum, the number of listeria cells decreased more efficiently than on the dark non-coated aluminum plate (**Table 2**). The illumination of surfaces prior to carrying out existing sanitizing practices could significantly accelerate the effectiveness of these methods and, in general, increase the microbiological safety of food processing environments.

Limitations and Future Research Aspects

The limitations in this study relate to the intensity and orientation of the illumination used. The light falls on the samples horizontally, which lowers the light intensity. For surface disinfection, illumination should be perpendicular to the surface to attain even sanitation across the surface so that possible uneven areas do not limit the effectiveness of the light and the nanomaterial. The research frame of our study was limited by the actual circumstances that are possible to achieve with the illumination method.

It is well known that the intensity of the light exposure affects to the results obtained. Therefore, the circumstances in the research frame were kept the same, including the distance and orientation of the LED to the plates. This, however, limits the number of strains and surface materials tested simultaneously. Well-established *L. monocytogenes* ATCC 7644 strain was chosen to enable international comparison of results between studies. Research by Manso et al. (2020) showed that different *Listeria monocytogenes* isolates differ in susceptibility for environmental stress; therefore, other listeria strains should be further investigated in the future to accomplish knowledge how different strains react to nanomaterials and illumination.

More studies are needed to gain deeper knowledge of the antibiofilm properties of nanoparticles on different materials and to identify the most optimal surfaces for different applications. In addition, for safety and user-friendly purposes, further optimization studies with different light exposures and nanomaterials are needed in order to achieve as high as possible antimicrobial efficacy at as low as possible light intensity. Furthermore, the durability of different nanomaterials against chemical and mechanical cleaning needs to be investigated and improved.

CONCLUSION

Listeria monocytogenes poses a threat to the food industry by forming biofilm that is difficult to inactivate; thus, new sanitation methods for biofilm control are being intensively investigated. Our study showed that visible light and TiO_2 nanocoating together are effective on aluminum surface to inactivate *L. monocytogenes* at chilled temperatures. On stainless steel, TiO_2 nanomaterial did not enhance the antibacterial effect of the surface compared to the non-coated surface. However, the amount of listeria was remarkably reduced by the effect of visible light. These results indicate that nanomaterial coating could increase the hygiene of certain surfaces compared to light exposure alone. This concept, known as the Hurdle effect, highlights the need to use multiple methods simultaneously to control or even eliminate the threat of *L. monocytogenes* in food environments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SP, KR, and JK contributed to the design and implementation of the research, to the analysis of the results, and to the writing

of the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Deciphering the Antibacterial Role of Peptide From *Bacillus subtilis* subsp. *spizizenii* Ba49 Against *Staphylococcus aureus*

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An increase in antibiotic resistance has led to escalating the need for the development of alternate therapy. Antimicrobial peptides (AMPs) are at the forefront of replacing conventional antibiotics, showing slower development of drug resistance, antibiofilm activity, and the ability to modulate the host immune response. The ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens that jeopardize most conventional antibiotics are known to be involved in severe respiratory tract, bloodstream, urinary tract, soft tissue, and skin infections. Among them, *S. aureus* is an insidious microbe and developed resistance against conventional antibiotics. In the present study, an AMP (named as peptide-Ba49) isolated from *Bacillus subtilis* subsp. *spizizenii* strain from *Allium cepa* (the common onion) exhibited strong antibacterial efficacy against *S. aureus* ATCC 25923. The mode of action of this peptide-Ba49 on *S. aureus* was deciphered through various sensitive probes, i.e., $DiSC_3$ (5) and H_2DCFDA, suggesting the peptide-Ba49 to be acting upon through change in membrane potential and by triggering the production of reactive oxygen species (ROS). This induced disruption of the cell membrane was further supported by morphological studies using scanning electron microscopy (SEM). Investigations on a possible post-antibiotic effect (PAE) of peptide-Ba49 showed prolonged PAE against *S. aureus*. Furthermore, the peptide-Ba49 prevented the formation of *S. aureus* biofilm at low concentration and showed its potential to degrade the mature biofilm of *S. aureus*. The peptide-Ba49 also exhibited intracellular killing potential against *S. aureus* ATCC 25923 in the macrophage cells, and moreover, peptide-Ba49 was found to bolster the fibroblast cell migration in the scratch assay at low concentration, exhibiting a wound healing efficacy of this peptide. These studies demonstrated that peptide-Ba49 isolated from the strain *B. subtilis* subsp. *spizizenii* could be a therapeutic candidate to combat the pathogenic *S. aureus* infections.

Keywords: antimicrobial peptides, *Staphylococcus aureus*, biofilm, intracellular activity, ROS, PAE, scratch assay

INTRODUCTION

The expeditious emergence of bacterial resistance toward conventional antibiotics, especially those related to staphylococcal infections, has become a serious healthcare concern worldwide (Mohamed et al., 2016). *Staphylococcus aureus* is an opportunistic pathogen found in humans and animals and is a major cause of morbidity and mortality in the community- and hospital-acquired infections (Gresham et al., 2000). Generally, *S. aureus* is considered an extracellular pathogen, but its invasion ability plays a critical role in cases of pertinacious and chronic infection (Clement et al., 2005). Also, it has the capability of causing skin and soft tissue infection, sepsis, mastitis, urinary tract infection, endocarditis, bone and joint infections, food poisoning, biofilm-associated infections, or septicemia (Lowy, 1998; Tong et al., 2015; Rollin et al., 2017). Infections like bacteremia and skin abscesses are generally caused by planktonic *S. aureus* cells by producing secreted toxins and exo-enzymes, whereas chronic infections are associated with *S. aureus* biofilm (Gordon and Lowy, 2008). This organism attaches and recurs on host tissues such as bone and heart valves causing osteomyelitis and endocarditis, respectively, and also on implanted materials like pacemakers, prosthetic joints, etc. (Parsek and Singh, 2003; Kiedrowski and Horswill, 2011; Barrett and Atkins, 2014; Chatterjee et al., 2014). During the implantation of medical devices or biomaterials within the host, the host proteins such as fibronectin, fibrinogen, or fibrin coat these implants and become a potential target of interaction with the matrix-binding proteins present on the surface of *S. aureus*, ultimately leading to the formation of biofilm on these implants (Cheung and Fischetti, 1990; Francois et al., 1996).

In nature, bacteria generally dwell a biofilm, a complex and dynamic surface-associated community. These are sessile microbial consortia that establish a three-dimensional structure (Costerton et al., 1999). These communities of microbes adhere to various surfaces and are confined in a self-produced extracellular matrix (Monroe, 2007). The biofilm growth displays the altered physiologies with respect to the expression of a gene and the production of proteins (Parsek and Singh, 2003; Archer et al., 2011; Kiedrowski and Horswill, 2011). *Staphylococci* are associated with the most persistent cause of biofilm-associated infections and present as a commensal bacterium on human and other mammalian mucous and skin surfaces (Vuong and Otto, 2002). The staphylococcal biofilms are developed in three main stages, i.e., (i) initial adhesion to the surface, (ii) biofilm formation and maturation, and (iii) dispersal and their maturation are linked to the EPS production, which allows the biofilm stabilization (Lister and Horswill, 2014). The dispersion phase related to the acquittal of cells that colonize the new sites.

Intriguingly, it has been observed that mature biofilm dynamically changes the niche at the site of formation, which ultimately prompts the survival of dormant and non-dividing cells within this biofilm and hampers the antibacterial efficacy of conventional antibiotics against it (Galdiero et al., 2019). Moreover, the cells of biofilm are less susceptible and show profound resistance against various available drugs and antimicrobial agents as compared to planktonic cells. There are various mechanisms that make the biofilm cell highly resistant to antibacterial agents, such as (i) reduction in growth and metabolic activity of cells residing deep in biofilm; (ii) the biofilm matrix, i.e., EPS, which acts as an adsorbent and limits the availability of antibiotics to the cells present within the biofilm; and (iii) biofilm cells protecting themselves using mechanisms such as multidrug efflux pumps and regulons (Brown et al., 1988; Gilbert et al., 2002). Generally, *S. aureus* is considered as an extracellular organism. However, it can survive and persist within the phagocytotic and non-phagocytotic cells and is responsible for frequent cause and relapsing of infections (Lemaire et al., 2007). The intracellular *S. aureus* escapes the extracellular host antibacterial defense mechanism and antibiotics have the tendency to infect other cells, and thus, it becomes more challenging to treat the infections (Mohamed et al., 2014).

Most drugs are poorly diffused into phagocytes or do not take up by the same intracellular compartment as the bacteria; thus, their antibacterial activity may be affected by the intracellular environment or by the change in bacterial metabolism (Brinch et al., 2009). Antibiotics like oxacillin, moxifloxacin, and levofloxacin etc., show poor intracellular penetration and are affected by low cellular level of accumulation (Wang et al., 2018). Antibiotics such as linezolid and gentamicin are partially and inconsistently active due to the acidic environment because of aminoglycosides and intra-lysosomal constituent binding (Van Der Auwera et al., 1991). This may lead to the use of some antibiotics at a higher extracellular concentration to get a significant activity, which further causes drug resistance and side effects. Thus, these problems have aggrandized the need for an improved or alternative therapeutics (Wang et al., 2018).

In recent years, antimicrobial peptides (AMPs) are at the forefront as alternatives to conventional antibiotics to reduce the effectiveness of this pathogenic infection. These AMPs are having multiple modes of actions such as membrane permeabilization, protein synthesis inhibition, and DNA binding etc., (Wimley, 2010; Wang et al., 2019). AMPs, produced by all living organisms, are small molecules (10–100 amino acids) that play an essential role in innate immunity (Di Somma et al., 2020). Naturally produced AMPs play a key role in host defense systems in prokaryotes and eukaryotes and act as weapons to fight against various pathogens (Yeaman and Yount, 2003; Ageitos et al., 2017). In addition to antimicrobial activity, these AMPs also represent a promising therapeutic option against biofilm infections (Lombardi et al., 2015). Furthermore, AMPs also deploy intracellular inhibitory activities as a supportive mechanism to achieve efficient killing (Le et al., 2017). There are various sources from which an AMP can be isolated, such as humans, animals, plants, bacteria, and fungi etc., (Yang et al., 2016; Huang et al., 2017; Huan et al., 2020). Among microorganisms, the genus *Bacillus* is a vast arsenal of antimicrobials and a promising host for screening new bioactive compounds (Lajis, 2020; Taggar et al., 2021).

Previously, we had reported screening and isolation of strain Ba49, i.e., *Bacillus subtilis* subsp. *spizizenii*. The peptide-Ba49 from this strain was successfully purified and characterized. The peptide-Ba49 showed a molecular weight of 3,319.2 Da

and was characterized as subtilin on the basis of *de novo* sequencing and antiSMASH results of whole genome of strain Ba49 (Taggar et al., 2021). Furthermore, peptide-Ba49 was found to be stable in a wide range of temperature and pH and showed low minimum inhibitory concentration (MIC) value against various *S. aureus* and notorious methicillin-resistant *S. aureus* (MRSA) strains, i.e., between 0.5 to 16 μM. Following 4 h of peptide-Ba49 treatment of *S. aureus* cells at 2 × MIC, a 5–6 log reduction of *S. aureus* cells was observed and this was further confirmed by using propidium iodide (PI) staining and transmission electron microscopy (TEM) studies. The above study also showed a low cytotoxicity effect against the mammalian cell lines at a concentration much higher than that of its MIC values (Taggar et al., 2021). Based on the above results, the present study was undertaken to explore the role of the peptide-Ba49 in inhibiting the formation of *S. aureus* ATCC 25923 biofilm and also in eradication of pre-formed biofilm. Furthermore, the post-antibiotic effect (PAE) of peptide-Ba49 against *S. aureus* planktonic cells at different time intervals and its intracellular killing efficacy against *S. aureus* were investigated. The fibroblast cell migration capability of peptide-Ba49, which putatively contributes to wound healing, was also evaluated.

MATERIALS AND METHODS

Bacterial Strains and Cell Line

The bacterial strain *B. subtilis* subsp. *spizizenii* MTCC 13006 was used for the production of peptide-Ba49 (Taggar et al., 2021). *S. aureus* (ATCC 25923) was used as a model organism to decipher the role of peptide-Ba49. RAW 264.7 cell line (ATCC® TIB-7) and L929 cell line (ATCC® CCL-1) were used to study the intracellular killing activity of peptide-Ba49 in macrophage and for cell migration assay, respectively.

Reagents

Muller-Hinton Broth (MHB), Muller-Hinton Agar (MHA), Nutrient Broth (NB), and crystal violet (CV) were purchased from HiMedia, India. Roswell Park Memorial Institute-1640 Medium (RPMI-1640) was procured from Sigma-Aldrich, United States. PI and SYTO 9 were purchased from Invitrogen (Thermo Fisher Scientific, India). 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), 3,3-dipropylthiacarbocyanine [$DiSC_3$ (5)], XTT sodium3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate, and calcofluor white M2R (CFW) were procured from Sigma-Aldrich, United States. Peptide-Ba49 used in all studies was produced by *B. subtilis* subsp. *spizizenii* MTCC 13006 using ZMB medium. Following harvest at 36 h, the peptide was extracted from fermentation broth using Diaion HP-20 resin followed by further purification using a series of chromatographic techniques, i.e., ion-exchange chromatography and RP-HPLC. Furthermore, the purity of purified peptide was confirmed through Tricine SDS PAGE and RP-HPLC (as single band and single active peak) as reported earlier (Taggar et al., 2021).

In vitro Antibacterial Activity of Peptide-Ba49 Against *S. aureus* ATCC 25923

Post-antibiotic Studies

The post-antibiotic studies were performed according to Tambat et al. (2019) with some modifications. *S. aureus* cells (∼10^5 CFU/ml) were treated with the two different concentrations, 4 μM (1 × MIC) and 8 μM (2 × MIC), of peptide-Ba49 in MHB medium. Followed by incubating the samples in a shaking incubator at 37°C and 150 rpm for 2 and 4 h, the samples were centrifuged and the pellets were further diluted after adjusting to the same cell density with fresh MHB medium in the ratio of 1:100 to minimize the effect of the peptide and transferred to 96-well plates. For studying the growth kinetics, the plates were incubated at 37°C and low shaking (infinite M plex TECAN) and the cell $O.D._{600}$ was measured at an interval of 30 min up to 12 h. The cells without any treatment were taken as positive control and processed similarly. The experiments were carried out in duplicate and cell growth at $O.D._{600}$ was measured in triplicate for each experiment.

Intracellular Killing Efficacy of Peptide-Ba49 in RAW 264.7 Cells

The intracellular activity of peptide-Ba49 was determined by seeding the RAW 264.7 macrophages (5 × 10^5cells/ml) into six-well plates containing RPMI-1640 and 10% FBS (without antibiotic) and incubating the plates in a carbon dioxide (CO_2) incubator at 37°C for 24 h. Later, the macrophages were infected with *S. aureus* using a 1:10 multiplicity of infection (MOI) in RPMI-1640 supplemented with 10% FBS (without antibiotic) for 2 h. After infection, 50 μg/ml of gentamicin was added to each well and the plates were incubated for 30 min to eliminate the extracellular bacteria. Furthermore, the macrophages were washed twice with 1 × PBS, followed by the treatment with different concentrations of peptide-Ba49, i.e., 4 μM (1 × MIC) and 8 μM (2 × MIC). Additionally, nisin in two different concentrations, i.e., 4 μM (1 × MIC) and 8 μM (2 × MIC), was taken as positive control and cells without any treatment were taken as negative control. Later, the cells were washed with 1 × PBS and then lysed with 0.1% saponin. The numbers of intracellular bacteria were measured at 12 and 24 h by colony counting. The experiment was carried out in triplicate with two individual repeats.

Studies on Mechanism of Action of Peptide-Ba49 Against *S. aureus* ATCC 25923

The mechanism of peptide-Ba49 against *S. aureus* was studied using fluorescence spectroscopy and scanning electron microscopy (SEM) as described in the following paragraphs.

Reactive Oxygen Species (ROS) Assay

The ROS production by *S. aureus* after the treatment with peptide-Ba49 was determined by using sensitive probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent

dye (Sigma-Aldrich), which could detect a broad range of ROS including nitric oxides and hydrogen peroxides (Arakha et al., 2015). *S. aureus* cells at concentration of 5×10^4 CFU/ml were treated with peptide-Ba49 at 4 μM (1 × MIC) and 8 μM (2 × MIC) for the sub-lethal stage (2 h). This was followed by pelleting the cells by centrifugation, washing the pellets with 1 × PBS, and transferring the cells to 96-well plates. Furthermore, the plates containing cells were mixed with H_2DCFDA at a final concentration of 5 μM and incubated at 37°C for 1 h. Untreated cells were taken as control whereas cells mixed with polymyxin B (60 μg/ml) was taken as a positive control and the fluorescence was measured at an excitation and emission of 485 and 525 nm, respectively. The experiment was done in triplicate, each with three individual repeats.

Cytoplasmic Membrane Disruption Assay

A modified method (Kwon et al., 2019) based on the membrane potential-sensitive dye, $DiSC_3$ (5), was used to evaluate the effect of peptide-Ba49 on the membrane disruption of *S. aureus* cells. Briefly, following the overnight culture of *S. aureus* in MHB medium at 37°C, the cells were washed with 5 mM HEPES buffer containing 20 mM glucose and resuspended to an $O.D_{600}$ of 0.05 in 5 mM HEPES buffer with 20 mM glucose and 100 mM KCl. Furthermore, $DiSC_3$ (5) was added at a final concentration of 0.4 μM to each well of 96-well plates and incubated for 1 h. The fluorescence was allowed to be stable and then peptide-Ba49 at concentrations of 4 μM (1 × MIC) and 8 μM (2 × MIC) was added to each well separately. The cells without any treatment were taken as control whereas the cells treated with polymyxin B were taken as a positive control. Following 1 h of incubation, fluorescence of the cells was measured at an excitation and emission of 622 and 670 nm, respectively. The experiments were carried out in triplicate with two independent repeats.

Scanning Electron Microscopy

SEM was used to examine the structural changes of *S. aureus* treated with peptide-Ba49. *S. aureus* cells at a concentration of 1×10^7 CFU/ml in the NB medium was mixed with 4 μM (1 × MIC) of purified peptide Ba-49 and incubated at 37°C for 240 min. Cells not treated with the purified AMP was taken as a control. Both peptide-Ba49 treated and untreated *S. aureus* cells were centrifuged at 5,000 rpm and 4°C for 10 min and washed twice with 1 × PBS (0.1 M, pH 7.4). Poly(L-lysine)-coated coverslip was used for cell attachment. Cells were then fixed on these coated coverslips with Karnovsky's fixative for 2 h at 4°C followed by twice washing with 1 × PBS. These cells were then dehydrated with gradients of ethanol (30, 50, 70, 90, and 100%) each for 30 min (David et al., 1973). Then, the cells were air-dried, coated with platinum, and observed under the SM-IT 300 LV scanning electron microscope (JEOL, Tokyo Japan).

S. aureus Biofilm Formation and Its Assessment

A static microtiter plate assay was performed to check the ability of the test strain to form biofilm. Briefly, the strains of *S. aureus* were grown overnight in LB supplemented with 1% (w/v) sucrose (LBS) at 37°C. Later, the cells were diluted to 1:100 in fresh LBS media and 100 μl of this diluted culture was inoculated into each well of a sterile 96-well microtiter plate. Additionally, 100 μl of LBS was used as a negative control followed by incubation of the plate at 37°C for 24 h to allow for biofilm formation (Shikha et al., 2020).

A variety of direct and indirect methods for quantification of cells in biofilms have been reported (Wilson et al., 2017). A modified method of O'toole (2011), based on the CV staining of the biofilm mass, was used (O'toole, 2011) for the determination of biofilm biomass. Following the removal of biofilm plates, the biofilm was washed gently with the 1 × PBS to remove the planktonic cells. Later, the remaining biofilm cells were fixed by adding 200 μl of 100% methanol into each well. Following incubation at room temperature for 15 min, the remaining methanol was aspired and the plate was air-dried for 15–20 min. Once fixed, biofilm was stained with 200 μl/well of 0.1% CV and the plates were incubated for 15 min at room temperature. Later, the stained plates were washed twice with distilled water to remove the extra stain followed by drying of the plates. This was followed by preparation of the biofilm suspension by adding 30% acetic acid and leaving the suspension at room temperature for 30 min to dissolve the stain properly. The absorbance was measured at 595 nm and the values obtained were considered as adhere biofilm index to the surface of the well and the extracellular mass produced by them. The percentage of biofilm was calculated by using a previously described formula (Shikha et al., 2020):

$$\%biofilm = (OD_{595}\ of\ treated\ cells / OD_{595}\ of\ untreated\ cells) * 100.$$

In the present study, XTT sodium3′-[1-[(phenylamino) -carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (Sigma-Aldrich, United States) was used to estimate the viability of biofilm (Haney et al., 2018). In this method, following the formation of the *S. aureus* biofilm (as describe above), the media was gently removed and the biofilm was washed with 1 × PBS to remove the planktonic cells. Later, the aspired cells were dissolved in 100 μl of 1 × PBS, followed by the addition of 30 μl of 1% sucrose-containing XTT-menadione solution and incubation in the dark at 37°C for 1–2 h. The absorbance was measured at 490 nm, which reflected the viability of the cells present in the biofilm (Shikha et al., 2020) and % viability of biofilm was estimated as follows:

$$\%viability\ of\ biofilm = (OD_{490}\ of\ treated\ cells / OD_{490}\ of\ untreated\ cells) * 100.$$

Evaluation of Inhibitory Effect of Peptide-Ba49 on *S. aureus* Biofilm Formation

The inhibitory effect of peptide-Ba49 on *S. aureus* biofilm formation was measured by taking 1:100 diluted *S. aureus* cells (in LBS medium) in a microtiter plate and adding different concentrations of peptide-Ba49 (2, 4, 8, and 16 μM) to each well separately at the beginning of the assay. The plates were incubated at 37°C for 24 h. The untreated cells were taken as control. Following incubation, the plate was removed and gently

washed with 1 × PBS and stained with 0.1% CV as described above. Optical density was measured at 595 nm to determine the final biomass of the biofilm. Data obtained in triplicate were analyzed and expressed as the mean plus standard deviation. The experiment was performed in three biological repeats.

The viability of the cells within the biofilm was measured using the XTT method as described above with the following modifications. Following the formation of biofilm in LBS media containing different concentrations of peptide-Ba49 (2, 4, 8, and 16 μM), the plates were removed and washed with 1 × PBS. XTT was added as described above and incubated in the dark for 1–2 h. The absorbance of the cell suspension was measured at 490 nm to determine the presence of viable cells in the biofilm. Data obtained in triplicate were analyzed, and the cell viability was expressed as the mean plus standard deviation. The experiment was performed in three biological repeats.

Confocal Laser Scanning Microscopy (CLSM) Imagining of *S. aureus* Biofilm

The effect of peptide-Ba49 on *S. aureus* biofilm formation was analyzed using confocal laser microscopy (CLSM). A previously described method (Haque et al., 2016) with some modifications was used for *S. aureus* biofilm formation. The *S. aureus* cells were grown overnight in LBS medium at 37°C and later the cells were diluted to 1:100 in fresh LBS medium. One thousand microliters of this diluted culture was poured into each 12 well-plate containing sterile coverslips and was further treated with 16 μM of peptide-Ba49, followed by incubation at 37°C for 24 h in a static condition. Cells without peptide treatment were taken as a control. The media was gently decanted after incubation, and the wells were washed twice with 1 × PBS to remove the planktonic cells. Three fluorescent dyes, i.e., SYTO 9, PI, and Calcofluor white M2R (CFW), were used for staining of the biofilm. Working solutions of SYTO 9, PI, and Calcofluor white M2R (CFW), in concentrations of 5, 30, and 0.15 μM, respectively, were prepared in 1 × PBS. The biofilms were stained with 500 μl of working solution of SYTO 9 (5 μM) and PI (30 μM) for 15 min in the dark, followed by staining with 500 μl of working solution of CFW (0.15 μM) for 3 min in the dark. Following staining, the coverslips were washed with sterile water to remove the extra stain and then placed over the glass slide with a drop of mounting oil in between to avoid the dryness and sealing the coverslips from any air contact. The stained biofilm was observed under CLSM with 40 × numerical aperture (NA) and excitation at 488 and 543 nm. Emissions at 495–535 nm (green color) and 580–700 nm (red color) were characterized for viable bacteria and dead bacteria, respectively. The EPS of biofilm when excited at 405 nm emitted light at 413–480 nm (blue color) (Hou et al., 2018).

Eradication of *S. aureus* Biofilm

Formation of *S. aureus* biofilm was carried out as previously described. Once the biofilm was established (24 h), it was gently washed with 1 × PBS to remove the planktonic cells and then mature biofilm was treated with different concentrations of peptide, i.e., 8, 16, 32, and 64 μM, and incubated at 37°C for 24 h. Following incubation, the plates were gently washed with 1 × PBS and then stained with 0.1% CV as described before. The absorbance was taken at 595 nm to determine the final biofilm biomass. Data obtained in triplicate were analyzed and expressed as the mean plus standard deviation. The experiment was performed in three biological repeats.

The viability of the cells within the pre-formed biofilm after peptide-Ba49 treatment was determined using the XTT method as described before. Briefly, following the treatment of biofilm with peptide-Ba49 at concentrations of 8, 16, 32, and 64 μM, the plates were gently washed with 1 × PBS, XTT was added, and the plates were incubated in the dark for 1–2 h. The absorbance was measured at 490 nm to determine the presence of viable cells within the pre-formed biofilm. Data obtained in triplicate were analyzed and expressed as the mean plus standard deviation. The experiment was performed in three biological repeats.

Cell Migration Assay of Peptide-Ba49

The cell migration capacity of purified peptide-Ba49 was carried out by performing *in vitro* cell migration studies of L929 cells (Balekar et al., 2012). A cell density of 5×10^5 cells/well were seeded into a six-well plate containing RPMI-1640 culture medium supplemented with 10% FBS and incubated overnight in a humidified carbon dioxide incubator at 37°C and 5% CO_2 to allow the cells to adhere on the surface. After incubation, the media was decanted and the adherent layer was scratched, followed by the removal of cellular debris by washing with 1 × PBS (pH 7.2). Later, fresh RPMI media supplemented with 10% FBS was added followed by the addition purified peptide-Ba49. Untreated cells were taken as a control. The cells were then incubated in a humidified carbon dioxide incubator at 37°C and 5% CO_2. The scratch area images were captured using a 40 × magnification microscope at different intervals of 0, 12, 24, and 36 h.

Statistical Analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests unless otherwise mentioned using GraphPad Prism 7 software. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and $^{*****}p < 0.00001$ were considered significant and $p > 0.05$ was considered as non-significant (ns).

RESULTS

Pharmacodynamic Studies of Peptide-Ba49 Against *S. aureus*

PAE of Peptide-Ba49

PAE is a phenomenon that describes the delayed regrowth of bacteria following treatment with any antimicrobial agent. It is one of the characteristics of pharmacodynamics being increasingly applied to the design of dosing regimens of antimicrobial agents. The inhibition in growth of the pathogen is followed by recovery and regain of their infectious potential, once the efficacy of antimicrobial agent was finished/reduced (Saravolatz et al., 2017). **Figure 1** suggested that *S. aureus* growth was delayed post 2 and 4 h treatment at two different

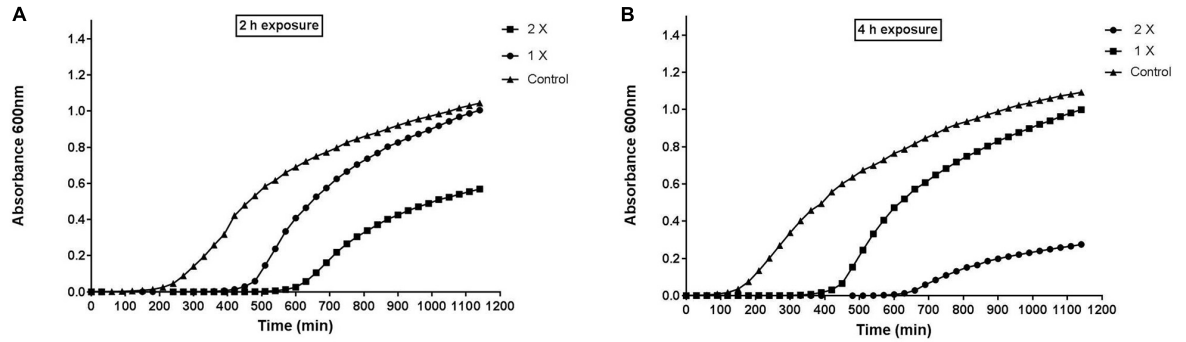

FIGURE 1 | Post-antibiotic effect of peptide-Ba49. **(A)** Post 2 h and **(B)** post 4 h of exposure of peptide-Ba49. The bacteria showed suppression for approximately 7 and 10 h at post 2 and 4 h exposure to peptide-Ba49 at a concentration of 1 × MIC and 2 × MIC, respectively. The bacterial repair was also reduced at post 4 h exposure to peptide-Ba49 at a concentration of 2 × MIC.

concentrations of peptide-Ba49. Interestingly, peptide-Ba49 at 1× and 2× MIC was found to suppress the growth of pathogen by approximately 7 and 10 h compared to control (no treatment). However, in the case of 4 h post-exposure at 2× MIC, the re-growth of the *S. aureus* cells was observed to be less as compared to post 2 h treatment at 2× MIC. These results indicated that higher cellular damage occurred within the *S. aureus* cells following 4 h of treatment of cells at 2× MIC of peptide-Ba49.

Intracellular Killing Efficacy of Peptide-Ba49 Against *S. aureus* in Macrophage Cell Line

The intracellular activity of peptide-Ba49 was evaluated by infecting the macrophage cell line, i.e., RAW 264.7 with *S. aureus*. Macrophage cells are one of the first line of defense innate cells that came into action during any infection in the host. It was observed that upon treating the infected macrophages with two different peptide concentrations, i.e., 4 μM (1 × MIC) and 8 μM (2 × MIC) for 12 and 24 h, there was a decline in $\log_{10}$CFU/ml. Intriguingly, there was significant decrease in bacterial burden in macrophages treated with 4 μM ($p < 0.0001$) and 8 μM ($p < 0.0001$) as compared to untreated cells at 12 h; a similar reduction in bacterial count was also observed in case of 24 h treated cells with 4 μM ($p < 0.0001$) and 8 μM ($p < 0.0001$). Also, there were significant declines in bacterial count in cases of *S. aureus* cells treated with peptide-Ba49 (1 × MIC and 2 × MIC) ($p < 0.001$, $p < 0.01$) as compared to positive control (cells treated with nisin at 1 × MIC and 2 × MIC). It depicted peptide-Ba49 to be having very potent killing activity against intracellular pathogens hidden and surviving inside the host cell. Furthermore, it also demonstrated the peptide-Ba49 to be having significantly higher antibacterial potency as compared to well-known AMPs such as nisin (**Figure 2**).

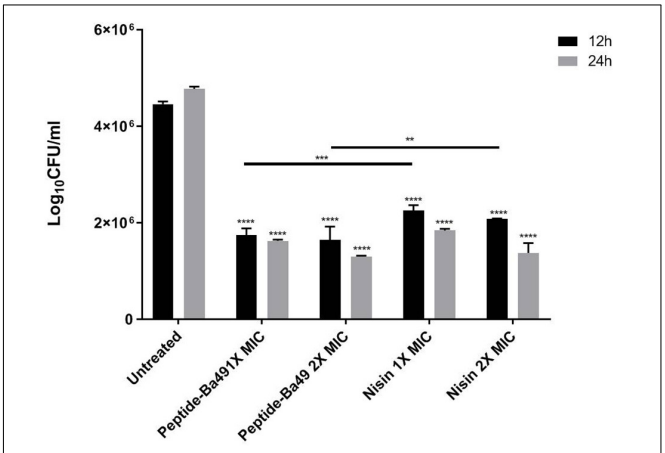

FIGURE 2 | Intracellular activity of peptide-Ba49 against *S. aureus*-infected RAW 264.7 cells: The figure showed the intracellular killing efficacy of peptide-Ba49 against *S. aureus* ATCC 25923 infected macrophage cells. After 24 h of incubation with the peptide-Ba49, there was a significant reduction in the $\log_{10}$ CFU/ml, as compared to the untreated one. The analysis was carried out in two-way ANOVA with Tukey's multiple comparisons test. $^{****}p < 0.0001$, $^{***}p < 0.0001$, $^{**}p < 0.01$ indicated the significant difference. The values "mean ± SD" were representative of two independent experiments done in triplicate.

Mechanism of Action of Peptide-Ba49 Against *S. aureus*

In case of the healthy untreated bacterial cell, ROS production is a natural side effect of aerobic respiration. The bacteria can produce enzymes like catalase and superoxide dismutase to prevent damage, which further detoxifies the ROS (Greenberg and Berger, 1989; Gasser et al., 2016). For determining the effect of the peptide on the enhancement of ROS production, *S. aureus* was treated with different concentrations of peptide in the presence of H_2DCFDA, an unspecific probe for ROS. It showed that the ROS production was enhanced when treated with 8 μM (2 × MIC) of peptide than control, whereas, at a peptide concentration of 4 μM (1 × MIC), ROS production was low (**Figure 3**). This suggested that an increase in the concentration of peptide resulted in an enhancement of ROS production, which indirectly affected the growth of *S. aureus* ATCC 25923. However, it is known that if the bacterial cells fail to minimize the excess of intracellular ROS production, membrane depolarization occurs, which ultimately leads to cell death (Yadav et al., 2020; Zhou et al., 2020). The same phenomenon was observed in our study. The effect of peptide-Ba49 on the membrane potential of *S. aureus*

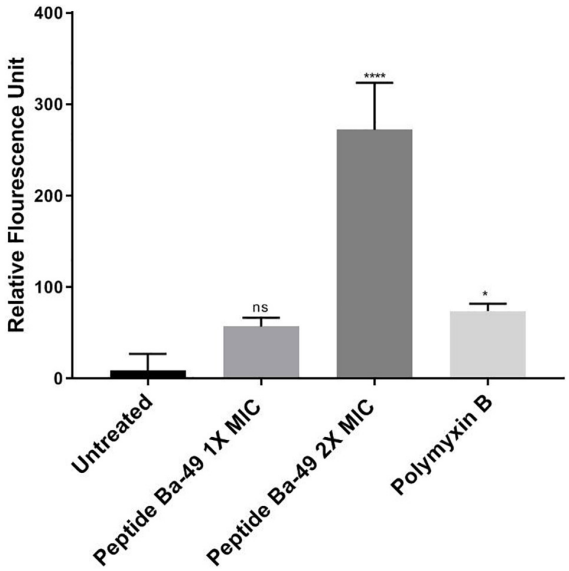

FIGURE 3 | Spectrometric analysis of ROS level alteration in *S. aureus*. Following treatment of *S. aureus* cells with varied concentrations of peptide-Ba49, the cells were stained with ROS marker 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA). The histogram represents fluorescence of H_2DCFDA dye normalized with untreated cells. One-way ANOVA followed by Holms–Sidak multiple comparisons test showed $^*p < 0.05$ significant difference between polymyxin B and the untreated one. Intriguingly, we observed a significant difference at 2 × MIC (with $^{****}p < 0.0001$) as compared to untreated.

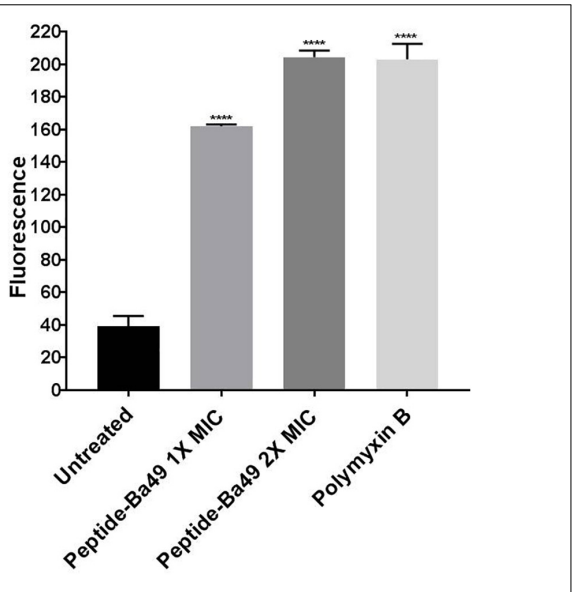

FIGURE 4 | Spectrometric analysis of membrane potential of *S. aureus* cells. Cell membrane permeability of *S. aureus* ATCC 25923 was evaluated with the release of voltage-sensitive dye $DiSC_3$-(5) during peptide-Ba49 treatment. Fluorescence was measured spectroscopically at 620–670 nm excitation and emission wavelengths. *S. aureus* cells were treated with peptide-Ba49 at concentrations of 4 μM (1 × MIC) and 8 μM (2 × MIC). Significant increase in fluorescence signal was observed in peptide-treated cells as compared to untreated cells. Polymyxin B-treated cells were taken as a positive control. The analysis was carried out in two-way ANOVA with Dunnett's multiple comparisons test. $^{****}p < 0.0001$ indicated the significant difference between control and treatment groups. The values "mean ± SD" were representative of two independent experiments done in triplicate.

was studied by determining the localization of a voltage-sensitive fluorescent cationic probe $DiSC_3$ (5) in *S. aureus* cells. Due to its cationic nature, $DiSC_3$ (5) accumulates on the polarized membranes, which results in self-quenching of fluorescence. However, during membrane depolarization, de-quenching of fluorescence dye has been reported (Boix-Lemonche et al., 2020). After treating *S. aureus* cells with peptide-Ba49 for 1 h, an increase in fluorescence was observed compared to that of the untreated one. This increased fluorescence in case of *S. aureus* cells treated with peptide-Ba49 at 1 × MIC and 2 × MIC was due to the depolarization of the cytoplasmic membrane of the *S. aureus* (**Figure 4**).

Subsequent studies on cell morphology by SEM showed that the *S. aureus* cells without peptide-Ba49 treatment had a smooth surface (**Figures 5A,B**) as compared to some apparent morphological alterations (like damaging the cell envelope and destruction of the cells) in the case of cells treated with peptide-Ba49 (**Figures 5C,D**).

Evaluation of Inhibitory Effect of Peptide-Ba49 on *S. aureus* Biofilm Formation

The effect of peptide-Ba49 on inhibition of *S. aureus* ATCC 25923 biofilm formation was evaluated at different concentrations of the peptide, i.e., 2, 4, 8, and 16 μM, as shown in **Figure 6A**. It could be observed that the biomass of *S. aureus* biofilm was significantly reduced in the presence of the peptide-Ba49. The peptide-Ba49 at concentrations of 8 and 16 μM inhibited about 90% of biofilm formation. Furthermore, the XTT assay was used to determine the viability of the cells in the biofilm, and as shown in **Figure 6B**, a significant reduction in viable cells within the biofilm in the presence of peptide-Ba49 was observed. At a peptide concentration of 8 μM, more than 50% of cells within the biofilm were viable, whereas at a concentration of 16 μM of peptide-Ba49, the cell viability was almost completely reduced.

CLSM of *S. aureus* Biofilm

CLSM studies showed that the peptide-Ba49-treated *S. aureus* biofilm could maintain the overall structure as compared to the untreated one. However, in the treated biofilm, the viable cells were less compared to the untreated biofilm (**Figure 7**). This could be due to the interaction of AMPs with the cytoplasmic membrane of the bacterial cells, causing the rupture of the cell membrane and leading to cell lysis (Bessa et al., 2019). Thus, in the peptide-treated biofilm, the red (PI labeled) cells are more as compared to the untreated one (**Figures 7C,G**). Calcofluor white M2R (CFW) dye was used to stain the EPS (extracellular polymeric substance), and a reduction in the blue signal was observed in the peptide-treated biofilm as compared to the untreated one (**Figures 7B,F**). This indicated that peptide-Ba49 prevented the biofilm formation of *S. aureus* by rupturing the cell membrane, and moreover, because of the low EPS formation, the adhesion to the surface was prevented.

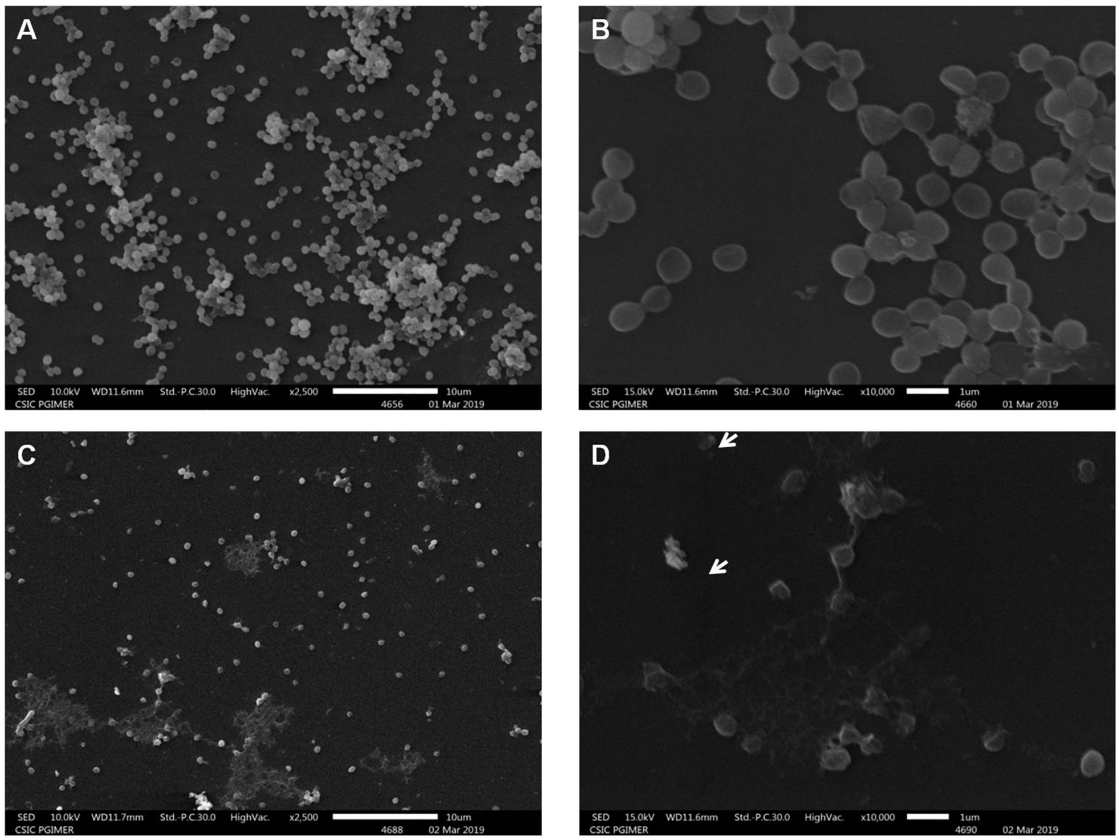

FIGURE 5 | Scanning electron microscopy of *S. aureus* cells treated with purified peptide-Ba49. **(A,B)** Untreated *S. aureus* cells from the control group at 2.5 and 10 K resolution. **(C,D)** Purified peptide-Ba49-treated *S. aureus* cells at 2.5 and 10 K resolution. A white arrow shows ruptured cells after peptide treatment for a duration of 240 min.

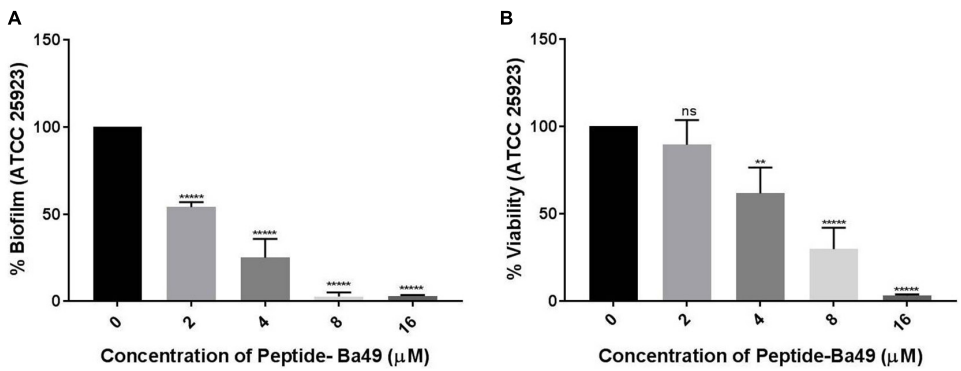

FIGURE 6 | Effects of peptide-Ba49 on inhibition of *S. aureus* biofilm formation. **(A)** *S. aureus* biofilm biomass was measured using crystal violet assay. **(B)** Viability was measured by using XTT assay. One-way ANOVA followed by Dunnett's test for multiple comparisons, $N = 2$ independent experiments with triplicates, $^{**}p < 0.01$, $^{*****}p < 0.00001$, and $p > 0.05$ were considered as non-significant (ns).

Effect of the Peptide-Ba49 on Eradication of Biofilm

The effects of peptide-Ba49 on a mature *S. aureus* biofilm was studied by treating it with varied concentrations of the peptide and observing the reductive effect, followed by additional 24 h incubation. In the case of the mature biofilm, the number of bacteria was increased up to 10-fold; thus, higher concentrations of peptide-Ba49 were used to treat the mature biofilm, i.e., 8, 16, 32, and 64 μM. Treatment with peptide-Ba49 at concentrations of 16–64 μM resulted in a significant reduction in preformed biofilm biomass (OD_{595}), as shown in **Figure 8A**. The colorimetric reduction assay of tetrazolium salt (XTT) also showed that about 80% of the cells in the biofilm

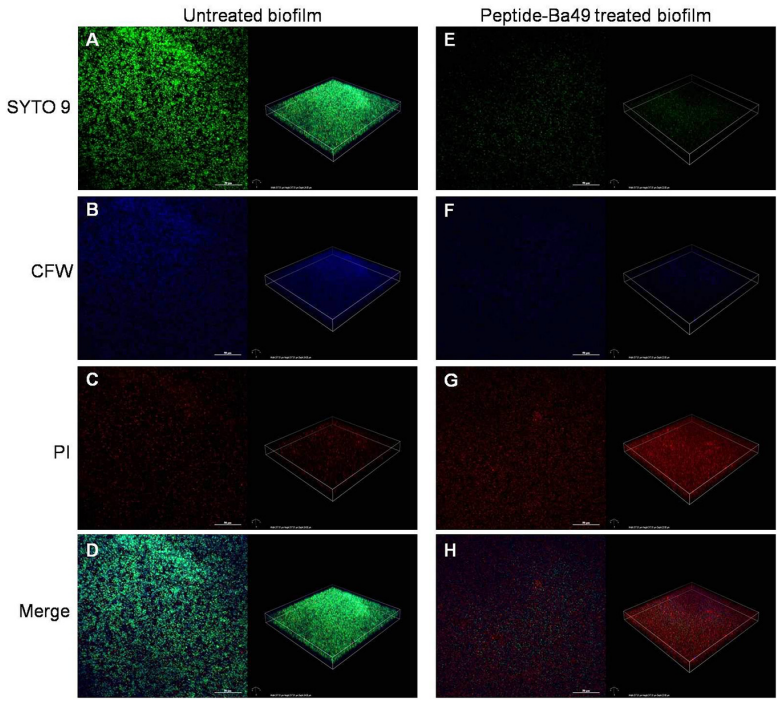

FIGURE 7 | Confocal imaging of live/dead stained *S. aureus* biofilm [left—green channel (live), middle—red channel (dead), and right—merged channel]. 2D and 3D images showing the *S. aureus* biofilm inhibition effect of peptide-Ba49; untreated *S. aureus* biofilm **(A–D)** and Peptide-Ba49-treated biofilm **(E–H)** were post stained with Syto 9 (live stain, green), CFW (EPS stain, blue), and PI (dead stain, red).

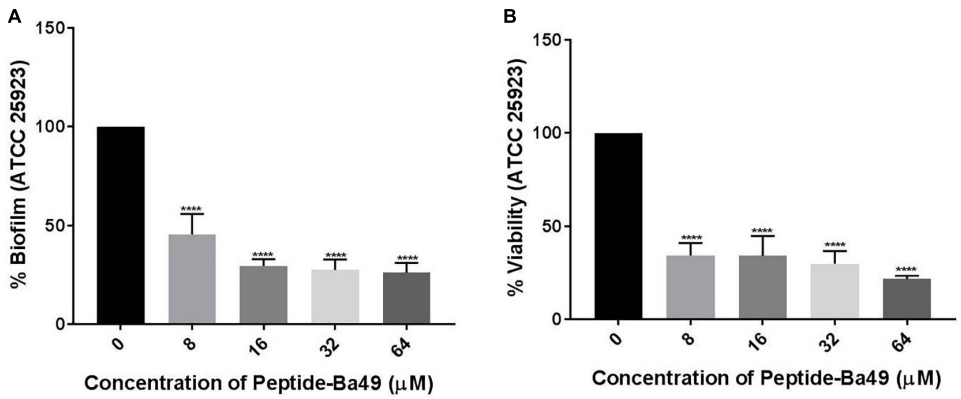

FIGURE 8 | Effects of peptide-Ba49 treatment on inhibition of pre-formed biofilm of *S. aureus*. **(A)** Pre-formed *S. aureus* biofilm biomass after peptide-Ba49 treatment was estimated using crystal violet assay. **(B)** Viability was evaluated using XTT assay. One-way ANOVA followed by Dunnett's test for multiple comparisons, $N = 2$ independent experiments with triplicates, $^{****}p < 0.0001$.

were metabolically inactive at a concentration of 64 μM of peptide-Ba49 (**Figure 8B**).

Cell Migration Assay

Cell migration is a rate-limiting factor in the wound healing process (Cappiello et al., 2018). The migration capacity of the murine fibroblast cells (L929) under the stimulation of peptide-Ba49 was determined by scratch assay to evaluate wound closure *in vitro*. It was observed that after the stimulation of L929 cells with peptide, the migration capacity became faster and the wound closure was approximately 50% at the 24 h time point. Moreover, following 36 h of treatment of the L929 fibroblast cell line with peptide-Ba49, almost complete wound closure was observed as compared to control, i.e., untreated cells (**Figure 9**).

DISCUSSION

The ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*

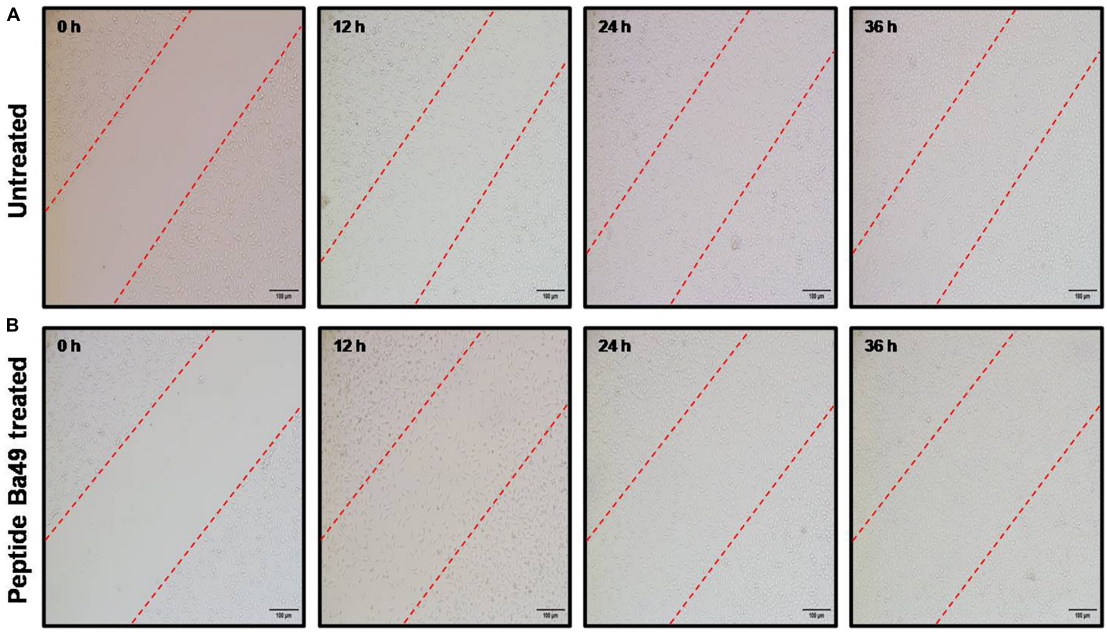

FIGURE 9 | Cell migration assay. The fibroblast cells L929 were treated with peptide-Ba49 and observed under microscope until 36 h to evaluate the cell migration: **(A)** Untreated L929 cells. **(B)** Peptide-Ba49-treated L929 cells.

aeruginosa, and *Enterobacter* species) are the most resistant to almost all the antibiotics and cause various chronic infections. *S. aureus* is one of the pathogens that infect the host and cause mild to chronic infections (Haddadin et al., 2002; Koch et al., 2014). Furthermore, the alarming factor related to *S. aureus* infection is their capability to form biofilm and become highly resistant to host attacks and antimicrobials (Lewis, 2001; Mah and O'toole, 2001; Savage et al., 2013; Koch et al., 2014). The biofilm cells are generally more than 1,000 times resistant to planktonic cells though it may vary from organism to organism. Various factors affect biofilm resistance, such as growth rate, temperature, nutritional value, and pH, and the most critical factor affecting biofilm resistance is the slower diffusion of drugs (Shikha et al., 2020). This necessitates the urgency for antibiotics that not only eradicates the planktonic cells but also has the potential to decimate the biofilm. *S. aureus* is also termed as a facultative intracellular pathogen and has the ability to survive within the host cells and escape the detection of professional phagocytes (Fraunholz and Sinha, 2012). Therefore, the intracellular bacteria enhance drug resistance development by protecting it from a higher concentration of extracellular antibiotics (Thwaites and Gant, 2011), thereby necessitating a need to develop new strategies to combat infectious pathogens. In that direction, AMPs are a new treatment strategy for bacterial infections that are rapidly in focus due to increased resistance to conventional antibiotics (Pfalzgraff et al., 2018).

In our previous study, an AMP, i.e., peptide-Ba49, was isolated and purified from *B. subtilis* subsp. *spizizenii* Ba49 (MTCC 13006), a strain isolated from *Allium cepa*. Later, based on whole genome analysis and *de novo* amino acid sequencing, the peptide-Ba49 was found to be identical to subtilin and shown to be having low MIC values in the range of 0.5–16 μM against different *Staphylococcus* strains and MRSA strains. Subsequently, the time kill studies showed a fast bactericidal efficacy against S. *aureus*, i.e., within 4 h (Taggar et al., 2021). In the present study, the role of purified peptide-Ba49 against methicillin-sensitive *S. aureus* ATCC 25923 strain was further deciphered. The potential of this peptide as a therapeutic candidate was evaluated by studying its PAE and *in vitro* intracellular infection. PAE is defined as the suppression period of bacterial growth that persists after a limited exposure of organisms to antimicrobials (Nedbalcova et al., 2019), and the degree of PAE relates to the degree of cellular damage done by the antimicrobial agent to the bacterial cell (Haukland and Vorland, 2001). The PAE of peptide-Ba49 indicated an extended PAE, i.e., 10 h against *S. aureus* after 4 h of exposure at a concentration of 8 μM (2 × MIC) (**Figure 1B**). This suggested that the damage to the cell could be immense and might take a long time to repair. In comparison, a naturally produced AMP DLP4 from hemolymph of *Hermetia illucens* has been reported to have a PAE value of 8.83 h against *S. aureus* (Li et al., 2020), and also a synthetic AMP, LTX-109, was shown to have 5.51 h of PAE against *S. aureus* (Saravolatz et al., 2017).

It is also well known that *S. aureus* can penetrate and survive within the host cell and cause chronic infections. Due to the difficulty in antibiotic passaging through cellular membranes, it becomes more challenging to treat the infection at the intracellular stage. So, in addition to the extracellular activity of the AMP, it is also required to have intracellular killing activity (Wang et al., 2018). Previously, it was shown that the peptide-Ba49 has low cytotoxic effect on the various cell lines (Taggar et al., 2021). The investigations on the intracellular killing activity of peptide-Ba49 showed a significant reduction in

bacterial burden upon treating *S. aureus*-infected macrophages with peptide-Ba49.

Furthermore, the mechanistic insight into peptide-Ba49-mediated killing of *S. aureus* was elucidated. The intracellular generation of ROS was enhanced after the treatment with the peptide at a concentration of 8 μM, as measured by using a ROS-sensitive probe, H_2DCFDA. It suggested that intracellular production of ROS increased the oxidative environment of the cell by altering the cell membrane resting potential. Moreover, oxidative stress plays a crucial role in altering the bacterial membrane permeability and damage the cell membranes (Shaikh et al., 2019). Furthermore, a voltage-sensitive $DiSC_3$ (5) probe was used to investigate the change in *S. aureus* cell membrane permeability after treatment of peptide-Ba49. Interestingly, an increase in fluorescence was observed, indicating a change in the membrane potential of *S. aureus* cells after peptide treatment. This was further confirmed by morphological changes analyzed by SEM, which showed membrane lesions (**Figure 5D**), indicating that the damage of the cell could have occurred by change in membrane potential of *S. aureus* cells following peptide treatment.

S. aureus biofilm infections are of a primary concern compared to planktonic cell infections, and this is also a cause for resistance to conventional antibiotics (Savage et al., 2013; Koch et al., 2014; Olsen, 2015). An infection associated with biofilm requires high drug concentrations and are extremely difficult to treat (Koch et al., 2014; Mcconoughey et al., 2014; Liu et al., 2015). Interestingly, it was found that the peptide-Ba49 could inhibit *S. aureus* biofilm formation at a concentration of 8 and 16 μM within 24 h. The confocal imaging using PI and SYTO-9 showed the inhibition of biofilm formation treated with peptide-Ba49 (**Figures 7E,G**) compared to the control, i.e., untreated cells (**Figure 7F**). This was further confirmed by less EPS production during biofilm formation in the presence of peptide-Ba49 as compared to control (**Figure 7B**), indicating inhibition of *S. aureus* growth in the presence of peptide-Ba49. However, at a concentration of 16 μM, there was a significant reduction in viability of bacterial cells within the biofilm. Peptide-Ba49 was also found to kill the bacterial cells in the mature biofilm of *S. aureus* ATCC 25923 after 24 h of peptide treatment of *S. aureus* cells at a concentration of 64 μM where the cell biomass and cell viability within the mature biofilm were significantly reduced. These results signified that peptide-Ba49 could be developed as an antimicrobial agent for treating *S. aureus* planktonic as well as *S. aureus* biofilm-associated infections.

In the wound healing phase, cell proliferation and migration are two essential features, whereas an *in vitro* scratch assay mimics a wound and evaluates the cell migration rate.

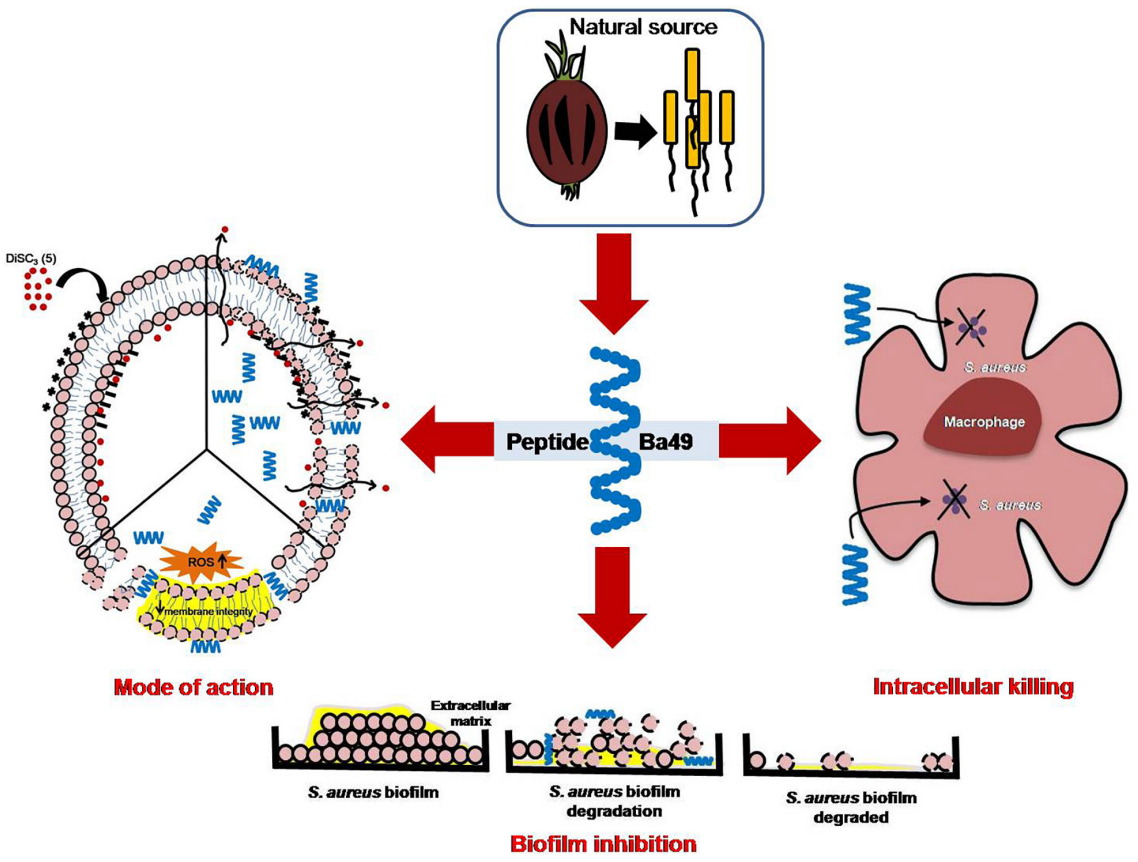

FIGURE 10 | Proposed mechanism of action of peptide-Ba49 isolated from *Bacillus subtilis* subsp. *spizizenii* strain from *Allium* cepa against *S. aureus*.

Due to the disruption of the cell monolayer, it loses cell–cell interaction, and increasing concentration of growth factors and cytokines at the edge of the wound further initiates cell migration and proliferation (Liang et al., 2007; Pitz Hda et al., 2016). Interestingly, peptide-Ba49 at a concentration of 2 × MIC prompted the L929 fibroblast cell proliferation. This is a positive event for the wound healing process, as fibroblast cells are essential cells because of their involvement in wound contraction and ECM production (Balekar et al., 2012).

In conclusion, the peptide-Ba49 showed antimicrobial activity against MRSA isolate. This peptide was effective against *S. aureus* planktonic cells and showed pre- and post-antibiofilm activity at low concentration. It also demonstrated intracellular activity in the MIC range studied and efficiently reduced the bacterial burden from *S. aureus*-infected macrophages (RAW264.7). All these properties of peptide-Ba49 established it as a potential candidate for therapeutic applications (**Figure 10**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DS, RT, and SS conceived the project. RT and SS performed the experiments. DS, RT, and SS analyzed the data, wrote and edited the manuscript, with input from all of the authors. All authors contributed to the article and approved the submitted version.

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Characterization of a Potent New-Generation Antimicrobial Peptide of *Bacillus*

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An antimicrobial peptide [*Bacillus* antimicrobial peptide (BAMP)] produced by *Bacillus paralicheniformis* was isolated from the Indian traditional fermented food and characterized. The antimicrobial peptide BAMP showed many unique features such as thermostability (4.0–125°C), pH tolerance (pH 2.0–9.0), and resistance to physiological enzymes (trypsin, chymotrypsin, pepsin, proteinase K, protease, and catalase), and food-grade metal salts do not inhibit the activity. The broad spectrum of BAMP (antimicrobial activity) makes it a suitable candidate for food preservation as well as antimicrobial therapy. BAMP was found to exhibit a bacteriostatic effect on *Salmonella typhi* and controls the viability of *Listeria monocytogenes* in chicken meat efficiently. BAMP was found to establish eubiosis, as it is not antagonistic to *Lactobacillus*. Its non-hemolytic nature makes it suitable for therapy. Various genome prediction tools were adopted and applied to understand their localization, gene arrangement, and type of antimicrobials. Founded on its superior functional attributes, BAMP is a potent new-generation antimicrobial peptide.

Keywords: bacteriocins, *Bacillus* antimicrobial peptides, GRAS organisms, probiotics, *Listeria* monocytogenes, *Vibrio harveyi*

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INTRODUCTION

A few bacteria produce low-molecular-weight antimicrobials known as Ocins. Bacteriocins are identified as ribosomally synthesized (RPS) antimicrobial peptides. Bacitracin is a non-ribosomal (NRPS) synthesized peptide, usually synthesized and secreted by *Bacillus* spp. The characteristics of various bacteriocins such as source, features, and use in the food industry are studied/reported (Choyam et al., 2019). During the process of making various foods for long durability, the food industry incorporates chemical additives that elicit toxic elements. The introduction of chemical-based food additives also brings a change of taste and flavor and lowers the quality of food, further causing a health hazard. Therefore, natural food additives/preservatives such as bacteriocins and bacitracin may play an essential role in the maintenance of food taste, color, and flavor in the long term. Thus, natural antimicrobials such as bacitracin and bacteriocins from probiotic or generally recognized as safe (GRAS) microorganisms promise safety in use as a preservative in meat, aquatic, vegetable, and dairy industries (Stevens et al., 1991; Cleveland et al., 2001; Deegan et al., 2006; Settanni and Corsetti, 2008).

There are four different classes of bacteriocins, namely, I, II, III, and IV. The class I bacteriocins are usually produced by LAB; their molecular weight ranges from 1 to 5 kDa (Boman, 1995), and posttranslational modifications are essential for their function. They are known as lantibiotics,

containing lanthionine, an unusual amino acid (Nissen and Nes, 1997). A few examples of class I are Nisin, Subtylin, and Variacin 8 (Gross and Morell, 1971; Gross and Kiltz, 1973; Gilmore et al., 1994). Class II bacteriocins are thermostable, and posttranslational modifications are not required and do not contain the lanthionines hence they are non-lantibiotics. Class II bacteriocins are further divided into three subclasses, namely, IIa, IIb, and IIc (Ennahar et al., 2000). A few well-known class II types are pediocin PA-1, lactacin F, and enterocin AS-48 (Nissen et al., 2009). The class III bacteriocins are thermolabile and larger than 10 kDa (Savadogo et al., 2006). They are subclassified as class IIIa or bacteriolysins and class IIIb or non-lytic bacteriocins. Lysostaphin is the best example (Bastos et al., 2010). Plantaricin S and leuconocin S belong to class IV bacteriocins; they are complex proteins containing lipid or carbohydrate moieties (Upreti and Hinsdill, 1975; Oman et al., 2011). Recently, Cotter et al. (2005) introduced a universal method of bacteriocin classification.

The most widely (commercially) used bacteriocins derived from *Lactobacillus* are Nisin, Pediocin (Broughton, 1990; Twomey et al., 2002), and antimicrobial peptides like bacitracin from *Bacillus* spp. However, the ability of Nisin to function in the presence of food preservatives is yet to be known. Besides, Nisin is thermolabile and protease sensitive, with a narrow (pH range 7.0–8.0) spectrum of activity and specifically, it does not work against *Salmonella*. Therefore, a few limitations still exist for its use in the food industry and human therapy. Thus, the present need is to discover potent and indigenous antimicrobials either bacteriocins or bacitracin. The other well-known antimicrobial peptide is bacitracin, which is a mixture of 10 cyclic dodecapeptides, non-ribosomally synthesized by *B. subtilis* and *B. paralicheniformis* (Azevedo et al., 1993; Ishihara et al., 2002). It is well investigated that bacitracin is primarily active against Gram-positive bacteria only (Rietkötter et al., 2008). Bacitracin is of different types with varying degrees of antibacterial activity. They include bacitracin A1, A2, B1, B2, B3, C, D1, D2, E, F, H1, H2, H3, I1, I2, and I3 with few amino acid differences.

The primary aim of the present manuscript lies in characterizing bacitracin derived from *B. paralicheniformis*, finally concluding their importance in the food industry. Based on its characteristics and functional attributes, we reason that it is a potent antimicrobial agent.

MATERIALS AND METHODS

Bacterial Strains Used in the Study

All the microorganisms, including indicator strains, were received from the Microbial Type Culture Collection (MTCC-IMT, India), and they were grown in Luria-Bertani (LB), De Man, Rogosa, and Sharpe (MRS), brain heart infusion (BHI) media (Hi-media), or nutrient broth (NB). *B. paralicheniformis*, a novel isolate showing antimicrobial activity (isolated from Himachal Pradesh traditional foods), was cultured in nutrient broth (Hi-media) at 37°C under shaking conditions (200 rpm). The list of microorganisms used in the study is shown in **Table 1**.

TABLE 1A | Strains used for the study.

Sl. No.	Indicator organisms	MTCC No.
1	*Pseudomonas putida* (−ve)	2492
2	*Corynebacterium callunae* (+ve)	700
3	*Pseudomonas aeruginosa* (−ve)	1934
4	*Salmonella typhi* (−ve)	New isolate
5	*Enterococcus gallinarum* (+ve)	7049
6	*Streptococcus thermophilus* (+ve)	New isolate
7	*Staphylococcus aureus* (+ve)	1430
8	*Vibrio harveyi* (−ve)	7954
9	*Streptococcus mutans* (+ve)	497
10	*Vibrio cholerae* (−ve)	3904
11	*Bacillus cereus* (+ve)	430
12	*Lis*teria monocytogenes (+ve)	839
13	*Clostridium perfringens*	450
14	*Lactobacillus plantarum*	Lab Isolate
15	*Enterococcus raffinosus*	Lab isolate

−ve represents Gram-negative and +ve represents Gram-positive bacteria.

Isolation of BAMP Producer

More than a hundred bacterial strains were isolated from the Himachal Pradesh Traditional Fermented Foods such as mango pickle, rice-based fermented foods, and bamboo shoots. The source material was dissolved (approximately 10 g/ml) in peptone water, and appropriate serial dilutions were made, spread to get an isolated colony. Subsequently, microorganisms such as *Salmonella* spp., *Listeria* spp., and *Vibrio* spp. were screened for their antimicrobial activity against common food spoilage. The isolated colonies were inoculated and grown in MRS, LB, BHI, and NB from 12 to 18 h. The cells were harvested by centrifugation (9,000 × *g* for 15 min at 4.0°C). The cell-free supernatant and/or crude extract of 15 isolates were subjected to well-diffusion assay with various food spoilage microorganisms as indicators.

Identification and Characterization of the Isolate

The isolated microorganism was grown in a nutrient medium, and genomic DNA was isolated using Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific). The genomic DNA was used for 16S rRNA-based identification. The universal forward and reverse primers were used for polymerase chain reaction (PCR) amplification of 16S rDNA, which were 27F/1492R (5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5′-GGTTACCTTGTTACGACTT-3′) with *Taq DNA polymerase* (Thermo Scientific DyNAzyme II DNA Polymerase). Thermal cycling parameters followed are as follows: hot-start at 95°C for 2–4 min, followed by 30 cycles of denaturing at 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 1 min. Final extension for 5 min at 72°C. Subsequently, the PCR product was subjected to 1.0% agarose gel, sequenced, and submitted to NCBI. DNA gyrase-based phylogenetic studies were also followed for taxonomic characterization (data not shown).

Growth Curve Studies of the Producer Strain

In all the studies, a single isolated colony was obtained by streaking cultures from the glycerol stock. The streaked agar plate was incubated for 12–16 h, at 37°C. The single isolated colony of *B. paralicheniformis* was inoculated in nutrient broth, incubated for 12 h at 37°C at 200 rpm shaking condition. Subsequently, the overnight culture was subcultured to obtain an optical density (OD) of 0.1 at 600 nm. The resulting fresh culture was grown until it reached the stationary phase. During the growth, the OD of the culture was measured at 600 nm after every hour using an ultraviolet (UV) spectrophotometer (Shimadzu analytical PVT LTD, Mumbai, India). The growth curve was expressed in a graph by plotting OD against time. As reported by Stevenson and Aalto-Araneda, we followed the measurement of OD as it is deemed suitable for the relative comparison of growth patterns of microorganisms than absorbance (Stevenson et al., 2016; Aalto-Araneda et al., 2020).

Antimicrobial Peptide Production Kinetics

B. paralicheniformis was grown in nutrient broth at 37°C under shaking conditions as explained above. The production kinetics of the antimicrobial compound was estimated by collecting the culture supernatant after every hour of growth for 12–16 h. The resulting supernatant (1.0 ml) was centrifuged at 9,000 × *g* for 20 min at 4.0°C which was later filtered using 0.22 μm sterilized cellulose membrane (Millipore). The supernatant was used for preliminary studies, later subjected to agar well-diffusion assay against the indicator strain *Vibrio harveyi*. The zone of inhibition (ZOI) (activity) was measured using an antibiotic zone measurement scale in millimeters (mm).

Well-Diffusion Assay With Different Indicator Strains

The cell-free supernatant produced as explained above was used for Agar well-diffusion assay (Choyam et al., 2015) to detect the antimicrobial activity. The cell-free supernatant (CFS) was centrifuged at 9,000 × *g* for 30 min at 4.0°C, and the supernatant was passed through a 0.22-μm membrane filter (vacuum-driven membrane filter, Hi-media), followed by ammonium sulfate precipitation, and dialyzed. The Gram-negative and Gram-positive pathogenic and food-spoilage microorganisms were used as indicators, as listed in **Table 1**. The culture of the indicator strain is grown overnight and mixed with BHI soft agar (0.75%), then subsequently poured over the previously prepared BHI agar and allowed to solidify. The wells (approximately 0.6 mm diameter) were made in the agar plate using a sterile borer. The filtered supernatant (30 μl) was added to the well and incubated for 12 h at 37°C. Nisin was used as a positive control (based on the indicator). The ZOI was measured after 12 h of incubation using an antibiotic zone measurement scale. MRS/NB agar with 0.1% calcium carbonate ($CaCO_3$) (neutralizes acidity caused by organic acids) was used as a control for well-diffusion assay (Sigma-Aldrich, Bengaluru, India).

Overlay Assay

The chromatography-purified BAMP was subjected to tricine-SDS-PAGE. The gel was made in two parts, one part of the gel contained the molecular weight marker (lane 1) and purified protein (lane 2). The other part of the gel was subjected to fixation, later washed with 100 mM sodium phosphate buffer. Subsequently, SDS was removed by washing thrice with 2.5% Triton-X 100 for 3 h. Finally, the gel was washed with Milli-Q water to remove Triton X-100. The resulting gel was overlayed onto the Petri plates seeded with *V. harveyi*, which were incubated at 37°C overnight.

Percent Viability of the BAMP-Treated *V. harveyi*

The overnight grown culture of the indicator microorganism (*V. harveyi*) was subcultured (1.0%) in a fresh MRS broth. The experiment was divided into four groups. One group was maintained as a control without the addition of any antimicrobials. BAMP CFS (crude) and BAMP powder were added to the second and third groups, respectively, soon after the subculture in a volume and quantities as per the requirement. The culture samples were collected at regular intervals (1 h each) for 12 h (for viability studies). They were serially diluted and plated to determine the colony-forming units (CFU/ml). The results were compared with negative and positive controls.

Effect of pH, Temperature, Different Proteolytic Enzymes, Food Preservatives, and Food-Grade Metal Salts

The BAMP protein solution was adjusted at pH 1.0–14.0 using 1.0 N HCl and 1.0 N NaOH and incubated at 37°C for 2 h. The activity against the indicator microorganism (*V. harveyi*) was checked by approximately neutralizing the pH to 8.0. To understand the effect of temperature, aliquots of the culture supernatant were taken and treated at different temperatures, including 80°C for 1 h and 100°C for 30 min, and were then autoclaved for 20 min. The untreated protein solution was used as a control.

The sensitivity of the antibacterial substance (BAMP) to various physiological enzymes was evaluated. Aliquots of the protein solution (CFS) at different pH values were incubated (1:1, *v*/*v*) with an enzyme (1.0 mg/ml) and their respective controls for 2 h at 37°C. Similar experiments were conducted with sodium dodecyl sulfate (SDS), Tween 20, Tween 80, and Triton X-100, which were incubated with the antimicrobial compound (0.1 mg/ml) at a final concentration of 1.0% (*v*/*v*) for 5 h at 37°C. The antimicrobial activity of BAMP was checked against the indicator microorganism, *V. harveyi*. The permitted concentrations of the detergents alone were tested for their antagonistic activity, and they were taken as –ve controls. All the experiments were conducted thrice, and the average was considered for theoretical performance.

To understand the effect of various metal salts (used as preservatives) on antagonistic activity, BAMP was incubated with various food-grade metal salts such as $MgSO_4$, $FeSO_4$,

$MnCl_2$, $AgNO_3$, $ZnSO_4$, $CdCl_2$, $CuSO_4$, and $CaCl_2$ at a final concentration of 1.0 mg/ml for 1 h at 37°C, and the activity was measured against the indicator microorganism. Untreated samples and metal salts at their final concentration were taken as controls (Kumar et al., 2015).

Finally, the effect of various food preservatives and their effect on antimicrobial activity of BAMP was followed (as above) by considering sodium chloride, sucrose, acetic acid, ascorbic acid, benzoic acid, sodium benzoate, and sodium sulfite. Here, BAMP was mixed with different concentrations of preservatives, and the activity checked against the most common food pathogens (*L. monocytogenes*, *V. harveyi*, *S. mutans*, and *S. aureus*). The ZOI around each well was measured using an antibiotic zone measuring scale. The residual activity and percentage activity reduction of BAMP upon different treatments were calculated, tabulated, and plotted separately (all the experiments were performed thrice).

Purification of the Antimicrobial Agent

The culture of *B. paralicheniformis*, grown overnight, was subcultured in 1 L of nutrient broth and incubated for 72 h at 37°C under shaking conditions (200 rpm). The culture was harvested by centrifugation at 9,000 × *g* (radius of rotor/diameter 100 mm) for 30 min at 4.0°C. The supernatant was filtered through a 0.22-μm filter. The filtered supernatant was subjected to 0–70% ammonium sulfate precipitation and dialyzed. The dialyzed sample was diluted with NaCl and phosphate buffer and pH adjusted to 7.0. Before, the gel filtration (Superdex 75, Sigma Aldrich) column was equilibrated with (one column volume) 50 mM phosphate buffer and 150 mM NaCl, pH 7.0 (Amersham Biosciences, FPLC, Uppsala, Sweden). Subsequently, the sample (2.5 ml) was loaded and its flow through was tested for the activity. Later, the column was washed with one column volume of wash buffer. Suitable flow rate (1.0 ml/min, 76.4 cm/h) such as the long column with a slow flow rate was considered as it may give good resolution. Finally, the protein was eluted with 150 mM phosphate buffer and 2.0 ml fractions were collected. The whole procedure of purification was followed for 130 min. Fraction collection was initiated at 40 min and terminated at fraction number 40. Each fraction was subjected to well-diffusion assay/antimicrobial activity.

Determination of Minimum Inhibitory Concentration of Purified BAMP

The minimum inhibitory concentration (MIC) of the purified antimicrobial peptide/protein (AMP) was (against Gram-negative and Gram-positive microorganisms, antagonistic activity against probiotic microorganism) determined using the microbroth dilution method (methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria) (Eucast) [Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution, 2003; Temitope et al., 2020]. The method is as follows; *V. harveyi* test strain was grown in MRS at 37°C at 180 rpm. The culture was grown until the mid-exponential growth phase with an OD_{600} of 0.4. Subsequently, the culture was adjusted to the final bacterial count of ∼4 × 105 CFU/ml. A hundred microliter of sterile media containing the twofold serial dilutions of the purified BAMP was added to each well. Then, 100 μl of prepared culture was added and the plate was incubated at 37°C for 18 h. The lowest concentration with no visible growth in the well was considered the MIC.

Hemolytic Assay

The method of hemolysis by Rodríguez et al. (2014) was followed. The method is as follows: human blood (5.0 ml) was collected and centrifuged and the pelleted erythrocytes were washed thrice with phosphate-buffered saline (PBS, 150 mM NaCl at pH 7.0); 20 % (*v*/*v*) of erythrocyte suspension was made in the PBS solution. Different concentrations of purified BAMP 2.5, 5, 10, 50, and 100 μg/ml were incubated with 1:5 dilutions of 20% (*v*/*v*) of RBC at 37°C for an hour. Later, the sample was centrifuged and the $OD_{450\ nm}$ was estimated. The positive control for the study was 1.0% (*v*/*v*) Tween 20 in PBS solution buffer alone. The following equation was used to calculate the percentage of hemolysis.

(A450 of the peptide-treated sample – A450 of the buffer-treated sample) × 100/(A450 of the Tween 20-treated sample – A450 of the buffer-treated sample).

Mass Spectra Studies of BAMP

Following gel filtration chromatography, the homogenous and purified BAMP was subjected to nano-LC-ESI-MS (Agilent 6550 I funnel QTOF).

Sample Preparation

Purified protein sample at 25.0 μm in 50 mM sodium phosphate buffer was subjected to zip tip with 0.6 μl of C18 resin (Merck Millipore, India). First, the column was regenerated with 10 μl of acetonitrile (MS-MS grade). Followed by equilibration with 0.1% trifluoroacetic acid (TFA) at 10 μl × 10 times. The protein sample was loaded, subsequently, washed with 0.1% TFA and finally eluted with 50 and 80% acetonitrile (ACN). The samples were speedVac dried, and the resulting pellet was resuspended in MS-grade water.

Mass Spectrometric Analysis of Protein

MS conditions are as follows: nitrogen as curtain gas, ISVF 5,500 V at 100°C, DP 100, and Tof range 100–2,000 *m*/*z* ion source. The selected multiple-charged ions were subjected to MS/MS analysis using collision energy at 30–45. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan.

Scanning Electron Microscope Analysis of the Indicator Strain *V. harveyi* After BAMP Treatment

The culture of the indicator strain (*V. harveyi*) grown overnight was subcultured to obtain 0.01 OD_{600} and mixed with the purified antimicrobial compound (0.01 mg/ml). The mixture was incubated for 12 h at 37°C under shaking conditions (200 rpm). One-milliliter sample was centrifuged at 9,000 × *g* for 10 min to pelletize the cells and fixed in 2.0% glutaraldehyde solution for 12 h. Subsequently, they were subjected to a series of alcohol washes (10–100%) and analyzed under scanning electron

microscopy (SEM). The surface images obtained were compared with the control. The experiments were performed in triplicate, along with the control (untreated).

The Bacteriostatic Effect of BAMP on Fluorescent-Labeled *S. typhi*

The indicator strain, *S. typhi*, was labeled with a green fluorescent protein (GFP) by transforming the pFU95 GFP plasmid (a kind gift of Dr. Tuli, IMT, Chandigarh). The labeled cells cultured overnight in LB–ampicillin broth. Then, the subcultured samples were allowed to reach 0.5 OD, after which BAMP was added. A *Salmonella* GFP without BAMP treatment was maintained as control, and samples were drawn at regular intervals and processed through fluorescence microscopy. The results were compared with the untreated control.

Impact of BAMP on Ground Chicken Meat and Reduced CFU

Fresh chicken meat (boneless) from the local market was minced using a meat mincer (sterile). The resulting 10 g of meat slurry (in 10 ml of phosphate buffer) was taken, and the experiment was divided into two batches representing two different temperatures 37°C and 4.0°C for 36 h and 16 days, respectively. One group was maintained as a fresh group, without the inoculation of the pathogenic strain. The other groups were mixed with 1.0% inoculum (0.5 OD) of *L. monocytogenes*, subdivided into three groups. The three subgroups were BAMP treated (3.0 μg/ml) and untreated (negative control). The volume and quantities of antimicrobials taken correspond to their MIC (3.0 μg/ml). An aliquot (representing) of 0.1 g from 10 g of each group was taken on days 1, 4, 7, 10, 13, and 16 from samples stored at 4.0°C and 12, 24, and 36 h from those incubated at 37°C. The *L. monocytogenes* CFUs were estimated from a serial dilution of each aliquot by plating. The results were compared with positive and negative controls.

Whole-Genome Sequencing, *de novo* Genome Assembly, Gene Prediction, and Functional Annotation

Genomic DNA was isolated from the *B. paralicheniformis* using the Qiagen gDNA extraction kit (Qiagen, Hilden, Germany), and the whole-genome sequencing was performed on the Illumina HiSeq 2,000 sequencing platform using a paired-end library, resulting in 150-base pair (bp) paired-end reads and an average coverage of 170-fold. Raw paired-end reads were evaluated, and quality control was carried out using FastQC[1] and Trimmomatic (Bolger et al., 2014) (parameters: ILLUMINACLIP: 2:30:10, LEADING: 3, TRAILING: 3, SLIDING WINDOW: 10:30, MINLEN: 100) to obtain a set of clean paired-end reads. After preprocessing, FastQC was again used to report features of the preprocessing libraries and to verify the effectiveness of read trimming. After filtering, short reads were assembled using the SPAdes Genome Assembler with default parameters (Bankevich et al., 2014).

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Prodigal (version 2.6.2) (Hyatt et al., 2010) was used for gene prediction in the *Bacillus* spp. sample draft genome, while BAGEL-4 (Medema et al., 2011) and anti-SMASH (van Heel et al., 2018) were used to predict the biosynthetic gene clusters for secondary metabolites and antimicrobial peptides with default parameters. The whole-genome sequence of *B. paralicheniformis* was submitted in NCBI, and the accession number is WHJA00000000. The above process followed may predict the presence of possible antimicrobials/bacteriocins in the genome.

RESULTS AND DISCUSSION

Identification of the Producer Strain Using 16S rDNA and Gyrase A Phylogenetic Analysis

The amplified full-length (1,500 bp) 16S rDNA and the resulting DNA sequences were analyzed by subjecting them to NCBI BLAST. BLAST results above 99.8% were identified as *B. paralicheniformis*. The 16S rRNA sequence was submitted to the NCBI sequence submission portal, and the accession number is MG183675. **Table 1** contains all the strains used in the study. For further confirmation of *Bacillus* speciation, phylogenetic analysis and taxonomic studies of 16S rRNA and gyrase A (Gyr) A-conserved sequences were followed (data not shown). The whole-genome sequence of the producer strain was submitted in the NCBI WGS portal (accession No. WHJA00000000).

Growth Curve of the Producer Strain

The growth curve of *B. paralicheniformis* is shown as OD_{600} for the culture against time (**Figure 1A**). The growth curve studies were conducted as per the materials and methods, and the growth curve was observed to be sigmoidal. However, a few unusual observations were made wherein as soon as the growth reaches the stationary phase, the formation of huge amounts of mucus-like material was observed. Furthermore, the mucous formation was observed particularly when the bacteria were grown in the static condition and for long periods above the stationary phase. The harvesting of the cells becomes difficult once mucous formation sets in. However, the mucoid material formation does not negatively affect either inhibition or prevention of the production of bacitracin. The presence of residual mucus affects obtaining pure cell-free supernatant and a major problem in downstream processing occurs.

Antimicrobial Production Kinetics

Growth and activity kinetics are shown in **Figure 1A** and inset, respectively, which demonstrate bacitracin synthesis and production set within 5 h of growth (inset). The secretion is rapid as it does not stop and continues even in a stationary phase. Therefore, the minimum growth of 5 h may be sufficient to synthesize the bacitracin. After 8–10 h of growth, harvesting is carried out to isolate the bacitracin. Inset plate shows the well assay of each bacterial supernatant collected at different time points and also the beginning of the secretion of BAMP and the end of secretion of BAMP. The collected CFS was tested through

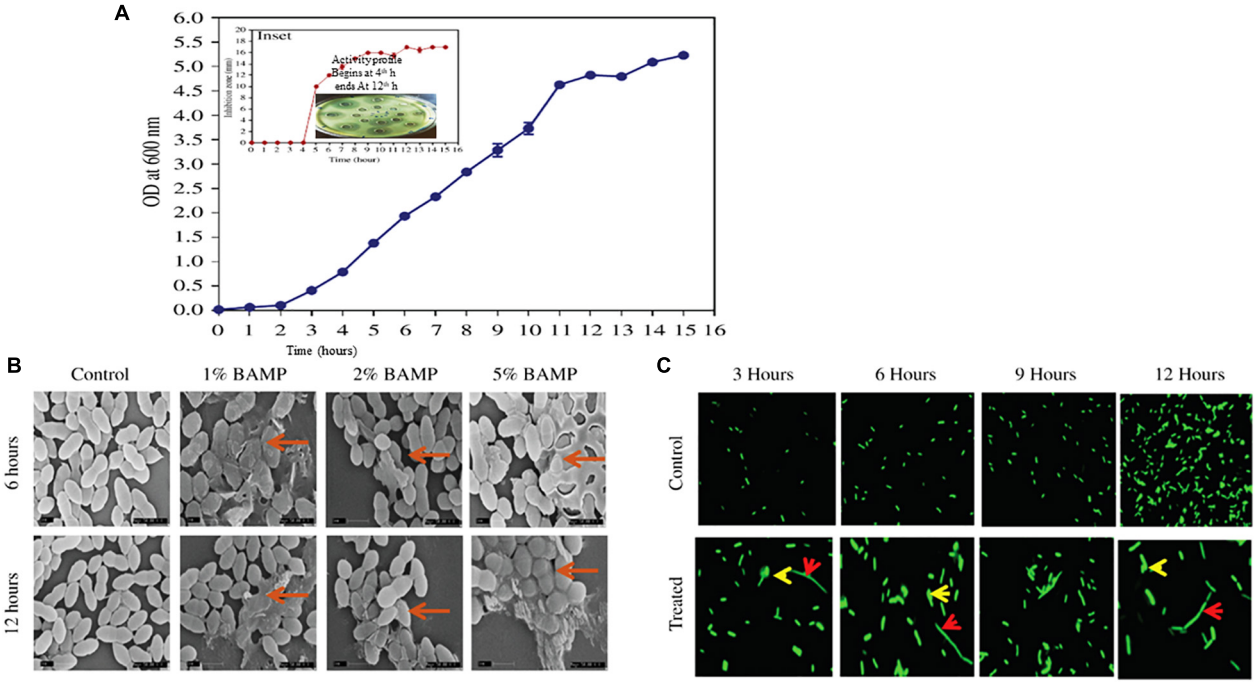

FIGURE 1 | (A) Growth curve of *B. paralicheniformis*. Inset shows antimicrobial protein production kinetics. The indicator strain used is *V. harveyi*. The inset shows diffusion assay of the zone formation which begins after 4 h of growth, and the peak ZOI continued to 12 h. **(B)** SEM images of the different percentages of BAMP-treated *V. harveyi* cells in its 6 and 12 h of growth. The arrows indicate the swollen and damaged cells. **(C)** Effect of BAMP on florescent-labeled *S. typhi* at regular intervals of growth is shown in comparison with the control. The elongated bacteria are shown with a red arrow; bulged cells are indicated with a yellow arrow.

a well-diffusion assay by using various Gram-positive and Gram-negative microorganisms as indicators. The results are enclosed in **Table 1B**.

Scanning Electron Microscope Analysis

The BAMP-treated cells showed morphological changes compared with the control. The cell samples treated with BAMP were found to be swollen and elongated; these may either be due to the inhibition of cell division or inhibition of separation of daughter cells after cell division. Cell bursting and leakage of the cytoplasmic contents were observed in the treated cells. The nature of morphological changes upon treatment with different percentages of BAMP is shown in **Figure 1B**. The activity of BAMP increases as the percentage of BAMP in the reaction increases. The highest BAMP activity was observed at the 5.0% level, where cell wall disruption and lysis, the presence of swollen cells, and cell debris may be observed (marked by the red arrow in **Figure 1B**). The PI-labeling studies show bright-colored diffused cells, indicating cell lysis by BAMP (data not shown). Cell lysis begins at 6 h of 1% BAMP exposure; as the concentration of BAMP increased from 1 to 5%, the incidence of lysis increased. The severe cases of cell lysis and aggregation were observed when the cells were incubated for 12 h with larger quantities of BAMP.

TABLE 1B | Inhibitory activity of BAMP against Gram-positive and Gram-negative indicator species.

Sl. No.	Indicator organisms	Zone of inhibition of BAMP (mm)
1	*Pseudomonas putida* (−ve)	10
2	*Corynebacterium callunae* (+ve)	12
3	*Pseudomonas aeruginosa* (−ve)	12
4	*Salmonella typhi* (−ve)	10
5	*Enterococcus gallinarum* (+ve)	15
6	*Streptococcus thermophilus* (+ve)	10
7	*Staphylococcus aureus* (+ve)	11
8	*Vibrio harveyi* (−ve)	13
9	*Streptococcus mutans* (+ve)	14
10	*Vibrio cholerae* (−ve)	10
11	*Bacillus cereus* (+ve)	11
12	*Listeria* monocytogenes (+ve)	16
13	*Clostridium perfringens*	32
14	*Lactobacillus plantarum*	NA
15	*Enterococcus raffinosus*	10

The Bacteriostatic Effect of BAMP on Fluorescent-Labeled *S. typhi*

The fluorescence microscopic images of the BAMP-treated cells showed it interfered with division and resulted in elongated cells when compared with the untreated control at regular intervals during growth. Two different kinds of cells were observed in lysed cells: elongated cells and diffused cells. This indicates a compromised cell wall and cell lysis. This shows the bacteriostatic effect of BAMP on *S. typhi* cells (**Figure 1C**). Here, we could

also see elongated, abnormal GFP-expressing cells. A few cells were ruptured due to BAMP action where the GFP is observed coming out of the cells (arrows in the figure indicates rupture). There is an overall decrease in the GFP levels after BAMP treatment in comparison with the control *Salmonella* GFP (**Figure 1C**, 12 h sample).

Effect of pH and Temperature

BAMP was subjected to different temperatures ranging from 4.0 to 121°C (autoclaving) for 20–60 min. The results show no change in the activity of BAMP. At 4.0–80°C, exposure does not reduce the activity, but upon exposure to 100°C, a loss of 10% activity was observed, which was further reduced to 20% at 121°C. This shows that BAMP is thermostable. To understand the effect of pH (1.0–13.0), BAMP was treated with different pH values. No drastic changes in BAMP activity and a very marginal reduction in the activity at pH 2.0 and 9.0 (the reduction was 20%) were observed. However, at pH 11.0 and higher, the activity was negligible. The purified antimicrobial peptide showed good activity. The temperature and pH do not negatively affect BAMP.

Effect of Physiological Enzymes on the Activity of BAMP

Even after treatment with physiological enzymes, including chymotrypsin, trypsin, pepsin, protease, proteinase K, and catalase for 5 h at 37°C, the antimicrobial compound continuously maintained its activity (**Table 2A**). **Table 2A** shows the residual activity of BAMP after treatment with physiological enzymes. Hyperactivity of BAMP was observed (83%) in the presence of proteinase K (ZOI 10.5 mm). The ZOI of the BAMP treated with physiological enzymes ranges from 10.5 to 12.0 mm. This is another factor that makes BAMP suitable for food industries that process hydrolysates and protein concentrates since it can be degradable.

TABLE 2A | Effect of physiological enzymes on the activity of BAMP.

ENZYME	BAMP ZOI (mm)
Control	13.0
Chymotrypsin	11.5
Trypsin	12.0
Pepsin	10.5
Protease	10.5
Proteinase K	10.5
Catalase	10.5

TABLE 2B | Effect of surfactants and chloroform on the activity of BAMP.

Treatment	BAMP ZOI (mm)
Control	13.0
SDS	12.5
Tween 20	10.5
Tween 80	10.5
Triton x-100	12.5
Chloroform	11.5

The percent residual activity of the physiological enzyme, surfactants, and metal salt-treated BAMP shows that there is a loss of 20% activity. The ZOI of the BAMP treated with various surfactants and chloroform ranges from 10.5 to 12.5 mm. Therefore, a few chemical food preservatives do not negatively affect their function (**Table 2B**).

BAMP Stability Test in the Presence of Various Detergents/Effect of Surfactants and Chloroform on the Activity

As per the rules of the Food Safety and Standards Authority of India (FSSAI), permitted amounts (of surfactants and chloroform in the food industry) were used for these studies. The antimicrobial compound maintained its activity in the presence of surfactants such as SDS, and Triton X-100 nominal loss of 10–20% activity was observed with the other surfactants (**Table 2B**). This further implies that BAMP is an efficient food preservative as well as an antimicrobial compound. The residual activity and the reduction of activity of BAMP treated with various detergents are shown in **Table 2B**.

Effect of Food-Grade Metal Salts

As per the regulations of the FSSAI and the United States Food and Drug Administration (USFDA), various metals were considered to understand their effect on the antagonistic activity of BAMP. Similarly, a few metals do negatively affect BAMP, e.g., $CuSO_4$ drastically inhibits its activity (no activity). However, BAMP is more effective in the presence of most of the metals. The BAMP activity was unaffected when subjected to seven different metals out of eight (**Table 2C**). The residual and percent reduction of the activity of the BAMP after treatment with food-grade metal salts are presented in **Table 2C**. The ZOI of the BAMP exposed to food-grade metal salts is not affected drastically wherein the ZOI in the case of BAMP ranges from 0 to 12.5 mm. Here, BAMP shows a superior function. The effect of BAMP on another infectious pathogen *V. harveyi* was evidenced through viability studies (data not shown). It is suggested not to use BAMP in food material that contains $CuSO_4$.

TABLE 2C | Effect of food-grade metal salts on the activity of BAMP.

Metal salts	BAMP ZOI (mm)
Control	13.0
$MgSO_4$	10.5
$FeSO_4$	10.5
$MnCl_2$	10.5
$AgNO_3$	12.5
$ZnCl_2$	11.5
$CdCl_2$	13.5
$CuSO_4$	0
$CaCl_2$	10.5

The values are the results of three independent experiments.
ZOI, Zone of Inhibition.

Effect of Food Preservatives on the BAMP Activity

As per the prescription by the FSSAI/USFDA, various food preservatives were considered for the study. The results showed that the antimicrobial compound maintained its activity in the presence of preservatives. Virtually, the food preservatives do not inhibit the action of either BAMP. **Tables 2A–C** shows 100% residual activity, taken into consideration during the final calculations of activity reduction. This value gives actual activity only.

Purification of BAMP

The ammonium sulfate-precipitated protein after dialysis was subjected to a Superdex 75 gel filtration column. **Figure 2A** shows the elution profile of BAMP. We can see that the peak is not steep but is very shallow; it shows a zigzag manner, and elution begins after 35 min and proceeds to 130 min. Almost all the fractions were subjected to Bradford analysis, and activity was observed only in the fractions that eluted after 90 min and continued for 115 min. The peak fractions could be observed from 25 to 40 min. The eluted peaks were collected and the activity checked (by a well-diffusion assay). The elution profile and activity profile (inset) of the purified fractions were plotted (**Figure 2A**, inset). The activity of gel filtration fractions begins with fraction numbers 28–31 in low activity at around 7.0–8.0 mm ZOI. The highest activity was observed between the fraction numbers 32 and 39. Here, the ZOI ranges from 10.0 to 12.0 mm. Therefore, the elution peak with activity is between 28 and 40 fractions of 2.0 ml each. The ZOI was seen from the fraction number 33 onwards and continuing to 39. It begins at the 95th minute and ends at the 115th minute of elution profile, as shown in **Figure 2A**. **Figure 2A** (inset) shows the beginning and the end of the active fractions with time.

BAMP purification is shown in **Figure 2A** (inset). **Table 3A** shows the purification table of BAMP. The homogenous purified and electrophoretically separated single protein band is shown in **Figure 2B** (lane 1). Subsequently, the purified protein was subjected to an in-gel activity assay—zymogram and shown in **Figure 2B** (lane 2). The gel, as well as the zymogram, shows an approximate molecular weight of 1.4 kDa. The BAMP CFS and BAMP powder were subjected to check their effective concentrations against various food contaminants. The results show that BAMP does not work against *Pseudomonas putida* but is antagonistic to *Pseudomonas aeruginosa*. The results also indicate that BAMP MIC ranging from 2.4 to 6.0 μg (**Table 3B**) varies with different indicator strains. The same protein also tested their ability to kill probiotic microorganisms such as *Lactobacillus plantarum* and *Enterococcus* spp. It was understood that BAMP (**Table 3C**) is not antagonistic to *Lactobacillus* and functional against *Enterococcus* spp. The specific target function of BAMP renders it suitable for human therapy and eubiosis. The eubiosis nature of BAMP further supports its suitability as a therapeutic protein in the maintenance of gut microbiota and elimination of pathogenic and disease-causing microorganisms.

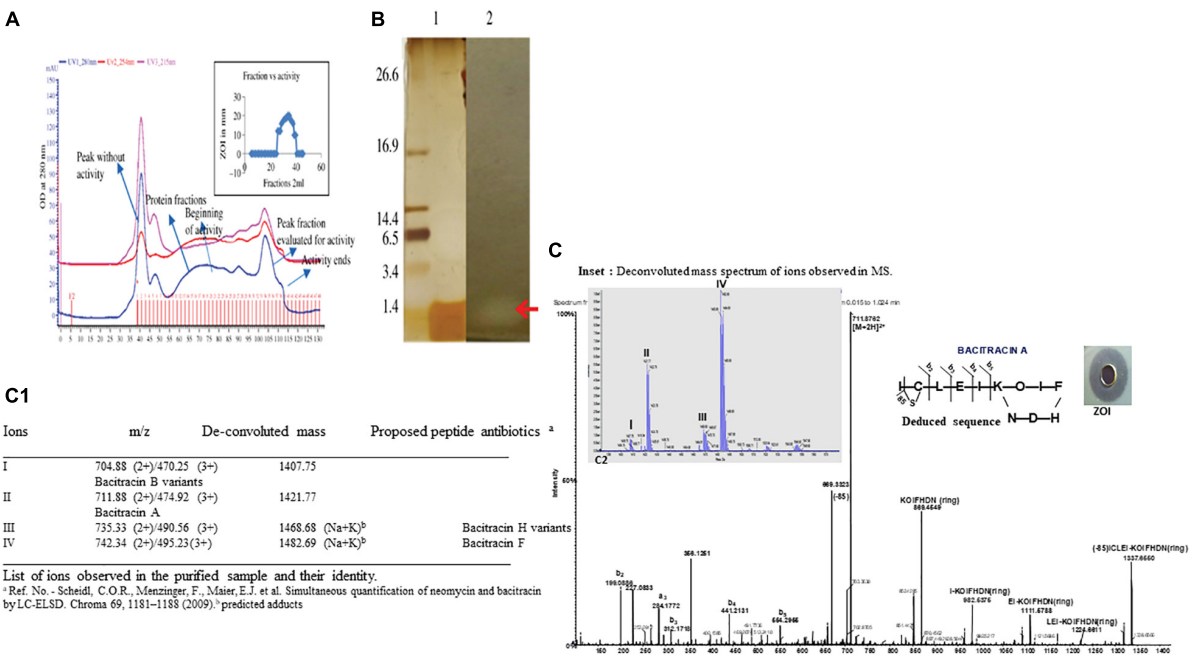

FIGURE 2 | (A) Elution profile and activity profile of the BAMP-purified fraction. The antimicrobial activity of the fractions is showed in the inset. **(B)** Tricine-SDS-PAGE of purified BAMP. Lane 1: marker; lane 2: purified BAMP; lane 3: direct overlay of tricine-SDS-PAGE gel demonstrating ZOI indicating the activity of purified BAMP. **(C)** MS-MS spectrum of purified BAMP (inset) showing the well-diffusion assay/ZOI. (a) LC-MS profile of active fraction of antimicrobial compound of BAMP. Assignment of b and y ions. Amino acid sequence deduced from the MSMS data. The inset shows the deconvoluted mass spectrum of peaks I, II, III, and IV and were observed and demarcated. **(C1)** The List of ions observed in the purified sample and their identity.

TABLE 3A | BAMP purification table.

Purification stage	Volume (ml)	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification (fold)	Yield (%)
Crude supernatant	1,000	100	33,320	333.2	1	100
Ammonium sulfate precipitation	20	23.6	10,662	451.77	1.335	31
Superdex-75 gel filtration	16	0.265	2,665	10039.9	30	7

The Minimum Inhibitory Concentration of BAMP

The MIC was observed to change based on the protein condition, and the crude supernatant MIC was 3.0 μg/ml, ammonium sulfate-precipitated protein MIC was 2.1 μg/ml, and MIC of the purified antimicrobial compound was 0.124 μg/ml. The MIC was determined using the microbroth dilution assay. The MIC was found to be 3.0 μg/ml against the indicator strain *V. harveyi*. Various indicator strains were used to find a suitable strain, and it was found that *V. harveyi* is the best indicator and consistent results were obtained. Most importantly, it is pathogenic, contagious, ubiquitous, and food contaminating. *Vibrio* spp. is one of the major contaminants in the dairy and aquatic system. We also used *Salmonella* as this is also one of the food contaminants. Outbreaks of *V. harveyi* are known to be very common as stated by Zhang et al. (2020). Subsequently, during the year 2020, Ogunbanwo et al. (2003) reported milk contaminated with *V. harveyi*. It is a major contaminant of dairy foods as well as the aquatic system; therefore, we chose *V. harveyi* for the study. MIC determined is shown in **Table 3B**.

TABLE 3B | MIC of BAMP supernatant and spray dried powder (concentration in micrograms).

Sl. No.	Indicator strain	BAMP supernatant (μg)	BAMP powder (μg)
1	*Enterococcus gallinarum*	3.0	6.0
2	*Corynebacterium callunae*	2.4	3.0
3	*Listeria monocytogenes*	2.4	3.0
4	*Streptococcus mutans*	2.4	6.0
5	*Staphylococcus aureus*	6.0	12
6	*Micrococcus luteus*	2.4	3.0
7	*E. coli*	3.6	6.0
8	*Vibrio harveyi*	3.0	1.2
9	*Pseudomonas putida*	–	–
10	*Pseudomonas aeruginosa*	–	12
11	*Streptococcus thermophilus*	Bacteriostatic	–
12	*Salmonella typhi*	Bacteriostatic	–
13	*Vibrio cholerae*	Bacteriostatic	–

TABLE 3C | The activity of the purified antimicrobial compound against probiotic microbes. The activity was expressed as a zone of inhibition.

Probiotic strain	BAMP
L. plantarum	NA
E. raffinosus	10 mm

NA, No Activity.

Mass Spectra/MS Analysis

The MS results of the purified BAMP showed the presence of a 1.4-kDa peptide (**Figures 2A,B**). Tricine-SDS-PAGE of the purified BAMP showed a protein band at the range of 1.4 kDa (**Figure 2B**, lane 1). The corresponding zymogram indicates the activity of the peptide (**Figure 2B**, lane 2). The LCMS data was processed using proteome discoverer software against UniProt *Bacillus* database at MS1 and MS2 tolerance of 10 ppm and 0.5 Da, respectively (**Figures 2C,C1**). The identified peptide is bacitracin as reported earlier (Singh et al., 2019).

MS analysis of peptide sample indicates the presence of majorly triply and doubly charged ions at *m*/*z* 470.25 (3+), 474.92 (3+), 495.23 (3+), 711.88 (2+), 735.33 (2+), and 742.34 (2+). The deconvolution of the spectrum revealed the ions with mass—1,407.75, 1,421.77, 1,468.68, and 1,482.69 Da (**Figure 2C**, inset a, C1), corroborating to the mass of the peptide determined through SDS-PAGE. Furthermore, the evaluation of 711.88 ion tandem spectra showed the presence of fragment ions that could match bacitracin A (**Figures 2C,C1**; Govaerts et al., 2003; Landeen et al., 2016). In the case of ions at 1,468.68 and 1,482.69 Da, we observed 61 Da increment compared with 1,407.75 and 1,421.77, respectively, that could correspond to $Na^{+}K$ adduct of keto-thiazole form. The list of identified bacitracin variants is given in **Figure 2C1**. Mass spectrum data indicate that there are four different kinds of peptide antibiotics/bacitracin antimicrobials as bacitracin A variants, bacitracin B variants, bacitracin F variants, and bacitracin H variants.

The compound was further analyzed through tandem MS and amino acid analysis, and the presence of isoleucine, cysteine, leucine, aspartic acid, glutamic acid, isoleucine, lysine, ornithine, isoleucine, phenylalanine, histidine, aspartic, and arginine was found. We observed the presence of one non-standard amino acid ornithine (**Figure 2C**). Subsequently, it was observed that the MS/MS data also supported the amino acid composition.

We studied the MS/MS data extensively and annotated all the b and y ions present in the raw spectrum (**Figure 2C**, deduced sequence, inset). Lockhart and Abraham (1954) and Stone and Strominger (1971) stated that bacitracin A is a cyclic peptide containing 12 amino acid residues. For further confirmation of the presence of bacitracin at the genome level, we looked into WGS analysis focusing on the genes encoding for the synthesis of the bacitracin.

The Effect of BAMP in Chicken Meat Contaminated With *Listeria*

The percent viability of BAMP-treated *L. monocytogenes* was found to reduce (20% in 20 h) compared with the untreated *L. monocytogenes*. The BAMP-treated *L. monocytogenes* showed

percentage viability similar to the negative control at 4.0°C. The viability of the cells gradually decreased at 37°C. The positive control turned blackish upon incubation, but the color of the treated sample was similar to the negative control. This shows the antimicrobial activity of samples against the indicator strain used (**Figure 3A**). BAMP shows 50% viability (50% dead) in 30 h (**Figure 3B**). Subsequently, around 10% viability was observed in 38 min, and 90% of *Listeria* was eliminated from the food at 37°C. A similar experiment at 4.0°C concluded an almost negligible number of viable cells. This further confirms the effect of BAMP and its use in the meat industry. The *Listeria* field trials envision that the use of BAMP does not change taste, color, and flavor. Without BAMP, the sample does not only lose its color but also its flavor and taste. This envisages BAMP as a potential biopreservation agent.

The wide activity range of BAMP against Gram-negative and Gram-positive microorganisms is shown in **Table 1B**. No activity of BAMP against probiotic microorganisms was detected (**Table 3C**). The activity of BAMP against *V. harveyi* was confirmed, and the viability percentage was analyzed using standard procedures (data not shown). The molecular characterization of BAMP such as tricine-SDS-PAGE, zymogram, and MS analysis predicted its molecular weight (**Figures 2A–C**). The BAMP was found internalized by the indicator strain through HPLC analysis, and the interaction of BAMP with DNA was confirmed by FTIR analysis (data not shown).

Hemolytic Assay

As per the guidelines of FSSAI/FDA, to consider BAMP as a food additive/supplement, the protein should be non-hemolytic in nature. The hemolytic assay of the BAMP showed 0.43% hemolysis. The result based on the studies conducted to understand its hemolytic activity is negative (**Figure 3C**). This property makes the BAMP phenomenal, suitable as a food additive, as well as for therapy. As per the report, hemolysis values above 6 are non-phenomenal and not considered for therapy. The hemolytic attribute of BAMP strengthens its applicability to therapy.

Whole-Genome Sequence Analysis and Prediction

Raw data sequences from Illumina HiSeq were assessed and filtered with FastQC and Trimmomatic to produce a high-quality set of reads. These whole-genome DNA samples yielded 4,374,835 raw paired-end reads with lengths of 150 bp. After the removal of adapters and low-quality data (quality scores $\leq$ 30), 2,256,832 clean reads (51.59% of the initial total) remained. The clean sequences were assembled into the draft genome of the *B. paralicheniformis* (accession No. WHJA00000000) in which 101 contigs were obtained with a total draft genome length of 4,200,259 bp (N50 of 287,402 bp and GC content of 45.86%). For functional analysis, we predicted 5,195 genes from the SPAdes-assembled contigs using Prodigal.

Moreover, we subjected the whole-genome sequence assembly to other tools like Antibiotics and Secondary Metabolites Analysis Shell (anti-SMASH) (Medema et al., 2011) and BAGEL-4 (van Heel et al., 2018) to discover the genes associated with the antimicrobials and secondary metabolites. Various bacteriocins were found in the WGS of *B. paralicheniformis* through BAGEL-4

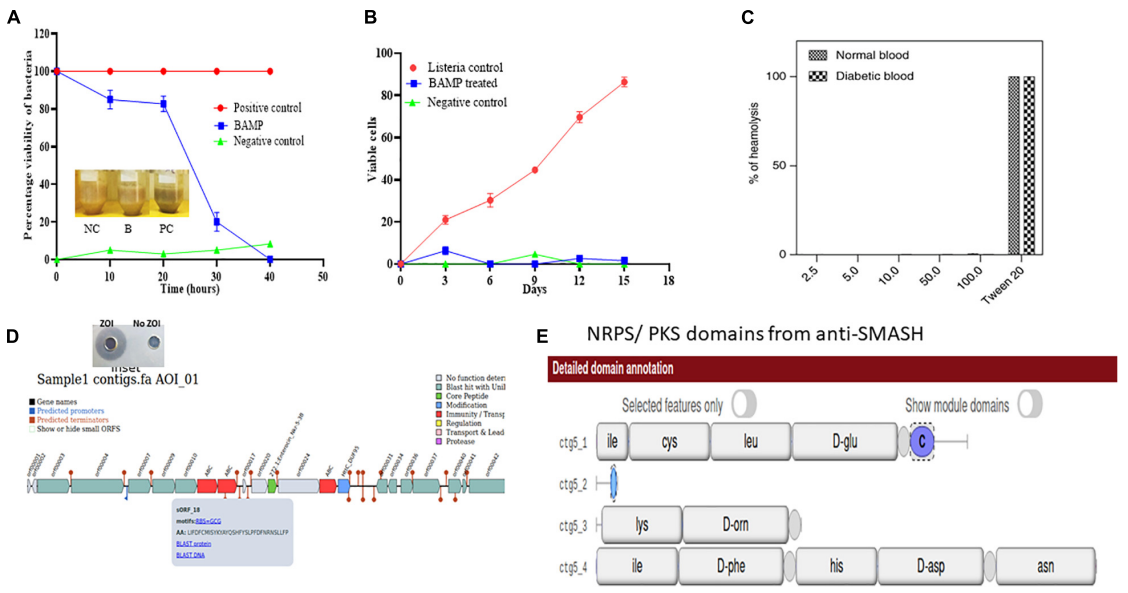

FIGURE 3 | (A) Viability of *L. monocytogenes* in chicken meat on treatment with Nisin and BAMP for 36 h at 37°C and 16 days at 4°C. The picture in the inlet of graph A shows the differences in appearance of treated and untreated samples. NC, negative control; B, BAMP-treated samples; PC, positive control. **(B)** *L. monocytogenes* viability curves after treatment with BAMP. **(C)** Hemolytic activity of BAMP on healthy and diabetic human blood samples. **(D)** Antimicrobial biosynthetic gene cluster in the genome sequence of *B. paralicheniformis.* The inset shows ZOI and No-ZOI, predicted through BAGEL 3. **(E)** Non-ribosomal peptide predicted using anti-SMASH.

and Anti-SMASH. Lichenisin and bacitracin are the two bacteriocins that are showing 100% similarity as per anti-SMASH data. According to anti-SMASH, bacitracin is localized in the contig number 6 and nucleotide position approximately from 15,000 to 45,000. Contig. Twenty-two comprise cystabiotic of 98 amino acids, whose sequence is identical to antimicrobial peptide LCI of *B. paralichemiformis*. Two bacitracins were observed at regions 2.2 and 22.2 and are NRPs with 100% similarity with the existing bacitracin. **Figures 3D,E** shows the gene arrangement in the genome sequence. The inset shows the well-diffusion assay of BAMP that was used for genome sequencing (zone of inhibition).

The bacitracin gene was also found in different species of *Bacillus* such as *B. licheniformis* ATCC 14580, *B. glycinifermentans*, even in *Paenibacillus* spp. and *B. paralicheniformis*. Therefore, the mass spectrum data coincide with the WGS data confirming the presence of a gene to the corresponding protein. Established along the gene arrangement shown in **Figures 3D,E**, it is noted that the bacitracin and its variants are arranged identically with other bacteriocins wherein it contains ABC transporters, hypothetical protein, and immunity protein in the same array. The genome sequence of the strain *B. paralicheniformis* has been deposited in the GenBank database, and Accession No. WHJA00000000 was obtained.

BAMP Is a Potent Antimicrobial Peptide

The purified BAMP is thermostable. The activity could be seen after incubation at various temperatures ranging from 4.0 to 121°C. BAMP functions in a very wide range of pH from 2.0 to 9.0, where its activity is not affected, but above 9.0 in alkaline conditions, its activity is drastically reduced. Upon the treatment of BAMP with various surfactants such as SDS, Tween 20, Tween 80, Triton X-100, and chloroform, it was found that Tween 20 and 80 reduces the activity around 20% (**Table 2B**). Various food-grade metal salts and preservatives do not affect BAMP (**Table 2C**). BAMP does not create dysbiosis because it is harmless to the gut bacteria such as *Lactobacillus* and *Enterococcus* spp. (**Table 3C**). The compromised, broken, and aggregated *V. harveyi* after the treatment with the various concentrations of BAMP is shown in **Figures 1B,C**. This envisages the effect of BAMP causing cell damage. The case of *S. typhi* was an indication of the bacteriostatic effect of BAMP against certain indicator strains (**Figure 1C**). It was further confirmed that BAMP can permeabilize the cell membrane (**Figure 1C**). *Salmonella* with GFP clearly shows the compromised cell membranes. The percent viability of *V. harveyi* treated with BAMP shows that BAMP requires just 3–4 h for 50% lysis (**Figures 3A,B**). The chromatographic peak fractions shown in the inset were used for subsequent studies such as MS analysis. Homogenous and pure protein corresponding to approximately 1.4 kDa is shown in **Figure 2B** (lanes 1 and 2). Finally, the purified protein was subjected to MS analysis.

The virtual and real/field experimental setup studies conclude that BAMP controls the *Listeria* population in the meat (**Figures 3A,B**). This shows better efficiency of BAMP and confirms that BAMP could function in a food matrix as well. The purified BAMP is hemolytic negative as the present threshold considered for the therapy is much less and considered for therapeutic purposes (**Figure 3C**). Even after using 2.5–100 μg/ml, negligible activity was observed wherein Tween 20, a controlled detergent, showed the activity of 100%. BAMP is observed to have a character wherein it does not establish gut dysbiosis, establishing eubiosis by not harming the beneficial microorganisms such as *Lactobacillus*. The potent antimicrobial peptide BAMP was found to have many superior characteristics, making it desirable for the preservation of food from pathogens and an ideal therapeutic agent.

CONCLUSION

The present work deals with the development of a potent next-generation antimicrobial agent that may be utilized as a food preservative. Although Nisin is extensively used as a preservative, there are a few features that limit its preservative functions, including its very limited antibacterial activity. Other limitations include its lack of function against very common food contaminants such as *S. typhi* and *S. thermophilus* that infect and spread rapidly in tropic regions.

In the end, we conclude that we successfully isolated, purified, and characterized a potential next-generation antimicrobial agent for the food industry. It is thermostable, physiologically enzyme resistant, and possesses wide spectrum activity. The molecule may be desirable for the various food industries, as it does not lose activity upon incorporation into the foods that contain metals, detergents, preservatives, and salts. The cloning, expression, and large-scale production/purification of the BAMP may help us to understand its therapeutic efficacy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the NCBI 16sr RNA and WGS repository under accession number WHJA00000000.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Biosafety Committee approval no. 012/IBSC/2020 reg. All methods were carried-out in accordance with relevant guidelines and regulations as well as a sentence confirming that informed consent was obtained from all subjects. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RK: conceptualization, methodology, data curation, and writing – original draft preparation and reviewing and editing. SC: data generation and experimental work. PJ: involving in LC-MS

studies. All authors contributed to the article and approved the submitted version.

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Exploring Phenolic Compounds as Quorum Sensing Inhibitors in Foodborne Bacteria

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The emergence of multidrug-resistant bacteria stimulates the search for new substitutes to traditional antimicrobial agents, especially molecules with antivirulence properties, such as those that interfere with quorum sensing (QS). This study aimed to evaluate the potential of phenolic compounds for QS inhibition in a QS biosensor strain (*Chromobacterium violaceum*) and three foodborne bacterial species (*Aeromonas hydrophila*, *Salmonella enterica* serovar Montevideo, and *Serratia marcescens*). Initially, an *in silico* molecular docking study was performed to select the compounds with the greatest potential for QS inhibition, using structural variants of the CviR QS regulator of *C. violaceum* as target. Curcumin, capsaicin, resveratrol, gallic acid, and phloridizin presented good affinity to at least four CviR structural variants. These phenolic compounds were tested for antimicrobial activity, inhibition of biofilm formation, and anti-QS activity. The antimicrobial activity when combined with kanamycin was also assessed. Curcumin, capsaicin, and resveratrol inhibited up to 50% of violacein production by *C. violaceum*. Biofilm formation was inhibited by resveratrol up to 80% in *A. hydrophila*, by capsaicin and curcumin up to 40% in *S.* Montevideo and by resveratrol and capsaicin up to 60% in *S. marcescens*. Curcumin completely inhibited swarming motility in *S. marcescens*. Additionally, curcumin and resveratrol increased the sensitivity of the tested bacteria to kanamycin. These results indicate that curcumin and resveratrol at concentrations as low as 6 μM are potential quorum sensing inhibitors besides having antimicrobial properties at higher concentrations, encouraging applications in the food and pharmaceutical industries.

Keywords: quorum sensing, phenolics, antivirulence, antimicrobial, antibiofilm

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INTRODUCTION

The microbial communication system called quorum sensing (QS) is used by a wide variety of bacteria allowing them to collectively modify their behavior in response to changes in cell density. This communication is mediated by small molecules accumulated during microbial multiplication and involves the production, secretion, and detection of extracellular signaling molecules, known as autoinducers (AI; Mukherjee and Bassler, 2019).

Gram-positive and Gram-negative bacteria exhibit different communication systems to regulate many physiological traits. In Gram-negative bacteria, signaling is usually mediated by acyl

homoserine lactone (AHL) molecules, also known as autoinducer-1 (AI-1; Vanetti et al., 2020). In Gram-positive organisms, communication is mediated by autoinducer peptides which are usually secreted by ABC-type carrier proteins (Monnet et al., 2014; Lima et al., 2020).

In addition to these main QS autoinducers, the furanosyl borate diester, also known as autoinducer-2 (AI-2), is associated with both Gram-positive and Gram-negative bacteria allowing intra and interspecific communications (Chen et al., 2002). The autoinducer-3 (AI-3), a metabolite involved in pathogenesis of enterohemorrhagic *Escherichia coli* (EHEC), was recently elucidated and characterized (Kim et al., 2020). In fact, AI-3 analogs belong to the pyrazinone family, and they are derived from threonine dehydrogenase (Tdh) products and "abortive" tRNA synthetase reactions, being present in a variety of Gram-negative and Gram-positive pathogens (Kim et al., 2020). A number of other extracellular bacterial metabolites that function as signals in a range of microorganisms have also been discovered recently (Vanetti et al., 2020).

The genes and functions regulated by QS are diverse and can be classified into four groups: (1) cellular behavior, such as biofilm formation and dispersion, motility, and adhesion; (2) cell maintenance and proliferation, such as exoenzyme and siderophore synthesis, sporulation, and acid resistance; (3) horizontal gene transfer, such as conjugation in *Agrobacterium tumefaciens*; and (4) interactions with the host and other microorganisms, such as production of virulence factors, antibiotics and exo-polysaccharides, and bioluminescence, among others (Jimenez et al., 2010; Monnet et al., 2014; Grandclément et al., 2016; Lima et al., 2020).

The communication mediated by QS can be interrupted in several ways: by inhibiting the autoinducer synthesis, through enzymatic degradation of autoinducers, or by competition for binding to receptor proteins, ultimately inhibiting the target gene expression, mediated by interfering molecules called quorum sensing inhibitors (QSI; Zhang and Li, 2015; Grandclément et al., 2016).

Plants are one of the main sources of natural QSI, including medicinal plants, vegetables, and edible fruits. Some compounds derived from the secondary metabolism of plants significantly increase their ability to adapt to unfavorable environments. These metabolites, such as alkaloids, phenols, flavonoids, quinones, tannins, terpenes, and lecithins, are well-known defense mechanisms against herbivores and microorganisms (Nazir et al., 2020). Phenolic compounds are the second largest family of plant nutraceuticals, after terpenoids (Gutiérrez-Del-Rio et al., 2018). Recent studies have shown that phenolic compounds in extracts of edible foods can act as QSI (Quecán et al., 2019; Rivera et al., 2019; Santos et al., 2020).

Food spoilage causes great concern to the food industry as it leads to significant economic losses. The detection of QS signaling molecules in spoiled foods, as well as microbial interactions in fermentation of foods, has added a new dimension to the understanding of the spoilage process (Bai and Rai, 2011; Nazzaro et al., 2013; Martins et al., 2018; Almeida et al., 2020). Interference in QS communication could be an additional target to delay bacterial food spoilage since some phenotypes regulated by QS are also related to food deterioration, such as production of pectinases, lipases and proteases, and biofilm formation.

Antimicrobial resistance is one of the greatest threats to global health (WHO, 2018), and new alternatives are needed to treat infections and stop the spread of multidrug-resistant bacteria (Singh et al., 2019). The rationale for using QS inhibitors as an anti-virulence strategy is the lower selective pressure when compared to traditional antibiotics (Maura et al., 2016; Saeki et al., 2020).

This study evaluated the ability of some phenolic compounds to inhibit bacterial quorum sensing regulated phenotypes. First, we applied molecular docking with the CviR QS regulator of *C. violaceum* in order to select potential QSI, and then, we tested these compounds against several quorum sensing-regulated phenotypes.

MATERIALS AND METHODS

In silico Analysis – Selection of Phenolic Compounds by Molecular Docking

Docking studies were performed according to Almeida et al. (2016, 2018). The potential anti-QS activity of 79 pre-selected compounds present in foods and plants, likely having antimicrobial and anti-QS activity (**Supplementary Table 1**), was evaluated, using six structural variants of the CviR QS regulator of *C. violaceum*. The crystallized structures of CviR (3QP1, 3QP2, 3QP4, 3QP5, 3QP6, and 3QP8) with different AHLs (Chen et al., 2011) were obtained from the RCSB Protein Data Bank database.[1] The molecular structure of phenolic compounds, homoserine lactones, and furanones was obtained from the PubChem database.[2] Molecular docking was performed using the "Dock Ligands" tool of the CLC Drug Discovery Workbench 4.0 software as described previously (Almeida et al., 2016, 2018). The five best docking scores of each compound were selected, allowing the inspection of the binding sites to the CviR protein.

Phenolic Compound Preparation

The next steps of the study were performed with the five phenolic compounds that presented the greatest inhibitory potential in *in silico* analyses: curcumin (PubChem CID: 969516), capsaicin (PubChem CID: 1548943), gallic acid (PubChem CID: 370), resveratrol (PubChem CID: 445154), and phloridizin (PubChem CID: 4789). The compounds (Sigma-Aldrich, Brazil) were dissolved in dimethyl sulfoxide (DMSO), so that the final solvent concentration did not exceed 1% in the tests (Rivera et al., 2019). Negative control comprised DMSO at the same concentration as in the tests with phenolic compounds.

Bacterial Strains and Growth Conditions

QS biosensor and foodborne bacterial species used in this study were *Chromobacterium violaceum* ATCC 12472,

[1]http://www.rcsb.org
[2]http://pubchem.ncbi.nlm.nih.gov

Chromobacterium violaceum 026 and *Serratia marcescens* MG1, cultivated at 30°C, as well as *Aeromonas hydrophila* IOC/FDA 110-36 and *Salmonella* Montevideo 163 (Monte et al., 2019), cultivated at 37°C. All strains were grown in Luria-Bertani (LB) broth (KASVI) for 18 h.

Antimicrobial Activity – Minimal Inhibitory Concentration

The antimicrobial activity of the selected phenolic compounds was evaluated by determination of the minimal inhibitory concentration (MIC), using the broth microdilution assay according to Wiegand et al. (2008), with some modifications. LB broth (100 µl) containing different concentrations of each phenolic compound was added to a 96-well microtiter plate, and each well was inoculated with an overnight culture of the tested microorganism, adjusted to contain 10^5 CFU/ml. The controls were bacterial cultures in LB broth without the compounds, LB broth with each compound in each tested concentration without bacteria (color control), and LB broth (sterility control). Growth curves were determined by measuring the optical density at 595 nm (OD_{595}) every 2 h, during 12 h on a spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, Massachussetts, United States). The MIC was the lowest concentration of the compound in which there was no bacterial growth, as observed by the growth curves. All QS assays were performed in concentrations that did not interfere with bacterial growth as recommended by Defoirdt et al. (2013).

Anti-QS Activity

Violacein Production

Quantification of violacein production was performed according to Santos et al. (2020), with modifications. The assay was performed in a 96-well microtiter plate containing 100 µl of LB broth with each phenolic compound at sub-MIC and 10 µl of inoculum containing 10^6 CFU/ml of *C. violaceum*. The plates were incubated for 24 h at 30°C at 150 rpm and then dried at 50°C in a BOD incubator. Subsequently, 100 µl of pure DMSO was added to each well. After 30 min at room temperature, the OD_{595} was measured using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, Massachussetts, United States). For the *C. violaceum* 026 assay, the autoinducer C6-HSL at 100 µM was added to each well. LB broth without phenolic compounds was used as negative control. Results were expressed as percentages, comparing OD_{595} measurements for the tested phenolic compound and the negative control, which was considered 100%.

Swarming and Swimming Motility

The test was performed according to Packiavathy et al. (2014) with some modifications. Swarming motility was tested using 3 ml of semi-solid LB agar 0.5% (w/v) to which the phenolic compounds were added at sub-MIC, and swimming motility was tested using 6 ml of semi-solid LB agar 0.3% (w/v) with the phenolic compounds at sub-MIC. These tests were performed in tubes containing melted semi-solid LB agar mixed with the tested phenolic compound. After vortexing, the mixtures were transferred to small Petri dishes (49 mm × 12 mm). After 10 min, 2 µl of a culture inoculum containing 10^8 CFU/ml of *A. hydrophila* or *S. marcescens* was spotted at the center of the plate, followed by 24 h incubation at the optimum growth temperature of the microorganism. Semi-solid agar without phenolic compound was used as control of absence of inhibition. The results of swarming and swimming motility inhibition were obtained comparing the bacterial growth diameters in the test and control plates.

Biofilm Formation

The analysis was performed according to Santos et al. (2020), using *S. marcescens* MG1, *A. hydrophila*, and *S.* Montevideo as target microorganisms. In a 96-well plate, 200 µl of LB broth with the phenolic compound at sub-MIC was mixed with an aliquot of 20 µl of an overnight culture of each tested bacterium, adjusted according to McFarland 0.5 solution (10^5 CFU/ml). The plates were incubated for 24 h at the optimal growth temperature of each bacterium. After removal of the culture medium, the plates were washed three times with 200 µl of sterile saline 0.9% (w/v). The sessile cells were stained with 200 µl of crystal violet 0.1% (w/v) for 30 min, the dye was removed, and the wells were rinsed three times with saline solution. Each test included a negative control, correspondent to LB broth without the addition of phenolic compounds. The crystal violet retained by the adhered cells was dissolved in 200 µl of 95% ethanol, and the OD_{595} nm was determined by spectrophotometry (Multiskan FC, Thermo Fisher Scientific, Waltham, Massachussetts, United States). Results were expressed as percentages, comparing OD_{595} measurements for the tested phenolic compound and the negative control, which was considered 100%.

Sensitivity to Kanamicin

The MIC and minimum bactericidal concentration (MBC) of kanamycin (Sigma-Aldrich, Brazil) were determined using the broth microdilution method (CLSI, 2007). In a 96-well plate, 100 µl of LB broth containing increasing concentrations of the antibiotic (4 to 512 µg/ml) and 10 µl of inoculum (10^5 CFU/ml, according to McFarland 0.5 solution) were added to each well. Negative controls, constituted of LB broth without antibiotic, were included in each test. The plates were incubated for 24 h at the optimal growth temperature of each bacterium, and the results were analyzed by visual inspection. The MIC corresponded to the concentration of the antibiotic that resulted in the absence of turbidity in the well. For confirmation of absence of viable cells, 2 µl of the content of the well was inoculated on LB agar for growth visualization. The MBC value corresponded to the lowest concentration of kanamycin that prevented bacterial growth on the LB agar.

Synergy Between Phenolic Compounds and Kanamycin

The inhibitory effect of curcumin and resveratrol in combination with kanamycin on the growth of *A. hydrophila* IOC/FDA 110-36, *S.* Montevideo 163 and *S. marcescens* MG1 was evaluated

by the checkerboard method, as described by Sanhueza et al. (2017). The tests were done in 96-well plates. The wells in rows contained phenolic compounds (resveratrol ranging from 25 to 100 μM and curcumin ranging from 1.5 to 50 μM), and the wells in columns contained kanamycin (4 to 512 μg/ml) plus LB broth, totaling 90 μl in each well. Subsequently, 10 μl of bacterial inoculum was added to each well (10^5 CFU/ml, according to McFarland 0.5 solution). The last row and column were used as controls, with only one agent and increasing concentrations of the second agent. The plates were incubated for 24 h at the optimal growth temperature of each bacterium, and growth curves were constructed based on OD_{595} measured every 2 h using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, Massachussetts, United States).

Statistical Analyses

Experiments were performed in three replicates, and results were submitted to ANOVA followed by Tukey's test using the GraphPad Prism 8.0 software. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Selection of Phenolic Compounds by Molecular Docking

Among the tested phenolic compounds, curcumin, capsaicin, resveratrol, and phloretin presented good molecular docking results, anchoring with good affinity to at least four of the six CviR structural variants. Based on their cost and commercial availability, they were selected for the *in vitro* studies. Gallic acid was also included due to its availability because it is a standard phenolic compound.

Figure 1 indicates how the tested phenolic compounds interacted with the 3QP1 protein. The interactions with the controls 3-oxo-C12-HSL (PubChem CID: 3246941) and Furanone C30 (PubChem CID: 10180544) are also shown. Ranking by the best binding affinities, 3-oxo-C12-HSL was in first place for structures 3QP1, 3QP4, and 3QP6, presenting GScores of −83.91, −85.61, and −89.6, respectively. The lower GScores (more negative) correspond to better binding affinities (Almeida et al., 2016). The phenolic compounds presented weaker binding affinity than 3-oxo-C12-HSL for all protein structures. However, the negative GScores for resveratrol (−57.30 to −66.62), capsaicin (−70.39 to −76.67), curcumin (−66.93 to −75.82), and phloretin (−63.10 to −70.19) indicated a good potential for binding to CviR QS regulator of *C. violaceum* (for the full list of GScores please see **Supplementary Tables 2 and 3**).

Antibacterial Activity

Minimal Inhibitory Concentration

Table 1 shows that gallic acid presented a MIC of 9.4 mM over the two strains of *C. violaceum* (ATCC 12472 and 026). The MIC of the other compounds was higher than the highest tested concentration. Concentrations above this limit could not be tested due to issues related to the solubility of the compounds in the assay media.

Anti-QS Activity of Phenolic Compounds

Violacein Production

The effect of the studied phenolic compounds on violacein production by *C. violaceum* ATCC 12472 is shown in **Figure 2**. The inhibition of pigment production by curcumin (12 μM) and capsaicin (70 μM) was 40%, while resveratrol reached 60% inhibition, regardless of the tested concentration (1.5, 3.0, or 6.0 μM), without affecting the growth of *C. violaceum* ATCC 12472. Gallic acid and phloridizin did not affect violacein production.

Figure 3 shows the effect of the phenolic compounds on violacein production by *C. violaceum* 026 in the presence of C6-HSL. *C. violaceum* 026 is not capable of producing the autoinducer C6-HSL due to a mutation in the *cviI* gene but maintains the ability to receive exogenous short-chain HSLs (4 to 6 carbons) as the *cviR* gene remains functional, resulting in production of the violacein pigment (Zhang et al., 2016). Among the evaluated phenolic compounds, only curcumin and capsaicin inhibited the production of violacein by this strain. The inhibition presented by curcumin was concentration-dependent, in which the highest tested concentration (25 μM) inhibited 75% of pigment production.

As phloridizin and gallic acid did not affect violacein production, indicating that they did not present any anti-QS activity, they were excluded from the tests for motility and biofilm formation.

Swarming and Swimming Motility

Table 2 shows the effect of phenolic compounds on the swarming motility of *A. hydrophila* and *S. marcescens*. *Salmonella* Montevideo was unable to perform swarming motility in our study. Capsaicin and resveratrol did not inhibit the swarming motility of *A. hydrophila* ($p > 0.05$) at the evaluated concentrations, while curcumin induced the motility in this organism ($p < 0.05$). Conversely, curcumin effectively inhibited the swarming motility of *S. marcescens*, indicating the anti-QS potential of curcumin in this organism. In contrast, capsaicin and resveratrol did not inhibit this phenotype ($p > 0.05$; **Supplementary Figure 1S**).

Regarding swimming motility, none of the tested phenolic compounds inhibited this phenotype in *A. hydrophila* and *S. marcescens* ($p > 0.05$; **Supplementary Figure 2S**).

Biofilm Formation

The effect of curcumin, capsaicin, and resveratrol on biofilm formation by *A. hydrophila* is shown in **Figure 4**. Curcumin (**Figure 4A**) and capsaicin (**Figure 4B**) did not inhibit this phenotype. In fact, capsaicin induced the biofilm formation in a concentration-dependent manner. In contrast, resveratrol (**Figure 4C**) significantly reduced the biofilm formation up to 80% at 100 μM.

Figure 5 shows the effect of curcumin, capsaicin, and resveratrol on biofilm formation by *S.* Montevideo. Inhibitory activity of curcumin (**Figure 5A**) and capsaicin (**Figure 5B**)

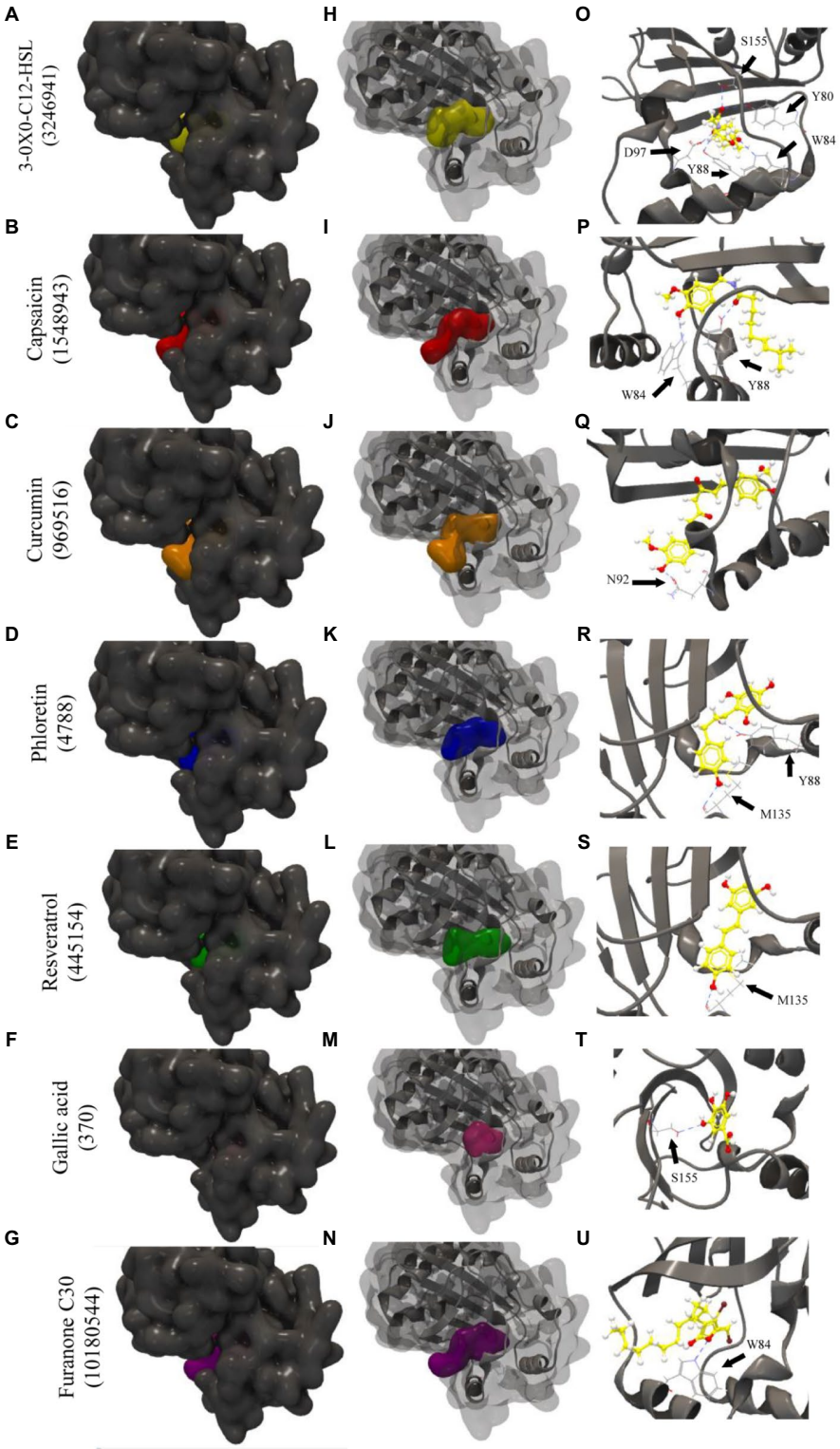

FIGURE 1 | Molecular docking of the 3QP1 protein of CviR QS regulator of *Chromobacterium violaceum* with 3-oxo-C12-HSL, capsaicin, curcumin, phloretin, resveratrol, gallic acid, and Furanone C30. Surface representation **(A–G)**; surface and backbone representations **(H–N)**; and backbone representations with hydrogen bonding between amino acid residues and phenolic compounds **(O–U)**. Gray surface representation: CviR protein; yellow surface representation: 3-oxo-C12-HSL; red surface representation: capsaicin; orange surface representation: curcumin; blue surface representation: phloretin; green surface representation: resveratrol; pink surface representation: gallic acid; and purple surface representation: Furanone C30. Black arrow indicates the binding site by hydrogen bonding.

TABLE 1 | Minimum inhibitory concentration (MIC) of phenolic compounds.

Target microorganism	MIC (mM)				
	Curcumin	Capsaicin	Phloridizin	Resveratrol	Gallic acid
Aeromonas hydrophila IOC/FDA 110-36	>0.1	>1.0	>0.9	>0.1	>9.4
Chromobacterium violaceum ATCC12472	>0.1	>1.0	>0.9	>0.1	9.4
Chromobacterium violaceum O26	>0.1	>1.0	>0.9	>0.1	9.4
Salmonella Montevideo 163	>0.1	>1.0	>0.9	>0.1	>9.4
Serratia marcescens MG1	>0.1	>1.0	>0.9	>0.1	>9.4

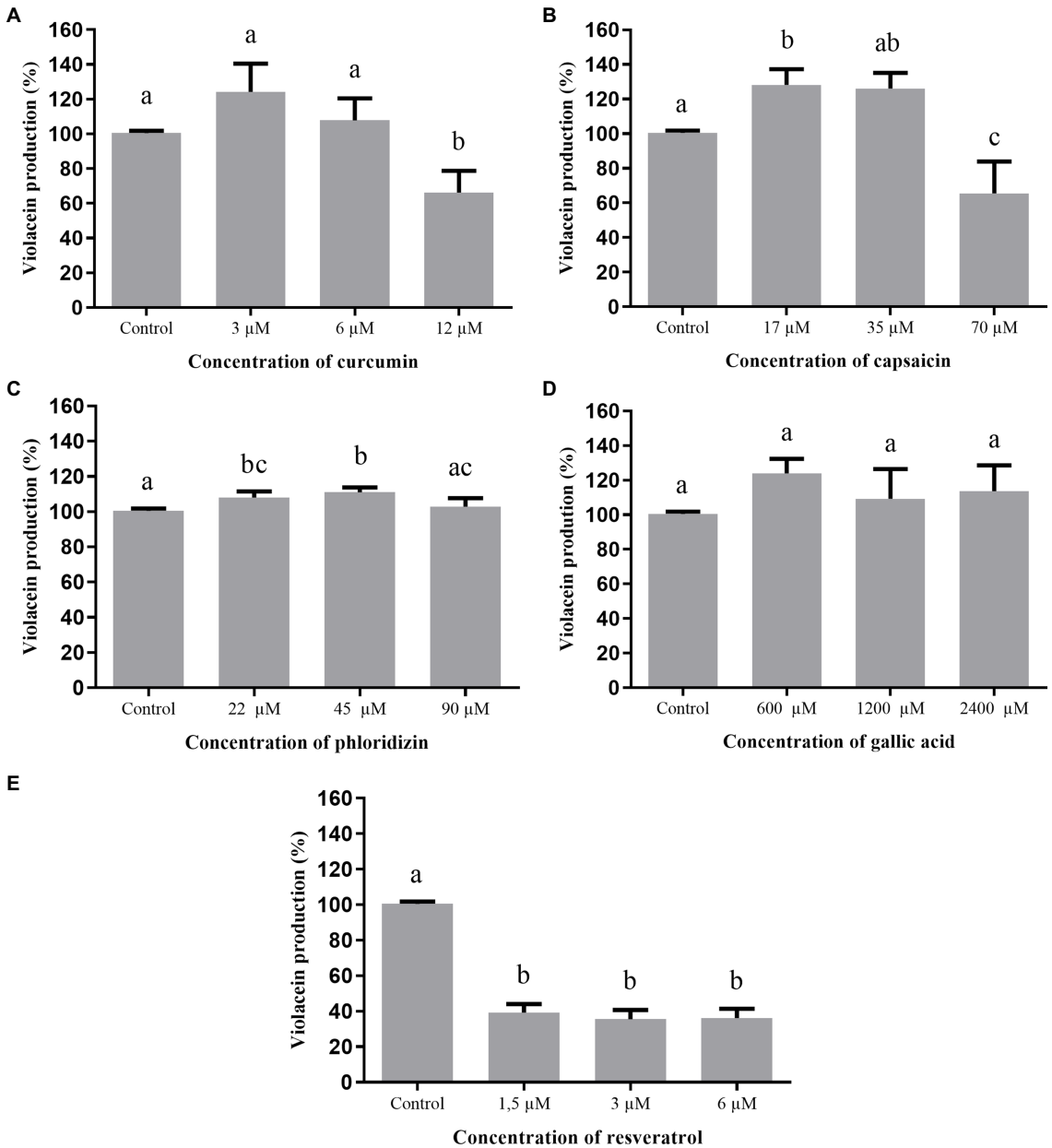

FIGURE 2 | Effect of phenolic compounds on violacein production by *Chromobacterium violaceum* ATCC 12472 expressed as percentage of production, when compared to the control (100%). Curcumin **(A)**; capsaicin **(B)**; phloridizin **(C)**; gallic acid **(D)**; resveratrol **(E)**; and control = violacein production in LB broth + dimethyl sulfoxide (DMSO; 1%).

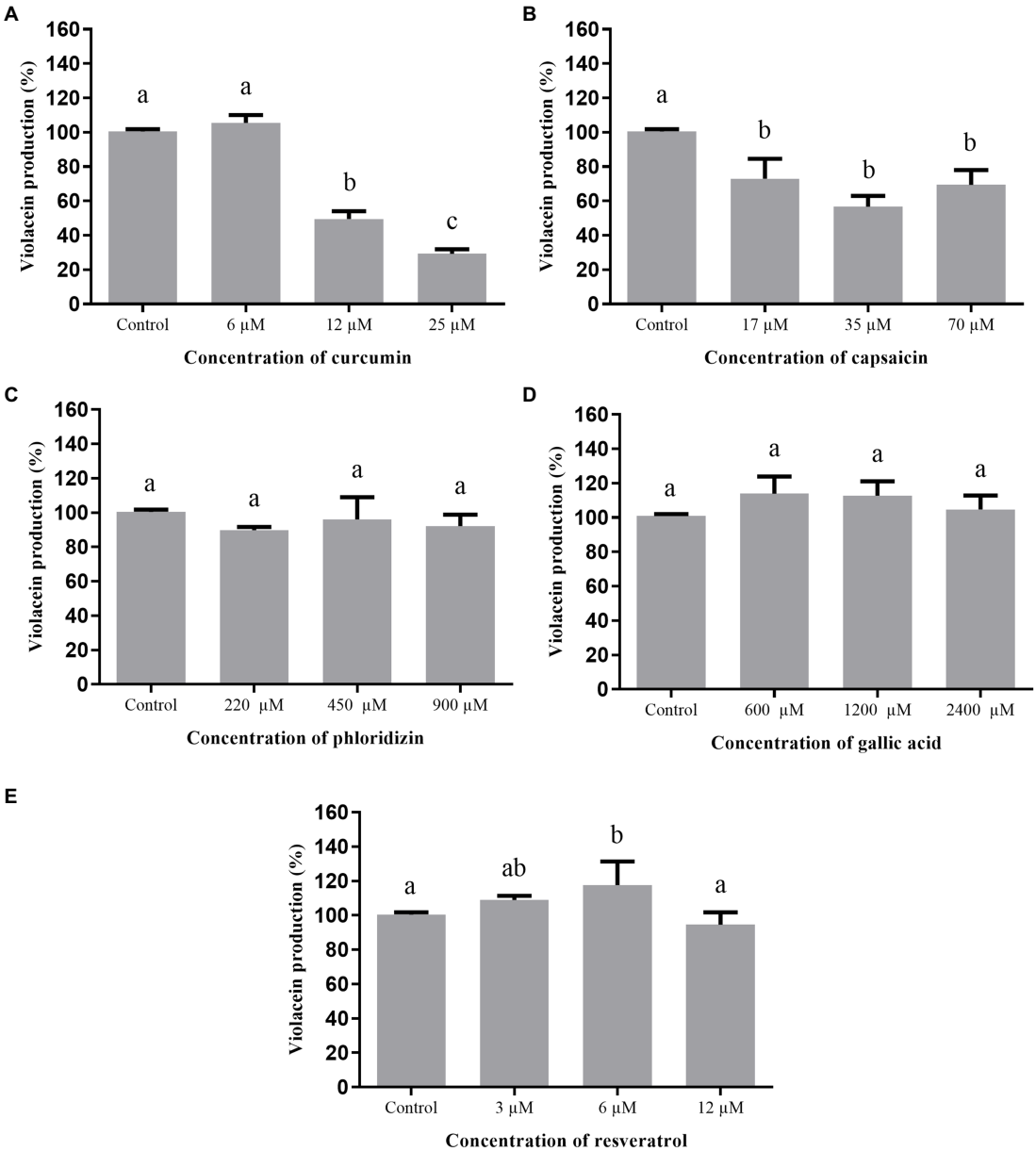

FIGURE 3 | Effect of phenolic compounds on violacein production by *Chromobacterium violaceum* 026 in the presence of C6-HSL, expressed as percentage of production, when compared to the control (100%). curcumin **(A)**; capsaicin **(B)**; phloridizin **(C)**; gallic acid **(D)**; resveratrol **(E)**; and control = violacein production in LB broth + DMSO (1%).

was similar; i.e., both were able to inhibit biofilm formation up to 27% when compared to the control ($p < 0.05$). In contrast, resveratrol (**Figure 5C**) did not inhibit biofilm formation at the tested concentrations ($p > 0.05$).

When the phenolic compounds were tested for inhibition of biofilm formation by *S. marcescens* (**Figure 6**), curcumin (**Figure 6A**) was ineffective ($p > 0.05$). Capsaicin (**Figure 6B**) and resveratrol (**Figure 6C**) were active at the highest tested concentrations; i.e., 1,000 µM capsaicin and 100 µM resveratrol were able to inhibit biofilm formation up to 43%.

Effect of Phenolic Compounds (Curcumin and Resveratrol) Combined With Kanamycin on Growth

Figure 7 presents the growth curves of *A. hydrophila*, *S.* Montevideo, and *S. marcescens* in LB broth containing curcumin or resveratrol in combination with kanamycin, at varied concentrations. The curves clearly indicate that the two phenolic compounds delayed the growth of all tested bacteria when combined with sub-inhibitory concentrations of the antibiotic, in a concentration-dependent pattern.

TABLE 2 | Effect of capsaicin, curcumin, and resveratrol on swarming motility of *Aeromonas hydrophila* IOC/FDA 110-36 and *Serratia marcescens* MG1.

Phenolic compound	Radius of swarming zone after 12 h (mm)			
	Tested concentration	*Aeromonas hydrophila*	Tested Concentration	*Serratia marcescens*
Capsaicin	0 μM	12.0 ± 0.0^{a}	0 μM	20.0 ± 0.0^{a}
	65 μM	12.2 ± 0.3^{a}	65 μM	30.0 ± 1.4^{b}
	130 μM	9.5 ± 2.1^{a}	130 μM	31.5 ± 0.7^{b}
	260 μM	8.7 ± 1.0^{a}	260 μM	32.5 ± 0.7^{b}
Curcumin	0 μM	12.5 ± 0.7^{ab}	0 μM	49.0 ± 0.0^{a}
	3 μM	15.2 ± 1.0^{abc}	12.5 μM	5.5 ± 0.7^{b}
	6 μM	16.5 ± 0.0^{bc}	25 μM	5.0 ± 0.0^{b}
	12 μM	18.7 ± 3.1^{c}	50 μM	5.0 ± 1.4^{b}
Resveratrol	0 μM	13.5 ± 2.1^{a}	0 μM	22.5 ± 2.1^{ab}
	25 μM	14.0 ± 0.0^{a}	25 μM	31.0 ± 1.4^{b}
	50 μM	13.7 ± 0.3^{a}	50 μM	27.0 ± 2.8^{b}
	100 μM	14.0 ± 0.0^{a}	100 μM	21.0 ± 0.0^{ab}

Results expressed as mean ± standard deviation. Means followed by different superscript letters (a,b,c) in the same column differ among themselves by Tukey's test ($p < 0.05$).

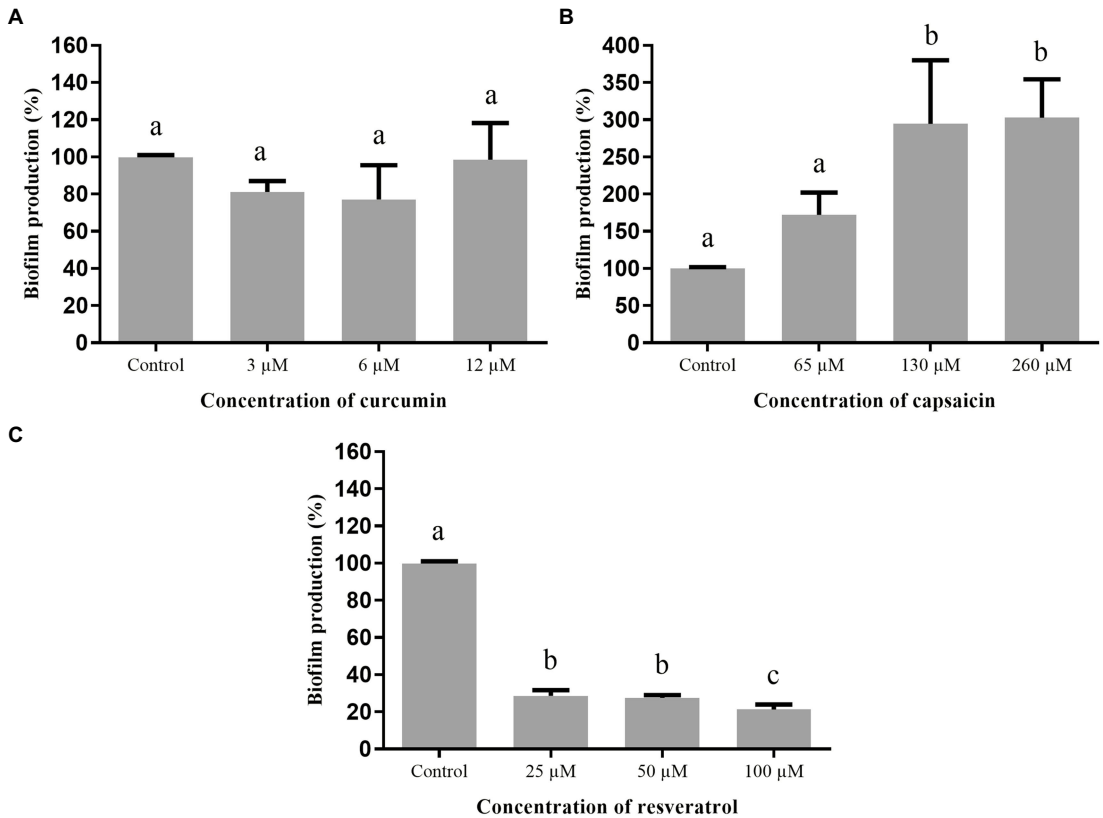

FIGURE 4 | Effect of phenolic compounds on biofilm formation by *Aeromonas hydrophila* IOC/FDA 110-36, expressed as percentage of production, when compared to the control (100%). curcumin **(A)**; capsaicin **(B)**; resveratrol **(C)**; and control = biofilm formation in LB broth + DMSO (1%). Means followed by different letters differ among themselves by Tukey's test ($p < 0.05$).

For *A. hydrophila*, 256 μg/ml of kanamycin held the bacterial growth for at least 14 h. Growth in the presence of curcumin (3–12 μM) was slowed for longer periods of time (**Figure 7A**). Lower tested concentrations of kanamycin (4–128 μg/ml) did not affect the growth of this bacterium (**Supplementary Figure 3S**). When the concentration of kanamycin was increased to 512 μg/ml, there was no bacterial growth, representing the MBC for this antibiotic.

In the tests with *S.* Montevideo, 6 μM of curcumin and 32 μg/ml of kanamycin completely inhibited the bacterial growth (**Figure 7B**). Lower concentrations of curcumin (0–3 μM) or kanamycin (4–16 μg/ml) had no pronounced effect on growth

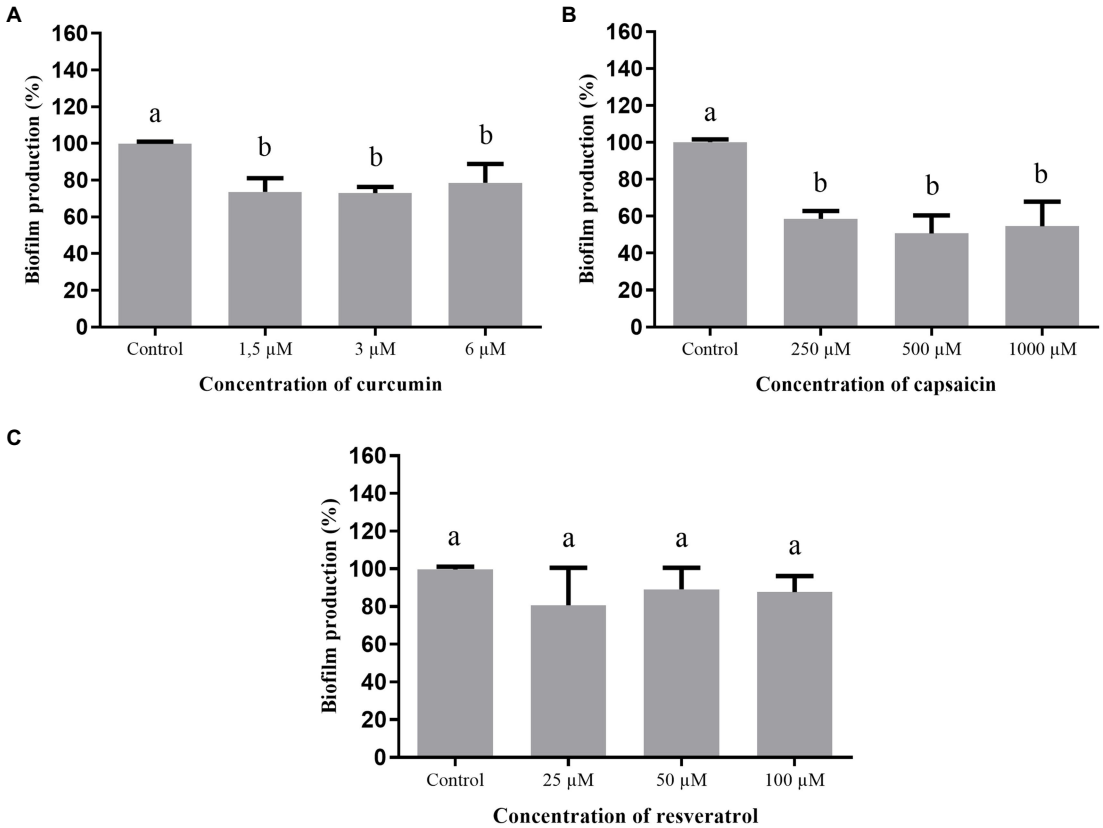

FIGURE 5 | Effect of phenolic compounds on biofilm formation by *Salmonella* Montevideo 163 expressed as percentage of production, when compared to the control (100%). curcumin **(A)**; capsaicin **(B)**; resveratrol **(C)**; and control = biofilm formation in LB broth + DMSO (1%). Means followed by different letters differ among themselves by Tukey's test ($p < 0.05$).

(**Supplementary Figure 4S**). Higher concentrations of kanamycin (64 to 512 µg/ml) caused the complete inhibition of bacterial growth (**Supplementary Figure 4S**).

For *S. marcescens*, the combination of curcumin with kanamycin in inhibiting growth was less effective than for *A. hydrophila* and *S.* Montevideo (**Figure 7C**). Evidence of inhibition was observed only when 32 µg/ml of kanamycin was combined with curcumin, being slightly more effective at 50 µM of the later. Lower concentrations of kanamycin (4 to 16 µg/ml) and curcumin (0–50 µM) resulted in no reduction in the microbial growth, while higher concentrations of kanamycin (64 to 512 µg/ml) resulted in stronger growth inhibition that was not affected by the presence of curcumin (**Supplementary Figure 5S**).

Results of the tests with resveratrol (0–100 µM) combined with kanamycin (4–512 µg/ml) are shown in **Figure 7D–F**. The growth curves of *A. hydrophila* (**Figure 7D**), *S.* Montevideo (**Figure 7E**), and *S. marcescens* (**Figure 7E**) indicate that the effects of these combinations were similar to those observed for the curcumin-kanamycin combinations.

The curves from assays with kanamycin (4 to 64 µg/ml) and resveratrol (0–100 µM) show partial inhibition of growth of *A. hydrophila* from 8 to 10 h, compared with the curve from assays using only kanamycin and the control (**Supplementary Figure 6S**). Kanamycin at 128 µg/ml combined with resveratrol presented a growth delay, in which the bacterial growth started at 14 h of incubation, while with the treatment containing only kanamycin the growth started at 8 h of incubation (**Figure 7D**). The results show that resveratrol made *A. hydrophila* more sensitive to kanamycin at the studied concentrations (**Supplementary Figure 6S**).

The lowest concentrations of kanamycin (4 and 8 µg/ml) did not inhibit the growth of *S.* Montevideo. In the concentration of 32 µg/ml of kanamycin, there was growth retardation in all concentrations of resveratrol evaluated, especially at 100 µM, with a delay of 6 h compared to the other treatments (**Figure 7E**). In the highest concentrations of antibiotic, there was no microbial growth; thus, 64 µg/ml is the MBC of this microorganism (**Supplementary Figure 7S**).

In the test with *S. marcescens*, at concentrations from 4 to 16 µg/ml of kanamycin, growth in the presence of resveratrol was strongly inhibited. The highest concentration of resveratrol (100 µM) reduced the MIC 3-fold when compared to the curve containing only kanamycin (**Figure 7F**). At concentrations of 32 to 128 µg/ml of kanamycin, resveratrol completely inhibited growth. It was observed that resveratrol made the microorganism more sensitive to the antibiotic. Finally, complete inhibition of growth was observed in the presence of 256 and 512 µg/ml kanamycin alone (**Supplementary Figure 8S**).

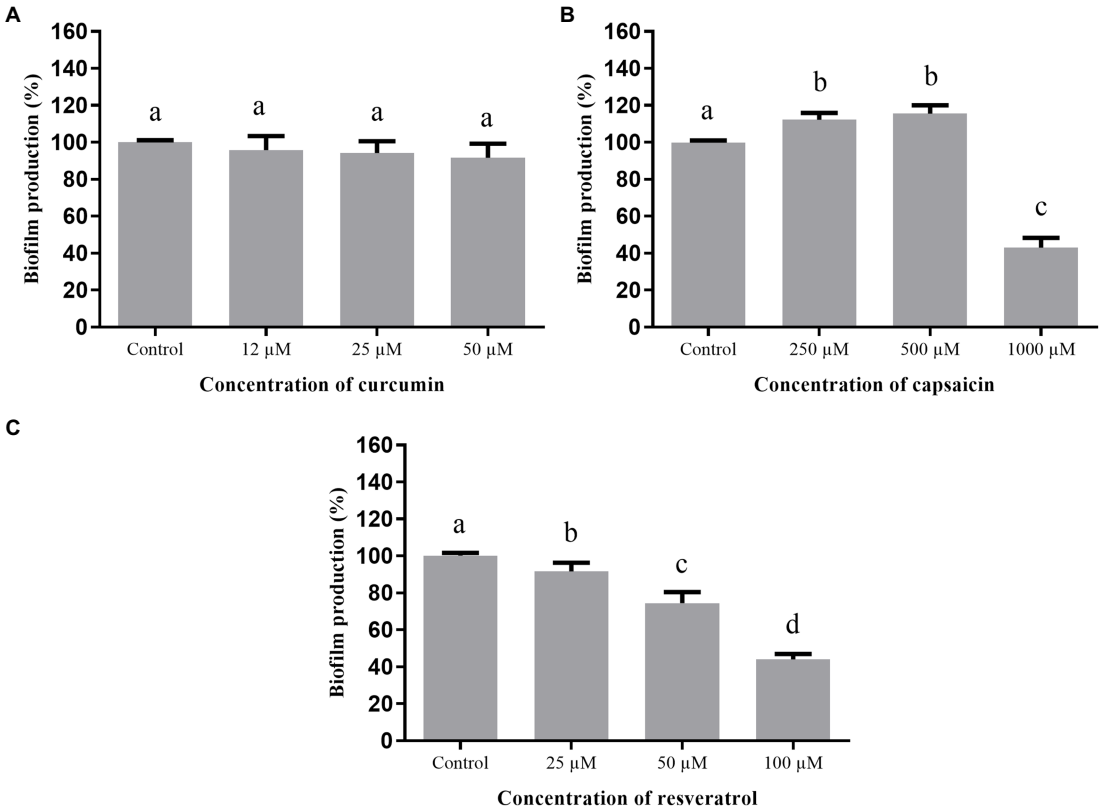

FIGURE 6 | Effect of phenolic compounds on biofilm formation by *Serratia marcescens* MG1 expressed as percentage of production, when compared to the control (100%). Curcumin **(A)**; capsaicin **(B)**; resveratrol **(C)**; and control = biofilm formation in LB broth + DMSO (1%). Means followed by different letters differ among themselves by Tukey's test ($p < 0.05$).

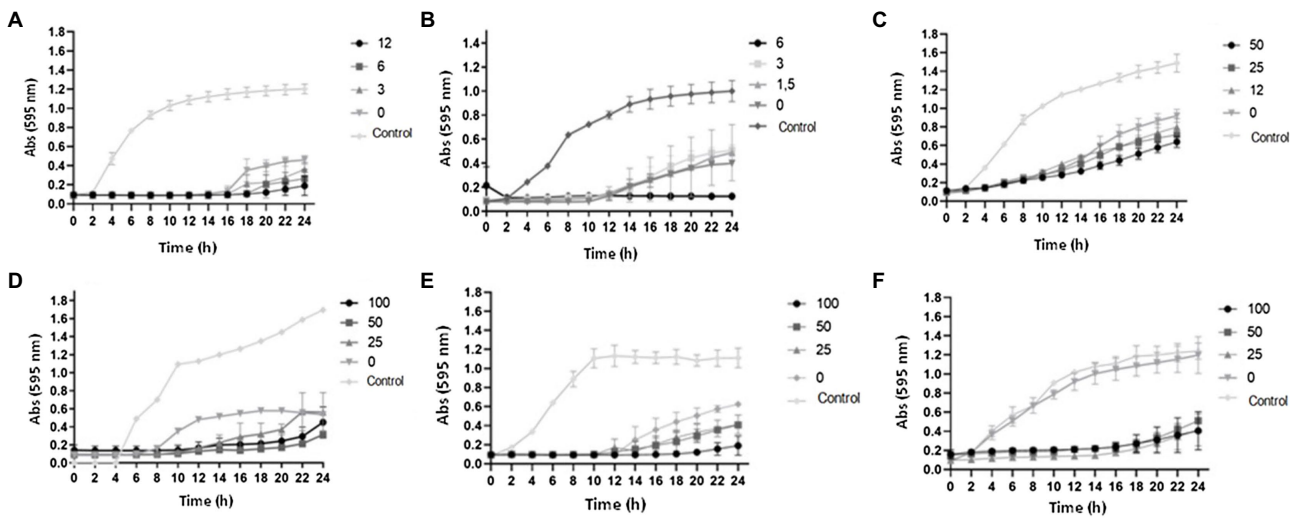

FIGURE 7 | Growth curves of bacteria treated with phenolic compounds and kanamycin. **(A)** *A. hydrophila* IOC/FDA 110-36 treated with curcumin (0, 3, 6, and 12 µM) and kanamycin (256 µg/ml). **(B)** *Salmonella* Montevideo 163 treated with curcumin (0, 1.5, 3, and 6 µM) and kanamycin (32 µg/ml). **(C)** *Serratia marcescens* MG1 treated with curcumin (0, 12, 25, and 50 µM) and kanamycin (32 µg/ml). **(D)** *A. hydrophila* IOC/FDA 110-36 treated with resveratrol (0, 25, 50, and 100 µM) and kanamycin (128 µg/ml). **(E)** *Salmonella* Montevideo treated with resveratrol (0, 25, 50, and 100 µM) and kanamycin (32 µg/ml). **(F)** *Serratia marcescens* treated with resveratrol (0, 25, 50, and 100 µM) and kanamycin (4 µg/ml). Control = bacterial growth in LB broth + DMSO (1%).

DISCUSSION

Phenolic compounds are known for their antimicrobial effects as a consequence of structural or functional damages to the bacterial cell membrane (Silva et al., 2018). Besides, phenolic acids can affect the physicochemical properties of the bacterial cell surface, especially hydrophobicity, in addition to altering electron receptors and polar and non-polar components of bacteria (Borges et al., 2013). Furthermore, phytochemicals, such as phenolic compounds, may compete with AHL signaling molecules for binding to QS receptors (Nazzaro et al., 2013; Almeida et al., 2018; Aswathanarayan and Vittal, 2018; Quecán et al., 2018; Santos et al., 2020), suggesting possible interactions of these compounds with LuxR-type proteins, like the CviR QS regulator of *Chromobacterium violaceum* (**Figure 1**).

Curcumin, one of the selected phenolic compounds for the *in vitro* studies, is the main polyphenol in the rhizome of *Curcuma longa*. Beyond its numerous pharmaceutical properties, such as antitumor, anti-HIV-1, and antibacterial activity, this compound also presents anti-QS activity, inhibiting several phenotypes in *Pseudomonas aeruginosa* PAO1, *Escherichia coli*, *Serratia marcescens*, and *Proteus mirabilis* (Ding et al., 2017). Resveratrol is also active against various pathogens and presents anti-QS activity, being capable of inhibiting biofilm formation and dispersing established biofilms (Duarte et al., 2015). Gallic acid is a phenolic acid present in numerous foods, and also an antimicrobial agent, affecting bacterial cell membranes and causing irreversible changes in permeability, rupture, and pore formation (Sorrentino et al., 2018). Capsaicin and luteolin are polyphenols abundant in several pepper species, especially those of the *Capsicum* genus, and have the ability to inhibit microbial growth, in addition to antioxidant, anti-inflammatory, cardioprotective, neuroprotective, and anticancer activity (Basith et al., 2016; Rivera et al., 2019).

The antimicrobial activity of phloridizin in the tested concentrations against the target microorganisms was lower when compared to the other phenolic compounds. Barreca et al. (2014) evaluated the antimicrobial activity of phloridizin and phloretin, its aglycone form, against several Gram-positive and Gram-negative bacteria, and observed that the presence of glucose in the basic chalcone structure caused a reduction in antimicrobial activity. Furthermore, they also observed that phloretin is particularly active against Gram-positive bacteria. The low activity against the tested Gram-negative microorganisms observed in this study corroborate the findings of Barreca et al. (2014). Interestingly, Quecán et al. (2019) also observed that glycosylation of quercetin reduces its antimicrobial and anti-QS effect.

Regarding the determination of the MIC of phenolic compounds, it is crucial to perform QS assays in concentrations that do not interfere with bacterial growth, since this would affect quantification of QS regulated phenotypes due to cell density differences (Defoirdt et al., 2013; Maura et al., 2016). Bali et al. (2019) evaluated the MIC of curcumin (1,500 μg/ml, equivalent at 4.1 mM) and gallic acid (>1,500 μg/ml, equivalent to >8.8 mM) in *C. violaceum* ATCC 12472, corroborating the results of the present work (**Table 1**). Packiavathy et al. (2014) also evaluated anti-QS activity of curcumin and reported an MIC of 384 μg/ml, equivalent to 1.04 mM, for *C. violaceum* 026, a value higher than that tested in the present study (0.1 mM). Rivera et al. (2019) studied compounds present in *Capsicum frutescens* pepper extract and found that MIC of capsaicin was higher than 100 μg/ml (equivalent to 0.34 mM). The growth of *C. violaceum* ATCC 12472 and 026 was partially inhibited by the compound at 0.1 mM, which is similar to the results found in the present work.

Curcumin inhibited violacein production by *C. violaceum* ATCC 12472 (**Figure 2**), as also observed by Bali et al. (2019). However, those authors evaluated higher concentrations (2 mM, 1.02 mM, and 0.51 mM) than those used in the present work (0.012 mM, 0.006 mM and 0.003 mM), indicating that even at lower concentrations, curcumin consistently inhibits this QS regulated phenotype. It should be noted that in concentrations higher than 0.050 mM, a partial inhibition of microbial growth was observed in our study (results not shown), which should affect violacein production in a QS-independent manner.

In the study of Rivera et al. (2019), capsaicin did not inhibit violacein production when the concentration was similar to the one used in this study. However, those authors did not test the concentration of 70 μM, which was the one with best anti-QS activity detected here. When working with resveratrol, Duarte et al. (2015) found positive results for violacein inhibition, corroborating the results of this work and the potential of this compound. They further suggested that the anti-QS activity of resveratrol is due to its ability to mimic QS signals and disrupt bacterial communication (Duarte et al., 2015). In the present study, phloridizin and gallic acid did not show anti-QS activity in *C. violaceum* ATCC 12472. Borges et al. (2014) and Bali et al. (2019) also reported that gallic acid did not exhibit anti-QS activity in the biosensor strain.

Tests performed with only wild-type *C. violaceum* strain are not enough to detect the QS inhibitory mechanism, since the inhibition of violacein production may be due to reduced production of the autoinducer (inhibition of AHL synthesis by CviI) or by interference with the AHL-dependent transcriptional activator, the CviR protein (Rivera et al., 2019). Thus, besides *C. violaceum* ATCC 12472, complementary assays performed with additional strains, such as *C. violaceum* 026, a mutant unable to produce AHLs but still capable to respond to exogenous AHLs, can be informative (Rivera et al., 2019). If the tested compounds interfere with C6-HSL detection by CviR, a reduced violacein production by *C. violaceum* 026 can be expected (LaSarre and Federle, 2013). Thus, curcumin and capsaicin likely present anti-QS activity *via* AHL detection by CviR since these compounds reduced violacein production in *C. violaceum* 026 (**Figure 3**). Brackman et al. (2009) and Packiavathy et al. (2014) also observed inhibition of violacein production by curcumin in *C. violaceum* 026, even though they have used higher concentrations than those used in this study.

Swarming motility is an organized microbial movement on surfaces, dependent on extensive flagellation, cell–cell contact, and QS. This type of motility is associated with virulence and antibiotic resistance of various microorganisms, and is considered

a favorable adaptation to the challenges that arise in dynamic environments, contributing to biofilm formation and infection (Rütschlin and Böttcher, 2019; Carette et al., 2020). Interference with this phenotype is an important alternative to reduce or prevent biofilm-based infections. In this study, curcumin effectively inhibited swarming motility (**Table 2** and **Supplementary Figure S1**), which corroborates results reported by Packiavathy et al. (2014), who observed the inhibition of swarming motility of *S. marcescens*, *E. coli*, *P. aeruginosa*, and *P. mirabilis* by curcumin at 100 μg/ml (135 μM). One explanation for the observed inhibition is the ability of curcumin in reducing the production of extracellular polysaccharides by some pathogens, negatively affecting their motility (Zheng et al., 2020).

Swimming-type inhibition by curcumin liposomes was also observed for *A. hydrophila* and *Serratia grimesii* (Ding et al., 2018) and for *S. marcescens*, *E. coli*, *P. aeruginosa*, and *P. mirabilis* (Packiavathy et al., 2014). Other studies also observed motility inhibition by phenolic compounds, like proanthocyanidins and tannins that completely inhibited swarming, but did not block swimming (O'May and Tufenkji, 2011), and trans-resveratrol, that had greater activity on swarming than on swimming motility, indicating that these movement phenotypes have different activation mechanisms.

Regarding the inhibition of biofilm formation by *A. hydrophila* (**Figure 4**), only resveratrol showed a significant effect. Similar results were found in other studies using different bacteria: *L. monocytogenes* (Vasquez-Armenta et al., 2018); *Campylobacter* spp. and *Arcobacter butzleri* (Duarte et al., 2015); methicillin-resistant *S. aureus* (MRSA; Qin et al., 2014); and *E. coli* O157: H7 (Lee et al., 2013), evidencing the broad inhibitory effect of resveratrol.

Mangoudehi et al. (2020) evaluated curcumin at different concentrations against biofilm formation of *A. hydrophila* ATCC 7966 and other three strains isolated from fish (silver carps) and found 67% reduction of biofilm formation at 43.4 μM. These results differ from those observed in the present study (**Figure 4A**), where the maximum concentration was 12 μM, indicating that biofilm inhibition is concentration-dependent. On the other hand, curcumin inhibited the biofilm formation by *S.* Montevideo (**Figure 5**). The efficacy of curcumin against various infectious organisms results from multiple mechanisms, including its ability to disrupt bacterial membranes, inhibit replication, and alter gene expression (Vaughn et al., 2017). Packiavathy et al. (2014) observed a visible reduction in microcolonies, disruption of biofilm architecture, and reduced biofilm biomass of *P. aeruginosa*, *S. marcescens*, *E. coli*, and *P. mirabilis* treated with curcumin.

Biofilm formation by *S.* Montevideo was also inhibited by capsaicin (**Figure 5**). This compound is used as a food additive and flavoring, and has important pharmacological actions, such as bacteriostatic activity against Gram-negative bacteria and anti-inflammatory effects (Zhou et al., 2014). Some studies have shown that capsaicin inhibits adhesion, growth, and biofilm formation of Gram-negative bacteria, such as *Pseudomonas putida*, *Vibrio matriegens*, *Vibrio parahaemolyticus* (Xu et al., 2005), and *Porphyromonas gingivalis* (Zhou et al., 2014).

Regarding the inhibition of biofilm formation of *S. marcescens* (**Figure 6**) by capsaicin at 1,000 μM and resveratrol at 25 μM or higher, interesting effects were observed. The capability of trans-resveratrol to inhibit biofilm formation by enterohemorrhagic *E. coli* O157:H7 ATCC43895 (Lee et al., 2013), *P. aeruginosa* PAO1 and PA14 (Cho et al., 2013), and *S. aureus* (Morán et al., 2014) has already been described. Thus, resveratrol can be considered a potential anti-biofilm compound for several biofilm-forming microorganisms and its use should be further investigated on food-contacting materials.

Antimicrobial resistance is a serious concern in clinical practice. One strategy that is useful in combating multidrug-resistant bacteria is the coadministration of antibiotics with adjuvants that modify bacterial resistance. Several medicines of great utility have been approved using this concept, such as the use of amoxicillin with beta-lactamase inhibitors. Other studies also show the effective combination of antibiotics with efflux-pump inhibitors (Ayaz et al., 2019). As biofilm formation is associated with reduced sensitivity to antibiotics, and it is known that QS regulates the expression of some genes associated with this phenotype, QS interference can be explored as an alternative to reduce bacterial virulence and increase the efficacy of antibiotic treatment (Mion et al., 2019). This study showed that curcumin at 6 μM reduced the MIC of kanamycin by half in *S.* Montevideo (**Figure 7B**). These results corroborate those of Packiavathy et al. (2014) who observed increased sensitivity of *S. marcencens* to different antibiotics when combined with curcumin. Mun et al. (2013) and Kali et al. (2016) also observed a decrease in the MIC of several antibiotics against MRSA and other bacteria, when applied in combination with curcumin. These studies demonstrate that this phenolic compound can increase the sensitivity of several pathogens to antibioticos and is a potential agent to be coadministered with these drugs.

Serratia marcescens may be resistant to several antibiotics, including beta-lactam, aminoglycosides, and fluoroquinolones, hindering the treatment against infections caused by this bacterium (Yang et al., 2012). Like other Enterobacteriaceae, the production of beta-lactamase enzyme which inactivate beta-lactam antibiotics is the most common resistance mechanism in this micro-organism (Yang et al., 2012). The increased sensitivity of *S. marcescens* to colistin when combined with resveratrol reported by Cannatelli et al. (2018), the potentiation of the effects of aminoglycosides against biofilms of *P. aeruginosa* PAO1 (Zhou et al., 2018), and the increased sensitivity of multidrug resistant *Klebsiella pneumoniae* and *E. coli* (Liu et al., 2020b) and *S. aureus* (Liu et al., 2020a) deserve to be better explored as effective therapeutic alternatives.

The combination of bioactive compounds with antimicrobials seems to be a promising approach to improve the efficacy of these drugs. However, there are several challenges to be faced before translating these *in vitro* findings into real-life applications. Some of the challenges we envision are related to interactions and metabolization of these compounds in the human body, which could hinder their antimicrobial and anti-quorum sensing activities *in vivo*. Additionally, as pointed out by Vipin et al. (2020) and observed in the present work, strain variation in response to bioactive compounds is another challenge in treating infections.

CONCLUSION

Phenolic compounds have potential to inhibit quorum sensing in foodborne bacteria. *In silico* analyses showed that gallic acid, capsaicin, curcumin, phloridizin, and resveratrol could interact with CviR QS receptor of *C. violaceum*. Curcumin and resveratrol presented better activity against violacein production, swarming motility, biofilm formation, and concomitant use with an antibiotic for increased microbial sensitivity. The present study and several others from the literature indicate that curcumin and resveratrol are potential QSI and have antimicrobial properties that encourage studies that could translate these findings to applications in the food and pharmaceutical industries. In order to characterize the specificity of quorum sensing inhibition, biochemical and genetic studies should be performed with these compounds and model organisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors have made direct and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.735931/full#supplementary-material

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REVIEW
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Antibacterial Activity of Pediocin and Pediocin-Producing Bacteria Against *Listeria monocytogenes* in Meat Products

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One of the most important challenges in the food industry is to produce healthy and safe food products, and this could be achieved through various processes as well as the use of different additives, especially chemical preservatives. However, consumer awareness and concern about chemical preservatives have led researchers to focus on the use of natural antimicrobial compounds such as bacteriocins. Pediocins, which belong to subclass IIa of bacteriocin characterized as small unmodified peptides with a low molecular weight (2.7–17 kDa), are produced by some of the *Pediococcus* bacteria. Pediocin and pediocin-like bacteriocins exert a broad spectrum of antimicrobial activity against Gram-positive bacteria, especially against pathogenic bacteria, such as *Listeria monocytogenes* through formation of pores in the cytoplasmic membrane and cell membrane dysfunction. Pediocins are sensitive to most protease enzymes such as papain, pepsin, and trypsin; however, they keep their antimicrobial activity during heat treatment, at low temperatures even at −80°C, and after treatment with lipase, lysozyme, phospholipase C, DNase, or RNase. Due to the anti-listeria activity of pediocin on the one hand and the potential health hazards associated with consumption of meat products on the other hand, this review aimed to investigate the possible application of pediocin in preservation of meat and meat products against *L. monocytogenes*.

Keywords: bacteriocin, pediocin, antimicrobial, *Listeria monocytogenes*, meat

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INTRODUCTION

Meat and meat products have an important role in the human diet, and their consumption has increased among animal food consumers as an excellent source of protein and other nutrients in recent years (Bohrer, 2017; Yousefi et al., 2018). These products have high nutritional value, but are highly perishable. In fact, meat and meat products are a suitable medium for growth of various pathogenic and spoilage microorganisms due to the presence of essential nutrients, absence of competing microorganisms, and desirable water activity and pH (Galvez et al., 2008). In

addition, environments of food production and ingredients applied in the recipes of products such as frankfurters and sausages can facilitate microbial proliferation (Mamber et al., 2020). Therefore, meat and meat products must be produced and stored under safe and hygienic conditions. However, microbial contamination of meat and meat products is likely to occur in some poor hygienic conditions of processing and storage of these products, which can lead to safety and spoilage problems (Kurpas et al., 2018; Yousefi et al., 2020).

Contamination of meat products with pathogenic microbes is one of the main health and economic concerns all over the world, since serious foodborne diseases can be caused by their consumption and production and healthcare costs increase for food manufacturers owing to loss of productivity (Abatcha, 2017; Yousefi et al., 2020). Various pathogens such as *Listeria monocytogenes*, *Escherichia coli*, O157:H7, *Campylobacter jejuni*, *Salmonella* spp., and *Clostridium* spp. participate in incidence of foodborne diseases through the consumption of meat and meat products that were produced and stored under inappropriate conditions (Laranjo et al., 2019). Among these, *L. monocytogenes* is the causative agent of listeriosis, which is known as a major virulent foodborne disease (Cherifi et al., 2020; Pilevar et al., 2020a; Yousefi et al., 2020). It has been indicated that 172,823 disability-adjusted life-years (DALYs), 23,150 illnesses, and 5,463 deaths occurred in 2010 worldwide as a consequence of listeriosis (De Noordhout et al., 2014). Also, it is estimated that approximately 1,600 cases of listeriosis occur each year in the United States, and nearly 2,500 cases of listeriosis have been reported in European Union (EU) countries with a high mortality rate of about 20% in endangered population (EFSA, 2019; Ceruso et al., 2020). Therefore, the presence of *L. monocytogenes* in foodstuff and incidence of listeriosis is still considered as one of the most important food safety challenges worldwide (Ceruso et al., 2020).

Due to the ability of *L. monocytogenes* to grow in harsh conditions of processing or storage including low water activity, high concentrations of salt, pH ranges of 4.1–9.6, and refrigeration temperatures, it is difficult to control *L. monocytogenes* by food preservation techniques, and since this microorganism is able to grow at low temperatures (2–4°C), there is a specific concern about the presence of *L. monocytogenes* in meat and meat products (Shukla et al., 2017; Bucur et al., 2018; Chen et al., 2019; Fang et al., 2021). Various thermal and non-thermal preservation strategies have been applied to ensure the safety of food. Furthermore, different preservatives such as synthetic antimicrobial agents have been used to prevent microbial contamination through processing, distribution, and storage of food products such as meat products (Amit et al., 2017; López et al., 2019; Bahrami et al., 2020). Although synthetic additives used in the food industry are food-grade and Generally Recognized as Safe (GRAS), there is a growing concern about the use of these additives by consumers (Carocho et al., 2014; Khorshidian et al., 2018; Yousefi et al., 2020). Hence, there is an increasing attention in utilizing natural antimicrobial agents such as lactoperoxidase, lactoferrin, lysozyme from animal sources, essential oils and herbal extract from plant sources, and bacteriocin from microbial sources (Del Nobile et al., 2012; Hayashi et al., 2013; Carocho et al., 2015).

Various studies have carried out the use of various bacteriocins as food biopreservatives to inhibit pathogenic microbes (De Souza de Azevedo et al., 2019; Furlaneto-Maia et al., 2020; Xu et al., 2021). Nisin and pediocin are the most studied bacteriocin that could be utilized commercially as natural preservatives (Acuña et al., 2011). Bacteriocins are antibacterial peptides or proteinaceous toxins synthesized in ribosomes of bacteriocinogenic strains including *Lactococcus lactis*, *Pediococcus acidilactici*, and *Enterococcus faecalis* (Song et al., 2017; Balandin et al., 2019; Pilevar et al., 2020b; Yoon and Kang, 2020). These natural food biopreservatives kill or prevent the growth of a wide panel of Gram-positive foodborne pathogens, as well as spoilage bacteria (Pilevar et al., 2020b). Several studies have reported that bacteriocins produced by *L. sakei* and *L. curvatus* can diminish the number of *L. monocytogenes* in meat products (De Souza Barbosa et al., 2015; Casaburi et al., 2016; Castellano et al., 2018). It seems that they operate their antimicrobial activities by forming pores on target cell membranes, inhibiting the synthesis of nucleic acids, changing the electrostatic potential of microorganisms, and inhibiting the activity of certain enzymes (Basanta et al., 2009; Sirsat et al., 2009).

The use of effective bacteriocins against meat pathogens has gained attention in the food industry, especially for ready-to-eat (RTE) or fresh-tasting foods because their utilization can decline the application of intense thermal methods and chemical preservatives (Hernández-Aquino et al., 2019). On the other hand, at a daily intake of 2.9 mg/person, they are recognized safe for humans (Cleveland et al., 2001).

One of the bacteriocins is pediocin, which is a heat-stable peptide (Deegan et al., 2006; López et al., 2008) secreted by *Pediococcus* bacteria (Porto et al., 2017; Balandin et al., 2019). It is also able to tolerate low temperatures and retain its activity in a wide range of pH (Porto et al., 2017; Niamah, 2018). Numerous reports have stated that pediocin has an anti-listeria effect and can reduce the population of *L. monocytogenes* in various food products (Loessner et al., 2003; Bari et al., 2005).

Considering the anti-listeria activity of pediocin and pediocin-like bacteriocins on the one hand and the listerial resistance to nisin on the other hand, it seems that pediocin can be applied in meat and meat products alone or in combination with other preservation methods to hinder *L. monocytogenes* growth (Naghmouchi et al., 2006; Maciel et al., 2017; Castro et al., 2018). Therefore, we aim in this study to review the pediocin and pediocin-like bacteriocin, antimicrobial activity of these bacteriocins and their application against *L. monocytogenes* in meat and meat products.

LISTERIA MONOCYTOGENES AND ITS PRESENCE IN MEAT PRODUCTS

Listeria spp. are Gram-positive, facultative anaerobes, oxidase-negative, catalase-positive, and non-spore-forming bacteria (Doijad et al., 2018). *L. monocytogenes* is a member of the

Listeria genus and a food-associated pathogen that is widely distributed in the nature (water, soil, and forage) and a broad range of foodstuffs (Shamloo Aghakhani et al., 2012; Jones and D'Orazio, 2013). Thirteen serotypes of it have been identified, and five of these serotypes (1.2a, 1.2b, 1/2c, 4c, and 4b) are most prevalent in food manufacturing plants or food (Meloni, 2015; Shamloo et al., 2019). Listeriosis is a human infection caused by consumption of contaminated foods with *L. monocytogenes* that can lead to severe symptoms such as spontaneous abortion in pregnant women, meningitis, and septicemia (Kim et al., 2018; Kurpas et al., 2018). Furthermore, it has been reported that foodborne listeriosis could present as a gastrointestinal illness with fever in non-immunocompromised patients (Aureli et al., 2000). It has been demonstrated that the large numbers of microbes ($>10^3$ cfu/g) are needed for the severe form of listeriosis (Churchill et al., 2019). One of the highest mortality rates are attributed to the foodborne disease listeriosis, so *L. monocytogenes* infections are considered as a life-threatening illness for high-risk groups including elderly, immunocompromised, pregnant women, and newborns (Zhang et al., 2021).

Listeria monocytogenes is a potential microbiological risk for raw meat and meat products due to its unusual ability to adapt at cold temperatures, even at 1°C (Conficoni et al., 2016). Additionally, this bacterium can multiply to threatening levels in meat products at any step of the food chain because (a) meat products with pH value above 5 are an appropriate medium for the growth of this organism (Zhu et al., 2005), (b) *L. monocytogenes* can tolerate nitrite and salt up to 12% (Gandhi and Chikindas, 2007; Camargo et al., 2017), (c) modified atmospheres have no impact on its survival (Meloni, 2019), and (d) its capability to form biofilms on food contact surfaces or with other bacteria in meat production establishments increases its resistance to UV light, sanitizers, and bactericide agents (Doijad et al., 2015; Kurpas et al., 2018). The highest adhesion to the surfaces is attributed to serotypes 1/2a, 1/2b, and 4b (Meloni et al., 2014). Therefore, the possibility of cross-contamination and the growth of *L. monocytogenes* are enhanced to an unsafe level in finished products. Many researches have been published about the incidence of *L. monocytogenes* in foods of animal origin around the world. Mohamed et al. (2016) investigated 150 samples of processed meat for the presence of *L. monocytogenes* from October 2013 to September 2014 in Egypt. They found that 4% of minced meat, beef burger, and luncheon samples were infected with *L. monocytogenes*. In a survey conducted by Benhalima et al. (2019) over a 10-year period in China, RTE meats and raw meats were contaminated with *L. monocytogenes* 3.2 and 8.5%, respectively, and meat products from northeastern and central China had the highest occurrence of *L. monocytogenes*. In East Algeria, the prevalence of *L. monocytogenes* in sausage was 33.3% (Liu et al., 2020). In another research in Spain, the incidence of *L. monocytogenes* in RTE meat products between 2012 and 2013 was 17.14% in cooked products, 36.84% in raw-cured products, and 24.32% in dry-cured, salted products (Gómez et al., 2015). Also, D'Ostuni et al. (2016) isolated *L. monocytogenes* from 4.2% of raw pork sausages and 2.4% of entrails lamb rolls examined from 2008 to 2014 in Southern Italy. It appears that bacterial contamination of meat products can occur in processing lines and equipment and during postprocessing phases (slicing and packaging). With regard to the sensitivity of *L. monocytogenes* to the thermal process and its inactivation after cooking (Martín et al., 2014), the major concern is recontamination of processed food with *L. monocytogenes*, which affects their shelf life (Bakhtiary et al., 2016). Thus, from the viewpoint of food safety, the development of appropriate control strategies against this organism is an important issue in food industry.

BACTERIOCIN

As mentioned before, production of safe food free from microorganism especially pathogens needs to considered as one of the most important priorities of the food industry. Due to the achievement to this important aim, various strategies have been examined, among which attention has been drawn to utilizing natural antimicrobial agents such as essential oils and bacteriocins (Carocho et al., 2015; Mokoena, 2017; Yousefi et al., 2020).

Bacteriocins as natural antimicrobial agents are ribosomally synthesized peptides or proteins that are produced by G+ and G– bacteria such as lactic acid bacteria (LAB), *Staphylococcus* strains, *Bacillus* strains, and *E. coli* strains. Without having an adverse effect on the bacteria that produce them, bacteriocins have inhibitory effects on various groups of undesirable microorganisms, and their activities are mostly targeted at the cell wall of microorganisms (Espitia et al., 2016; Gutiérrez-Cortés et al., 2018; Lopetuso et al., 2019; Ng et al., 2020; Benítez-Chao et al., 2021). They are small cationic molecules made up of approximately 30–60 amino acids forming amphiphilic helices showing good stability at 100°C for 10 min. They are different in terms of genetic origin, biochemical properties, molecular weight (MW), and activity spectrum (Parada et al., 2007; Mokoena, 2017; Sidooski et al., 2019). The bacteria that are taxonomically close may be inhibited by narrow-spectrum bacteriocins, while a wide variety of bacteria are inhibited by broad-spectrum bacteriocins (Cotter et al., 2005; O'Connor et al., 2020). Based on the producer organism, MW, chemical structure, existence of modified amino acids, and thermal stability, the bacteriocins can be classified into four groups (Class I, II, III, and IV) (López-Cuellar et al., 2016; Johnson et al., 2018; Kumariya et al., 2019; Cui et al., 2021). The main classes and sub-classes of bacteriocin and their characteristics are shown in **Figure 1**. Nisin and pediocin are the most studied bacteriocin that could be utilized commercially as natural preservatives (Acuña et al., 2011). Nisin is currently the only bacteriocin that can be used as an authorized additive. However, pediocin as a food ingredient produced by *P. acidilactici*, a pediocin-producing strain, can be commercially exploited for food preservation, and its application is covered by several US and European patents (El-Ghaish et al., 2011; Espitia et al., 2016).

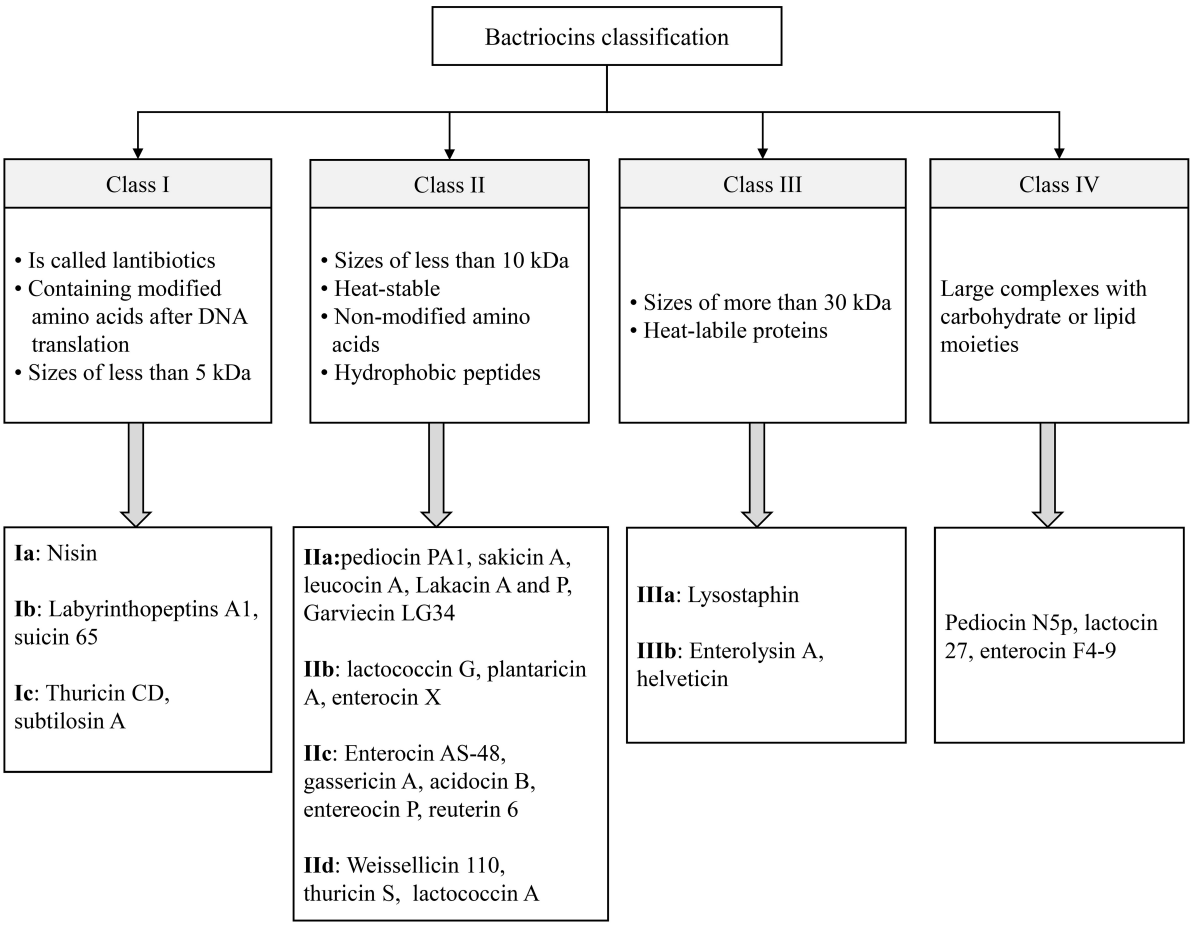

FIGURE 1 | The main classes and sub-classes of bacteriocins.

PEDIOCIN AS NATURAL ANTIMICROBIAL AGENT

Pediocin belongs to subclass IIa and is produced from *Pediococcus* spp. such as *P. acidilactici*, *P. claussenii*, *P. cellicola*, *P. damnosus*, *P. ethanolidurans*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*, and *P. stilesii* (Haakensen et al., 2009; Porto et al., 2017). The bacteriocins generated from these species are generally called pediocin. However, based on the isomer and producing strain, the symbols have been added with the pediocin word such as pediocin AcH, pediocin SJ-, pediocin JD, and pediocin PA-1 (Bhunia et al., 1991; Christensen and Hutkins, 1994; Rodríguez et al., 2002; Niamah, 2018).

Pediocins as natural bacteriocins are the biomolecules that exert a broad spectrum of antimicrobial activity against Gram-positive bacteria, especially against pathogenic bacteria, such as *L. monocytogenes*. Due to its activity against *L. monocytogenes*, it could be applied in various food products to control the growth of this food-borne pathogen that is a concern in the food industry (Mokoena, 2017; Porto et al., 2017). Pediocin can be utilized in the food industry through two approaches, either through the *in situ* method by adding *Pediococcus*, *Enterococcus*, or *Lactobacillus* strains to the food matrix to produce pediocin under controlled conditions to prevent the growth of pathogens in food, or direct use of the optimal concentration of pediocin to the food matrix (Silva et al., 2018; Ng et al., 2020).

PROPERTIES AND STRUCTURAL PROPERTIES OF PEDIOCIN

As mentioned, Class II of bacteriocins is described as the compounds with sizes of less than 10 kDa, heat-stable, non-modified, and hydrophobic peptides. Among this, Class IIa (pediocin-like or *Listeria*-active) has shown to exert high specific activity against the food pathogen *L. monocytogenes* (Espitia et al., 2016).

Pediocins are characterized as small unmodified peptides that have a MW of lower than 5 kDa (Papagianni and Anastasiadou, 2009). Pediocin peptides are composed of 40–44 amino acids of both aliphatic and aromatic amino acid with no posttranslational modification. The amino acid similarity in the sequence of pediocin-like bacteriocins is about 40–60%, and this sequence of amino acid presents a conserved N-terminal hydrophobic region

in the YGNGV motif and a variable C-terminal hydrophobic or amphiphilic region (Papagianni, 2003; Porto et al., 2017; Niamah, 2018). A significant reduction in anti-listeria activity occurred by the substitution of a single amino acid residue (Sun et al., 2015).

Pediocin is principally present in unstructured conformations as random coils in watery solutions, while, in non-aqueous solutions, it makes a partly helical structure with different amounts of hydrophobicity (Ennahar et al., 2000). The structure of pediocin basically consisted of two regions: a hydrophilic cationic region (N-terminal) and a hydrophobic/amphiphilic region (C-terminal) (Johnsen et al., 2005).

The N-terminal region shows the three-stranded antiparallel β-sheet supported by a disulfide bridge consisting of two cysteine residues (C9 and C14) (Drider et al., 2006; Porto et al., 2017). At the end of the structure, the hairpin domain is created by a C-terminal tail with two cysteine residues that fold back onto the central α-helix by a disulfide bridge. Furthermore, a flexible hinge that presents among the N-terminal region and the hairpin domain in the C-terminal region makes two of these regions to move relative to each other (Espitia et al., 2016).

The known pediocins produced from *P. acidilactici*, *P. damnosus*, and *P. pentosaceus* strains are heat-resistant small-structure hydrophobic peptides. Their bactericidal activities can be maintained during heat treatment, sometimes even at sterilization temperatures. Furthermore, pediocins are able to tolerate low temperatures even at −80°C (Anastasiadou et al., 2008b; Papagianni and Anastasiadou, 2009; Porto et al., 2017; Niamah, 2018; Ghosh et al., 2019). They also maintain their activity after treatments with lipase, lysozyme, phospholipase C, DNase, or RNase, while they are sensitive to most protease enzymes such as papain, pepsin, and trypsin (Wu et al., 2004; Anastasiadou et al., 2008a,b; Papagianni and Anastasiadou, 2009; Espitia et al., 2016; Niamah, 2018). Pediocin activity is retained in a wide range of pH. The isoelectric point of pediocin is 8.6–10, and it has a positive charge between (+3) and (+7) in pH 6 (Venema et al., 1997; Porto et al., 2017; Niamah, 2018). It has been reported by Papagianni and Anastasiadou (2009) that most isoforms of pediocin are thermally stable and remain active in a wide range of pH (2–10). It has been indicated that the main difference between the pediocin isoforms is related to their sensitivity against protease enzymes such as chymotrypsin, papain, pepsin, pronase E, proteinase K, and trypsin (Porto et al., 2017).

The effect of various treatments on antimicrobial activity of pediocin isoform is summarized in **Supplementary Table 1**.

ANTIMICROBIAL ACTIVITY OF PEDIOCIN AND MODE OF ACTION

Antimicrobial activities of bacteriocins are carried out through various mechanisms such as destruction of cell wall and membrane integrity, interference with cell wall formation, inhibition synthesis of protein, and inhibition of gene expression. These bactericidal mechanisms depend on the class of bacteriocins and indicator bacteria (Balciunas et al., 2013; Cavera et al., 2015; Porto et al., 2017; Cui et al., 2021).

Pediocin, like other Class II bacteriocins, participates in creating partial or total imbalance of transmembrane proton distribution in sensitive cells (Bruno and Montville, 1993; Cleveland et al., 2001; Papagianni, 2003). In fact, the cytoplasmic membrane of bacteria is the pediocin's target. All pediocin variants are known for their inhibitory activities against Gram-positive bacteria, especially *L. monocytogenes*. However, it has been indicated that pediocin S and L from *P. pentosaceus* S and L were also effective against Gram-negative microorganisms (Yin et al., 2003). The antimicrobial activity of pediocin depends on its structure and is carried out by formation of pores in the target membrane. These pores lead to leakage of ions and other cell compounds, release of cytoplasmic adenosine triphosphate (ATP), and inhibition of proton motive force (PMF) for energy production, and finally, when the leakages are more than the limit, cell death occurs (Montville and Chen, 1998). An initial connection between the target bacteria and pediocin PA-1 occurs before a pore is made in the bacterial membrane. This initial connection is principally occurred through electrostatic interactions created by the cationic and anti-parallel region of the β-sheet in the N-terminal region of pediocin with the lipoteichoic acid, the main component on the surface of Gram-positive bacteria (Espitia et al., 2016). The native divalent cations from cell surface is removed by this electrostatic interactions and makes the outer membrane unstable. Therefore, the entrance of the peptide and further peptide contact with the cytoplasmic membrane is facilitated (Powers and Hancock, 2003; Espitia et al., 2016). It has been indicated by Nissen-Meyer and Nes (1997) that the lipid composition of the target cell membrane is probably a substantial factor in the sensitivity of the bacteria to the pediocin PA-1 and other bacteriocins. It has also been demonstrated by Chen et al. (1997) that the affinity between phospholipid vesicles and pediocin PA-1 was enhanced by the presence of anionic lipids. After contacting and distribution of pediocin on the surface of the bacterial cytoplasmic membrane, the pores are formed by insertion of the hydrophobic, C-terminal, hairpin-like domain into the membrane (Johnsen et al., 2005; Drider et al., 2006). Furthermore, structural flexibility that is provided by the hinge makes the C-terminal, hairpin domain penetrate the hydrophobic part of the membrane (Espitia et al., 2016). It has been indicated that salts and amino acid cellular efflux were affected by pediocin PA-1 and the transmembrane electrical potential is also dissipated by this bacteriocin (Chikindas et al., 1993). Furthermore, it has been demonstrated by Bauer et al. (2005) that K+ loss occurred as a consequence of purified PD-1 pediocins and formation of pores on the cytoplasmic membrane in *Oenococcus oeni* cells. They also found that K+ loss is dependent on pH, and when reduced from 7 to 5, membrane disruption and K+ loss increased. Additionally, it has been stated that beside pore formation and cell membrane dysfunction, pediocin molecules enter the cell and create complexes between pediocin and cell components such as DNA, proteins, and enzymes (Montville and Chen, 1998). The antimicrobial activity of pediocin and formation of pores in the cytoplasmic membrane of bacterial cell are illustrated in **Figure 2**.

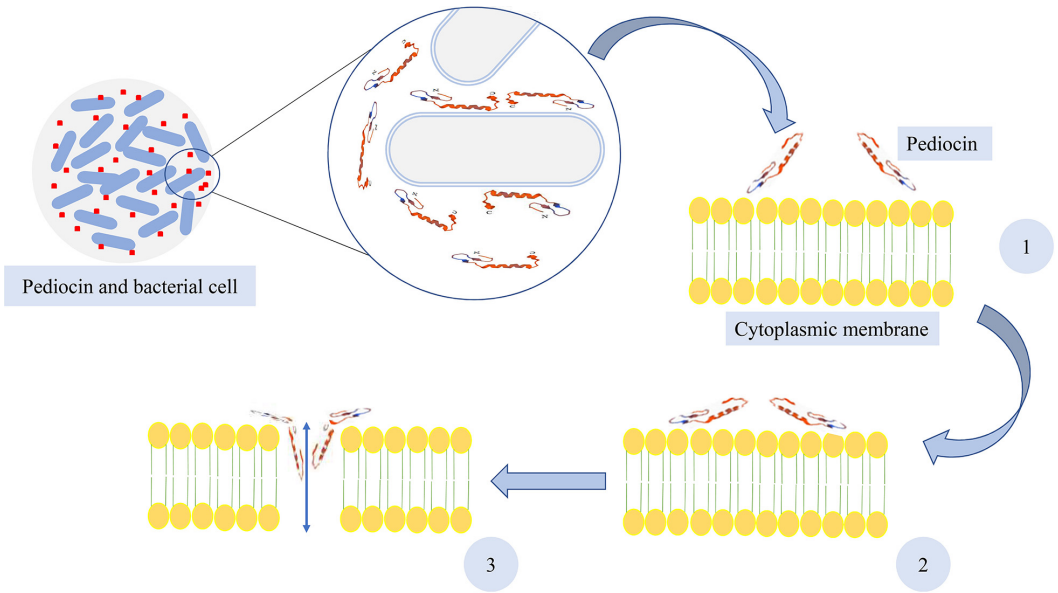

FIGURE 2 | Pore formation of pediocin in bacterial cell membrane. (1) Adsorption of pediocin, (2) distribution and interaction of pediocin on the surface of the cytoplasmic membrane, and (3) pore formation in the membrane.

ANTI-LISTERIA ACTIVITY OF PEDIOCIN AND PEDIOCIN-PRODUCING BACTERIA IN MEAT PRODUCTS

Generally, bacteriocins such as pediocins can be applied in food preservation through two methods: (a) inoculation of the bacteriocin-producing strain into the food matrix under desirable conditions to produce antimicrobial peptide *in situ* and (b) direct addition of bacteriocins to the food matrix (Perez Espitia et al., 2012). The first strategy and the inoculation of pediocin-producing strains into the food matrix can be considered as an effective alternative method to preserve meat products, due to pediocin's ability to inhibit *L. monocytogenes*, *Clostridium perfringens*, and *Clostridium botulinum* (Okereke and Montville, 1991; Foegeding et al., 1992; Nieto-Lozano et al., 2006, 2010; Bagenda et al., 2008). However, it is necessary to be careful in selecting pediocin-producing strains based on the type of food in order to produce pediocin in adequate amount. In another method, commercially available pediocin that is produced at a laboratory or industrial scale is directly added into food products (Espitia et al., 2016). However, some limitations such as degradation by proteolytic enzymes, adsorption to food components, and variation in the solubility of pediocin should be taken into consideration when it is directly utilized in food products (Coma, 2008). In this section, the anti-listeria activities of direct addition of pediocin as well as pediocin-producing bacteria in the meat and meat product are reviewed.

In a study that is carried out by Nielsen et al. (1990), the use of bacteriocin produced by *P. acidilactici* was evaluated to inhibit *L. monocytogenes* in fresh meat. They figured out that the pediocin-treated meat showed 1–2.5 log cycles fewer in attachment of the *L. monocytogenes* than the control. Furthermore, their results revealed that after 2-min exposure with pediocin, a decrease of 0.5–2.2 log cfu/ml occurred in the population of attached bacteria depending on bacteriocin concentration. They also reported that the tested bacteriocin was able to exert an inhibitory effect against *L. monocytogenes* after 28 days at 5°C, and its residual activity was detected on the meat surface for at least 28 days at refrigerated storage.

It has been demonstrated by Berry et al. (1991) that the ability of bacteriocin-producing *P. acidilactici* JD1–23 to control *L. monocytogenes* contamination of frankfurters was dependent on the concentration of *Pediococcus*, the atmosphere, and the temperature of packaging. They reported that coinoculation of high levels (10^7 cfu/g) of *P. acidilactici* JD1–23 and *L. monocytogenes* in frankfurters under vacuum packaging at 4°C inhibited the growth of pathogen up to 60 days. Similarly, Motlagh et al. (1992) studied the effect of pediocin AcH in controlling tree strains of *Listeria* in various food products and found that the bacteriocin action was dependent on concentration and strain. They reported a decrease of 1, 3, and 7 log cfu/g occurred in the population of *L. monocytogenes* ScottA, *L. monocytogenes* Ohio2, and *L. ivanovii* ATCC 19119, respectively, as a consequence of using 1,350 AU/ml of pediocin. They also indicated that this activity was immediate and independent of food types.

The inhibition of *L. monocytogenes* using the pediocin-producing strain *P. acidilactici* JD1–23 as starter culture during the manufacture of fermented semidry sausage was studied by Berry et al. (1990). They found that a 2 log cfu/g reduction of *L. monocytogenes* occurred during fermentation, while less than 1 log cfu/g decrease was observed in the *L. monocytogenes* population in the sausage fermented with a non-inhibitory *Pediococcus* strain. They also stated that

L. monocytogenes was also inhibited in the sausage with pH more than 5.5, indicating that bacteriocin is produced independently of carbohydrate fermentation.

Furthermore, *in situ* production of pediocin using *P. acidilactici* during dry fermentation of sausage in order to control *L. monocytogenes* was studied by Foegeding et al. (1992). They understood that the *L. monocytogenes* population was reduced over dry sausage fermentation process, and an effective inactivation of *L. monocytogenes* was obtained when an adequate pH drop (below 4.9) occurred at the end of the fermentation process. Furthermore, they indicated that when pH was not lowered sufficiently during the fermentation, bacteriocin production also facilitated the reduction of any remaining *L. monocytogenes*. They also recommended that it is useful to apply bacteriocin-producing starter cultures to enhance control of *L. monocytogenes* in meat fermentations.

The effect of *P. acidilactici* H or Pediocin AcH on the behavior of *L. monocytogenes* strains in wiener sausage exudates was evaluated by Yousef et al. (1991). They recognized that both methods were effective in decreasing the growth of pathogens during storage of wiener sausage exudates at 4 or 25°C. They also mention that, although rapid initial reduction occurred in a numbers of pathogens, using the producing strain resulted in the lower final levels. This reduction in counts might be due to the production of pediocin AcH during late logarithmic growth.

Similarly, the anti-listeria ability of *P. acidilactici* JBL1095 (pediocin AcH producer) and a non-bacteriocin producer (*P. acidilactici* LB42) in the vacuum-packaged wieners was evaluated by Degnan et al. (1992). The results showed that there were no significant changes in the *L. monocytogenes* or *P. acidilactici* population in the treated and untreated samples during 72 days of storage at 4°C. On the other hand, during 8 days of storage at 25°C, a slight (0.33 log10 cfu/g) and significant (2.7 log cfu/g) decrease in the counts of *L. monocytogenes* occurred in packages containing strain LB42 and JBL1095 held at 25°C, respectively. They stated that the bacteriocin producer was not able to grow or produce the bacteriocin at 4°C, while a remarkable anti-listeria effect was observed at 25°C due to production of bacteriocin.

Additionally, genomic analysis and anti-listeria activity of three pediocin-producing (Ped+) and two non-pediocin-producing (Ped–) strains of *P. acidilactici* in the preparation of turkey summer sausage were studied by Luchansky et al. (1992). The results showed that equivalent amounts of acid were produced by all the starter culture acid during fermentation. However, the present of Ped+ starter culture resulted in greater reduction of *L. monocytogenes* (3.4 log cfu/g) in comparison to the samples containing Ped- starter culture (0.9 log cfu/g) and remarkable pediocin activity was determined from sausages prepared with the Ped+ strain during at least 60 days storage at 4°C. They claimed that all the commercially available starter cultures are not equal in encoding pediocins or as effective as each other in controlling *L. monocytogenes*. Therefore, selection of an effective strain should be taken into consideration.

The anti-listeria activity of pediocin (3,000 AU/ml) in the slurries of beef muscle tissue and beef tallow that were contaminated with 2.5×10^5 cfu/ml of two *L. monocytogenes* strains was studied by Degnan et al. (1993). The greatest reduction in *Listeria* counts was carried out within 1.5 min of pediocin addition, and after that, the population of *Listeria* did not change; however, the activity of pediocin continued to decrease in the treatment for up to 60 min. They noted that this reduction might be driven by the cumulative effects of proteolysis and association with both protein and lipid. They also indicated that encapsulation of pediocin within phosphatidyl-choline-based liposomes before addition to the slurries resulted in higher activity of pediocin in comparison to free pediocin (Degnan et al., 1993). Furthermore, it has been claimed that cold storage of *L. monocytogenes*-contaminated ground pork (10^3 cfu/g) in the presence of pediocin (8,192 AU/g) led to a 2 log cfu/ml decrease in the population of *L. monocytogenes* in comparison to the sample free from pediocin, regardless of whether the samples were stored in air, vacuum, or modified atmosphere (Khojasteh and Murano, 1996).

The anti-listeria activity of pediocin AcH bound to heat-killed *P. acidilactici* cells in irradiation-sterilized raw chicken breast meat that was contaminated with *L. monocytogenes* Scott was studied by Goff et al. (1996). They figured out that pediocin-treated samples showed anti-listeria activity both before and after cooking. They claimed that this might be useful in protecting the consumers from bacterial post-processing recontaminations and/or undercooking. Similarly, Mattila et al. (2003) bound pediocin AcH to heat-killed producer cells of *Lactobacillus plantarum* WHE 92 by adjusting the pH of the medium to 6.0, and the preparation was added on the sliced cooked sausage that inoculated with *L. monocytogenes* ATCC 7644 (2.7 log cfu/g). Their results showed that there were no significant differences among the treated and control samples in terms of pH value, flavor, and growth of LAB. However, in the pediocin-treated sample, *L. monocytogenes* population was decreased to <2 log cfu/g after 6 days of storage and no more reduction occurred through remaining storage time, while in the control sausage, the counts of *L. monocytogenes* remained at the inoculated level. They indicated that due to the presence of *Listeria* in the treated sample at the end of storage, pediocin was not efficient enough to kill all *L. monocytogenes*. Therefore, it seems that this bacteriocin should be applied in hurdle technology to improve the efficiency (Mattila et al., 2003).

Furthermore, Chen et al. (2004c), investigated the ability of pediocin (ALTA 2341) in controlling *L. monocytogenes* on frankfurters. In this regard, the surface of frankfurters was inoculated with a five-strain mixture of *L. monocytogenes* (3.40 or 5.20 log cfu/g) and treated with 3,000 or 6,000 AU of pediocin per link. The treated samples were vacuum-packaged (as 1, 5, or 10 links per package) and stored at different temperatures (4, 10, and 25°C) for 12 weeks. The results revealed that the extent of *Listeria* inhibition was highly temperature-dependent and the most effective reduction occurred in the pediocin-treated frankfurters. They stated that the frankfurters treated with 6,000 AU of pediocin and stored at 4°C prevented the growth of *L. monocytogenes* for at least 7 weeks and reduced the growth of this pathogen for up to 12 weeks. They claimed that in order to achieve sufficient inhibition of *Listeria*, pediocin should

be applied in combination with well-controlled temperature or other complementary inhibitory methods (Chen et al., 2004c).

Nieto-Lozano et al. (2006) studied the anti-listeria effect of bacteriocin, produced by *P. acidilactici* on Spanish raw meat. The pediocin-treated meat samples (500, 1,000, or 5,000 bacteriocin U/ml) were contaminated with *L. monocytogenes* and stored at 15°C for 72 h or at 4°C for 21 days. They figured out that application of 500, 1,000, or 5,000 pediocin U/ml (BU/ml) led to a 1, 2, or 3 log cfu/g decrease in the population of *L. monocytogenes* after 72 h storage at 15°C, respectively, indicating that this reduction was dependent on the concentration of pediocin. They also reported that the treatment with 1,000 or 5,000 BU/ml decreased the counts of *L. monocytogenes* by 2.5 and 3.5 cfu/ml after 21 days storage at 4°C, respectively, compared to the control (Nieto-Lozano et al., 2006). Similarly, Nielsen et al. (1990) demonstrated that reduction in *L. monocytogenes* population was dependent on the inoculum of *L. monocytogenes* and the pediocin PA-1 concentration.

In the other study, which was carried out by Nieto-Lozano et al. (2010), anti-listeria activities of pediocin PA-1 and the *P. acidilactici* MCH14 pediocin-producing strain were investigated in frankfurters and Spanish dry-fermented sausages. Their results showed a 2 log cycles decrease in treated Spanish dry-fermented sausages in comparison to the control sample after 30 days storage (Nieto-Lozano et al., 2010). It has been suggested that the bactericidal effect of the pediocin-producing strain of *P. acidilactici* was due to bacterial lysis (Heo et al., 2007). It has also been reported that application of 5,000 BU/ml pediocin PA-1 led to a 2 and 0.6 log cycle decrease in *Listeria* counts in the frankfurters stored at 4°C for 60 days and at 15°C for 30 days, respectively, when compared to the control samples (Nieto-Lozano et al., 2010). Their results also revealed the importance of storage temperature in the effectiveness of bacteriocin such as pediocin as stated in previous investigations (Degnan and Luchansky, 1992; Nieto-Lozano et al., 2006). Therefore, due to the greater inhibitory effect of pediocin PA-1, this bacteriocin and related pediocin-producing strain could be applied in refrigerated products (Nieto-Lozano et al., 2010).

Furthermore, the ability of bacteriocin-producing *P. acidilactici* 13 and its antimicrobial substance against *L. monocytogenes* during ripening of dry fermented sausage (sucuk) and storage of sliced turkey breast was investigated by Cosansu et al. (2010). *P. acidilactici* 13 was isolated from naturally fermented sucuk, and its application as a starter culture for sucuk production resulted in a 3.32 log cfu/g decrease in population of the pathogen during 8 days of storage. On the other hand, in the control sample, 1.37 log cfu/g occurred, while a purified antimicrobial substance (6,400 AU/ml) produced by *P. acidilactici* 13 led to an instant reduction of *L. monocytogenes* (1.03 log cfu/cm^{-2}) in turkey breast slices stored at 12°C for 10 days. However, application of partially purified antimicrobial substance was not able to inhibit growth of *L. monocytogenes* for 10 days of storage at 12°C. Therefore, they proposed that this partially purified substance of *P. acidilactici* 13 could be utilized in combination with other preventative strategies against *L. monocytogenes* in meat products that do not undergo a fermentation step (Cosansu et al., 2010).

Kingcha et al. (2012) found that inoculation of *P. pentosaceus* BCC 3772 in Nham, a Thai traditional fermented pork sausage, resulted in a significant reduction in the growth of *L. monocytogenes* ATCC 19115 over 18–24 h of fermentation without any significant changes in sensory properties of the final fermented Nham products. They also observed a correlation among *P. pentosaceus* BCC3772 inoculum and anti-listeria effect, indicating the importance of pediocin concentration in the prevention the growth of *Listeria*. It seems that the differences in anti-listeria activity of various concentrations of pediocin or the loss of antimicrobial activity of pediocin, especially during long times, could be attributed to the binding of the pediocin to the food matrix and proteolytic degradation by enzyme. In this regard, Kingcha et al. (2012) pointed out that inoculation of 10^4 cfu/g *P. pentosaceus* BCC 3772 in Nham led to a rapid reduction of *L. monocytogenes* counts to <2 log cfu/g over 18–24 h of fermentation. However, a slight increase in the population of *L. monocytogenes* was observed after 36 h of fermentation, indicating the possible loss of pediocin.

Furthermore, Kiran and Osmanagaoglu (2014) studied the anti-listeria activity of purified pediocin AcH/PA-1, produced by *P. pentosaceus* OZF in chicken meat products that were radiated and inoculated with 10^5 cfu/g of *L. monocytogenes*. They reported that there was significant reduction in the *Listeria* counts (3.8 log cfu/g) in the pediocin-treated sample in comparison to control after 14 days of storage at 4°C. On the other hand, re-growth of *L. monocytogenes* was observed after more storage time (3–4 weeks), indicating the possible degradation of the tested pediocin by proteases derived from raw meat products or its interaction with different compounds of meat. They suggested that more effective protection of bacteriocin such as pediocin can be achieved through multi-hurdle preservation methods (Kiran and Osmanagaoglu, 2014). **Figure 3** shows the factors that affect the efficacy of anti-listeria activity of pediocin in meat and meat products.

As mentioned before, various studies have been using pediocin, the bacteriocin-like inhibitory substance of *Pediococcus* strain, and pediocin-producing bacteria in various meat products and explored the anti-listeria activity of these components (Mattila et al., 2003; Cosansu et al., 2010; Nieto-Lozano et al., 2010; De Azevedo et al., 2020). However, it has been noted that due to the sensitivity of these compounds to proteolytic degradation, binding to food compounds and variation in the solubility of this bacteriocin have limited the direct application of pediocin, especially in low concentration or during long-term storage at improper temperatures. Therefore, in addition to these applications, pediocin has shown potential for use as a part of hurdle technology along with other preventative strategies or as part of antimicrobial agents incorporated into packaging materials.

As aforementioned, Chen et al. (2004c) investigated the ability of pediocin (ALTA 2341) in controlling *L. monocytogenes* on frankfurters and presented that in order to achieve sufficient inhibition of *Listeria*, pediocin should be applied in combination with well-controlled temperature or other complementary inhibitory methods (Chen et al., 2004c). Therefore, in the next investigation carried out with same authors, irradiation was

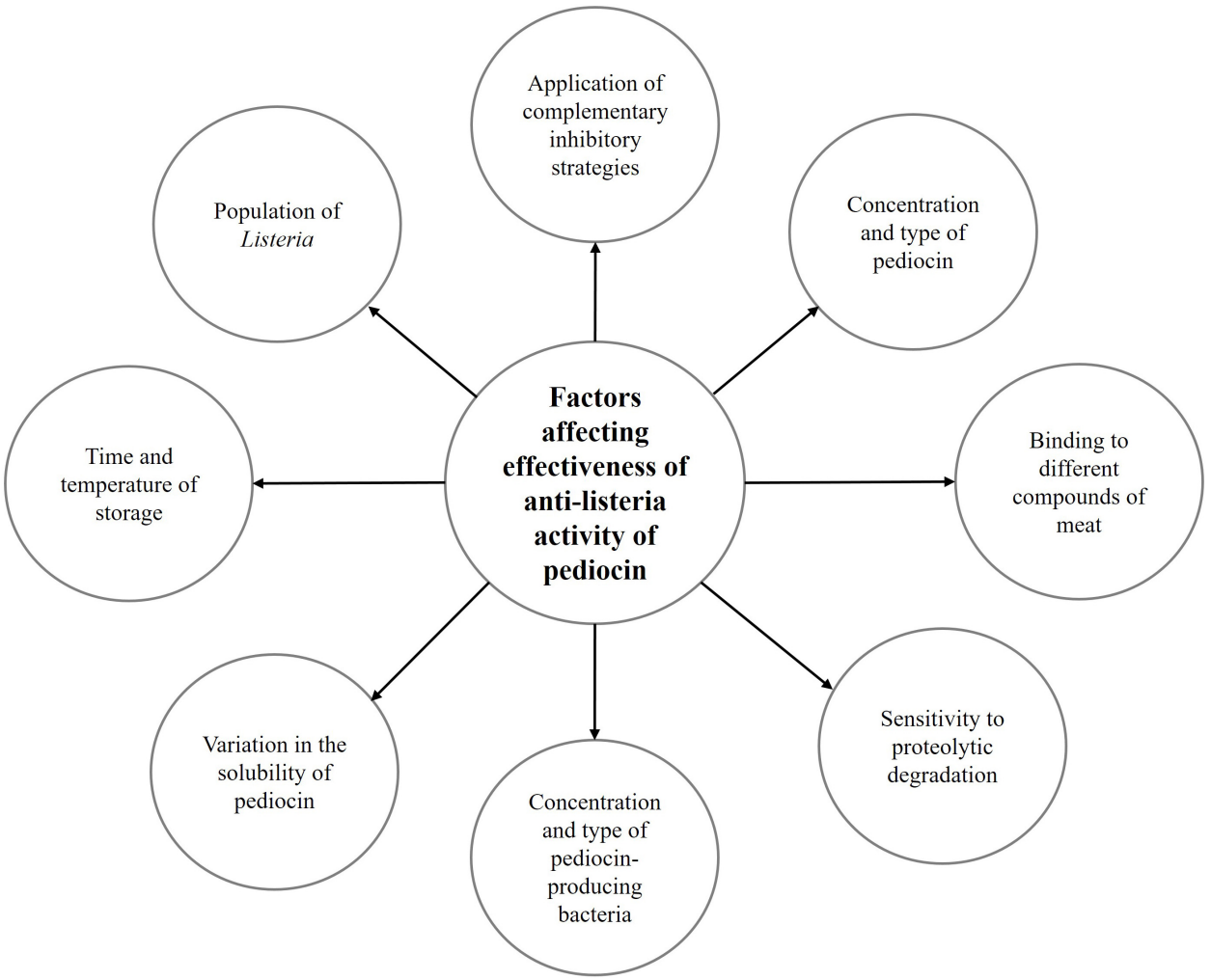

FIGURE 3 | Factors affecting the effectiveness of pediocin in inhibition of *Listeria* in meat and meat products.

applied in combination with pediocin (ALTA 2341) to control the growth of *L. monocytogenes* on frankfurters. Similar to a previous study, the artificially contaminated frankfurters were treated with 3,000 or 6,000 AU of pediocin per link and vacuum packaged as 1, 5, or 10 links per package. After packaging, 1.2 or 2.3 kGy irradiation dose was applied for 1-link and 5-link packages, while the 10-link packages were irradiated at 1.4 or 3.5 kGy. The treated and untreated samples were stored at 4, 10, and 25°C for 12 weeks. The results showed that in order to reach a 50% reduction of *L. monocytogenes* on frankfurters in 1-link or 5-link packages, pediocin should be applied with postpackaging irradiation at 1.2 kGy or more. They demonstrated that application of 6,000 AU pediocin in combination with 2.3 kGy or higher irradiation dose was effective in preventing the pathogen in all package sizes stored at 4 and 10°C for 12 weeks. They claimed that application of pediocin did not have an adverse effect on the sensory properties of frankfurters, and these synergistic effects between pediocin and irradiation in combination with cold storage (4°C) led to little or no growth of the *L. monocytogenes* in the 1-link or 5-link packages during 12 weeks of storage (Chen et al., 2004b). In another similar study performed by the same authors, instead of irradiation, postpackaging thermal pasteurization was exerted in combination with pediocin (ALTA 2341) to inhibit *L. monocytogenes* on frankfurters. For this purpose, the vacuum-packed frankfurters were heated in hot water at 71, 81, and 96°C for 30, 60, and 120 s, respectively. They figured out that the anti-listeria effect of thermal pasteurization was dependent on package size, and the most and least effective heat treatment for pediocin-treated samples were observed in 1-link and 10-link packages, respectively. Their results showed that a 50% reduction of initial inoculations was obtained in the treatment with 6,000 AU/g pediocin utilized in combination with heat treatment of 81°C or more for at least 1 min. They also indicated that little or no growth of *L. monocytogenes* was observed on the surface of frankfurters stored at 4 or 10°C for 12 weeks and at 25°C for 12 days. They concluded that application of pediocin along with postpackaging thermal treatment can be considered as an efficient

treatment to enhance control of *L. monocytogenes* on frankfurters (Chen et al., 2004a). Furthermore, the combined effect of food additives and pediocin in inhibition of *L. monocytogenes* has shown that the anti-listeria activity of food additives increased by combination use with pediocin. For example, the anti-listeria effects of sodium diacetate in combination with pediocin (5,000 AU/ml) in turkey slurries were studied by Schlyter et al. (1993). Their results revealed a listericidal effect (ca. 7 log cfu/ml) in treatments containing pediocin with 0.5% diacetate at 25°C and pediocin with 0.3% diacetate at 4°C. They stated that the increased anti-listeria activity of diacetate in combination with pediocin was due to synergistic effects. They concluded that it is recommended to utilize multiple barriers such as diacetate in combination with pediocin for increased control against *L. monocytogenes* in turkey. Additionally, Maks et al. (2010) studied the interaction effect of temperature (56.3–60°C) and pediocin (0–10,000 AU) on the thermal inactivation of *L. monocytogenes* on bologna. They figured out that by increasing the concentration of pediocin from 0 to 5,000 AU, *D*-values decreased, while a further increase (to 7,500 and 10,000 AU) has a protective effect on thermal inactivation. They also mentioned that application of 10,000 AU pediocin in combination with sodium lactate or sodium diacetate at 56.3 and 60°C, respectively, led to a slight increase in predicted *D*-values. Their results revealed that the interaction effects between additives could be different at various temperatures/concentrations and therefore food manufacturers should carefully modify food formulations and evaluate them with sufficient tests to ensure that the safety of the product is not compromised. Similarly, Grosulescu et al. (2011) developed a predictive model in order to interpret the effect and interaction of sodium diacetate (0–2.5%), sodium lactate (0–4.8%), and pediocin (0–10,000) on thermal resistance (56.3–60°C) of starved *L. monocytogenes* on bologna. They found that addition of pediocin (up to 5,000 AU) by increasing temperature and sodium diacetate decreased *D*-values. On the other hand, a slight increase in the *D*-value occurred when pediocin was utilized in concentrations of 7,500 or 10,000 AU (Grosulescu et al., 2011). In accordance with these studies, Gupta (2005) found that the addition of pediocin up to a concentration of 7,500 AU decreased thermal resistance of *L. monocytogenes*, whereas further addition of pediocin (10,000 AU) led to a slight increase in heat resistance of *L. monocytogenes*.

In the other hurdle technology, mild high hydrostatic pressure (HHP) (300 MPa, 10°C, 5 min) and *P. acidilactici* HA-6111-2 or its bacteriocin, pediocin PA-1 (1,280 AU/g), was utilized as a potential hurdle technology to control *L. monocytogenes* in Portuguese traditional fermented meat sausages. They understood that *L. monocytogenes* was undetectable at 14 and 21 days of refrigerated storage in the samples treated only with PA-1 or *P. acidilactici* HA-6112, respectively. However, application of pediocin PA-1 or *P. acidilactici* HA-6112 along with HHP led to elimination of the pathogen immediately or 72 h after HHP, indicating the point that there is synergistic effect among pediocin and HHP (Maciel et al., 2017). Similarly, Castro et al. (2018) demonstrated that application of pediocin bacHA-6111-2 (*in situ* and *ex situ*) in combination with HHP was able to effectively control *L. innocua* in fermented meat products.

Furthermore, pediocin can be incorporated to food packaging materials to achieve a possible alternative approach to control *L. monocytogenes* in meats and poultry products. Ming et al. (1997) studied the anti-listeria effect of pediocin addition (at 7.75 μg/cm) into packaging material on turkey breast, ham, and beef. They found that *L. monocytogenes* growth was completely prevented in the samples coated with plastic packaging bags containing pediocin powder during 12 weeks of storage at 4°C. In addition, Woraprayote et al. (2013) observed that *L. monocytogenes* was decreased by 2 log cfu/g in the raw sliced pork that was packed with poly(lactic acid)/sawdust particle biocomposite film incorporated with pediocin PA-1/AcH. Similarly, Santiago-Silva et al. (2009) reported a 2 log cfu/ml reduction on the sliced ham coated with cellulose-based film impregnated with 50% pediocin during 15 days of storage at 12°C. Likewise, it has been observed that application of commercially available pediocin (ALTA^{TM} 2341) in the cellulosic film-forming solution at concentrations of 50% (w/w) resulted in a 1.2 log cfu/g reduction in *L. monocytogenes* population on sliced bologna and hindered biofilm formation on packaging and bologna surfaces (Espitia et al., 2013). Therefore, it seems that pediocin could be a packaging material, as part of the hurdle technology system for the barrier of *L. monocytogenes* in meat and meat products.

As mentioned above, various studies have demonstrated the anti-listeria activity of pediocin in meat and meat products, and this activity is driven by the destruction of bacterial membranes. In fact, the presence of cysteine residues in the structure of the bacteriocin molecule often shows the characteristics of an amphipathic helix, which allows the bacteriocin molecules to begin rearranging the membrane and create pores in the membrane (Nissen-Meyer et al., 1992; Miller et al., 1998; Montville and Chen, 1998). This process is part of membrane lysis, dissipation of PMF and prevention of energy production, inhibition of glucose uptake, and release of cytoplasmic ATP (Montville and Bruno, 1994; Montville and Chen, 1998). It has been indicated that dissipation of PMF and efflux of inorganic phosphate were dependent on the concentration of pediocin and time (Chen and Montville, 1995). As observed in different studies, the effectiveness of the anti-listeria activity of pediocin was affected by various factors such as type of pediocin, type of products, the population of *Listeria*, and initial contamination, time and temperature of storage, and application of complementary inhibitory strategies (Nielsen et al., 1990; Degnan et al., 1992; Luchansky et al., 1992; Chen et al., 2004c; Nieto-Lozano et al., 2006, 2010; Grosulescu et al., 2011; Kingcha et al., 2012; Castro et al., 2018). Furthermore, inactivation of pediocin by protease enzymes, limited diffusion in a solid matrix, binding to food components, and limited effect on Gram-negative bacteria should be considered as the most important challenges regarding the use of pediocin in meat and meat products (Schved et al., 1994; Murray and Richard, 1997). Therefore, most of the studies indicated that pediocin should be used in combination with other inhibitory supplementation methods, especially in the multi-hurdle technology to achieve the highest anti-listeria activity.

CONCLUSION

Class IIa bacteriocins are small and cationic proteins with anti-listeria activity. Among these, pediocin presents a conserved N-terminal hydrophobic region in the YGNGV motif and a variable C-terminal hydrophobic or amphiphilic region. Pediocin and pediocin-like bacteriocins show various important technological features such as thermostability and retaining activity at a wide range of pH, which, when accompanied by antibacterial activity against Gram-positive food spoilage and pathogenic bacteria, make them a main class of biopreservatives. This study has revealed that pediocin has promising antimicrobial activity and could be potentially utilized as a natural anti-listeria agent in meat and meat products. The cytoplasmic membrane of bacteria is the target of pediocin, and after contact of pediocin on the surface of the bacterial cytoplasmic membrane, it forms the pores by insertion of the hydrophobic, C-terminal, hairpin-like domain into the membrane. Formation of pores leads to efflux of ions and other cell compounds, release of cytoplasmic ATP, prevention of PMF for energy production, and finally death of cells. Purified pediocin, pediocin in killed cells, and inoculation with pediocin-producing starter cultures are the methods utilized to add this bacteriocin to meat and meat products. Due to the sensitivity of pediocin to proteolytic degradation as well as its binding to food compounds, direct application of pediocin is associated with limitations, particularly in low concentration or during long-term storage at improper temperatures. In this regard, encapsulation of pediocin or its application in combination with other preventative methods could be useful. Generally, it can be stated that pediocin and pediocin-like bacteriocin could be potentially used as part of the hurdle technology along with other preventative strategies or as part of antimicrobial agents incorporated into packaging materials. Furthermore, the application of pediocin with novel technologies such as cold plasma, irradiation, high-intensity pulsed electric field, and HHP against *Listeria* should be investigated in future studies.

AUTHOR CONTRIBUTIONS

MY and NK designed this study. EK, NK, and MM wrote the manuscript. MY and AM critically revised the manuscript, and finally, all authors listed have approved it for publication. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.709959/full#supplementary-material

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Water-Soluble Ruthenium (II) Complex Derived From Optically Pure Limonene and Its Microencapsulation Are Efficient Tools Against Bacterial Food Pathogen Biofilms: *Escherichia coli*, *Staphylococcus aureus*, *Enteroccocus faecalis*, and *Listeria monocytogenes*

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Bioactive aminooxime ligands based on optically pure (R)-limonene have been synthesized in two steps. Their ruthenium (II) cationic water-soluble complex was prepared by a reaction between dichloro (para-cymene) ruthenium (II) dimers and aminooxime ligands in a 1:2 molar ratio. Antibacterial and antibiofilm activities of the synthetized complex were assessed against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis.* The results revealed that the ruthenium (II) complex has higher antibacterial and antibiofilm activities in comparison with free ligands or the enantiopure (R)-limonene. Moreover, microencapsulation of this complex reduced its cytotoxicity and improved their minimum inhibitory concentration and antibiofilm activity toward the considered bacteria. The ruthenium (II) complex targets the bacterial cell membrane, which leads to rapid leakage of intracellular potassium. Our study suggests that the developed ruthenium (II) complexes could be useful as an alternative to conventional disinfectants.

Keywords: ruthenium (II), limonene, complexation, microencapsulation, biofilm, antibacterial agent, food pathogen, biofilm removal

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INTRODUCTION

Unit operation hygiene is a major challenge for healthcare establishments and agro-food industries as the surface contamination can lead to infectious diseases and food poisoning (Abdallah et al., 2014). Despite the implementation of preventive measures such as frequent cleaning and disinfection procedures, persistent bacteria can form biofilms on abiotic surfaces if the growth conditions are suitable. Biofilms allow bacteria to increase resistance to environmental stresses, cleaning, and disinfection treatments compared with planktonic form bacteria (Bridier et al., 2011; Khelissa et al., 2017, 2018, 2019; Singh et al., 2017; Cadena et al., 2019).

Biosourced antimicrobials represent a promising alternative to chemically synthesized antimicrobials, such as quaternary ammonium compounds. Terpenes from aromatic plant essential oils show a good antibacterial activity (Guimarães et al., 2019). However, there are many challenges to be used as antimicrobial faces, such as high volatility, non-water miscibility, and cytotoxicity (Fisher and Phillips, 2008; Mahizan et al., 2019). Mediterranean countries produce high amounts of citrus fruits and aromatic plants. The valuation of citrus byproducts, as a source of renewable and biodegradable raw materials, through the production of essential oils, constitutes a major challenge for sustainable development. The chemical composition of the essential oil extracted from the peel of citrus fruits mainly contains low-priced enantiopure limonene (80–95%) as well as other terpenoids and aromatic compounds (Bourgou et al., 2012).

Limonene has many applications related to its antimicrobial activity (Bomgardner, 2011; Ciriminna et al., 2014). The degree of toxicity of limonene depends on its level of oxidation. Indeed, several studies have shown that oxidation derivatives of limonene exhibit greater antibacterial toxicity than limonene (Chubukov et al., 2015). Terpene (abundant agro-resource) use can be an effective tool to reduce the microbiological risk related to contamination of the surfaces of materials in contact with food-based products. Furthermore, it has been reported that the compounds containing amine and oxime functions in their structures have good antibacterial activities (Sahyoun et al., 2019), as well as the complexes of various functionalized ligands. It has been also reported that the antibacterial activity of some ligands and their associated metal complexes show higher activity than free ligands against bacteria (Samota and Seth, 2010). In addition, it has been reported that some metal complexes are water soluble (Bayón Castañón et al., 2018). This characteristic constitutes an advantage for their use over the use of terpenes as antibacterial compound.

Bacterial biofilms are encased in a self-produced matrix composed of extracellular polymeric substances (EPS) such as polysaccharides, proteins, lipids, and extracellular DNA. The biofilm matrix represents a diffusion barrier, which can delay or prevent the interaction of biocides with microbial cells. Thus, the interactions between the EPS and biocides can lead to biocide sequestration or repulsion with a significant decrease in the bioactive concentration of the used biocides (Pereira and Vieira, 2001). The encapsulation of biocides could prevent the interactions between biocides and the EPS of the biofilm matrix. This can enhance the effectiveness of the antimicrobials. In addition, microencapsulation could lead to the reduction of the amount of biocide used and minimize their environmental impact. The microencapsulation of biosourced terpenes and their derivative complexes as biocides could be also an efficient tool to overcome their high volatility, non-water miscibility, and cytotoxicity.

In this context, the aim of this work was to assess the antibacterial and the antibiofilm activities of a chiral aminooxime ligand L3 [(1S, 4R)-1-benzylamino-p-menth-8-en-2-one oxime] obtained by the functionalization of R-limonene and its water-soluble ruthenium complex. In addition, the microencapsulation was used as an innovative tool to enhance the antimicrobial and the antibiofilm activities of the limonene and its derivative and to reduce the amount of the biocide used in order to set up efficient and environmentally friendly disinfection procedures for closed and open food contact surfaces.

MATERIALS AND METHODS

Synthesis of the Aminooxime Ligand L3 and the RuL3 Complex

The synthesis of the aminooxime ligand L3 and the RuL3 complex were performed according to the procedures described in the literature (Ibn El Alami et al., 2012; Benabdelouahab et al., 2015; El Alami et al., 2015).

Ligand (1S,4R)-1-Benzylamino-p-Menth-8-en-2-One Oxime Ligand: L3

Isopentyl nitrite (isoamyl nitrite) $(CH_3)_2CH(CH_2)_3ONO$ and concentrated hydrochloric acid (37%) were added to (R)- or (S)-limonene at −5°C. The reaction mixture was stirred at the same temperature for 1 h. The precipitate formed was washed with cold methanol and dried to obtain a solid corresponding to the nitrosochloride A of (R)- or (S)-limonene (yield = 47%). A mixture consisting of nitrosochloride A (13 g, 32.5 mmol) and benzylamine (13 ml, 119.6 mmol) in ethanol (20 ml) was heated until a clear solution was obtained. This solution was cooled to −5°C, and hydrochloric acid (37%) was added dropwise (up to acid pH). The white obtained solid, corresponding to L3 hydrochloride, was washed successively with ethanol and diethyl ether, then basified with triethylamine. The solution was recovered with diethyl ether, washed with water, and dried on $MgSO_4$. Evaporation of the solvent provides the final aminooxime L3 as a white solid (7 g, yield = 71%).

Elemental analysis (%): $C_{17}H_{24}N_2O$
Calculated: C, 74.96; H, 8.88; N, 10.28.
Found: C, 74.59; H, 9.064; N, 10.17.

Complex {RuCl[(Para-Cymene)][Aminooxime L3]}+Cl⁻: RuL3

(1S,4R)-Benzylaminooxime L3 (0.133 g; 0.48 mmol) and $[RuCl_2(p\text{-cymene})]_2$ (0.15 g; 0.24 mmol) were introduced into a Schlenk tube and solubilized in anhydrous dichloromethane (7 ml). The reaction mixture was stirred for 30 min at room temperature. Diethylether (4.5 ml) was added dropwise, and the mixture was maintained at −5°C overnight. After filtration, the residue was dried under reduced pressure to give RuL3 complex as a yellow powder (0.26 g, yield = 81%).

Elemental analysis (%): $RuC_{27}H_{38}N_2OCl_2$
Calculated: C, 56.05; H, 6.62; N, 4.84.
Found: C, 55.65; H, 6.90; N, 4.77.

Bacterial Strains

Staphylococcus aureus CIP 4.83, *Escherichia coli* CIP 54127, *Enterococcus faecalis* isolated from French cheese, and *Listeria monocytogenes* ATCC 35152 were used in this study. Strains were maintained at −20°C in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France), supplemented with 40% (v/v) glycerol. For preculture preparation, each strain was grown in 5 ml of TSB for 24 h at 37°C. The main cultures were started by inoculating 10^4 CFU/ml from the preculture in 50 ml. Bacterial cultures were incubated overnight at 37°C, under continuous shaking (160 rpm).

Determination of Minimum Inhibitory Concentrations

The microdilution method was used to determine the minimum inhibitory concentration (MIC). Limonene was dissolved in TSB supplemented with 2% (v/v) dimethyl sulfoxide (DMSO). Twofold serial dilutions of each tested compound was prepared in a Bioscreen well microtiter plate over the range of 0–100 mg/ml (in TSB). Bacterial suspensions were adjusted to 10^6 CFU/ml. In addition, wells containing each strain inoculum in TSB without the tested component were measured as positive control, DMSO as vehicle control, and only TSB as negative control. The microdilution plates were incubated at 37°C under continuous shaking. The optic density (OD) at 600 nm was measured every 2 h for 24 h. The MIC was defined as the lowest concentration that prevented growth as measured by optical density. All experiments were done in triplicate using different microplates.

Antibiofilm Assays

Bacterial cells were harvested from overnight cultures by centrifugation (5,000 × g, 5 min, 20°C) and washed twice with potassium phosphate buffer (PPB; 100 mM, pH 7). Bacterial suspensions were adjusted to 10^7 CFU/ml in PPB. Three milliliters of suspension cells was deposed on sterile stainless steel (SS) slides. After 1 h of static incubation at 20°C, SS slides were rinsed with 5 ml of PPB then covered with 3 ml of TSB before incubation for 24 h at 37°C to allow biofilm formation on SS. Biofilms were rinsed with PPB then treated with the different considered compounds (at the MIC) for 10 min. Treated biofilms were immersed in 20 ml of neutralizing solution (Toté et al., 2010) containing 1 g of 1-mm diameter glass beads in 100-ml sterile pots. Pots were vortexed for 30 s followed by sonication for 5 min (37 kHz, 20°C) (Elmasonic S60H, Elma, Germany). Tenfold serial dilutions were made in PPB. Samples of 100 µl were spread onto TSA plates and incubated at 37°C for 24 h. The colony number was counted, and results were expressed in log CFU/cm^2. PPB was used as negative control. The results represent the means of three independent experiments, and in each experiment three slides were used.

Scanning Electron Microscope Observation

The inner structures of RuL3 microcapsules were investigated by scanning electron microscope (SEM-JEOL-JSM-7800FLV, Japan). The powder containing RuL3 microcapsules was fractured by moving a razor blade perpendicularly through a layer of microcapsules. The morphology of bacterial cells after treatment with different compounds was assessed by SEM. One-milliliter volume of treated and untreated cells was filtered through a 0.2-µm polycarbonate membrane filter (Schleicher and Schuell, Dassel, Germany) then fixed at 4°C for 4 h in 2% glutaraldehyde in cacodylate buffer 0.1 M pH 7. Fixed samples were then dehydrated in an ascending ethanol series (50, 70, 95, and 2 × 100% (v/v) ethanol) for 15 min at each concentration. Samples were critical point dried and coated with thin carbon film before examination in the SEM. Microscopy was performed with a JEOL JSM-7800F LV microscope at 3 kV.

Epifluorescence Microscopy Observation

Biofilms were treated with microencapsulated RuL3. For control condition, biofilms were treated with PPB. Biofilms were stained with LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, United States), according to the instruction of the manufacturer for 15 min, then washed by gently dipping in sterile distillated water, air dried in the dark before being observed under an epifluorescence microscope (Olympus BX43, Germany). The green cells were considered viable, and the red ones were defined as non-viable.

Cytotoxicity Assay

The HeLa cell line (ATCC® CCL-2™, ECACC, Sigma Aldrich, Saint-Quentin Fallavier, France) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco®) supplemented with 10% fetal bovine serum (FBS, Gibco®) and 1% penicillin–streptomycin (Gibco®) in a humidified incubator at 37°C and 5% CO_2. Cells were seeded at a density of 10^4 cells/well in a 96-well plate and grown for 24 h before assay. The culture medium was replaced with a fresh medium that contains the compounds from 12.5 to 800 µg/ml. After 24 h, the old medium was removed, and cells were washed with PBS. The cell viability was evaluated using the resazurin cell viability method. Briefly, 100 µl of the resazurin solution (11 µg/mL) in complete medium was added to each well, and the plate was incubated for 4 h in a humidified incubator. The fluorescence emission of each well

was measured at 593 nm (20-nm bandwidth) with an excitation at 554 nm (18-nm bandwidth) using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments SAS, France). Each condition was replicated three times, and the mean fluorescence value of non-exposed cells was taken as 100% cellular viability.

Ruthenium Complex-Induced Cell Membrane Permeabilization

E. coli grown overnight at 37°C cells was concentrated to 10^{10} CFU/ml (5,000 × g, 15 min, 20°C). The concentrated bacterial suspension was mixed with free or microencapsulated RuL3 (prepared in 50 mM MOPS buffer at MIC as final concentration) or TSB, prepared in 50 mM MOPS buffer (negative control). The K^+ concentration at time zero was measured in a 10-fold dilution of the bacterial suspension filtrate (0.2 μm, Sartorius™ Minisart™ NML Syringe Filters, France) before contact with different solutions. Tested compounds were introduced to cell suspension after 20 min. Samples were filter sterilized at 10, 20, 30, 40, 50, 70, and 90 min. Each sample was removed using a sterile plastic syringe attached to a sterile needle to enable easy access to the reaction mixture suspension through a silicon cap. The K^+ concentration in filtrate samples was determined using a Varian SpectrAA 55/B atomic absorption spectrometer in flame emission mode (wavelength 766.5 nm; slit 0.7-nm high; air–acetylene flame).

Extracellular GFP Intensity Assessment

The extracellular GFP intensity assessment was carried out according to Khelissa et al. (2021b). An overnight culture (37°C) of *Escherichia coli* GFP (ATCC 25922GFP) in TSB supplemented with 100 mg/ml of ampicillin was used. After centrifugation (5,000 × *g*, 5 min, 20°C), pelleted cells were washed with HEPES buffer (5 mM, pH 7.2). A 5 ml of concentrated bacterial cell suspensions (1,010 CFU/ml) was added to 45 ml of free DTAC, free or encapsulated RUL3 prepared in HEPES buffer (final concentration corresponding to the MIC in a final volume of 50 ml) or HEPES buffer as a negative control. At 0, 5, and 10 min before cells were exposed to DTAC, free or encapsulated RUL3, 10-fold dilutions of the concentrated inoculum were made in HEPES buffer, then filter sterilized (Sartorius™ Minisart™ NML 0.2-ml Syringe Filters, France). These filtrates were used to assess the extracellular GFP fluorescence intensity before the antibacterial treatment. Samples were filter sterilized at 5, 10, 15, 20, and 30 min after the addition of bacterial cells to the reaction vessel containing the antibacterial solution. Aliquots (200 ml) of the filtered supernatant samples were transferred to a 96-well microplate, and GFP fluorescence was quantified using a BioTek fluorescence spectrophotometer (BioTek Instruments SAS, France) with excitation at 485 nm and emission at 510 nm. The fluorescence intensity ratio of samples to HEPES buffer was plotted vs. contact time. The results represent the average of three independent experiments.

Zeta Potential Measurement

The electric charges (ζ-potential) of RuL3 at different pH values were determined using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, United Kingdom). The samples were diluted with imidazole acetate buffer adjusted to the adequate pH. The mean ζ-potential (ZP) values [± SD (standard deviation)] were obtained from the instrument. The measurements were repeated three times for each suspension.

Microencapsulation of RuL3 by Spray Drying

A stock maltodextrin solution was added to an aqueous solution of RuL3 in order to have a final composition (w/w) of 20% maltodextrin DE 19 and 0.4% RuL3 in imidazole/acetate buffer (5 mM, pH 7). Maltodextrin DE 19 (dextrose equivalent value of 19) were obtained from Roquette-freres SA (Lestrem, France). The mixture was stirred for 30 min and then spray dried using a laboratory scale device equipped with a 0.5-nm nozzle atomizer (Mini Spray-Dryer Büchi B-290, Switzerland). The operational conditions of the drying process were feed flow rate 0.5 L/h, inlet air temperature 180 ± 2°C, and outlet air temperature 80 ± 5°C. After spray drying, the powder was collected in sealed containers and stored at 4°C until microbiological tests. Spray-dried microcapsules without RuL3 (empty capsules) were used as control.

Statistical Analysis

All experiments were carried out at least three times. Statistical analysis was performed with IBM SPSS 19 statistics software following one-way ANOVA. Results were considered significantly different when $p < 0.05$.

RESULTS

Zeta Potential of RuL3 as a Function of pH

To assess the charge of the RuL3 in our conditions of bacterial cells and biofilm treatments, the zeta potential of RuL3 as a function of pH was measured as shown in **Figure 1**. The results show that the zeta potential of RuL3 is largely pH dependent and that the charge of the molecule changes from positive to negative as the pH increases. The point at which the overall charge of the molecule is neutral was between 5.5 and 6 (**Figure 1**).

Morphology and Inner Structure of Spray-Dried Microcapsules

Figure 2 shows the scanning electron micrographs of microcapsules obtained by spray drying the RuL3 solutions in the presence of maltodextrin DE19. The microcapsules consisted of well-separated spherical particles, having heterogeneous diameters, rounded shape, and generally dented surfaces. In fact, the viscosity of feed solutions was relatively high, and consequently, the particles were not well formed during the drying process. They tend to stick to each other and were not very well separated (**Figure 2A**), and some solid bridges were formed between individual microcapsules. High viscosity not only prevents well-defined particle formation but also tends to form irregular clumps. The microcapsule micrographs obtained

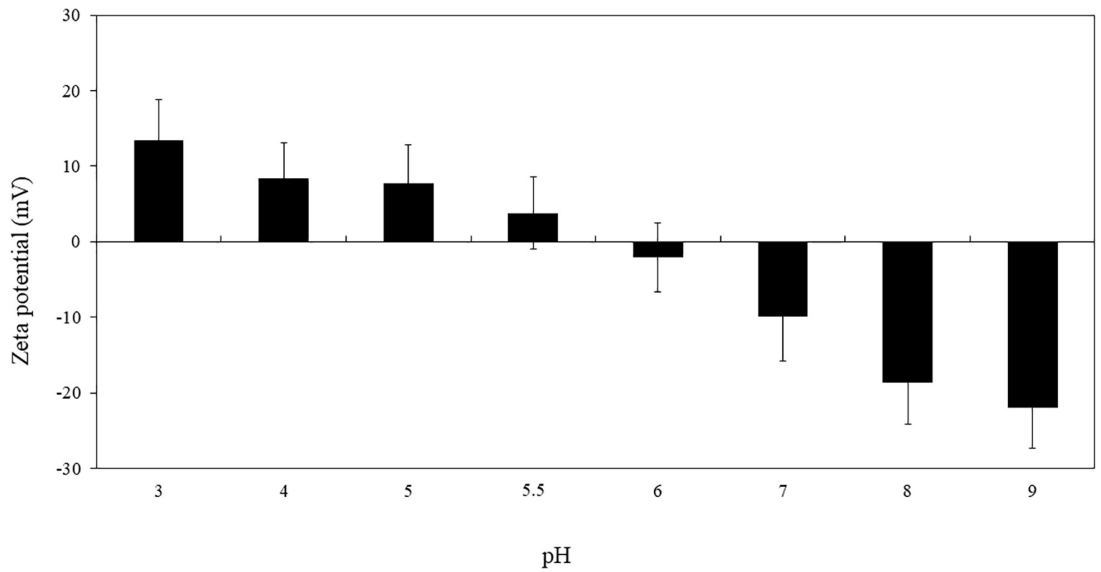

FIGURE 1 | Zeta potential of RuL3 as a function of pH (imidazole/acetate buffer, 5 mM). Data are presented as means (± SD) of three independent repextineats in triplicate.

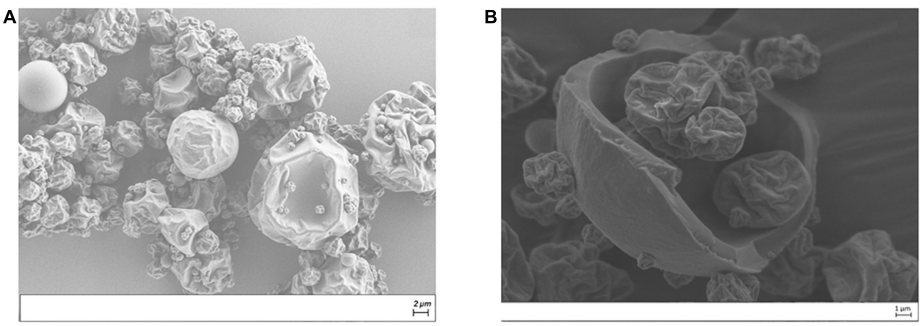

FIGURE 2 | Scanning electron microscope (SEM) micrographs of spray-dried solution containing 1 g of RuL3 and 50 g of maltodextrin DE 19 in imidazole acetate buffer at pH 7 [external **(A)** and internal **(B)** structures].

show also that indentation and roughness of the surface was more prevalent in smaller particles than in larger ones suggesting that solidification of the walls happened prior to expansion of the microcapsules. The internal structure of the obtained microcapsules show a thicker shell matrix and an air bubble (i.e., void) at the center of the microcapsule (**Figure 2B**).

Determination of Minimum Inhibitory Concentrations

In order to compare the antimicrobial activity of limonene, RuL3, and the microencapsulated RuL3, their minimum inhibitory concentrations were measured against *Escherichia coli*. *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis* (**Table 1**). DMSO (2%) used to enhance the water miscibility of limonene, did not affect the growth of the considered strains at this DMSO concentration (data not shown). The MIC values for limonene against all considered strains were 12.5 mg/ml. Compared with limonene, the MICs (0.4 mg/ml) of the RuL3 complex was approximately 30-fold lower. Thus, the complexation of limonene with 233 Ruthenium enhanced its antibacterial potential. In addition, **Table 1** shows that the MIC values of microencapsulated RuL3 were fourfold lower against all studied strains than those of free RuL3. These results clearly show that the Ru–limonene complexation and microencapsulation led to the improvement of the antibacterial efficacy of limonene.

Antibiofilm Activity of Studied Compounds

The antibiofilm efficacy of limonene, free and microencapsulated RuL3 were investigated on *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* biofilms (**Table 2**). The results show that treatment with limonene slightly reduced ($p > 0.05$) the initial population of *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* biofilms (**Table 2**). This reduction did not exceed the 0.6 log CFU/cm^2 regardless of the considered strain (**Table 2**). However, the RuL3 treatment reduced the initial cell counts of *E. coli*, *S. aureus*,

TABLE 1 | Minimum inhibitory concentration values (mg/ml) of limonene, RuL3, and microencapsulated RuL3 against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*.

	MIC mg/ml			
	E. coli	***S. aureus***	***L. monocytogenes***	***E. faecalis***
Limonene	12.5	12.5	12.5	12.5
RuL3	0.4	0.4	0.4	0.8
Microencapsulated RuL3	0.1	0.1	0.1	0.4

TABLE 2 | *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis* biofilm log counts after treatment for 10 min with limonene, free and microencapsulated RuL3 at their respective MICs.

	E. coli	***S. aureus***	***L. monocytogenes***	***E. faecalis***
Log CFU/cm^2				
Control	8.1 ± 0.3	8.3 ± 0.2	7.8 ± 0.2	7.4 ± 0.3
Limonene	7.5 ± 0.1	7.7 ± 0.2	7.3 ± 0.3	6.9 ± 0.1
RuL3	5.5 ± 0.2	5.0 ± 0.2	4.8 ± 0.3	5.4 ± 0.1
Microen-capsulated RuL3	3.7 ± 0.3	2.8 ± 0.1	3.0 ± 0.1	4.2 ± 0.2

For the control, biofilms were treated with potassium phosphate buffer (100 mM, pH 7) for 10 min.

L. monocytogenes, and *E. faecalis*, respectively, to 5.5, 5.0, 4.8, and 5.4 log CFU/cm^2 (**Table 2**). Moreover, our results show that the treatment with microencapsulated RuL3 led to a reduction in the initial biomass of *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis*, respectively, to 3.7, 2.8, 3.0, and 4.2 log CFU/cm^2. This reduction represents reduction of *ca* 90% of the biofilm biomass (**Table 2**). The log reduction effect of microencapsulated RuL3 was *ca* 2 log higher than that of free RuL3 for *E. coli*, *S. aureus*, and *L. monocytogenes*. However, this difference between free and microencapsulated RuL3 was 1.2 log for *E. faecalis* biofilm. The direct effect of the microencapsulated Rul3 on biofilms (**Figure 3**) was assessed by using epifluorescence microscopy on 24-h biofilm age of *L. monocytogenes*, *E. faecalis*, *E. coli*, and *S. aureus*. The staining was performed with SYTO9 and PI after treatment with microencapsulated RuL3. Biofilms treated with TS (control) show a dense and covering layer of viable biomass mainly stained with SYTO9. Treatment with RuL3 microcapsules resulted in a significant dispersion of biofilm biomass from the surface of SS coupon (**Figure 3**). Furthermore, the majority of the cells in the remaining biofilm was stained with PI, reflecting the significant biofilm elimination action of the proposed RuL3 microcapsules.

Impact of Free and Microencapsulated RuL3 Treatment on *E. coli* Morphology

Escherichia coli was used in order to measure the effect of free and microencapsulated RuL3 treatments on the bacterial cells. The bacterial cells were exposed to the MIC values of free and microencapsulated RuL3. The structural morphology of the treated *E. coli* was observed by SEM (**Figure 4**). **Figure 4** shows that untreated cells (control) had a regular and normal bacilliform structure. However, the micrographs of the cells treated with free and microencapsulated RuL3 shows different forms of distortion and deformation (**Figure 4**). Furthermore, treated cells endured a complete collapse of the morphology of the cells accompanied by intercellular pool leakage (**Figure 4**). Similar results were obtained with *Staphylococcus aureus* (data not shown). These results provide evidence that the microencapsulated RuL3 was endowed with a stronger antimicrobial activity against the *E. coli* compared with its free form.

Escherichia coli Cytoplasmic Membrane Permeabilization by Free and Microencapsulated RuL3

The effect of free and microencapsulated RuL3 on the permeability of *E. coli* membranes was investigated (**Figure 5**). In this context, the leakage of intracellular K^+ ions was monitored. Our results show that the addition of TSB (prepared in 50 mM MOPS buffer) had no effect on K^+ efflux, which remained stable (control). However, when *E. coli* cells were exposed to the free (0.4 mg/ml) and microencapsulated RuL3 (0.1 mg/ml) (final concentration corresponding to the MIC), the result was an immediate and rapid increase in K^+ concentration in the suspension medium. Seventy minutes after exposing cells to free and microencapsulated RuL3, the K^+ concentration in the suspension medium increased to 14 and 24 mg/L, respectively (**Figure 5**). These results show that the microencapsulated RuL3 (0.1 mg/ml) was more efficient than the free RuL3 (0.4 mg/ml) even at a concentration that was four times lower.

To check out if the membrane damage leads to intracellular protein leakage, an *E. coli* GFP strain was used to monitor extracellular GFP fluorescent intensity after the exposure of the bacterial cells grown at 37°C to free and microencapsulated RUL3. Dodecyltrimethylammonium chloride (DTAC) was used as the positive control. The results showed no significant effect on the fluorescence intensity (**Figure 6**) after the addition of TSB (prepared in 50 mM MOPS buffer) as a negative control. After the exposure of bacterial cells to DTAC, an instantaneous and significant increase in the extracellular fluorescence intensity was measured (**Figure 6**). After 10 min, the GFP fluorescence intensity increased by 1.5-fold in the supernatant filtrates of the bacterial suspension treated with DTAC. However, no fluorescence increase was measured after the addition of the free microencapsulated RUL3. These results were in agreement with these obtained by an analysis, using SDS page electrophoresis, of intracellular proteins in the supernatant before and after the addition of the free or microencapsulated RUL3 (data not shown). These results show that the free or microencapsulated RUL3 induce potassium release but not protein leakage from the cytoplasm.

Cytotoxicity of Free and Microencapsulated RuL3

The viability of *HeLa* cells was assessed after exposure for 24 h to free or microencapsulated RuL3 at twofold serial dilutions. The final concentration ranged from 12.5 to 800 μg/ml (**Figure 7**).

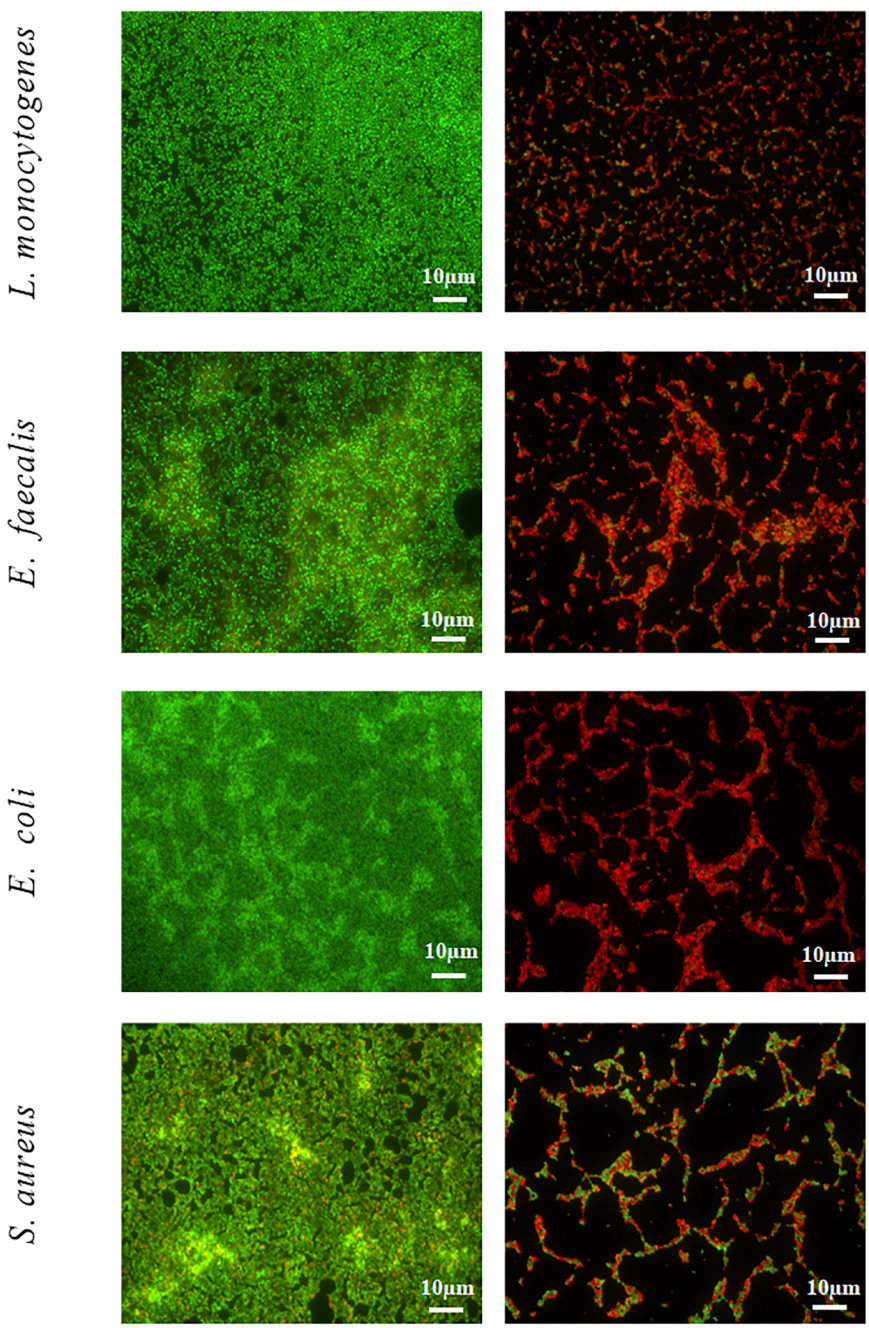

FIGURE 3 | Fluorescence microscope images of *Listeria monocytogenes*, *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus aureus* biofilm cells grown at 37°C for 24 h on stainless steel after treatment with microencapsulated RuL3 at the minimum inhibitory concentration (MIC). Cells were visualized after staining with SYTO-9 (green fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). The control represents cells treated with tryptone salt buffer.

The DMEM with 10% FBS was used as a negative control. An additional control was used consisting of microcapsules formulated without RuL3. The treatment with empty capsules had no cytotoxic effect as the viability of *HeLa* cells remained at *ca* 100% regardless of the considered concentration (**Figure 7**). Contact with 12.5 µg/ml of free RuL3 shows no negative effect on *HeLa* cell viability, which remained at 100% (**Figure 7**). However, when incubated in the presence of 25 µg/ml of free RuL3, the viability percentage was significantly ($p < 0.05$) reduced to 5% (**Figure 7**). At concentrations ≥ 50 µg/ml, the viability

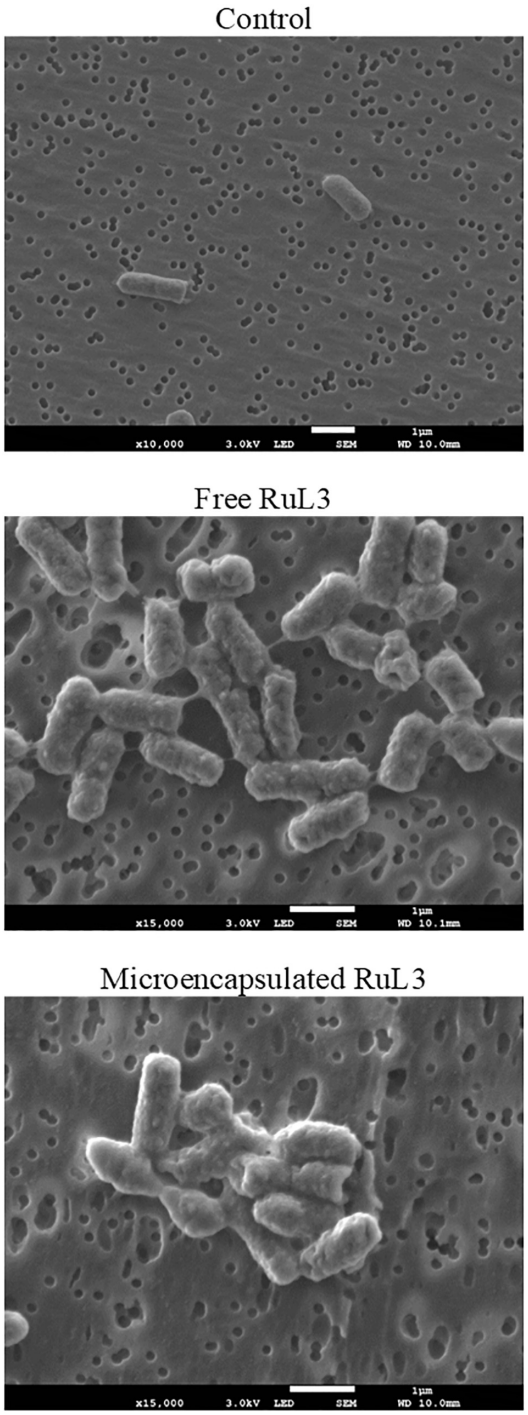

FIGURE 4 | Scanning electron micrographs of *Escherichia coli* biofilm cells, after treatment with free and micoencapsulated RuL3 at the MIC. The control represents cells treated with potassium phosphate buffer.

percentage was reduced to 0% (**Figure 7**). **Figure 7** shows that microencapsulated RuL3 had lower cytotoxicity toward *HeLa* cells compared with its free form ($p < 0.05$). At a concentration of 50 μg/ml, the cell viability was of 96% ($p < 0.05$). *HeLa* cell viability was reduced to a mean value of 32% in contact with 800 μg/ml of encapsulated RuL3.

DISCUSSION

Ruthenium complexes (Ru), initially developed for anticancer treatment purposes (Benabdelaouahab et al., 2016), have recently attracted interest as a new class of antimicrobial agents (Li et al., 2015; Southam et al., 2017; Yang et al., 2018). Moreover, ruthenium complexes are being pursued to help overcome the multidrug resistance bacteria (Claudel et al., 2020; Di Natale et al., 2020).

This study explored the antibacterial and the antibiofilm activity of free and microencapsulated RuL3 compared with limonene against four foodborne pathogenic bacteria. The RuL3 was synthesized, and its zeta potential was studied as a function of pH. The point at which the overall charge of RuL3 was neutral was between 5.5 and 6 as shown in our study. The information provided by this result was important because the antimicrobial activity depends on the interactions between the active molecule and the membrane of the target microorganisms, which, in turn, is a function of the electric charge of the molecule. Thus, we worked at pH 7, a condition in which the molecule had an overall negative charge, which can play an important role in the intensity of its antimicrobial activity (Khelissa et al., 2021a,b).

The MIC values indicated that limonene had an inhibitory activity against *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis*. However, the MICs of limonene dissolved in DMSO has shown a very high value of *ca* 12.5 mg/ml. Moreover, our results show that free RuL3 significantly inhibits the growth of *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* at a concentration up to 30-fold lower than those of limonene (**Table 1**). The microencapsulation of RuL3 reduced the initial MIC of free RuL3 by fourfold against *E. coli*, *S. aureus*, and *L. monocytogenes* and by twofold against *E. faecalis*. Among the studied bacteria, the highest MICs were observed against *E. faecalis* whatever the tested compound is. These results are consistent with our previous studies, which show that the microencapsulation of different disinfectants may enhance their antibacterial activity against several food pathogens (Khelissa et al., 2021a,b). Furthermore, the most interesting feature was that the microencapsulated RuL3 antibacterial activity against *E. coli*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* biofilm cells was significantly ($p < 0.05$) higher than that of free RuL3. These results are in agreement with several studies reporting that the structure of the antimicrobial agent influences its interactions with biofilm matrix components and, thus, its disinfection efficacy (Singh et al., 2017; Karygianni et al., 2020).

The SEM micrographs clearly show that both free and microencapsulated RuL3 were able to damage the morphology of *E. coli*. The destruction of cell wall and cell membrane could lead to an irreversible leakage of the intracellular pool, resulting in cell death. In fact, the bacterial cell membrane acts as a natural protective barrier against many antimicrobials (Claessen and Errington, 2019). Exposure of bacteria to microencapsulated

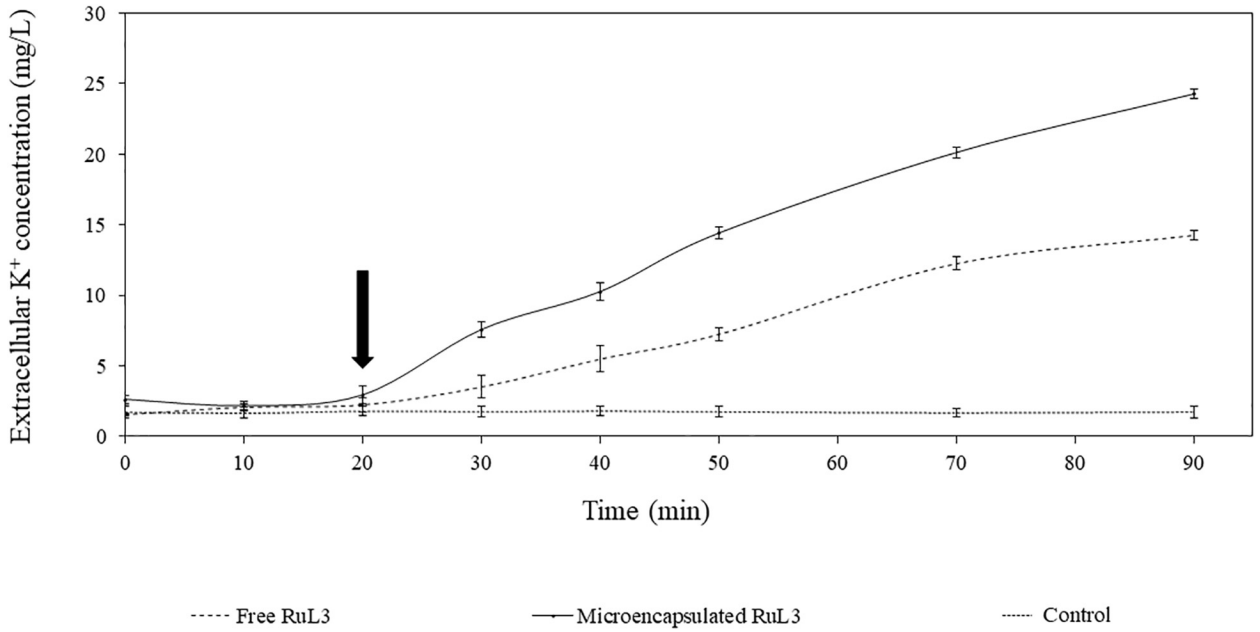

FIGURE 5 | Kinetics of intracellular potassium leakage from *Escherichia coli* cells after treatment with either free or microencapsulated RuL3 at the minimum inhibitory concentration. The K^+ concentration is presented in milligrams per liter. The treatment with TSB prepared in 50 mM MOPS buffer represents the negative control. The arrow indicates when free or microencapsulated RuL3 was added. Data are presented as means (± SD) of three independent repeats in triplicate.

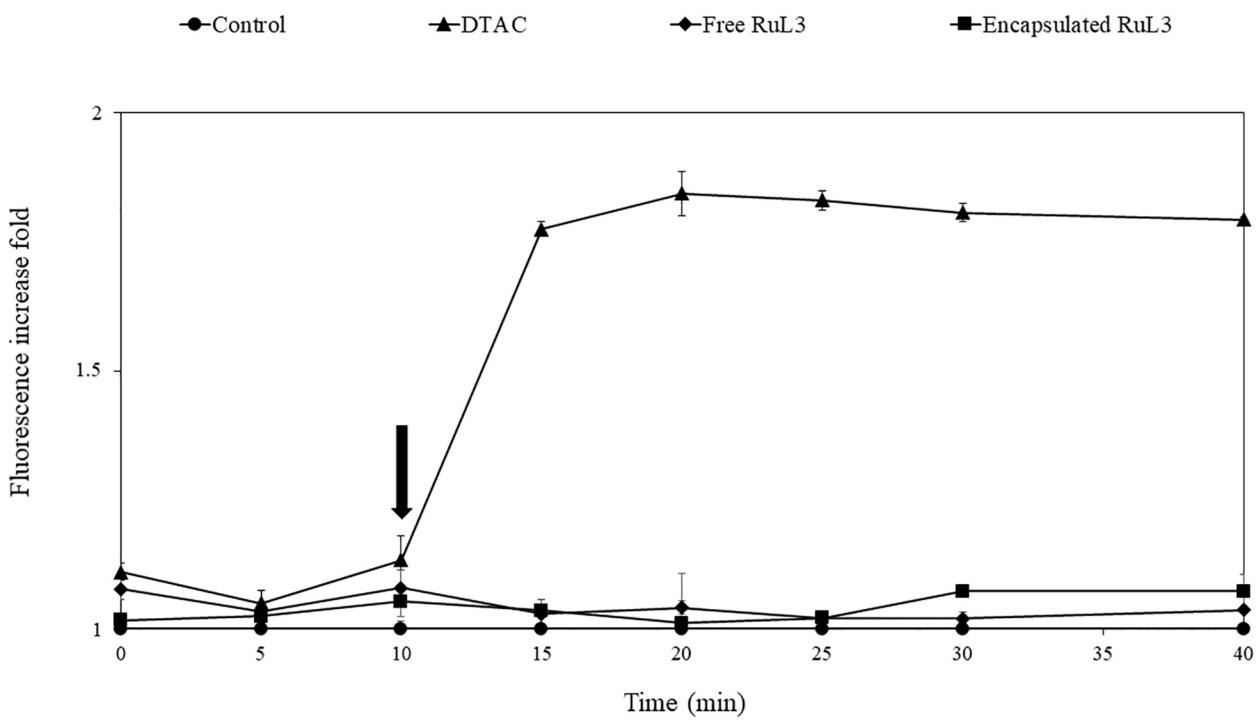

FIGURE 6 | Assessment of *Escherichia coli* (ATCC 25922GFP) cytoplasmic membrane permeability when exposed to dodecyltrimethylammonium chloride (DTAC), free RuL3, or encapsulated RuL3 at the minimal inhibitory concentration. The black arrow indicates the time at which (DTAC) free RuL3 or encapsulated RuL3 was added. Fluorescence of the green fluorescent protein was measured spectroscopically at 485- to 510-nm excitation and emission wavelengths. Data are presented as means (± SD) of three independent repeats in triplicate. The control represents cells treated with HEPES buffer.

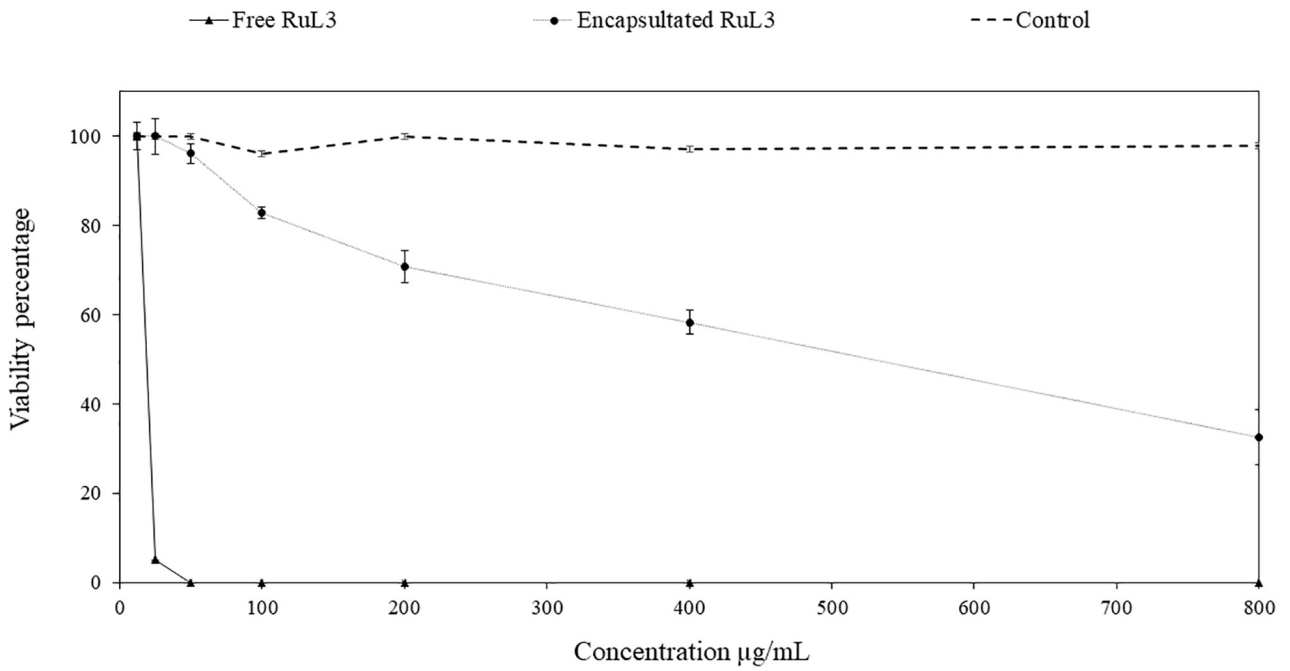

FIGURE 7 | *HeLa* cell viability percentage after 24-h exposure to concentrations (between 12.5 and 800 µg/ ml) of free and microencapsulated RuL3. The control represents the spray-dried microcapsules without RuL3 (empty capsules). Data are presented as means (± SD) of three independent repeats in triplicate.

RuL3 resulted in significant biofilm dispersal and an increase in cell death. The results are consistent with our previous studies conducted on microencapsulated quaternary ammonium compounds (Khelissa et al., 2021a,b). When cell membranes of bacteria are destroyed, after treatment with disinfectant, the internal electrolyte leaks into the culture medium (Han et al., 2019). It is known that under normal physiological conditions, the intracellular concentration of K^+ is higher than that in the extracellular medium (Stratford et al., 2019). In this study, the exposure of *E. coli* to RuL3 under its free and microencapsulated forms was observed to significantly increase the extracellular K^+ concentration, which reflects the permeabilization of the cell membrane and the irreversible leakage of bacterial intracellular pool. Nevertheless, the final extracellular concentration of K^+ after *E. coli* treatment with microencapsulated RuL3, was 1.7-fold higher than that measured after treatment with free RuL3. Thus, the results underlined that microencapsulation enhances the antibacterial action of RuL3. These finding are in agreement with our previous study reporting that microencapsulation of DTAC significantly enhanced its membrane-disturbing action compared with free DTAC (Khelissa et al., 2021b). Taken together, the results show that RuL3 is likely to target the bacterial cytoplasmic membrane and disturb the integrity of its phospholipidic bilayer. These results are consistent with the results of SEM observation insofar as they highlighted the bacterial membrane collapse. In addition, these results are in agreement with those from our previous studies reporting that spray-drying microencapsulation can be used as an effective tool to enhance the activity of the antibacterial agent while reducing the required concentration and the associated cytotoxicity (Khelissa et al., 2021a,b). Our finding also showed that the free or microencapsulated RUL3 induced no protein leakage from the cytoplasm in contrast to DTAC, which, when added to *E. coli* GFP resulted in a leakage of intracellular proteins as reported in our previous results (Khelissa et al., 2021b).

Ruthenium complexes are toxic and selective toward cancer cells as reported (Benabdelaouahab et al., 2016). They have exhibited low *in vitro* toxicity associated with a good *in vivo* antimetastatic characteristics (Scolaro et al., 2005). Our results are in agreement with these reports as, at a concentration of 25 µg/ml, the free RuL3 reduced cell viability by 95% of *HeLa* cells (**Figure 7**). The current results show that the microencapsulation of RuL3 significantly reduced its cytotoxicity toward *HeLa* cells. These results show that biosourced terpenes could be used to synthetize complexes, such as RuL3, which have a great antibacterial and antibiofilm activity. The encapsulation of the RuL3 appears as a promising approach, to reduce the amount of the antimicrobial used and enhancing at the same time its efficiency toward biofilm and biofouling. The encapsulation of RuL3 allows reducing its cytotoxicity. This will help to set up an efficient disinfection strategy with less negative environmental impact. In addition, our original study reports the successful synthesis and microencapsulation of RuL3 complex using spray-drying process. Additionally, results illustrate that the microencapsulation of the RuL3 complex improves its efficiency against biofilms of food pathogenic bacteria and promotes a significant decrease in its cytotoxicity. The RuL3 has likely targeted the bacterial cytoplasmic membrane and promoted its irreversible collapse. Thus, these important feature

makes this ruthenium complex an interesting alternative for the conventional range of commercialized antibacterial agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SK contributed to the conception and design of the study and manuscript redaction and performed the microbiology experiments. YF and M-AA performed the chemistry experiments and contributed to manuscript redaction. SM performed the membrane damage experiments using Escherichia coli GFP strain. SA contributed to manuscript writing and reviewing its final version. AG performed the microencapsulation experiments and contributed to the process of writing of the manuscript. AB performed the cytotoxicity assays. N-EC supervised the work, designed the study, and contributed to the redaction and revision of the manuscript. All authors approved the submitted version.

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Nanoencapsulation-Based Edible Coating of Essential Oils as a Novel Green Strategy Against Fungal Spoilage, Mycotoxin Contamination, and Quality Deterioration of Stored Fruits: An Overview

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Currently, applications of essential oils for protection of postharvest fruits against fungal infestation and mycotoxin contamination are of immense interest and research hot spot in view of their natural origin and possibly being an alternative to hazardous synthetic preservatives. However, the practical applications of essential oils in broad-scale industrial sectors have some limitations due to their volatility, less solubility, hydrophobic nature, and easy oxidation in environmental conditions. Implementation of nanotechnology for efficient incorporation of essential oils into polymeric matrices is an emerging and novel strategy to extend its applicability by controlled release and to overcome its major limitations. Moreover, different nano-engineered structures (nanoemulsion, suspension, colloidal dispersion, and nanoparticles) developed by applying a variety of nanoencapsulation processes improved essential oil efficacy along with targeted delivery, maintaining the characteristics of food ingredients. Nanoemulsion-based edible coating of essential oils in fruits poses an innovative green alternative against fungal infestation and mycotoxin contamination. Encapsulation-based coating of essential oils also improves antifungal, antimycotoxigenic, and antioxidant properties, a prerequisite for long-term enhancement of fruit shelf life. Furthermore, emulsion-based coating of essential oil is also efficient in the protection of physicochemical characteristics, *viz*., firmness, titrable acidity, pH, weight loss, respiration rate, and total phenolic contents, along with maintenance of organoleptic attributes and nutritional qualities of stored fruits. Based on this scenario, the present article deals with the advancement in nanoencapsulation-based edible coating of essential oil with efficient utilization as a novel safe green preservative and develops a green insight into sustainable protection of fruits against fungal- and mycotoxin-mediated quality deterioration.

Keywords: essential oil, nanoencapsulation, antifungal, antimycotoxigenic, preservative

INTRODUCTION

In the busy lifestyle of the current generation, consumers have become increasingly focused on healthy foods, especially on fresh fruits because they are rich sources of micronutrients, minerals, and bioactive constituents, which provide maximum health benefits such as antioxidant, antidiabetic, anti-inflammatory, and anti-allergic activities (Prakash et al., 2018). Fruits are also regarded as protective foods, suggested to be incorporated into the human daily diet due to the presence of vitamins, proteins, and trace elements such as manganese, copper, and zinc, which actively participate in the metabolic functioning of the body (Jahan et al., 2011). During storage, the quality of fruits deteriorates, resulting in unexpected flavors, softening of the outer surface, browning, water loss, and breakdown of surface textures. Furthermore, storage conditions also facilitate the infestation of fungal flora and secretion of mycotoxin, which is a major problem especially affecting the fruits' nutritional properties (Pessu et al., 2011). Consequently, mycotoxin secretion reinforces the production of reactive oxygen species, which could lead to lipid peroxidation (Kalagatur et al., 2018) and the ultimate shortening of the shelf life of fruits along with subsequent reduction in consumer acceptance. In addition to nutritional loss, postharvest fungal contamination may collapse the fruits' market value by increasing transport and storage costs. Synthetic preservatives or chemical disinfectants are commonly employed to combat fungal spoilage and mycotoxin contamination of fruits. Disinfectants containing chlorine and hypochlorite are not very effective in reducing fungal proliferation; however, their excessive application may cause skin irritation, respiratory and gastrointestinal problems (Issa-Zacharia et al., 2010). Moreover, ozone, peroxyacetic acid, organic acid, and hydrogen sulfide are not able to achieve maximum inhibition and also possess potential side effects and toxicity (Ramos et al., 2013). Essential oils, a major group of higher plant secondary metabolites, allow for the retardation of fungal spoilage and mycotoxin contamination and eventually help in the replacement of synthetic antimicrobials (Das et al., 2021a). However, the practical application of essential oils as a fumigant has several limitations such as easy volatility, rapid biodegradability, less solubility, less stability, and negative effect on organoleptic properties (Barradas and de Holanda e Silva, 2021). Hence, there is a pressing need for control delivery of essential oil components, which can be processed through a novel nanoencapsulation technology. Nanoencapsulation simply demonstrates the entrapment of essential oils into a carrier matrix providing stability and also protection from external fluctuating environmental conditions, leading to improvement in biological efficacy (Das et al., 2021a). Proteins, polysaccharides, and lipids are the most commonly used encapsulating matrix for efficient imprisoning of essential oils, having the qualities of high water solubility, biodegradability, and easy availability, which are obtained from plants (starch, cellulose, and gluten), animals (dextrin, chitosan, and casein), and marine and microbial sources (Martín et al., 2010; Vishwakarma et al., 2016). Various encapsulation techniques such as ionic gelation, coacervation, liposome, nanoemulsion, nanoprecipitation, and drying processes are commonly employed for encapsulation of essential oil and bioactive compounds to enhance their bioefficacy (Ezhilarasi et al., 2013). Currently, modern food scientists have suggested the implementation of nanoemulsion-based edible coating of essential oils around various stored food products for controlling food physicochemical characteristics such as respiration, firmness, color, and fungal infestation (Hasan and Nicolai, 2014). Edible coating constitutes a thin layer after dipping the food materials into an essential oil-based emulsion system which provides an extra protection similar to modified atmospheric packaging (Maringgal et al., 2020). Nanoformulation-based edible coating opens a new window for application of essential oils in protection of fruits, especially during postharvest storage conditions. Among different synthesized formulations of essential oils, nanoemulsion is an innovative approach for enhancement in quality attributes of fruits by maintaining their flavor, color, and antioxidant properties (Hasan et al., 2020).

A very limited number of studies have been demonstrated for nanoemulsion-based coating of essential oils for protection against fungal and mycotoxin contamination and for maintenance of the physicochemical properties of fruits. On the basis of this consideration, the present review has focused on updated information regarding nanoformulation-based edible coating of essential oil on fruits to improve bioefficacy, particularly focusing on the mechanism of action, improvement in antioxidant activity, sensory attributes, and possibility for future industrial implementation as novel preservatives.

FUNGAL AND MYCOTOXIN CONTAMINATION OF STORED FRUITS

Fruits are recognized as a prime substrate for fungal spoilage in postharvest conditions. The minimally processed fruits are more prone to fungal proliferation due to different processing such as peeling, slicing, and cutting, which expose the surface and release some nutritive constituents, leading to maximum contamination (Rojas-Graü et al., 2007; Siroli et al., 2015). Species of *Aspergillus*, *Penicillium*, and *Alternaria* are major fungal flora infesting the surface of fruits and produce some host-specific mycotoxins causing effective biodeterioration of nutritional quality, which has added a new dimension of postharvest problem for fruits (Soliman et al., 2015). Occurrence of different fungal flora in fruits depends on the water activity; for example, when the water activity is higher than 0.9, there is more chances of *Fusarium* infestation, whereas, at maturity, the fruits become dehydrated with prevalent occurrence of Aspergilli (Magan and Aldred, 2007; Sanzani et al., 2016). In storage conditions, species of *Alternaria* and *Penicillium* are of great concern, causing maximum spoilage of apples by decay and mycotoxin contamination (Doores and Splittstoesser, 1983; Ostry et al., 2004). Patulin is a common mycotoxin in apples during storage produced by *Penicillium expansum*. Association of patulin was observed at the cracked

surfaces of apples, and its limit often exceeded 50 ppb after postharvest managements (Ostry et al., 2004). Barkai-Golan and Paster (2008) reported *Aspergillus flavus* and *Aspergillus parasiticus* infestation in grape fruits along with production of ochratoxin A and aflatoxin, leading to the deterioration of organoleptic and physicochemical properties. Senyuva et al. (2007) described the occurrence of aflatoxins in dried figs (collected from Turkey) due to poor postharvest preservation processes. Ahmed and Robinson (1997) demonstrated rising contamination of dates collected from the United Arab Emirates by aflatoxin B_1 and G_1 during the storage periods. Higher relative humidity (66–93%) has been pointed out as a major factor for *A. parasiticus* infestation and aflatoxin contamination in the storage conditions. *Alternaria* is a commonly occurring storage fungal flora, basically contaminating stored fruits by production of altertoxin, alternuene, and tenuazonic acid (Scott, 2004). A number of alternaria toxins such as alternariol monomethyl ether, alternariol, and alternuene have been shown to contaminate oranges, apples, melons, and lemons. Scott and Kanhere (2001) reported the level of tenuazonic acid and alternariol in between 500 and 58,800 ng/g in apples. Delgado and Gómez-Cordovés (1998) and Lau et al. (2003) presented the contamination of alternariol up to 6 ng/g in raspberry, grapes, and apple juice due to the existence of alternariol during improper postharvest storage practices. Elamin and Sakuda (2021) reported the contamination of *Ziziphus jujuba* var *spinosa* fruit by *A. flavus* and AFB_1 in storage, especially during the ripening stage. Infestation of *Fusarium proliferatum* on banana fruits leading to excessive contamination with fumonisin B_1 and deterioration of fruit quality (due to the decreasing effect of chitinase, β-1,3-glucanase, and phenylalanine ammonia lyase) has been recently demonstrated by Xie et al. (2021). Li et al. (2020) reported the occurrence and co-occurrence of alternariol, altenuene, tenuazonic acid, and tentoxin in rotten apples. Moreover, the synergistic, antagonistic, and additive effects of different mycotoxins determine the severity of quality deterioration in apples. Considerable information has been reported for ochratoxin A contamination in grape fruits. Higher moisture content during postharvest preservation influences the infestation of *Aspergillus carbonarius* with resultant production of ochratoxin A (Wagacha and Muthomi, 2008). Wang et al. (2018) reported the contamination of different mycotoxins such as ochratoxins, aflatoxins, alternariol, tentoxin, patulin, zearalenone, T2 toxin, and trichothecenes from 10 kinds of dried fruits and nuts (chestnut, hazelnuts, almonds, figs, pine nuts, jujubes, longans, raisins, walnuts, and persimmons) with a contamination frequency of 124/253 (level of quantification < 473.16 μg/kg). Occurrences of multi-mycotoxins, *viz.*, ochratoxins, aflatoxins, patulin, trichothecenes, and alternaria toxins, in 104 different fruit samples (21 pears, 28 melons, 30 grapes, and 25 jujubes) collected from Xinjiang regions of China have been investigated, and the level of contamination was determined through ultra-performance liquid chromatography coupled to ion mobility quadrupole time-of-flight mass spectrometry with a level of detection and level of quantification at 0.06–2.22 and 0.2–7.39 μg/kg, respectively (Fan et al., 2022).

FACTORS AFFECTING FUNGAL SPOILAGE AND MYCOTOXIN SECRETION IN FRUITS

Fungal colonization and mycotoxin production depend on the variety of intrinsic and extrinsic factors in fruits. Intrinsic factors mainly include the initial factors contributing to contamination in postharvest conditions such as pH, water activity, nutritional status, and texture of the fruits. However, relative humidity, storage temperature, and atmospheric compositions are recognized as major extrinsic factors responsible for fungal spoilage and mycotoxin contamination.

Growth of storage fungi is markedly influenced by water activity (a_w), which correlates its interaction with substrates. An investigation of Romero et al. (2007) described the maximum growth of *A. carbonarius* at a_w 0.95 and produced ochratoxin. Bellı et al. (2004) reported the highest growth rate of 9.11 mm/day at 0.98 a_w and the slowest growth rate at 0.88 a_w for *Aspergillus* section Nigri. Mitchell et al. (2004) also agreed with between a_w 0.95–0.99 maximum contamination of *A. carbonarius* in grapes. Esteban et al. (2006) demonstrated the highest ochratoxin production by *Aspergillus ochraceous* at 0.96–0.99 a_w. More importantly, the *in vitro* studies illustrated the variation in a_w levels at initial and later stages in different growth media such as Yeast Extract Agar (YEA) and Czapek yeast extract agar (CYA) with variation in ochratoxin production. Mannaa and Kim (2017) reported infestation of three different types of fungal species based on the a_w level during initial, middle, and last phases of storage. Hydrophilic fungal species were firstly dominated followed by mesophilic and xerophilic fungal species. The optimum a_w for growth and proliferation of xerophiles, mesophiles, and hydrophiles has been recognized as 0.95, 0.95–1.00, and 1.00, respectively.

Low pH of fruits during storage particularly facilitates the infestation of molds and yeasts, causing maximum spoilage, because most of the bacteria are eliminated as they prefer a near-neutral pH (Zhao et al., 2020).

Temperature plays an important role in fungal growth and mycotoxin production during storage conditions. In a study of Hill et al. (1983), maximum contamination of *A. flavus* by growth and sporulation at 35°C has been demonstrated, while aflatoxin production occurred even at 33°C. Klich et al. (2009) suggested the variation in aflatoxin level between 24 and 30°C depending on the substrate and potentiality of the strain. During storage, high moisture content also facilitates the production of fumonisin B_1 by different species of *Fusarium*, and the optimum temperature for synthesis of this mycotoxin was reported as 15–30°C. In addition to water activity, pH, temperature, solute concentration, time, atmospheric conditions, inoculum density, and potential are major factors for maximum contamination along with the production of mycotoxins. More importantly, mycotoxins are synthesized by the toxigenic fungal species as a response to environmental stress conditions (da Cruz Cabral et al., 2013). Recent investigation suggested optimum growth conditions, storage temperatures, and substrate properties as prime factors for excessive proliferation and sporulation of fungal species in fruits (Marín et al., 2021). Hussein et al. (2020) reported

the ability of *A. flavus*, *A. niger*, and *Penicillium citrinum* for production of pectinase enzyme, leading to quality deterioration of strawberries. The highest level of pectinase activity was reported at pH 8.0, while at more acidity and alkalinity, pectinase production was considerably reduced. Osmotic pressure of the substrate is another important factor responsible for fungal proliferation and mycotoxin secretion (Daou et al., 2021). Most notably, foods (especially fruits) containing maximum sugar provide a suitable podium for fungal invasion with the ability to hydrolyze it and support the metabolic activities (Hamad et al., 2014). Liu et al. (2016) illustrated that an increment in concentration of soluble sugars such as maltose, sucrose, and glucose from 3.0 to 6.0% promoted AFB_1 production in foods.

ESSENTIAL OIL FOR PRESERVATION PURPOSES

Properties

A number of synthetic fungicides have been employed for inhibition of fungal spoilage and mycotoxin secretion in fruits. However, the emergence of resistant fungal races, limitations of government approval for continuously effective fungicides, and huge public concern regarding the hazardous effects of synthetic fungicides on human health and environmental non-sustainability have increased the attention for their sufficient replacement with non-chemical methods. Essential oils are a complex mixture of 20–60 different phenolic and terpenoid components and isolated from a number of aromatic plants (Basak and Guha, 2018). They are critically synthesized in plants as defense regulators and can also exert antibacterial, antifungal, antimutagenic, immunomodulatory, anti-inflammatory, and antioxidant activities (Bakkali et al., 2008). Essential oils have been used in agriculture, health, food, and cosmetic industries with widescale acceptability. The composition of essential oils widely differs among different plant parts, phonological stages, climatic factors, edaphic factors, seasons, light intensity, photoperiods, and extraction methods (Mazzarrino et al., 2015). *In vitro* studies pointed out that terpene as a single component is less efficient for fungal inhibition, while terpenoids are active against a number of food-contaminating microorganisms (Hyldgaard et al., 2012).

Fabrication of Essential Oil-Based Nanoformulation and Their Physicochemical Characterization

Essential oils are highly volatile, less water soluble, and easily oxidized in direct environmental conditions; therefore, it is hardly possible to maintain the fruit quality by inhibition of fungal infestation and mycotoxin contamination and their nutritional values during practical application in fruits. Moreover, the direct interaction of essential oils with fruit surfaces may cause alteration in the sensory properties of fruit, which could lead to consumer unacceptability in commercial markets (Prakash et al., 2018). In this context, nanoencapsulation of essential oil into biodegradable and biocompatible polymers strengthens the practical efficacy and helps in the controlled release of essential oil, which will lead to the enhancement of the shelf life of fruits (Mohammadi et al., 2015; Ansarifar and Moradinezhad, 2021). Different strategies have been applied regarding nanoencapsulation of essential oils in food industries, such as nanoemulsion, nanogels, nanocapsules, and nanoparticles with subcellular-size particles (de Matos et al., 2019). A number of proteins and carbohydrate polymers are capable of encapsulating essential oil through a cross-linking interaction and nanostructural compaction (Das et al., 2021b). However, the utmost criteria for the selection of biopolymer as an encapsulating agent are its inclusion under the Generally Recognized As Safe (GRAS) category with a biodegradable and environmentally friendly nature (Adeyeye et al., 2019). Three different types of biopolymers, *viz.*, polypeptides, polynucleotide, and polysaccharides, are broadly used with paramount significance to develop a variety of encapsulated materials (Rehman et al., 2020). Among different biopolymers, polysaccharides, especially chitosan, alginate, starch, and pectin, are recognized as excellent bioadhesive polymers and capable of binding with essential oils and have controlled- as well as targeted-release property (Martău et al., 2019).

Majority of the studies represent nanoemulsion as a suitable strategy for coating of fruits and enhancing their nutritional qualities (Al-Tayyar et al., 2020). Nanoemulsion is developed by two immiscible liquids by incorporation of the dispersion phase of essential oils into dispersed media of water especially synthesized as an oil-in-water emulsion (Espitia et al., 2019). The size of essential oil droplets in the emulsion ranges between 10 and 1,000 nm. Owing to small-size droplets, nanoemulsion can act as a suitable delivery vehicle with a targeted mechanism of action. Bioavailability of essential oil is increased by the emulsion system of encapsulation due to the greater surface-to-volume ratio and subcellular-size particles (Das et al., 2020b). Moreover, the essential oil-based nanoemulsion is a stable system due to increased gravitational separation and Brownian motion (Gharenaghadeh et al., 2017). The oil phase of nanoemulsion was prepared by dissolving essential oil into lipophilic components such as Tween-20, Tween-80, and dichloromethane. Two different methods, *viz.*, high energy and low energy, have been applied for synthesis of essential oil-loaded nanoemulsion. High-energy methods are constituted with intensive disruptive forces for breakdown of large particles into subcellular-size particles by high-pressure homogenizers, sonicators, and microfluidizers. Low-energy methods involve the spontaneous as well as phase inversion methods without involvement of high-speed homogenization by the help of an oil–water–emulsifier system (Sugumar et al., 2016a).

After synthesis of essential oil-loaded nanoemulsion, physicochemical characterizations of the prepared nanoemulsion are performed through dynamic light scattering (DLS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), atomic force microscopy (AFM), differential scanning colorimetry (DSC), and thermogravimetric analysis (TGA). A DLS study simply measures the size of particles in nanoscale regimes (Das et al., 2021c). SEM analysis confirms the nanometric size of particles

with a greater surface-to-volume ratio and smooth surface with somewhat agglomeration at some places (Hasheminejad et al., 2019). It has also been reported that the size of nanoparticles was higher in the DLS technique as compared to SEM, which has been associated with aggregation and swilling of particles during dispersion in water (Esmaeili and Asgari, 2015). XRD analysis confirms the destruction of crystallinity after incorporation of the essential oil into the matrix polymer, leading to the development of amorphous structures of nanoparticles (Hadidi et al., 2020). In order to understand the molecular interaction between the matrix polymer and essential oils, FTIR is considered as an important parameter. Changing peak intensity along with variation in wave number suggests proper entrapment of essential oil into the biopolymer matrix (Yilmaz et al., 2019). In addition to size and shapes, AFM is also indicative of surface morphology, showing three-dimensional structures of essential oil-loaded nanoparticles and nanocomposites (Anand et al., 2021). DSC is a common method for examining the thermal sensitivity/stability and formation of solid-state complexes after proper encompassment of essential oils into the biopolymer matrix (Hadidi et al., 2020). Mass loss of nanoparticles with a maximum degradation rate at different steps has been observed in TGA thermogram. Decomposition of nanoparticles/nanocomplexes/nanocomposites at higher temperatures as compared to their free forms indicated the thermal stability of essential oils after proper encapsulation (Hadidi et al., 2020). For instance, to measure the nanometric size of linalool-loaded chitosan nanoemulsion composite, a DLS-based Zeta sizer, SEM, and AFM have been used (Das et al., 2021a). Liu and Liu (2020) measured the stability of chitosan nanoemulsion loaded with thymol and thyme essential oil by TGA. Thermal stability has been pointed out as an important property of nanoemulsion for food preservation because of the direct relationship between temperature and manufacturer's processing during practical applications. The characteristic thermogram peak in DSC also revealed proper entrapment of oregano essential oil into the chitosan biopolymer through the electrospraying method. Wu et al. (2016) presented the nano-range size of emulsion particles in edible coating of citrus essential oil-loaded nanoemulsion by SEM and TEM analyses.

Procedure for Edible Coating of Essential Oil Nanoemulsion in Fruits

After preparation of essential oil nanoemulsion-based coating dispersion, the fruits are well mixed with the solution to attain homogeneous coating around the surface. Four different procedures, *viz*., dipping, brushing, spraying, and film hydration methods, are applied for successful coating of fruits (Ju et al., 2019). In the case of the dipping technique, the fruits are directly immersed into the coating dispersion for 2–4 min followed by drying for a certain period of time (Chaudhary et al., 2020). This technique usually forms high-thickness coating, maintaining the viscosity, density, and surface tension of the coating solution. Tharanathan (2003) applied the foam method for fruit coating with uniform distribution and repeated rolling action. Mastromatteo et al. (2012) developed coating of carrot by sodium alginate and sodium hydroxide and extended the shelf life by 7 days. In the spreading process, the coating dispersion is directly brushed on the surface of the fruit to form a uniform layer (Chaudhary et al., 2020). The spraying technique includes low-viscosity homogeneous coating of fruits by using high pressure. In the case of the film hydration method, essential oil-loaded emulsion is mixed with organic solvent followed by evaporation of the solvent through a rotatory evaporator and finally hydration by the addition of an appropriate buffer. This is a basic method of coating with high encapsulation efficiency. Alikhani-Koupaei (2015) demonstrated lemon essential oil-loaded liposome coating on spinach to maintain nutritional qualities. **Figure 1** presents essential oil nanoemulsion coating of fruits by different methods and their practical application for fruit quality maintenance.

ESSENTIAL OIL-BASED EDIBLE COATING ON FRUITS AGAINST FUNGAL INFESTATION AND MYCOTOXIN CONTAMINATION

Judgment of fruit quality has been done through freshness as well as external appearance at the time of purchase. Minimal processing can alter the integrity of fruits, resulting in a negative impact on fruit quality like browning, breakdown of texture, and off-flavor that may lead to fungal occurrence, compromising the safety of the fruits. Emulsion-based edible coating has shown promising effects to maintain the physicochemical qualities and nutritional behaviors of fruits during postharvest storage. **Table 1** presents the essential oil-based nanoemulsion edible coating of different fruits by using different coating wall polymers and their effects against microbial contamination.

Pear (*Pyrus malus* L.) is an economically important fruit in temperate zones of the world (Silva et al., 2014). The fruits maintain acid balance in the human body and are a prime source of pectin (Dave et al., 2017). During the postharvest period, rapid softening and senescence of the outer tissue with resultant fungal proliferation pose a serious problem for marketing. Gago et al. (2020) investigated the effect of alginate-based (2% w/w) nanoemulsion incorporated with lemongrass essential oil (1.25% w/w) for the preservation of Rocha pear (at 22°C and 70% relative humidity for 7 days), maintaining the fruit's color, soluble solid contents, titrable acidity, and firmness. More importantly, the nanoemulsion retarded the ripening process of fruits and exhibited a positive impact on fruit qualities.

Papaya (*Carica papaya*) is a common fruit and has been reported to possess high amounts of carotenoids, calcium, and ascorbic acid (Zillo et al., 2018). Papaya has broad nutritional and health beneficial effects, including prevention of arteriosclerosis, cancer, and heart problems (Ali et al., 2011). Due to its climacteric nature, the fruit has a very short postharvest life and is generally susceptible to fungal infestation, increased softening of pulp, and excessive accumulation of sugar and water (González-Aguilar et al., 2009). Generally, papaya is consumed after peeling, seeding, and cutting, which induce the respiration rate, leading to biochemical changes and desiccation

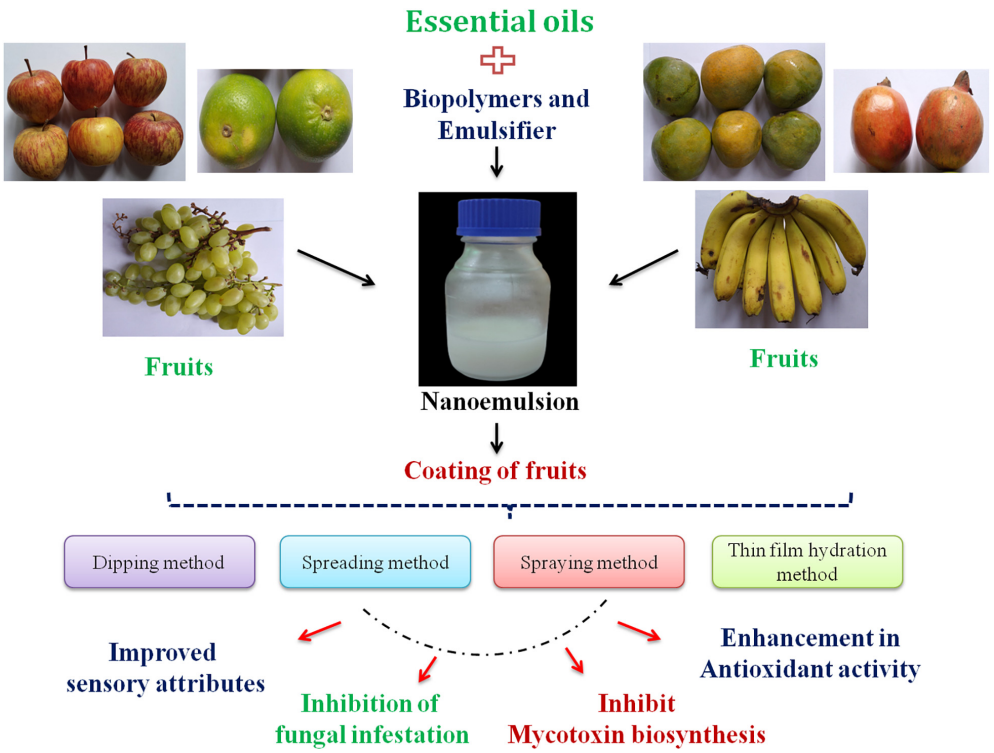

FIGURE 1 | Essential oil nanoemulsion coating of fruits by different methods and their practical application for fruit quality maintenance.

causing effective fungal spoilage. Essential oil nanoemulsion-based edible coating provides a primary packaging to papaya which effectively improves its internal atmosphere and control the moisture and gas content of the fruit. Tabassum and Khan (2020) studied the impact of alginate-mediated (2% w/v) edible coating incorporating *Thymus vulgaris* and *Origanum vulgare* essential oil (0.5, 1.0, and 2.0%) nanoemulsion in papaya on preservation against molds and yeast spoilage over a period of 12 days at 4°C. Yeast and mold counts of uncoated fruit samples reached up to 5.90–8.40 CFU/g, which was much higher than the consumable limit.

Strawberries are one of the economically important fruits and recognized worldwide due to its desirable flavor and taste, with rich bioactive components such as vitamin E, vitamin C, anthocyanin, and β-carotene providing a number of health benefits (Van De Velde et al., 2013). However, the perishability and high respiration rate lead to rapid senescence and reduce the shelf life of fruits. Moreover, the mechanical injuries cause infestation of several mold species, culminating into effective changes in firmness, color, and quality of fruits (Treviño-Garza et al., 2015). Chu et al. (2020) synthesized the pullulan coating (2% w/v) by incorporation of cinnamon essential oil nanoemulsion for strawberry fruits and studied its effect against mold and yeast contamination. The results indicated a reduction in decay percentage as compared to control during 6 days of storage period at 20 ± 2°C. In the case of control and pure pullulan-coated fruits, decay has started after 2 days, while the decay percentage reached up to 65% within 6 days. The essential oil-based pullulan coating of fruits reduced decay percentage by 30% after 6 days, suggesting the excellent antifungal activity of the essential oil nanoemulsion due to cinnamaldehyde as the major component, and inhibited fungal growth by membrane disintegration. The pullulan-incorporated cinnamon essential oil nanoemulsion coating retarded the fungal growth from 10^4 to 1.958 CFU/g, which has been associated with the nanometric size of essential oil droplets with increased surface area and better membrane accessibility. Similar to the previous investigation, Robledo et al. (2018) demonstrated the effectiveness of thymol nanoemulsion-based (1:5 w/v) edible coating of strawberry fruits by using chitosan and quinoa protein as a wall matrix. During the whole course of storage periods, thymol nanoemulsion-mediated packaging maintained a lower yeast and mold count as compared to control (uncoated fruits) up to 10 days of storage. Fruits only coated with quinoa protein and chitosan showed lower effectiveness, which could have been due to the interaction of chitosan and quinoa protein. The greater mold and yeast inhibitory effects of thymol nanoemulsion have been associated with the controlled release of thymol from the biopolymer. Moreover, thymol nanoemulsion coating reduced the fungal decay of fruits by 41.7% after 16 days. The author demonstrated that chitosan and quinoa protein are the best coating material for synthesis of edible coating in strawberry fruits, with great reduction capability of fungal contamination.

Blueberries are considered as "super food" due to the presence of different beneficial and bioactive compounds facilitating healthy growth and development. The fruits are highly

TABLE 1 | Essential oil-based nanoemulsion edible coating of different fruits by using different coating wall polymer and their effects against microbial contamination.

Type of coating	Essential oils	Fruits	Application methods	Major findings	References
Chitosan	Mentha piperita and Mentha villosa	Grape	Dipping	Significant efficacy against phytopathogenic fungi	Guerra et al., 2016
	Lemongrass essential oil			Effectively inhibit growth of yeast	Oh et al., 2017
	Bergamot essential oil			Maintained the quality of grapes	Sánchez-González et al., 2011
Hsian-tsao leaf gum and tapioca starch	Cinnamon essential oil	Apple	Dipping and spraying	Delay in browning	Pan et al., 2013
Gellan and alginate	Rosemary and cinnamon essential oil			Effective inhibition of microbial growth	Rojas-Argudo et al., 2009; Chiabrando and Giacalone, 2015
Sodium alginate and Tween-80	Lemongrass			Effective inhibition of microbial growth	Salvia-Trujillo et al., 2015
Carrageenan and alginate	Lemongrass essential oil	Pine apple	Spreading	Active against microbial deterioration and also maintain sensory attributes	Azarakhsh et al., 2014
Flaxseed gum	Lemongrass essential oil	Pomegranate aril	Dipping	Inhibition of yeast and mold growth	Yousuf and Srivastava, 2017
Starch	Nigella sativa			Reduced loss of anthocyanin and vitamins	Oz and Ulukanli, 2012
Basil seed gum	Origanum vulgare	Apricot	Spraying	Inhibition of yeast growth and facilitation of shelf life extension	Hashemi et al., 2017
Chitosan	Lemon essential oil	Strawberry	Dipping	Reduction in respiration rate during storage and inhibition of fungal growth	Perdones et al., 2012
Carnauba wax and Tween-80	Lemongrass	Plums	Spreading	Inhibition of microbial growth	Kim et al., 2013
Polysorbate 20/sodium lauryl sulfate	Thymol	Blueberry	Dipping	Inhibition of foodborne biofilm	Li et al., 2017
Chitosan and cassava starch	Myrcia ovata	Mangaba	Dipping, spraying, and spreading	Inhibition of foodborne microbe during storage	Frazão et al., 2017
Alginate and pectin	Lemon essential oil	Raspberries	–	Coating maintained raspberry color and reduced weight loss	Guerreiro et al., 2015
Rhamnolipid and sodium carbonate	Cinnamon essential oil	Cherry	Dipping	Inhibit growth of microorganism and lipid peroxidation	Xu, 2016

susceptible to fungal association, *viz.*, species of *Penicillium*, *Alternaria*, *Cladosporium*, *Botrytis*, and *Fusarium* and their associated mycotoxin contamination (Stinson et al., 1980; Tournas and Katsoudas, 2005; Munitz et al., 2013). In a study of Umagiliyage et al. (2017), D-limonene (10–50 μM) encapsulated into liposome showed prominent inhibition of fungal decay (7.5%) over a period of 63 days. Moreover, the phospholipid layer around D-limonene in the liposome system restricted the degradation and facilitated controlled volatilization with targeted delivery and better action.

Citrus fruits are severely infested by different storage fungi such as *P. expansum*, *Penicillium digitatum*, and *A. flavus*, leading to 90% of postharvest loss. Edible coating of essential oils and their component-assisted nanoemulsion with high water solubility developed a potential strategy for inhibition of fungal proliferation by preventing degradation of active constituents along with improved bioavailability. Yang et al. (2021) reported the antifungal effect of stable nanoemulsion containing carvacrol, eugenol, and cinnamaldehyde (0.0078–0.5 mg/ml) against *P. digitatum* with a resultant increase in fruit shelf life. Results represented that the fungal decay rate was reduced to 4.1% after nanoemulsion coating of fruits as compared to control groups (7.4%) after 60 days of storage. Nanoemulsion coating also reduced the weight loss from 4.12 to 3.14%, which has been associated with the nano-range size with greater surface-to-volume ratio and better fungal inhibitory efficacy.

Grapes are rich source of phenolic compounds possessing antioxidant activities and play an active role against cardiovascular and neurodegenerative diseases (Sun et al., 2010; Mattioli et al., 2020). During postharvest storage, table grapes are infested by a number of mold species such as *A. niger*, *Botrytis cinerea*, *Rhizopus stolonifer*, and *P. expansum*, leading to loss in fruit quality. A recent investigation of Guerra et al. (2016) suggested the significant protection of table grapes against *B. cinerea*, *R. stolonifer*, and *P. expansum* (32–38% of fruit was infected) by chitosan-incorporated edible coating of *M. piperita* and *Mentha villosa* essential oils (1.25, 2.5, and 5.0 μl/ml).

ANTIFUNGAL AND ANTIMYCOTOXIGENIC MECHANISM OF ACTION

As essential oils are mixtures of variable bioactive components, a number of target sites have been demonstrated for antifungal and antimycotoxigenic activity by different researchers. Moreover, hydrophobic essential oils easily traverse the lipid bilayer of the plasma membrane and affect the synthesis of ergosterol

along with enhanced efflux of vital cellular ions, leading to disintegration of membrane stability and permeability. The lipophilic nature of essential oils also allows disruption of the cell wall compatibility by targeting polysaccharide back bones (Rammanee and Hongpattarakere, 2011). Improvement in antifungal activity of essential oils in a nanoemulsion system has been associated with increased solubility and changes in permeability of the plasma membrane by modification of the cellular pH and affected osmotic pressure (Chaudhari et al., 2021). Manso et al. (2013) reported the disruption in hyphal structure and conidial wall of *A. flavus* after fumigation with cinnamon essential oil (0.1 mg/ml). Abd-Elsalam and Khokhlov (2015) reported antifungal activity of nanoencapsulated eugenol (2%) against *Fusarium oxysporum* due to the inhibition of conidial germination. Long et al. (2020) investigated the effect of garlic essential oil nanoemulsion (3.7%) against the growth of *Penicillium italicum* due to the penetration of active components and interaction with fungal cellular enzyme, culminating in cell death. A little increment in extracellular conductivity after fumigation with garlic essential oil nanoemulsion has been linked with the inhibition of spore germination. A recent investigation of Das et al. (2021c) suggested dose-dependent retardation of cellular methylglyoxal by eugenol-loaded chitosan nanoemulsion (0.07 μl/ml), leading to the inhibition of cellular aflatoxin biosynthesis in *A. flavus*. Wan et al. (2019) reported a remarkable negative impact of peppermint (2.5–25 mg/g), thyme, cinnamon, clove, and lemongrass essential oil (2–10 mg/g) nanoemulsion on the growth of *Fusarium* sp. and production of deoxynivalenol, 15-acetyldeoxynivalenol, and 3-acetyldeoxynivalenol based on changes in fungal metabolic activities. Hu et al. (2017) demonstrated an antiaflatoxigenic mechanism of action of *Curcuma longa* essential oil due to the downregulation of five structural genes, *viz*., Afl O, Afl M, Afl D, Afl P, and Afl Q, in *A. flavus*. El Khoury et al. (2016) pointed out the inhibition of ochratoxin A biosynthesis by essential oils (1.0 and 5.0 μl/ml) due to downregulation of polyketide synthase, acpks, and acOTAnrps genes in fungal cells.

Recently, an *in silico* modeling study also revealed the binding interaction of essential oil components with mycotoxin-biosynthesizing proteins, demonstrating a new horizon for molecular target sites of action. The *in silico* interaction of α-pinene, apiol, elemicin, *p*-cymene, and fenchone with the ergosterol-biosynthesizing protein lanosterol-14α-demethylase and the aflatoxin-producing proteins polyketide synthase and Ver-1 suggested a molecular target site for antifungal and antiaflatoxigenic activities (Das et al., 2021d). Pani et al. (2016) suggested a better understanding of the structure–activity relationship of phenolic components isolated from higher plants with the trichothecene biosynthetic gene trichodiene synthase TR 15 by molecular docking for the inhibition of trichothecene production in *Fusarium culmorum*. *In silico* molecular interaction of phytochemicals, *viz*., hexanoic acid and quercetin, with seven different domains of polyketide synthase for the inhibition of aflatoxin biosynthesis in *A. flavus* has been recently investigated by Tiwari et al. (2019). Conclusively, better elucidation of complex molecular interactions of essential oil components and target proteins could provide a new basis for synthesizing novel green antifungal components. **Table 2** represents the antifungal and antimycotoxigenic mechanisms of action of essential oil-loaded nanoemulsion.

IMPACT OF EDIBLE COATING ON ANTIOXIDANTS OF FRUITS

Fruits are good sources of antioxidant due to the presence of higher amounts of phenolic constituents and anthocyanins. Phenolic components provide the sensory and nutritional properties of many fruits. During the storage period, the oxidation of phenolic components by polyphenol oxidase is evidenced by dark-colored pigments, and the overall antioxidant activity is reduced (Orak, 2007). Moreover, the subsequent degradation and decline in phenolic content are also associated with cell structure breakdown and progressive fruit senescence. Edible coating of essential oils has the potentiality to preserve flavonoids, phenolics, carotenoids, lycopenes, and glucosinolates by reducing fruit deterioration (Maringgal et al., 2020). Essential oils facilitate the reduction of gas exchange and inhibition of carbohydrate decomposition by retarding the respiration process. Essential oil-incorporated nanoemulsion of edible coating is an innovative strategy for controlled delivery by immobilization of droplets, allowing stability against aggregation and increased bioavailability on the surface of the fruits and significantly maintaining the antioxidant activity. The particle size of essential oil-based nanoemulsion has been found as an important index for regular distribution and homogeneous edible coating. Sometimes, the phenolic content of essential oil may induce the overall phenol level in fruits. Retention of phenolic components in fruits by essential oil nanoemulsion coating may also depend on the type and concentration of essential oils in nanoformulation. Additionally, an increase in cellular phenylalanine ammonia lyase activity may also induce the total phenolic content of fruits (Guerreiro et al., 2017).

Moreover, during postharvest storage, the toxic free radical molecules are accumulated, causing maximum loss of nutritional quality of fruits, whereas catalase, peroxidase, superoxide dismutase, and ascorbate peroxidase are important antioxidant defense enzymes that can easily scavenge the ROS and make the fruits fresh with good antioxidant ability. Yang et al. (2021) reported that carvacrol, eugenol, and cinnamaldehyde nanoemulsion-based (0.0078–0.5 mg/ml) coating of citrus fruits was effective in increasing catalase, peroxidase, and superoxide dismutase by 22.29, 51.49, and 18.12%, respectively, after a period of 40 days of storage.

Antioxidant activity in fruits is easily measured through inhibition of the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical. Edible coating may act as an effective barrier against oxygen uptake and significantly reduce antioxidative agents (Bonilla et al., 2012). Essential oil-based nanoemulsion also provide pronounced effects on antioxidant potency of fruits due to increased antioxidant compounds with greater ROS scavenging ability. Cenobio-Galindo et al. (2019) studied the effect of *Citrus sinensis* essential oil coating on total phenolics, flavonoids, and antioxidant activity of avocado fruits.

TABLE 2 | Antifungal and antimycotoxigenic mechanisms of action of essential oil/components-loaded nanoemulsion.

Essential oils/components nanoemulsion	Active against	Mechanism of action	References
Garlic oil	*Penicillium italicum*	Destruction of membrane permeability and stability	Long et al., 2020
Cinnamon oil	*Fusarium graminearum*	Loss of cytoplasmic constituents	Wu et al., 2019a
Cinnamon oil	*Rhizopus* sp.	Membrane disruption	Yousef et al., 2019
Myristica fragrans	*Aspergillus flavus*	Leakage of vital ions and UV-absorbing materials	Das et al., 2020a
Eugenol	*Fusarium oxysporum* f. sp. vasinfectum	Inhibition of conidial germination	Abd-Elsalam and Khokhlov, 2015
Eugenol, cinnamaldehyde, and carvacrol	*Penicillium digitatum*	Destruction of membrane permeability	Yang et al., 2021
Lemongrass and clove	*Fusarium oxysporum* f. sp. *lycopersici*	Destruction of membrane integrity	Sharma et al., 2018
Illicium verum	*Aspergillus flavus*	Leakage of Ca^{2+}, K^{+}, and Mg^{2+} ions	Dwivedy et al., 2018
Cleome viscosa	*Candida albicans*	Inhibition of cell wall biosynthesis	Krishnamoorthy et al., 2021
Citrus sinensis	*Saccharomyces cerevisiae*	Distortion in cellular morphology	Sugumar et al., 2016b
Zataria multiflora and Carum copticum	*Byssochlamys fulva*	Inhibition of mycelia growth	Sahraneshin Samani et al., 2019
Coriandrum sativum	*Aspergillus flavus* and *aflatoxin*	Disruption of membrane permeability and inhibition of methylglyoxal biosynthesis	Das et al., 2019
Origanum majorana	*Aspergillus flavus* and *aflatoxin*	Leakage of Ca^{2+}, K^{+}, and Mg^{2+} ions and inhibition of methylglyoxal biosynthesis	Chaudhari et al., 2020
Anethum graveolens	*Aspergillus flavus* and *aflatoxin*	Inhibition of spore germination	Das et al., 2021b
Clove, black seed, lemon and orange essential oil	*Alternaria tenuissima, Galactomyces candidum*, and *Fusarium solani*	Inhibition of conidial sporulation	Mossa et al., 2021
Cinnamomum zeylanicum	*Fusarium graminearum*	Inhibition of conidial germination	Wu et al., 2019b
Citrus reticulata	*Aspergillus niger* and *Zygosaccharomyces rouxii*	Inhibition of mycelial growth	Mahdi et al., 2021
Orange essential oil	*Aspergillus flavus*	Inhibition of mycelial growth	Kringel et al., 2021
Zataria multiflora and Bunium persicum	Yeasts and molds	Slowdown of spore germination, radial growth, and germ tube elongation	Keykhosravy et al., 2020
Limonin	*Penicillium italicum*	Inhibition of ergosterol biosynthesis	Li et al., 2021
Litsea cubeba	*Penicillium italicum* and *Penicillium digitatum*	Inhibition of mycelial growth	Wang et al., 2020

They designed the coating experiments on the basis of four different treatments, *viz*., concentrated nanoemulsion (CN), 50% diluted nanoemulsion (N50), 25% diluted nanoemulsion (N25), and the control set (C). Among different treatment sets, the highest phenolic (240.15 and 214.29 mg gallic acid equivalent/100 g) and flavonoid contents (47.77 and 48.18 mg quercetin equivalent/100 g) were recognized for N50 and N25 after 60 days of storage. Increment in DPPH and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant activity of avocado mesocarp was also exhibited for N50 and N25 treatments as compared to the control. Authors demonstrated the antioxidant activity maintenance of fruits due to controlled delivery of *C. sinensis* essential oil with greater surface-to-volume ratio in the nanoemulsion system.

IMPACT OF EDIBLE COATING ON PHYSICOCHEMICAL PARAMETERS AND SENSORY PROPERTIES OF FRUITS

Encapsulation of essential oil and their controlled as well as targeted delivery can maintain the chemical attributes of fruits during storage. The edible coating has a positive impact on fruit freshness without affecting total soluble solids (TSS), titrable acidity, and pH. Chu et al. (2020) represented significant maintenance of TSS by cinnamon essential oil-loaded pullulan coating in strawberry during storage. The stable nature of TSS after essential oil-based edible coating has been associated with considerable retardation of conversion of reducing sugar in strawberry. Moreover, the pullulan–cinnamon essential oil nanoemulsion slowed down the respiration rate and metabolic activities possibly related with the interactions of cinnamon essential oil and the plasma membrane of strawberry. Sometimes, the edible coating of fruits also induces the TSS value, which may be due to the water loss during the storage periods and rapid ripening process. Therefore, the decline or stable nature of TSS value in fruits after nanoemulsion coating suggests effective barrier properties against water loss. Decreasing firmness and consistent weight loss are major problems for the preservation of tomato fruits. The investigation of Athayde et al. (2016) suggested that weight loss of tomato by treatment with chitosan-incorporated *Cymbopogon citratus* essential oil coating was lower as compared to uncoated tomato fruits. Moreover, the coating of tomato fruits also facilitated increment of ascorbic acid, pH, soluble solid, and titrable acidity, leading to better acceptance of market value.

In spite of better preservation potentiality, the effect of essential oil-based edible coating on sensory properties of fruits is one of the major aspects that deal with the decision of consumers during purchase. As essential oils contain strong

aroma, any changes in flavor and taste of the fruits may obstruct its quality attributes resulting from interaction with components in fruit tissue. The recent investigation of Mohammadi et al. (2021) reported *Aloe vera* gel coating enriched with *Ocimum basillicum* essential oil to conserve the sensory properties of strawberry fruits. Treatment of fruits with *A. vera* gel alone and two different concentrations, *viz.*, 500 and 1,000 μl/L, of *O. basillicum* essential oil displayed the highest scores as compared to distilled water-treated and control fruit samples in the storage conditions. Saki et al. (2019) also revealed the decreasing sensory scores of fig fruits over 20 days of storage, whereas fig fruits fumigated with 200 mg/L thymol incorporated into 0.5% chitosan showed maximum overall acceptability (score 3.66) as compared to separate fumigation of 200 mg/L thymol (score 1.66) and 0.5% chitosan (score 2.00). They also demonstrated the improvement in sensory attributes due to lower water loss from the fruit surfaces and maximum balance between acids and sugars of fruits.

CONCLUSION AND FUTURE DIRECTION OF RESEARCH

The safety and quality of fruits are of increasing concern because contaminated fruits can act as a vehicle for transmission of hazardous diseases. Essential oils have been utilized as a green alternative of synthetic preservatives with proven antifungal and antimycotoxigenic activities. Encapsulation of essential oils into biodegradable and biocompatible polymers forming a nanoemulsion system improves their solubility, stability, and efficacy and minimizes fungal as well as mycotoxin contamination. Moreover, nanoemulsion-mediated edible coating of fruits further ensures the maintenance of antioxidant activities, physicochemical properties, and organoleptic attributes with resultant enhancement in shelf life.

Encapsulated essential oils in a nanoemulsion system offer a number of benefits for fruit preservation; however, several challenges have to be focused during commercial exploitation. For instance, the combinatorial action of essential oil and components in a synergistic nanoemulsion system, judicious selection of essential oils, and more importantly, the safety profile of essential oil nanoemulsion should be worked out for large-scale practical recommendation as a novel shelf-life enhancer for stored fruits.

AUTHOR CONTRIBUTIONS

SD gave the idea and concept of the review article and wrote the original manuscript. AG and AM performed the review of literature. All authors read and approved the manuscript.

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Essential Oil Nanoemulsion as Eco-Friendly and Safe Preservative: Bioefficacy Against Microbial Food Deterioration and Toxin Secretion, Mode of Action, and Future Opportunities

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Microbes are the biggest shareholder for the quantitative and qualitative deterioration of food commodities at different stages of production, transportation, and storage, along with the secretion of toxic secondary metabolites. Indiscriminate application of synthetic preservatives may develop resistance in microbial strains and associated complications in human health with broad-spectrum environmental non-sustainability. The application of essential oils (EOs) as a natural antimicrobial and their efficacy for the preservation of foods has been of present interest and growing consumer demand in the current generation. However, the loss in bioactivity of EOs from fluctuating environmental conditions is a major limitation during their practical application, which could be overcome by encapsulating them in a suitable biodegradable and biocompatible polymer matrix with enhancement to their efficacy and stability. Among different nanoencapsulated systems, nanoemulsions effectively contribute to the practical applications of EOs by expanding their dispersibility and foster their controlled delivery in food systems. In line with the above background, this review aims to present the practical application of nanoemulsions (a) by addressing their direct and indirect (EO nanoemulsion coating leading to active packaging) consistent support in a real food system, (b) biochemical actions related to antimicrobial mechanisms, (c) effectiveness of nanoemulsion as bio-nanosensor with large scale practical applicability, (d) critical evaluation of toxicity, safety, and regulatory issues, and (e) market demand of nanoemulsion in pharmaceuticals and nutraceuticals along with the current challenges and future opportunities.

Keywords: essential oil, biodeterioration, toxins, nanoemulsion, food preservative, eco-friendly

INTRODUCTION

Nowadays, global food safety is one of the major public health issues. Presently, most of the food products *viz*. bakery, dairy, meat, fruits, and vegetables are heavily contaminated due to the presence of various toxigenic species of bacteria and fungi and their associated toxins leading to severe human illness and deaths. Therefore, consumers are demanding healthy, high-quality, and safer food products. Moreover, in tropical and subtropical regions, contamination of food items by various microbes and their associated toxic metabolites not only deteriorates the food but can also alter the nutritional qualities (Barba et al., 2017; Chaudhari et al., 2021a). Consequently, consumption patterns are changing toward the intake of healthy food products with preserved nutritional values (Güneş and Turan, 2017). Therefore, special attention has been given to improve the quality, safety, and security of food systems against microbial spoilage and their associated toxins.

In order to control bacterial and fungal growth, various synthetic preservatives have been commonly applied. However, indiscriminate utilization of these preservatives may cause several negative perceptions in terms of their toxicity to non-target organisms, development of microbial resistance, and degradation of environmental sustainability (Calvo et al., 2017). Therefore, to ensure microbial food safety, contemporary consumers are demanding "safer alternatives" with a green image and possible non-toxic effects on humans and animals (Castro-Rosas et al., 2017; Das et al., 2021c). Currently, plant essential oils (EOs) and components are garnering more attention in the commercial food sector due to their unique aroma, flavors, and antimicrobial properties without affecting organoleptic and nutritional attributes of food items (Burt, 2004). EOs are secondary metabolites of aromatic plants and are considered under the 'generally recognized as safe' (GRAS) label by the US- FDA (Ju et al., 2018). The major implication of EOs for the preservation of food commodities along with other chemical components lies in the possible synergistic effect during long term preservation (Jiang et al., 2009). However, applications of EOs are still restricted due to intense aroma, low water solubility, and less stability in fluctuating environmental conditions such as temperature, light, and oxygen (Maurya et al., 2021; Singh et al., 2021). Moreover, some of the EOs only perform their antimicrobial efficacy at higher concentrations leading to negative impact on the organoleptic properties of food products (Olatunde and Benjakul, 2018). Therefore, nanoencapsulation of EOs into different carrier matrices has been regarded as a novel green strategy to overcome the drawbacks with improvement in EOs functionalities and their practical application in food industries (Salvia-Trujillo et al., 2015a; Chaudhari et al., 2021b).

Hydrophobic EOs and their components may be encapsulated into suitable biopolymers to make them more available in watery environments as well as to enhance the antimicrobial efficacy of EOs by increasing uniform distribution on food surfaces (Salvia-Trujillo et al., 2015b; Das A. K. et al., 2020; Lugani et al., 2021). Different nanoencapsulation systems like nanoemulsion, solid lipid nanoparticles, nanofibers, liposomes, and edible films are available with practical utility in food preservation (Aswathanarayan and Vittal, 2019). Among them, nanoemulsion has been considered as more effective (Anwer et al., 2014) and widely used nanometric systems due to at least one dimension less than 100 nm (Hasan et al., 2020; Amiri et al., 2021). Consequently, nanoencapsulation has some remarkable advantages over other encapsulation systems for certain applications such as improved stability of encapsulated active compounds, large surface area to volume ratio, higher bioavailability, mass transfer behavior (Zhang et al., 2021), enhanced bioactivity (Donsì and Ferrari, 2016), and better diffusion to target food systems (McClements et al., 2021). Recently, extensive investigations have been performed on the EOs encapsulation and their potential application in the food industry (Dwivedy et al., 2018; Pabast et al., 2018; Aswathanarayan and Vittal, 2019; Zhu et al., 2020; Chaudhari et al., 2021a). However, to date, there is a lack of knowledge about the utilization of EOs nanoemulsion against microbial contamination, deterioration, and toxin secretion in food for long-term sustainable preservation. Therefore, the present review has briefly discussed (a) processes for the fabrication of nanoemulsion with critical analyses for antibacterial and antifungal activity, (b) diverse mechanisms associated with antimicrobial activity, (c) potential practical applications, (d) the toxicity and safety of nanoemulsions, and (e) future perspectives addressing the research gaps and current challenges.

MICROBIAL CONTAMINATION OF FOODS

A number of investigations have explained that food commodities, especially vegetables, fruits, meat, and other high fat-containing products are maximally spoiled or wasted due to infection of bacterial pathogens such as *Listeria* spp., *Escherichia* spp., *Campylobacter* spp., *Bacillus* spp., *Salmonella* spp., and *Klebsiella* spp. (Hadian et al., 2017; Kawacka et al., 2021; Lages et al., 2021). Recently, *Campylobacter* species, especially *C. coli* and *C. jejuni,* have been recognized as the most prevalent pathogens associated with chicken and meat products in both developed and developing countries, causing campylobacteriosis in humans (Guirin et al., 2020). After *Campylobacter* spp., the second most important bacterial pathogen is *Salmonella* spp. isolated from various foodstuffs causing vomiting, diarrhea, fever, abdominal cramps, headache, and blood in feces. According to the World Health Organization (WHO), in the United States of America, *Salmonella typhi* was responsible for the infection of 750,000 people, leading to more than 52,000 deaths annually (World Health Organization [WHO], 2015). *Listeria monocytogenes* is another major food contaminating pathogen causing listeriosis (Välimaa et al., 2015). Risks of listeriosis are commonly observed in pregnant women, infants, and immunocompromised patients, while healthy people showed mild symptoms (Gholipour et al., 2020). *Escherichia coli* is another important food contaminating pathogen associated with beef and beef products secreting the Shigha toxin (Shigha toxin-producing strain of *E. coli*- STEC) eventually causing

bloody diarrhea and low platelet counts in affected patients (Niveditha et al., 2021).

Apart from bacterial contamination, several fungal species *viz*. *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Cladosporium*, *Rizopus*, and *Mucur* are also registered to be responsible for spoilage of fresh fruits, vegetables, stored grains, meats, and other essential food products (Axel et al., 2017; Hu et al., 2019; Atif et al., 2020; Das S. et al., 2020). In addition to food spoilage, fungal pathogens also deteriorate food products by producing health-hazardous toxic secondary metabolites, called mycotoxins (Alizadeh et al., 2020). Moreover, frequent contamination of preharvest and postharvest stored food products by *Aspergillus*, *Penicillium*, and *Fusarium* secretes more than one mycotoxin leading to significant economic losses (Chaudhari et al., 2020). Among all 400 reported mycotoxins, aflatoxin, zearelenone, fumonisins, ochratoxins, and deoxynivalenol are major health-hazardous mycotoxins posing adverse effects on mammalian health after consumption of contaminated foods (Aldars-Garcia et al., 2016).

Foodborne pathogens deteriorate the food quality, reduce the content of important nutrients and vitamins, and shorten the shelf life of food products by releasing extracellular enzymes and changing the odor, texture, and overall appearance of foodstuffs (Deepika et al., 2021). Spoilage of food products is rarely investigated unless it has taken the form of an outbreak. Thus, the microbial spoilage and wastage of food and food products demands extensive research in the area of natural products including the application of EOs with an objective to secure public health through long-term preservation of different food commodities.

NANOENCAPSULATED ESSENTIAL OILS FOR FOOD PRESERVATION

Essential oils are volatile secondary metabolites obtained from different parts of medicinal and aromatic plants such as the leaves, stem, flowers, fruits, and buds (Falleh et al., 2019). Nearly, 3000 EOs have been isolated from 2000 plant species, and of them, 300 EOs are known to be utilized for commercial purposes. EOs are complex mixture of several bioactive components such as terpenes, terpenoids, alcohols, esters, ketones, phenylpropanoids, and aldehydes. Many synthetic preservatives have been used against microbial contamination in stored food items, but these preservatives have several adverse effects on human health and the environment. Therefore, current researchers are focused toward plant-based preservatives having negligible side effects.

In line with the non-toxic nature of EOs, some of them have been used as food preservatives. For instance, Eco-SMART is one of the most popular EOs based preservatives used in industry. Eugenol Tween® and Eugenol ethoxylate, Pycnogenol® and Hebalox®, DMC Base Natural, and Protecta One and Protecta Two are EO-based preservatives used for the protection of harmful pathogens commonly encountered in food items (Prakash and Kiran, 2016).

The current studies in food safety have witnessed the success of EOs in controlling food spoilage by Gram-positive and Gram-negative bacteria, fungi, and the toxins secreted by them. However, the wide-scale application of EOs as free form in food is limited due to:

(a) Rapid release from applied surfaces,
(b) Possibility in the changes of food organoleptic properties caused by intense aroma,
(c) Oxidation of EO components by environmental factors like temperature, irradiation, and moisture, and
(d) Considerable loss in EO biological activity.

Nanoencapsulation has emerged as an important technique to entrap EOs and bioactive compounds with an objective to improve the microbial inhibitory activities, antioxidant properties, and utilization in real food systems (Dwivedy et al., 2018; Chaudhari et al., 2021a). Nanoencapsulation encompasses the natural products or compounds of interest in a compatible polymeric matrix with a minimum of one dimension below 100 nanometers (Prakash et al., 2018). Loss in the availability of free EO at applied food surfaces after short durations restricts their potentiality in the food system. Controlled release, therefore, could be of considerable interest to improve the longevity of shelved food products. Controlled release of EOs loaded on zein nanoparticles has been presented to preserve meat (Xavier et al., 2021). The authors also demonstrated preserved antioxidant activity of *Cinnamodendron dinisii* EO after incorporation in a chitosan matrix. Mahdi et al. (2021) reported an enhancement in the oxidative stability of *Citrus reticulata* EO after encapsulation in composite wall materials comprising of whey protein, maltodextrin, and gum arabic. In addition, nanoencapsulated EOs are registered to exhibit enhanced antioxidant activity and solubility, as well as stability against increased temperature (Hadidi et al., 2021; Yang et al., 2021). Since the strong flavor and aroma of some EOs may modify the sensorial attributes of treated food, nanoencapsulation may be performed to facilitate the controlled release and hence the preservation of sensory properties. The positive influence of nanoformulated *Cinnamomum zeylanicum* EO in comparison to free EO on sensory attributes of beef patties has been demonstrated by Ghaderi-Ghahfarokhi et al. (2017). The patties color was more stable after EO encapsulation, suggesting the effectiveness of fabricated formulation in improving the organoleptic attributes of treated meat. Therefore, nanoencapsulation is a promising approach to augment stability, distribution, and delivery of EOs and visible characteristics of treated foods.

METHODS OF ENCAPSULATION OF ESSENTIAL OILS (FABRICATION OF NANOEMULSION)

Fabrication of nanoemulsion can be classified into:

(i) Methods involving nanoemulsion synthesis, and
(ii) Ingredients/major components of nanoemulsion.

Methods for Fabrication of Nanoemulsion

Nanoencapsulation of EO into a suitable polymer matrix in the form of nanoemulsion has been performed by different processes *viz*. nanoprecipitation, inclusion complexation, solvent evaporation-emulsification, coacervation, and supercritical fluid.

Nanoprecipitation is a common technique for the encompassment of lipophilic components like EOs into semipolar solvents and polymers through interfacial deposition. The method is easy, reproducible, and commonly used due to the its minimal energy use and simplicity (Eisner, 2014). Commonly used polymers for encapsulation of lipiophilic components involve poly (lactic-co-glycolic acid), poly (alkylcyanoacrylate) (PACA), polylactic acid (PLA), and poly(ε-caprolactone). More importantly, for improvement in functionalities of core material, controlled delivery, and cellular uptake, multiple biopolymer matrices have been used (Walia et al., 2019).

Inclusion complexation is another important method of encapsulation involving molecular linkage between the core material and matrix polymer. Molecular linkage during the encapsulation process includes Van der Waal forces, hydrogen bonding, and hydrophobic interactions with a high yield of nanoparticles (Aree and Jongrungruangchok, 2016). In this method, β-cyclodextrin and β-lactoglobin have especially been recognized as suitable nanocarriers for encapsulation of lipophilic components (Walia et al., 2019).

Solvent evaporation-emulsification involves polymer solution emulsification followed by solvent evaporation and subsequent formation of nanoparticles. Ethyl cellulose, polycaprolactone, polylactic-co-glycolic acid (PLGA), and polylactic acid (PLA) are commonly used polymers for the development of nanospheres by employing high-speed homogenization and ultrasonication (Fornaguera et al., 2015). Tsai et al. (2011, 2012) reported high-pressure emulsification for effective incorporation of curcumin in PLGA with improved bioavailability.

The coacervation process allows differentiation and phase separation of a single polymer matrix or a mixture of polymer matrices followed by encircling of the core phase. Cross-linking in hydrocolloid shells has occurred in presence of enzymatic and chemical cross-linkers such as transglutaminase and glutaraldehyde that help to increase the coacervate robustness (Ezhilarasi et al., 2013). On the basis of the number of polymers used, the process has been differentiated into simple and complex coacervation. More importantly, encircling/coacervate strength depends on chemical/enzymatic cross-linkers, ionic strength, pH, biopolymer type, concentration, and the nature of the complex formed (de Kruif et al., 2004). Hu et al. (2018) synthesized cinnamon-thyme-ginger composite essential oil nanocapsule by complex coacervation with the involvement of chitosan as biopolymer and tripolyphosphate as cross-linking agent.

A supercritical fluid is used for encapsulation of thermally sensitive bioactive compounds, followed by evaporation of fluid by spraying process and further precipitation of the solute particles. Arango-Ruiz et al. (2018) reported encapsulation of curcumin into polyvinylpyrrolidone by supercritical antisolvent technology. In an investigation by Türk and Lietzow (2004), nanoencapsulation of phytosterol was performed by supercritical fluid with particle size less than 500 nm.

Ingredients and Components of Nanoemulsion

Major ingredients of nanoemulsion are EO, surfactant, and water (Dasgupta et al., 2019). Proper mixing of these components regulates the properties and stability of the emulsion. The selection of surfactants during nanoemulsion formation is dependent on the surface's active nature emphasizing the stability, pH, temperature, and ionic strength of the nanoemulsion system (McClements et al., 2017). Moreover, the water-to-oil ratio determines the stability and size of particles in nanoemulsion systems. In addition to EO, surfactant and water, thickening agent, weighting agent, emulsification, antioxidants, and polyunsaturated fats also improve the dispersion stability of nanoemulsion (Dasgupta and Ranjan, 2018). The amount of water, as well as its unique properties, greatly influence the organoleptic property of foods. Water crystals in emulsion have a significant effect on the texture and taste of food products. Emulsifier also helps in the prevention of coalescence and flocculation in nanoemulsion by interfacial interaction. Emulsifier facilitates in droplet break up leading to the formation of small size particles. The concentration of emulsifier is decided on the basis of the amount of biopolymer to cover all the oil- water interfaces and the rate of coating. EOs are used as oil phase and have more tendency to protect themselves from oxidative degradation after encapsulation into biopolymer in nanoemulsion system (Dasgupta et al., 2016a).

ROLE OF ESSENTIAL OILS NANOEMULSION AS FOOD ADDITIVES

Recently, consumers as well as modern food industries are focusing on nanoengineered EOs in the forms of nanoemulsion to avoid the drawbacks of unencapsulated EOs for practical utilization with maximum stability and compatibility. Further, nanoemulsions provide maximum benefits associated with the use of EOs in food items such as:

(a) Increased dispersion within the food surfaces where microbes generally multiply.
(b) Reduced sensorial effects.
(c) Increased antimicrobial activity of nanoemulsions containing bioactive molecules of EO (Donsì and Ferrari, 2016).
(d) Due to multiple targets sites in microbial cells, the emulsion-based delivery systems containing EOs may easily interact with microbes, thus interfering with the normal biological activities.
(e) Most importantly, different components present in EOs either individually or in combination with other components may exhibit synergism and conceivably play a crucial effect in membrane disruption, leakage of cytoplasmic constituents, and metabolic alterations.

Antibacterial Efficacy of Essential Oil Nanoemulsion

The antibacterial efficacy of EOs is a research hotspot to fulfill the need for natural antibacterial compounds. However, in most of the studies, EOs showed higher activity against Gram-positive than Gram-negative bacteria. This mainly happens because of structural differences in the membrane of Gram-positive and Gram-negative bacteria in terms of the association of hydrophobic compounds (Franklyne et al., 2016). A number of researchers have added valuable information about the antibacterial activity of EO nanoemulsions against a wide range of food contaminating bacteria.

Efficacy of Essential Oil Nanoemulsion Against *Listeria monocytogenes*

Listeria monocytogenes is the major bacteria responsible for foodborne illnesses in the food sector, especially ready-to-eat foods causing disease outbreaks mainly in the US and in other parts of the world (Harris et al., 2003). *L. monocytogenes* is the main causal agent of listeriosis, a very common foodborne illness. In this context, nanoencapsulated EOs are an effective agent and have been substantially used to control *L. monocytogenes* growth in vegetables, fruits, and ready-to-eat foods. Bhargava et al. (2015) artificially inoculated fresh lettuce with *L. monocytogenes* and dipped it in *Origanum* spp. oil nanoemulsions for 1 min. At 0.1% concentration, they observed up to 3.57 log CFU/g reduction that was further confirmed by disruption in the cell membrane as revealed through SEM observation. Paudel et al. (2019) in a similar way artificially inoculated honeydew and cantaloupe with *L. monocytogenes* followed by treatment with cinnamon oil nanoemulsions for one minute. The treatment showed up to 7.7 log reductions in bacterial growth at 0.5% nanoemulsion. In another study, Maté et al. (2016) reported the combined effect of d-Limonene nanoemulsion with heat stress for inhibition of *L. monocytogenes*. The thermal resistance of *L. monocytogenes* was reduced two to five times when 0.5 mM D-limonene was added to the heating medium. Moreover, when the same concentration of D-limonene nanoemulsion was added to the heating medium, the resistance was reduced by more than one hundred times and showed very promising results on the inactivation of microorganisms by the combined effect of nanoemulsified D-limonene and thermal treatments. These studies suggested that EO-based nanoemulsions can be used as effective natural antibacterial agents against *L. monocytogenes* contamination in the food industry.

Efficacy of Essential Oils Nanoemulsion Against *Salmonella* Species

In a study, Moghimi et al. (2016a) tested Sage (*Salvia officinalis*) EO nanoemulsion against *S. typhi* and found four times higher activity than the unencapsulated EO. The nanoemulsion treatment showed extensive cell membrane damage as determined by efflux of cellular protein, DNA/RNA, and Mg^{2+}, K^{+}, and Ca^{2+} in the extracellular media during *in vitro* testing. In another study, Kang and Song (2018) spot-inoculated red mustard leaves with *Salmonella typhimurium* and treated them with nanoemulsions containing different EOs. The investigation observed higher reductions as compared to 0.02% NaOCl. More importantly, the nanoemulsion significantly maintained the sensory properties of red mustard along with nutritional qualities. Hadian et al. (2017) demonstrated the antibacterial activity of nanogel encapsulated *Rosmarinus officinalis* EO (REO) against *S. typhimurium* on beef cutlet samples. They reported that encapsulated REOs coating on beef cutlets were more effective as compared to free REOs in controlling the *Salmonella* population under refrigerated storage. Nanoencapsulation effectively reduced the microbial population by 2 mg/g beef cutlet.

Efficacy of Essential Oil Nanoemulsion Against *Escherichia coli*

He et al. (2021) observed the stronger microbial inhibitory activity of thyme (*Thymus daenensis*) EO nanoemulsion as compared to the EO alone or coarse emulsion against *E. coli* O157:H7. Further, the combination of the synergistic actions of ultrasound (US) and EO nanoemulsion showed remarkable decontamination of *E. coli* on contaminated cherry tomatoes without affecting firmness and color. Similarly, Guo et al. (2020) observed the morphological changes in *E. coli* cells after treatment as observed through SEM, TEM, and Laser scanning confocal microscopy. The synergistic effects of the ultrasound and oil nanoemulsion caused changes in the morphology, interior microstructure of cells, and permeability of cell membranes leading to increased release of nucleic acids and proteins. The study provided valuable information with reference to the potential of EO nanoemulsions in food preservation. However, in a similar study, Salvia-Trujillo et al. (2014) proposed that microfluidization rather than ultrasounds seemed to have an improved antimicrobial activity of *Cymbopogon citratus* EO against *E. coli*. Moraes-Lovison et al. (2017) evaluated the antibacterial and physiochemical stability of nanoencapsulated *Origanum vulgare* EO. In this investigation, *in vitro* minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *E. coli* at were recorded as 0.60 and 3.32 mg/mL, respectively. They also observed the physiochemical characteristics of meat products by incorporation of nanoemulsion in chicken pâté and found negligible changes in meat products. Moghimi et al. (2016b) developed high-intensity ultrasound-based water-dispersible *Thymus daenensis* EO nanoemulsion (diameter = 143 nm). The EO nanoemulsion showed a high inhibitory efficacy against *E. coli* with MIC at 0.4 mg/mL. The nanoemulsion exhibited 10 times more antibacterial activity than the free EO, thus the conversion of EO into nano-scale particles greatly enhanced the bactericidal activity.

Efficacy of Essential Oil Nanoemulsion Against *Bacillus* Species

Although many *Bacillus* spp. are non-pathogenic, they can exhibit hemolysis and are raising concern because of their extensive use as a model organism. Several case studies have confirmed severe health impacts associated with the consumption of *Bacillus* spp. contaminated food (Singh N. et al., 2020). There are many reports related with the *in vitro*

studies of EO nanoemulsions against different *Bacillus* spp. Hassanshahian et al. (2020) observed MIC and MBC values of *Alhagi maurorum* EO nanoemulsion as 1.75 and 6.25 mg/ml, respectively against *B. cereus* and found it two times more effective than that of the EO alone using the macro-broth dilution method. Ghosh et al. (2013) observed a significant reduction in *B. cereus* population even at a higher dilution of *Cinnamomum zeylanicum* EO nanoemulsion. They also demonstrated membrane distortion in treated cells as observed through vital cellular constituents leakage, EtBr staining, and SEM analysis. In a similar study, Zhang et al. (2017) observed that encapsulated cloves/cinnamon EO nanoemulsions displayed prominent antimicrobial effectiveness against *B. subtilis* as compared to the non-nanoemulsion counterparts. Further, the addition of EO nanoemulsion in a sauce prepared from mushrooms did not alter the flavor, offering practical applicability for food preservation. Frank et al. (2018) evaluated the antibacterial activity of Cinnamon EO nanoemulsion (CEO-NE) incorporated into alginate against *B. cereus*. Thus, they reported increased inhibition of *Bacillus* by enhancing the concentration of CEO-NE from 20–40%, respectively.

Supplementary Table 1 presents the antibacterial efficacy of EOs nanoemulsion focusing on the type of EO with their active constituents, microbes categorization, and effective mechanisms in foods.

Antifungal Activity of Essential Oils Nanoemulsion

As most of the studies dealing with antimicrobial activity of EO nanoemulsions have focused on bacteria, the study with respect to antifungal activity is sparse. However, some valuable information on the antifungal activity of EO nanoemulsions against some food-borne fungal strains has been added by some researchers during the last decades. The food items are most commonly contaminated with *Aspergillus*, *Fusarium,* and *Penicillium* causing severe health hazards to consumers, oxidative deterioration, and lipid peroxidation of contaminated food due to their mycotoxin-producing ability (Ribeiro et al., 2019).

Efficacy of Essential Oil Nanoemulsion Against *Aspergillus* Species

Among different mycotoxins, aflatoxins secreted by *Aspergillus* are the most common and serious food contaminant. During *in vitro* studies, Das S. et al. (2020) observed better fungitoxic efficacy of *Myristica fragrans* EO nanoemulsion (1.75 μl/ml) than the EO alone (2.75 μl/ml) against a toxigenic strain of *A. flavus*. A similar finding was also observed when tested *in vivo* in rice food samples stored for 6 months. The authors further suggested that the superior activity of nanoemulsion was due to the subcellular size particles with targeted delivery of components. In another study, Ribes et al. (2017) observed enhanced inhibitory effect of *Cinnamomum* spp. leaf EO nanoemulsions than free EO against *A. niger* mycelial growth and spore germination. The authors further suggested that the antifungal action was correlated with the loss of cytoplasm in hyphae and hyphal tip as observed in *A. niger*. Another interesting finding of the work has been associated with the implication of nanoemulsions for the preparation of effective and stable natural antifungal agents in food-based applications.

Efficacy of Essential Oil Nanoemulsion Against *Penicillium* Species

Mahajan et al. (2021) tested *Ocimum gratissimum* EO and its nanoemulsions against *P. digitatum* of kinnow mandarin fruit by the poisoned food technique. The nanoemulsion exhibited stronger growth inhibition (1×10^4 CFU ml^{-1}, 96%) than unencapsulated oil (13×10^4 CFU ml^{-1}, 85%) on the 15^{th} day of incubation. Further, the SEM and optical microscopy suggested stronger suppressive activity of EO nanoemulsions for germination of spore and elongation of hyphae in *P. digitatum*. In a similar study of interest, Long et al. (2020) observed about 300 times more bioactivity as MIC value changed from 3.7% to 0.013% when garlic oil nanoemulsion was tested in comparison to garlic oil alone against *P. italicum*, a common contaminant causing postharvest decay of fruits and vegetables. The antifungal mode of action of oil nanoemulsion showed malformation in cell structure with destroyed lipids, nucleic acids, and proteins. Further, the oil nanoemulsion also successfully inhibited *P. italicum* infestation in citrus during *in vivo* trials, thereby strengthening its use as a suitable alternative to fungal contamination in fruits and vegetables.

Efficacy of Essential Oil Nanoemulsion Against *Fusarium* Species

Wan et al. (2019) tested fungitoxic potentiality of lemongrass, clove, peppermint, thyme, and cinnamon EO nanoemulsions against *F. graminearum* using the agar dilution method. The thyme oil nanoemulsion showed the strongest antifungal activity (EC_{90} = 7.25–7.61 mg/g), while the peppermint oil nanoemulsion showed the lowest (EC_{90} = 23.67–23.84 mg/g) activity against mycelial growth of both strains. The authors also suggested that the strong activity of thyme EO nanoemulsion was due to the presence of phenol, i.e., thymol causing disruption in ergosterol biosynthesis and membrane integrity. In another study, Abd-Elsalam and Khokhlov (2015) observed suppression of mycelial proliferation in *F. oxysporum* f. sp. vasinfectum, the causal agent of wilt of cotton by eugenol loaded nanoemulsion.

Efficacy of Essential Oils Nanoemulsion Against *Rhizopus* spp.

Rhizopus is the main causal agent of soft rot in fruits and vegetables during storage. In a study, Yousef et al. (2019) observed MIC value 1000 μl/L of cinnamon EO nanoemulsion in potato dextrose agar medium against *R. stolonifera*, causing rot of strawberry. In general, the EO nanoemulsion showed stronger antifungal activity than the EO alone and the common synthetic fungicide thiabendazole. In addition, the EO nanoemulsion also caused significant fruit decay reduction and the lowest fruit infection (5.43%) at 0.2% concentration. In a similar study, cinnamon EO nanoemulsion also showed better efficacy than the EO coarse emulsion and a common antifungal drug Amphotericin B against *R. arrhizus* when examined through the disk diffusion method (Pongsumpun et al., 2020). The authors suggested better efficacy of nanoemulsion facilitated by small

sizes particles delivering EOs to the fungal cell membrane, while the coarse emulsion was due to low solubility in water, and could not interact with the cell membrane properly. These studies proved the efficacy of cinnamon EO nanoemulsion as natural fungicides for the control of postharvest losses caused by *Rhizopus* spp. A list of important studies indicating the inhibitory potential of EOs nanoemulsion against different storage fungi along with the key findings is presented in **Supplementary Table 2**.

ANTIMICROBIAL MECHANISM OF ACTION OF ESSENTIAL OILS NANOEMULSION

Essential oils recognized as secondary metabolites have been obtained from aromatic plant families such as *Lamiaceae*, *Asteraceae*, *Myrtaceae*, *Apiaceae*, *Rutaceae*, *Zingiberaceae,* and many others. Naturally, EOs are lipophilic or hydrophilic in nature and are complex mixtures of hundreds of unstable and non-volatile active organic compounds and may be characterized into terpene (e.g., limonene and myrcene), terpenoids (linalool and Thymol), and phenylpropanoids (anethole, eugenol) (Hassoun and Çoban, 2017; Pateiro et al., 2021). Major bioactive components responsible for antimicrobial activity in various EOs include the presence of phenolic components such as carvacrol, eugenol, and thymol in EOs which exhibit strong antimicrobial properties, followed by terpenes and ketones (Li et al., 2015; Pathania et al., 2018). In addition to major components, minor components of EOs also play a significant role against microbes owing to synergistic activity between minor and major components (Bhavaniramya et al., 2019). However, the mechanism of action of EOs and their components is largely restricted due to low solubility, poor bioavailability, and quick release. The drawbacks associated with EOs alone could be overcome by encapsulation. Therefore, nanoencapsulated EOs having enhanced antimicrobial activity can be used in various food sectors against different food spoilage microbes.

Benjemaa et al. (2018) reported that the antibacterial activity of EOs was enhanced and prolonged after nanoencapsulation. Usually, the antibacterial activity of nanoencapsulated EOs cannot be assigned to a single mechanism of action due to the presence of different bioactive components of EOs having multiple functional groups in their chemical composition (Diao et al., 2014), facilitating different routes for their possible action on microbial cells. Many researchers have presumed an enhancement in the specific mechanism of action of nanoencapsulated EOs as compared to free EOs (Moghimi et al., 2016b; Lu et al., 2018; Chu et al., 2020; El-Sayed and El-Sayed, 2021). In this reference, nanoencapsulated EOs provoked the disorganization of the phospholipid bilayer of bacterial cell membrane and mitochondria, damage to membrane proteins, followed by the increase in cellular permeability, instability of cellular structure, and the depletion of proton motive force, electron flow, and active transport (Benjemaa et al., 2018; Bhavaniramya et al., 2019; Yang et al., 2021). Consequently, the presence of EO enhances the leakage of vital cellular ions (Na^+, Mg^{2+}, K^+) and 260 and 280 nm absorbing cellular constituents such as DNA, RNA, and proteins (**Figure 1**), leading to significant changes in the bacterial cell responsible for cell death.

Notably, nanoencapsulated EOs have also been shown to exhibit strong activity against food-borne fungal pathogens. Due to their complex mixture of bioactive components, the exact antifungal mechanism of action is not fully unveiled. A possible antifungal mechanism of action of nanoencapsulated EOs is schematically presented in **Figure 2**. Several studies have indicated that the fungitoxic efficacy of nanoencapsulated EOs and their active constituents are the outcome of interferences in the biosynthesis of the cell wall rendered by the larger surface area of nanoparticles and modulation in ionic permeability of the fungal plasma membrane. Primarily, nanoencapsulated EOs target cell membrane due to the lipophilic nature of EOs allowing mobilization across the fungal cell membrane leading to contraction in the partitioning of the lipid bilayer, damage of cellular integrity and alteration in membrane permeability, leakage of vital intracellular components (Ca^{2+}, K^+, and Mg^{2+}), inhibition of mitochondrial electron transport system, reduction in the membrane potential by inhibiting the proton pump with subsequent loss in the ATP pool, and eventually apoptosis (Kalagatur et al., 2018; Singh A. et al., 2020). More importantly, ergosterol is maximally affected by EO nanoemulsions leading to destabilization of membrane integrity and stability (Chaudhari et al., 2021a). An investigation conducted by Singh et al. (2019) and Li et al. (2020), revealed that *Ocimum sanctum* and *Illicium verum* EOs, respectively can induce considerable impairment in ergosterol biosynthesis in *Aspergillus flavus.* Thus, nanoencapsulated EOs can be utilized as possible natural antimicrobial agents against food spoilage pathogens.

APPLICATION OF NANOEMULSION

Nanoemulsions have various applications for encapsulation, protection, and delivery of bioactive components such as nutraceuticals (food components having health benefits), pharmaceuticals (drugs), flavor improvement, and antioxidant qualities in foods.

Nanoemulsions containing lipophilic functional compounds *viz*. flavonoids, phytosterol, carotenoids, and fat-soluble vitamins have multiple applications in biomedical and health aspects. Guttoff et al. (2015) synthesized vitamin D nanoemulsion by spontaneous emulsification and studied the effect of composition and preparatory conditions on emulsion stability. Meghani et al. (2018) reported the potentiality of vitamin D encapsulated cinnamon essential oil nanoemulsion to arrest the cell cycle progression, increased caspase activity, and decreased expression of BcL2 protein leading to loss of mitochondrial membrane potential in human alveolar carcinoma cells. Encapsulations of bioactive components of *Tinospora cordifolia* extract *viz*. berberine, palmatine, and palmatoside into whey protein through electrospraying nanospheres with controlled delivery for anti-diabetic activity have been recently investigated by Jain et al. (2021). The synthesis of silver nanoparticles with the involvement of black cumin seed extract and its affectivity as an

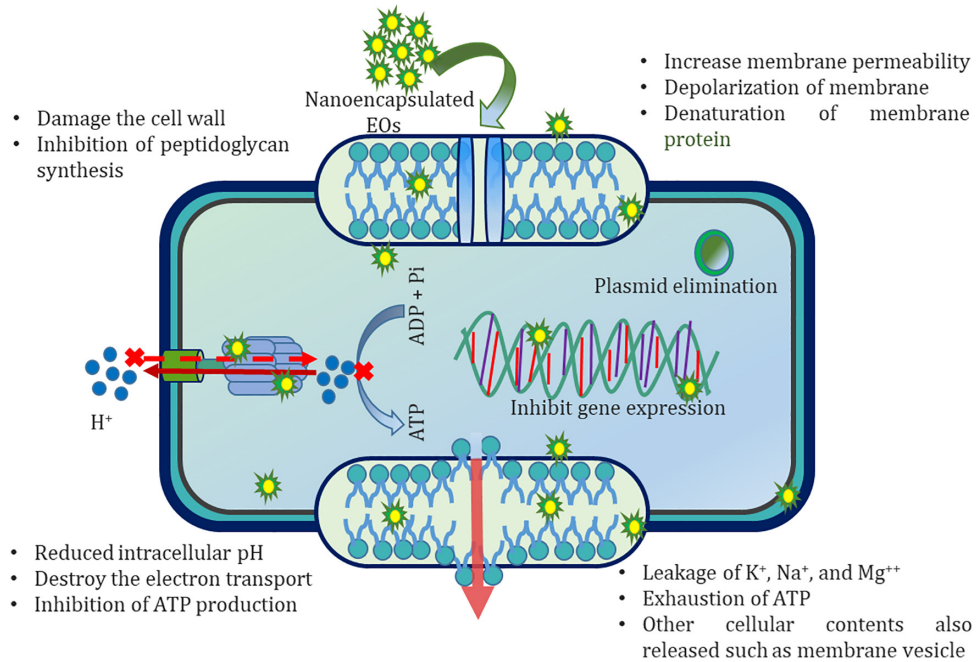

FIGURE 1 | Schematic representation of possible antibacterial mechanisms of action of EO nanoemulsion.

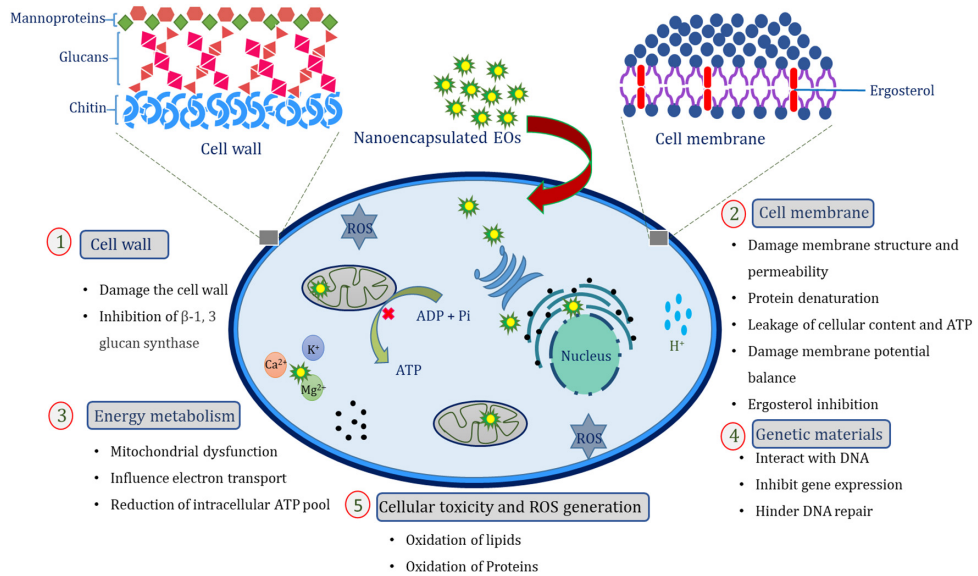

FIGURE 2 | Schematic representation of possible antifungal mechanisms of action of EO nanoemulsion.

antidiabetic and anti-inflammatory agent has been investigated by Vijayakumar et al. (2021a,b). Agarwal et al. (2021) developed curcumin-loaded polycaprolactone/polyvinyl alcohol silk-fibroin based electrospun nanofibrous mat for improved antidiabetic activity with controlled delivery. In addition to nanoemulsion, metal nanoparticles also have a prime role in biomedical applications such as drug delivery, imaging, gene delivery, bio-labeling, and tissue engineering. Vijayakumar et al. (2021b) synthesized zinc oxide nanoparticles by involving neem gum as a capping agent and analyzed the activity for inhibition of cell cycle proliferation in Hep G2 human cancer cell line.

Nanoemulsions have extended their applicability for improvement in air dispersion and foam stability in sugar and flour confectionery products. The non-fat particles of chocolate such as cocoa, milk, and sugar are suspended in cocoa butter (fat phase). Moreover, in the nanoemulsion form, emulsifiers help in

the prevention of blooming, a major cause of sensorial alteration. The cream of bakery products in sandwiches and cakes poses huge market demands in the form of emulsions. Nanoemulsion has been used in ice cream for effective improvement in texture and uniformity (Prasad et al., 2017).

Nanotechnology has more promising applications in the field of agriculture, food preservation, and biomedicals in the form of nanosensors. In addition to EO-loaded nanoparticles, copper, silica, and zinc nanoparticles showed effective potentiality for disease management, systemic acquired resistance, and also act as novel antimicrobial agents for the management of pathogens affecting agricultural crops, animals, and humans (Chhipa and Joshi, 2016; Gandhi et al., 2021). Plant-based nanoemulsions in conjunction with polymers develop smart nanosensors that can be used in food packaging and detection of agricultural food quality. Most notably, two different types of nanosensors *viz*. phytonanosensor and electrical nanosensors are being used in agriculture. Copper nanoparticles with nanogold electrodes have been used to detect salicylic acid levels in oilseed rape infected by fungal pathogen *Sclerotinia sclerotiorum* (Wang et al., 2010). Phyto/bionanosensor in combination with nanoparticles interpreted the quality of food by color changes without any laboratory testing. Owing to high toxicity, solubility, and indiscriminate utilization of pesticides in the agriculture and food industries, there is an urgent need for nanosensor technology for residue analysis of these pollutants (Valdés et al., 2009). Moreover, nanoparticle-based nanosensors can act as a smart delivery vehicle to protect agricultural crops from pathogens and facilitate an improvement in agrochemicals in low proportions (Thiruvengadam et al., 2018).

Practical application of EO nanoemulsion in food products as an antimicrobial agent is a challenging task. Nanoemulsions containing EO display a greater surface-to-volume ratio and more easily controlled delivery, with an improvement in their antimicrobial properties. In particular, the food-based application of nanoemulsions has been classified into different frontier areas:

(a) Direct mixing with food products,
(b) Infusion in porous food matrices,
(c) Food surface washing with antimicrobial nanoemulsion, and
(d) Coating of food products by nanoemulsion.

The application of nanoencapsulated EOs in the active packaging of food products is presented in **Supplementary Table 3**.

In a recent investigation by Singh P. et al. (2020), it has been observed that clove oil nanoemulsion effectively reduced the growth of *Fusarium proliferatum* and secretion of fumonisin B_1 and B_2 in maize kernels during storage conditions. More importantly, nanoemulsion not only enabled controlled delivery of EO or antimicrobial compounds, but also facilitated incorporation into complex food systems. Dasgupta et al. (2016a) reported food grade vitamin E acetate nanoemulsion using edible mustard oil with improved antimicrobial and antioxidant efficacy. Strong antibacterial and antibiofilm impact of nano-silver decorated *Ocimum basilicum* leaf extract has been reported by Muthulakshmi et al. (2021). Corresponding to complex mechanism, nanoemulsion enhanced the passive cellular absorption, reduced the mass transfer resistance, and as a result increased the antimicrobial activity (Ranjan et al., 2016). Additionally, nanotechnology has a prime role in nanofood packaging, particularly in smart and active packaging with resultant inhibition of microbial infection (Singh et al., 2018).

SAFETY PROFILE OF ESSENTIAL OILS AND NANOEMULSION

In addition to microbial infestation and toxin inhibitory efficacy, large scale practical recommendation of EOs and nanoemulsions as an effective food preservative require safety assessment without any toxic effects on mammals make them healthier for consumers. To address this issue, different international organizations such as the Food Chemical Codex, International Organization of Flavor Industries, Food and Drug Administration, Codex Alimentarium and the Council of Europe, and the Flavor and Extract Manufacturers Association have confirmed specific procedures for toxicological and chemical characterization of EOs. They also reported antagonistic and synergistic effects for specific EOs in mammals during practical application (Falleh et al., 2020; Chaudhari et al., 2021b). Generally, mammalian safety/toxicity of EOs and nanoemulsions were performed on experimental mice/rats which permits the determination of safety assessment in terms of Median Lethal Dose or LD_{50} value. In this context, Deepika et al. (2021) performed acute toxicity assay of *Petroselinum crispum* essential oil (PEO) and chitosan encapsulated PEO in mice and the LD_{50} value was found to be 10,765 and 26,830 mg/kg body weight, respectively. Similarly, Das et al. (2021b) in a study assessed the acute oral toxicity of *Pimpinella anisum* essential oil (PAEO) and chitosan nanostructured PAEO in mice, and LD_{50} were displayed as 19,879.89 and 13,641.35 μl/kg body weight, respectively. They suggested that the lower LD_{50} value of nanostructured PAEO might be due to a small-sized nanoemulsion with more EO in each nanocapsule and reduced the Median Lethal Dose. In another research, Dwivedy et al. (2018) reported the acute toxicity of *Illicium verum* essential oil (IvEO) on male mice in terms of Median Lethal Dose and found it to be 11,257.14 μl/kg body weight. EOs and nanoemulsions having higher LD_{50} values as compared to other synthetic food preservatives like bavistin (1500 mg/kg), nystatin (8000 mg/kg), and lindane (59-562 mg/kg) strengthen their application in food and agricultural industries as eco-friendly and safe preservatives.

In spite of proven safety in different model organisms, toxicities have also been reported. Synthesis of nanoemulsionic particles of tin oxide by using *Piper nigrum* seed extract and toxic effects on cancer cell lines has been investigated by Tammina et al. (2017). More importantly, the toxicity of nanoparticles is very complex and depends on different physico-chemical properties such as shape, size, charge, and reactivity (Fadeel and Garcia-Bennett, 2010). Direct ingestion of nanomaterials may occur through drug delivery and food

packaging-based applications. Nanomaterials after ingestion have been translocated to the intestinal lumen by blood circulation (Pietroiusti et al., 2013). In addition to size, the shape of nanomaterials also determines the toxicity, for example triangular-shaped nanoparticles are more toxic as compared to spherical nanoparticles (Dasgupta et al., 2016b). There are a number of risks that have been associated with the use of nanoparticles which have been focused by different regulatory agencies. The Royal Society and Royal Academy of Engineering in the UK significantly anticipate and regulate health-related problems associated with nanoparticles. The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks has also realized the nanoparticle toxicity problems in human health as well as the environment (Sharma et al., 2012). The European Commission's Scientific Committee on Consumer Products (SCCP) suggested the inappropriateness of excessive nanoparticle utilization and that there was inadequate information of uptake and absorption of nanoparticles by the skin and other cell organs. Mosa et al. (2018) reported that the toxicity of nanoparticles has been associated with liver complications and severe ill health. Most notably, nanoparticles by virtue of their small size, induce the chances of genotoxicity by directly interacting with DNA/RNA or causing indirect damage by ROS (Ranjan et al., 2019). Nanoparticles also interacte with nuclear and cytoplasmic proteins leading to interruption of antioxidant defense mechanisms (Jain et al., 2018). Therefore, there is a need for comprehensive investigation of nanoparticle uptake, entry into the food chain, and distribution both under *in vitro* and *in vivo* conditions to extend the applicability of EOs based nanoemulsion in food safety.

MARKET DEMAND OF NANOEMULSION BASED NUTRACEUTICALS AND PHARMACEUTICALS

The application of nanoemulsion in nutraceuticals, pharmaceuticals, and food products has huge market demands along with consumer preferences. Nanoemulsion basically contributes a lower optical transparency than the wavelength of light in the production of beverages and foods (Dasgupta et al., 2019). Furthermore, nanoemulsion also facilitates in the expansion of the functional food market by incorporating lipophilic bioactive components. The field of nutraceuticals is considering the advantages of the incorporation of innovative nanotechnology with controlled delivery from nano-nutraceuticals, nanoemulsion, and more importantly liposome-based delivery. Moreover, nanoemulsion-based delivery improves bioavailability and fulfills the gap between active substance content and bioaccessibility (Daliu et al., 2019). Aquanova, a commercial nanotech industry developed a number of nanocarrier systems with the encapsulation of vitamin E, vitamin C, and fatty acids for pharmaceutical and nutraceutical applications. Zyme and Aquanova synthesized omega-3 fatty acid nanocapsules as a commercial product with high market demand. NutraLease, a similar company, like Aquanova developed a nanoemulsion containing various functional compounds like lycopenes, isoflavones, vitamins (A, D3, and E), and phytosterol and have been found stable at various stages of processing (Silva et al., 2012; Ranjan et al., 2014; Dasgupta et al., 2015). Nano-self-assembled structured liquids (NSSLs), a flavor encapsulating nanoemulsion developed by NutraLease is reported to the enhance bioaccessibility and bioavailability of nutraceuticals (Jaiswal et al., 2015).

CONCLUDING REMARKS AND FUTURE RESEARCH OPPORTUNITIES

Microbial food spoilage caused by bacterial and fungal contamination has documented the rising cases of diseases outbreaks and massive human deaths globally. Although the employment of synthetic chemicals to control microbial food deterioration has received considerable success, the toxicity to human health and the environment, induction of resistance development, and the presence of residues in treated food samples have necessitated the search for preservatives of natural origin. Among natural products, EOs derived from aromatic plants have received considerable attention from the food industry because of their safety, negligible chances of residual toxicity, considerable antimicrobial activity, and promising antioxidant activity. Nevertheless, poor water solubility, high volatility, and the intense aroma of EOs have restricted their application in the food system. The limitations associated with EO could be resolved by encapsulation in suitable polymeric matrices including chitosan, alginate, zein, carrageenan, polycaprolactone, and cyclodextrins. Various studies have reported the improved antimicrobial of encapsulated EOs against food infesting bacteria, fungi, and associated toxins. The improved antimicrobial potential of nanoencapsulated EOs has been ascribed to controlled released of bioactives and easy access to food regions supporting the microbial proliferation rendered by the subcellular size of the emulsionic particles. The antimicrobial mechanism of action of encapsulated EO was attributed to the inhibitory action on ergosterol biosynthesis, release of biologically important ions including calcium, potassium, and magnesium, 260 and 280 nm absorbing materials, loss in ATP pool caused by disturbances in proton motive force, and oxidation of biomolecules caused by ROS. The antimicrobial activity of nanoencapsulated EOs has been reported to be influenced by fabrication process parameters including pH, temperature, concentration, and chemical composition of polymer matrix and tripolyphosphate content, homogenization speed, time of sonication, surfactant, and most notably the chemical characteristics of natural products employed.

Although, the EOs and non-encapsulated formulation thereof has achieved great success in preventing the growth of food spoilage bacteria, fungi, and microbial toxins, the commercialized formulations for application in food industries are still lacking. Most importantly, a future potential model with emphasis on the release and delivery of bioactive components/constituents from nanoemulsion needs to be evaluated before practical application

in food and agricultural industries. The time taken to release the volatile component greatly depends on the concentration of wall material. In the case of lipid carriers, low-fat product displayed burst release of the volatile component, while, high fat gave sustained-release (Dasgupta and Ranjan, 2018). Release of the non-volatile component occurs due to simple dilution. After nanoemulsion dilution, some of the bioactive components could move from oil droplets to the aqueous phase which may be considered as an efficient release mechanism of non-volatiles (Maher et al., 2015). A number of intrinsic and extrinsic factors *viz*. pH, dilution, ion strength, enzyme activity, and temperature determine the release rate of bioactives in the nanoemulsion system. Interestingly, a decrease in particle size in the nanoemulsion system regulates the oil-water partition coefficient which further affects the bioavailability and release profile. Walia et al. (2017) demonstrated encapsulation efficiency, loading capacity, and viscosity of vitamin-D is a prime factor for stable and efficient release from nanoemulsion. Additionally, droplets encapsulated into hydrogel particles increased the path length for diffusion and minimized the release rate (Komaiko and McClements, 2014). Some of the important future research opportunities linked with the application of EO based nanoformulation:

(a) Fabrication of nanoemulsions with enhanced stability under fluctuating conditions during food processing.
(b) Development of nanoemulsions with improved nanoencapsulation efficiency and loading capacity.
(c) The fate and transport of nanoemulsions in the natural environment needs to be extensively studied in order to safeguard environmental homeostasis.
(d) The search for newer wall materials as well as the design of composite materials having suitability for food applications to encapsulate the EOs is of immense importance in food industries.
(e) Appraisal of the cost-benefit ratio for the fabrication of nanoemulsion could be helpful in the development of cost-effective formulations.
(f) The effect on gut microflora is an important aspect while thinking about the supplementation of EO-based nanoemulsion to prevent the spoilage of food and toxin secretion by bacterial and fungal pathogens.
(g) As extremely small-sized nanoemulsions may get rapid access to human cellular systems and may exert undesirable effects after consumption of treated food, extensive *in vivo* and *in vitro* investigations should be conducted to avoid undesirable effects on human health.

Current challenges and research gaps of nanoencapsulated bioactive components for practical applications are:

(a) Minimal use of chemical substances during encapsulation.
(b) Long-lasting controlled release of encapsulated bioactive compounds.
(c) Use of chief plant-based polymeric matrix for encapsulation, and
(d) Maximization of loading of bioactive compounds in encapsulating matrix.

AUTHOR CONTRIBUTIONS

ND and AD conceptualized the idea of the manuscript and supervised the writing of the manuscript. AM wrote the manuscript, designed the figures and table along with contributions from VS, JP, and AK. VS, SD, and NU did the manuscript editing, formatting and gave the final approval for its submission in its present form. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.751062/full#supplementary-material

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Deinococcus radiodurans Exopolysaccharide Inhibits *Staphylococcus aureus* Biofilm Formation

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Deinococcus radiodurans is an extremely resistant bacterium against extracellular stress owing to on its unique physiological functions and the structure of its cellular constituents. Interestingly, it has been reported that the pattern of alteration in *Deinococcus* proportion on the skin is negatively correlated with skin inflammatory diseases, whereas the proportion of *Staphylococcus aureus* was increased in patients with chronic skin inflammatory diseases. However, the biological mechanisms of deinococcal interactions with other skin commensal bacteria have not been studied. In this study, we hypothesized that deinococcal cellular constituents play a pivotal role in preventing *S. aureus* colonization by inhibiting biofilm formation. To prove this, we first isolated cellular constituents, such as exopolysaccharide (DeinoPol), cell wall (DeinoWall), and cell membrane (DeinoMem), from *D. radiodurans* and investigated their inhibitory effects on *S. aureus* colonization and biofilm formation *in vitro* and *in vivo*. Among them, only DeinoPol exhibited an anti-biofilm effect without affecting bacterial growth and inhibiting staphylococcal colonization and inflammation in a mouse skin infection model. Moreover, the inhibitory effect was impaired in the Δ*dra0033* strain, a mutant that cannot produce DeinoPol. Remarkably, DeinoPol not only interfered with *S. aureus* biofilm formation at early and late stages but also disrupted a preexisting biofilm by inhibiting the production of poly-*N*-acetylglucosamine (PNAG), a key molecule required for *S. aureus* biofilm formation. Taken together, the present study suggests that DeinoPol is a key molecule in the negative regulation of *S. aureus* biofilm formation by *D. radiodurans*. Therefore, DeinoPol could be applied to prevent and/or treat infections or inflammatory diseases associated with *S. aureus* biofilms.

Keywords: exopolysaccharide, *Deinococcus radiodurans*, *Staphylococcus aureus*, biofilm formation, infection

INTRODUCTION

Deinococcus radiodurans is a non-pathogenic bacterium extremely resistant to extracellular stresses, such as ionizing radiation, desiccation, UV radiation, and oxidizing agents (Fredrickson et al., 2008; Slade and Radman, 2011; Farci et al., 2016; Schmier et al., 2017). This exceptional resistance to multiple stresses results from the concerted actions of various physiological functions and well-regulated molecular mechanisms, including efficient DNA repair systems and enzymatic/non-enzymatic antioxidant systems (Makarova et al., 2001; Lim et al., 2019). In particular, *D. radiodurans* can protect against oxidative damage to important cellular components, such as proteins, nucleic acids, and lipids, *via* effective redox control and reactive oxygen species (ROS) scavenging (Chen et al., 2020). These features have been used in various industrial applications, such as decontamination of radioactive waste and development of cosmetic ingredients with the antioxidant and anti-aging functions (Xu et al., 2018; Lin et al., 2020).

Deinococcus has been reported as a commensal bacterium in various human tissues. Resident *D. radiodurans* has been found in healthy human skin (Chng et al., 2016). The Deinococcus-Thermus phylum has been reported in 23 gastric endoscopic biopsy samples and vaginal microbiota of healthy women (Bik et al., 2006; Diop et al., 2019). In addition, the beneficial role of *Deinococcus* in human skin has been speculated to be due to the quiet immune responses of host cells; however, its exact function has not been clarified (Ott et al., 2019). Interestingly, the proportion of *Deinococcus* is negatively correlated with skin inflammatory diseases, such as psoriasis or allergic skin inflammation, which may play a role in maintaining healthy skin (Yau et al., 2019; Guo et al., 2020). In contrast, other skin commensal bacteria, such as *Staphylococcus aureus, Corynebacterium*, and *Actinobacteria,* were detected at a high proportion in lesions in patients suffering from chronic skin inflammatory diseases, and it has been reported to be implicated in this etiology (Daly, 2009; Leyva-Castillo et al., 2020; Quan et al., 2020). However, the interactive function of *D. radiodurans* with other commensals and its effect on disease propensity has not been investigated.

Staphylococcus aureus is a Gram-positive opportunistic bacterium that commonly colonizes the skin, nose, and mucosal surfaces of healthy individuals (Schwartz et al., 2012; Truong-Bolduc et al., 2014; Mulcahy and McLoughlin, 2016). Approximately 20–30% of individuals are asymptomatically colonized by *S. aureus*, and 30% are intermittently colonized (Sakr et al., 2018). *S. aureus* is a leading cause of various infectious diseases, including pneumonia, sepsis, endocarditis, osteomyelitis, and skin and soft tissue infections (SSTIs). Skin tissue is the most common site for *S. aureus* colonization and infection, causing SSTI from minor, self-limiting, superficial infections to life-threatening diseases. *S. aureus*-associated SSTI may progress to invasive diseases, such as sepsis, endocarditis, and osteomyelitis (Olaniyi et al., 2017). *S. aureus* colonization and biofilm formation have been reported as universal behaviors that are significant risk factors for subsequent skin infections (Kwiecinski, 2015). When *S. aureus* forms a biofilm on the site of infection, it is 10–1,000 times more tolerant to antibiotics, antimicrobial peptides, and immune cell-mediated phagocytosis than the planktonic stage (Mah, 2012; Vestby et al., 2020).

There have been reports on the regulation of *S. aureus* biofilms *via* interactions with other commensal species. The negative regulatory effect of serine protease (ESP) from *Staphylococcus epidermidis*, a commensal bacterium, against *S. aureus* colonization has been shown in the nasal cavities of human volunteers (Iwase et al., 2010). We also previously reported that *S. aureus* biofilms were significantly inhibited by lipoteichoic acid derived from *Lactobacillus plantarum*, a probiotic commensal in the skin, gut, and oral cavity (Ahn et al., 2018). Understanding the interspecies interaction between commensals is important for understanding the pathology of inflammatory diseases or developing therapeutic strategies against these diseases. We hypothesized that *D. radiodurans* or its cellular constituents have beneficial effects on the host by playing a preventive role against *S. aureus* colonization and biofilm formation. Thus, we investigated the effect of *D. radiodurans* on *S. aureus* biofilm formation, including the underlying molecular mechanisms, and assessed its therapeutic potential to control *S. aureus* infection.

MATERIALS AND METHODS

Bacteria and Reagents

The bacteria used in this study are listed in **Supplementary Table 1**. *D. radiodurans* strains were cultured at 30°C in tryptone glucose yeast extract (TGY) broth containing 0.5% tryptone, 0.3% yeast extract, and 0.1% glucose. *S. aureus* strains were cultured at 37°C in Luria-Bertani (LB) broth. All culture media used in this study were purchased from Difco (Franklin Lakes, NJ, United States). The LIVE/DEAD bacterial viability kit was purchased from Thermo Fisher Scientific (Waltham, MA, United States). The RNeasy Mini Kit was purchased from Qiagen GmbH (Hilden, Germany). The Primescript 1st strand cDNA synthesis kit was purchased from Takara Bio (Kusatsu, Japan). DNase I, RNase A, crystal violet solution, and Gram staining kit were purchased from Sigma-Aldrich (St. Louis, MO, United States). Wheat germ agglutinin conjugated with biotin (WGA-biotin) was purchased from GeneTex (Irvine, CA, United States). Horseradish peroxidase-conjugated streptavidin (streptavidin-HRP) was purchased from BD Biosciences (San Jose, CA, United States).

Purification of DeinoPol

DeinoPols were prepared from *D. radiodurans* R1, KCTC13953BP, KCTC13954BP, or KCTC13955BP, as previously described (Lin et al., 2020). Briefly, *D. radiodurans* was cultured in TGY broth at 30°C for 48 h under shaking conditions. After incubation, 0.1% deoxycholate was added to the bacteria to lyse the cell wall, and the bacterial suspension was then heated at 100°C for 10 min to inactivate the enzymes. The supernatant was harvested by centrifugation (10,000 × *g*, 30min, 4°C), concentrated, and dialyzed using a minimal tangential flow filtration system with 30K Minimate capsules (Pall Life Sciences,

Port Washington, NY, United States). The concentrate was precipitated with four volumes of 95% ethanol (Daejungchem, Seoul, Korea) at 4°C for 12 h to yield the crude polysaccharide. To remove proteins, the precipitate was suspended in distilled water and mixed with three volumes of chloroform:*n*-butanol (4:1v/v) for 20 min, and the aqueous phase was collected, followed by precipitation with 80% ethanol. Finally, it was filtered with a 0.22-μm Millex-GP syringe filter unit (Merck Millipore, Burlington, MA, United States) and lyophilized. In some experiments, DeinoPol was further treated with proteinase K (50 μg/ml) or DNase I (50 μg/ml) at 37°C for 1 h, or heat at 100°C for 10 min.

Isolation of *Deinococcus radiodurans* Cell Wall Fraction (DeinoWall) and Cell Membrane Fraction (DeinoMem)

DeinoWall was isolated as previously described method (Wallinder and Neujahr, 1971), with some modifications. *D. radiodurans* was suspended in 1 M NaCl and disrupted using an ultrasonicator (Sanyo, Osaka, Japan), followed by removal of undisrupted cells or heavy cell debris from the bacterial lysates by centrifugation at 1,000 × *g* for 15 min. Next, the cytosolic proteins or light cell debris were removed by centrifugation at 18,800 × *g* for 15 min. The pellet containing DeinoWall was resuspended in 0.5% SDS in PBS and incubated at 60°C for 30 min to remove the cell membranous fraction. After centrifugation at 18,800 × *g* for 15 min, the pellets were resuspended in 1 M Tris-HCl and treated with 10 μg/ml DNase I and 50 μg/ml RNase A at 37°C for 2 h. After centrifugation at 18,800 × *g* for 15 min, the pellet was resuspended in 1 M Tris-HCl and incubated with 10 mM $CaCl_2$ in the presence or absence of 200 μg/ml trypsin at 37°C for 18 h. After lyophilization, the quantity was determined by measuring the dry weight of DeinoWall and suspended in pyrogen-free water. To isolate DeinoMem, *D. radiodurans* was suspended in 0.1 M sodium citrate buffer (pH 4.7) and disrupted using an ultrasonicator. Bacterial lysates were vigorously mixed with an equal volume of *n*-butanol at RT for 30 min, and the aqueous phase was separated by centrifugation at 13,000 × *g* for 15 min. Butanol extraction was repeated three times. The aqueous phase was dialyzed against pyrogen-free water, followed by lyophilization and dissolution with pyrogen-free water.

Preparation of Culture Supernatant (DRsup) and Heat-Killed Bacteria (HKDR) of *Deinococcus radiodurans*

To prepare DRsup, *D. radiodurans* was cultured in TGY broth at 30°C for 16 h under shaking condition, followed by dilution to prepare OD_{600} of 1 corresponds to 10^8 CFU/ml. The culture was centrifuged at 10,410 × g for 10 min at 4°C. The culture supernatants were filtered through a 0.2 μm membrane filter to remove the remaining bacteria and debris, and then stored at −80°C. To prepare HKDR, the culture of *D. radiodurans* was centrifuged at 10,410 × g for 10 min at 4°C. After removal of the culture supernatant, the bacteria were washed and resuspended in PBS to a density of 10^9 CFU/ml. The bacteria were heat-killed at 70°C for 30 min, and then stored in aliquots at −80°C. Complete killing was examined by plating on an TGY-agar plate overnight.

Biofilm Assay With Crystal Violet Staining

S. aureus (5 × 10^7 CFU/ml) was grown in 96-well plates (SPL, Pocheon, Korea) at 37°C for 24 h in LB broth in the presence or absence of DeinoPol. In some experiments, to investigate the biofilm prevention effect of DeinoPol, DeinoPol suspended in PBS was pre-incubated onto 96-well plates for 12 h at RT. *S. aureus* (5 × 10^7 CFU/ml) was then grown in 96-well plates pre-coated with DeinoPol at 37°C for 24 h in LB broth. After incubation, planktonic bacteria were removed, and the biofilm was gently washed twice with PBS. The biofilms of *S. aureus* were stained with 0.1% crystal violet solution at RT for 30 min, followed by washing with PBS to remove non-specific stain. The adhered dye was dissolved in a solution (95% ethanol and 0.1% acetic acid), and absorbance was measured at 600 nm using a microplate reader (Biotec, Winooski, VT, United States).

Confocal Laser Scanning Microscopy

S. aureus (5 × 10^7 CFU/ml) was grown on sterile glass coverslips at 37°C for 24 h in LB broth in the presence or absence of DeinoPol. Planktonic bacteria were removed, and the biofilm was gently washed twice with PBS, followed by staining of the bacterial biofilm with SYTO9 and propidium iodide using the LIVE/DEAD bacterial viability kit according to the manufacturer's instructions. After washing with PBS to remove non-specific stain, the biofilm was visualized using an LSM800 confocal laser scanning microscope (Zeiss, Jena, Germany). For quantification of the confocal microscopy data, 10 random independent fields of view per each group were selected and the mean fluorescence intensity (MFI) was analyzed using the ImageJ software.

Quantitative Real-Time Reverse Transcription Chain Reaction

S. aureus (1 × 10^8 CFU/ml) was grown on cell culture dishes (100 × 20 mm) for 12 h in LB broth in the presence or absence of DeinoPol (50 μg/ml). Bacteria were harvested, and total RNA was prepared using the RNeasy mini kit. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA using the Primescript 1st strand cDNA synthesis kit. qRT-PCR was performed using SYBR Premix EX Taq (Takara Bio) in a real-time PCR system (Bio-Rad Laboratories, Hercules, CA, United States). The expression levels of these genes were normalized to the expression of *gyrB*. All primers were synthesized by Bionics (Seoul, Korea). The primer sequences are listed in **Supplementary Table 2**.

Poly-*N*-Acetylglucosamine Detection

A crude PNAG extract was prepared as described previously (Toledo-Arana et al., 2005). Briefly, *S. aureus* (1 × 10^8 CFU/ml) was grown in 1.7-ml microtubes at 37°C in LB broth in the presence or absence of DeinoPol, DeinoWall, or DeinoMem for 12 h. *S. aureus* was harvested by centrifugation at 10,000 × *g*

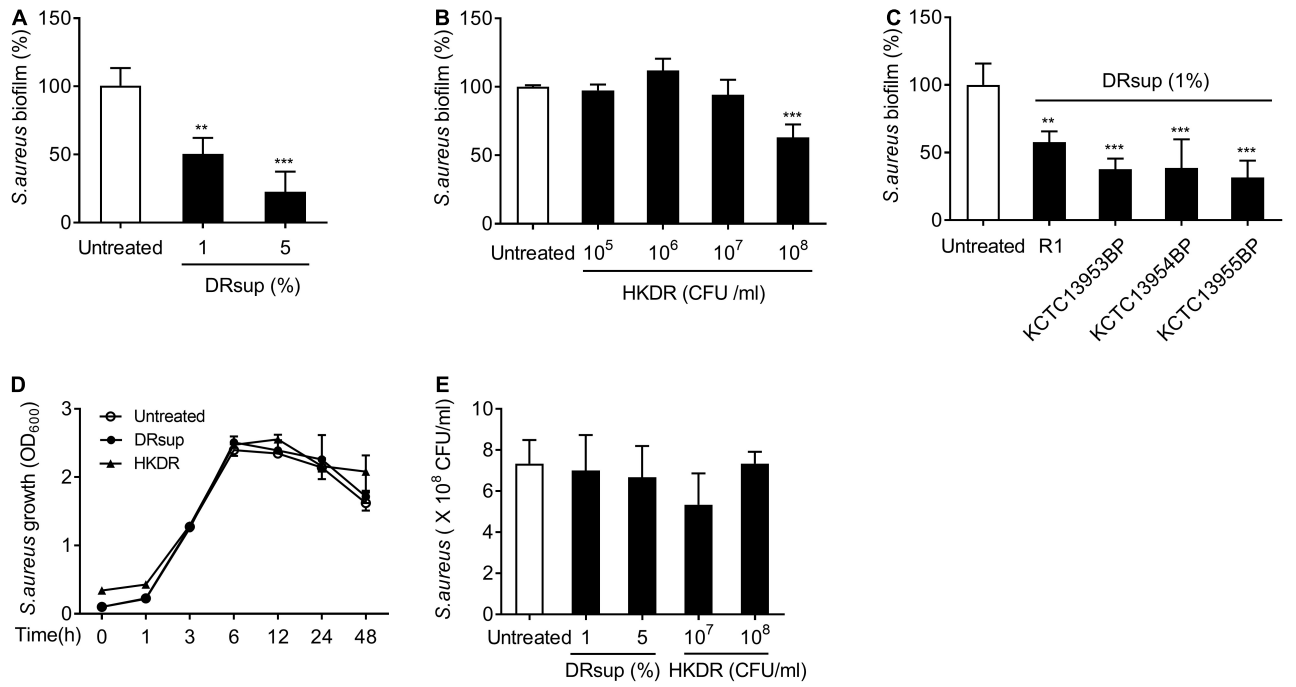

FIGURE 1 | *D. radiodurans* inhibits *S. aureus* biofilm formation. **(A–C)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates at 37°C for 24 h in the presence or absence of **(A)** culture supernatants (DRsup) (1 or 5%) or **(B)** Heat-killed cells (HKDR) (10^5, 10^6, 10^7, or 10^8 CFU/ml) of *D. radiodurans* R1, or **(C)** DRsup of *D. radiodurans* KCTC13953BP, KCTC13954BP, or KCTC13955BP strains. Biofilms were determined by crystal violet assay. **(D)** *S. aureus* (1×10^7 CFU/ml) was grown for 1, 3, 6, 12, 24, or 48 h in the presence or absence of HKDR (10^8CFU/ml) or DRsup (5%) under shaking culture conditions. The growth of *S. aureus* was determined by optical density (O.D.) at 600 nm. **(E)** *S. aureus* (5×10^7 CFU/ml) was grown at 37°C for 24 in the presence or absence of HKDR (10^7 or 10^8/ml) or DRsup (1 or 5%) under static culture conditions. Bacterial viability was measured by counting CFU. Data are the mean values ± S.D. of triplicate samples. Significant differences are indicated by asterisks (**$P < 0.01$ and ***$P < 0.001$).

for 5 min and washed five times with PBS. The pellets were then resuspended in 0.5 M EDTA (pH 8.0) and incubated at 100°C for 5 min. The supernatant was harvested by centrifugation at 10,000 × *g* for 10 min and treated with proteinase K (20 mg/ml) at 37°C for 30 min. The crude PNAG extracts were spotted onto a nitrocellulose membrane, and the blot was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h. The membrane was then incubated overnight with 10 μg/ml WGA-biotin. After washing three times with TBST, PNAG was detected with streptavidin-HRP, followed by chemiluminescence detection using the ChemiDoc Touch Imaging System (Bio-Rad). The densities of PNAG were quantified using densitometry analysis in the ImageJ software.

Biofilm Formation on HaCaT Cells Monolayer

HaCaT cells (5×10^4 cells/well) were plated in a 96-well plate and grown until they reached confluence. *S. aureus* (5×10^5 CFU/well) was added to the cell monolayer in the presence or absence of 30 μg/ml of DeinoPol. The cells were then incubated at 37°C for 8 h in a humidified 5% CO_2 incubator to form biofilms. After incubation, the biofilm was washed twice with PBS, and the cells were lysed with 0.1% Triton X-100. The number of the bacterial colonies was then counted.

Staphylococcal Wound Infection Model

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Atomic Energy Research Institute (KAERI, IACUC-2019-03) and performed according to accepted veterinary standards by the KAERI Animal Care Center. Seven-week-old female BALB/c mice were obtained from Orient Bio (Seongnam, Korea). After 1 week of acclimatization, the mice were anesthetized by intraperitoneal administration of avertin (250 mg/kg), and a flat head of tack preheated in boiling water for 30 min was applied to the shaved dorsal of then mice for 30 s. *S. aureus* (1×10^7 CFU) was then applied locally to the site of the burn wound in the presence or absence of DeinoPol. At 48 h post-infection, the mouse skin tissues were collected and subjected to Gram staining or homogenization for counting the bacterial CFU.

Macrophage-Biofilm Interaction Assay

The experiment was performed as previously described (Ahn et al., 2018). Briefly, *S. aureus* (5×10^7 CFU/ml) was incubated in LB broth on sterile glass coverslips at 37°C for 24 h in the presence or absence of DeinoPol. The biofilm was washed with PBS to remove LB broth and the planktonic bacteria. RAW264.7 murine macrophage cell line in FBS (fetal bovine serum) free-DMEM medium was added at 1×10^6 cells/ml to the preformed biofilm and further incubated at 37°C for 2 h in

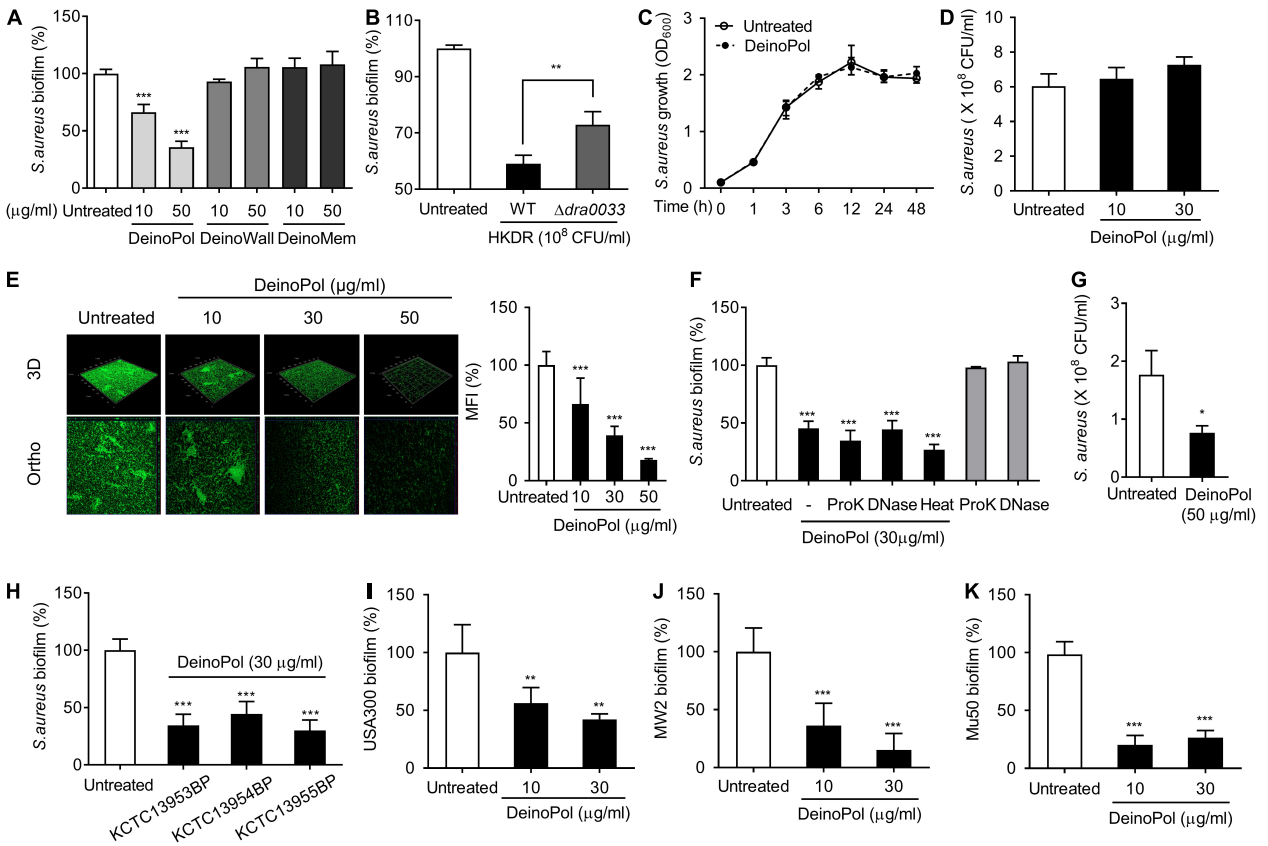

FIGURE 2 | DeinoPol inhibits biofilm formation of *S. aureus*. **(A,B)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates at 37°C for 24 h in the presence or absence of **(A)** DeinoPol (10 and 50 μg/ml), DeinoWall (10 and 50 μg/ml), or DeinoMem (10 and 50 μg/ml) isolated from *D. radiodurans* R1, or **(B)** HKDR (1×10^8 CFU/ml) of WT or Δ*dra0033* (DeinoPol-deficient strain) strains. Biofilms were determined by crystal violet assay. **(C)** *S. aureus* (1×10^7 CFU/ml) was grown for 1, 3, 6, 12, 24, or 48 h in the presence or absence of DeinoPol (30 μg/ml) under shaking conditions. The growth of *S. aureus* was determined by O.D. at 600 nm. **(D)** *S. aureus* (5×10^7 CFU/ml) was grown at 37°C for 24 h in the presence or absence of DeinoPol (10 or 30 μg/ml) under static culture condition. Bacterial viability was measured by counting CFU. Data are the mean values ± S.D. of triplicate samples. **(E)** *S. aureus* (5×10^7 CFU/ml) was grown on coverslips at 37°C for 24 h in the presence or absence of DeinoPol (10, 30 or 50 μg/ml) and DeinoPol-treated *S. aureus* biofilms were visualized by confocal laser scanning microscopy (green from SYTO9 and red from propidium iodide). One of three similar results is shown. In the right panel, the MFI values of each group were quantified by ImageJ software. **(F)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates at 37°C for 24 h in the presence or absence of DeinoPol (30 μg/ml), 50 μg/ml of proteinase K (ProK)-treated DeinoPol (30 μg/ml), 50 μg/ml of DNase I (DNase)-treated DeinoPol (30 μg/ml), or heat-treated DeinoPol (30 μg/ml). Biofilms were determined by crystal violet assay. Data are the mean values ± S.D. of triplicate samples. **(G)** HaCaT cells monolayer was exposed to *S. aureus* (MOI = 10) at 37°C for 8 h in the presence or absence of 30 μg/ml DeinoPol. After removing the supernatant containing the planktonic bacteria, biofilms or bacteria adherent onto the cell monolayer were measured by counting CFU. **(H)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates at 37°C for 24 h in the presence or absence of DeinoPol (30 μg/ml) purified from *D. radiodurans* KCTC13953BP, KCTC13954BP, or KCTC13955BP strains. **(I)** *S. aureus* USA300, **(J)** MV2, or **(K)** Mu50 (5×10^7 CFU/ml) were grown on polystyrene plates at 37°C for 24 h in the presence or absence of DeinoPol (10 or 30 μg/ml). Biofilms were determined by crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

a humidified incubator with 5% CO_2, followed by washing with PBS to remove the left suspension bacteria and RAW 264.7 cells. Then, the remaining biofilm was detached, suspended in PBS, and plated onto LB agar plates. After 24 h of incubation, the number of bacterial colonies was determined.

Statistical Analysis

The mean value ± standard deviation (S.D.) was obtained from triplicate samples for each treatment group. Statistical significance was determined by one-way ANOVA and Tukey post-test. Asterisks indicate significant induction compared with the control group (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

RESULTS

Deinococcus radiodurans Inhibits *Staphylococcus aureus* Biofilm Formation

To determine whether *D. radiodurans* can inhibit staphylococcal biofilm formation, we compared the biofilm formation of *S. aureus* in the presence of the indicated concentrations of deinococcal culture supernatant (DRsup) or heat-killed *D. radiodurans* R1 (HKDR). As shown in **Figures 1A,B**, DRsup potently inhibited biofilm formation by *S. aureus* in a dose-dependent manner and HKDR significantly inhibited biofilm

formation at 10^8 CFU/ml. To confirm, whether *Deinococcus* sp. inhibits staphylococcal biofilm formation, we tested three additional deinococcal strains, namely *D. radiodurans* KCTC13953BP, KCTC13954BP, and KCTC13955BP. All DRsup showed significant inhibitory effect on the biofilm production of *S. aureus* (**Figure 1C**). Next, we examined whether the anti-biofilm effect of *D. radiodurans* was due to its bactericidal effect on *S. aureus*. *S. aureus* was treated with DRsup or HKDR, and its growth and survival were measured. As shown in **Figures 1D,E**, neither DRsup nor HKDR altered *S. aureus* growth and viability. These data indicated that the inhibitory effect of *D. radiodurans* on staphylococcal biofilm formation was not due to a direct bactericidal effect, but may be caused by an indirect effect.

DeinoPol Inhibits *Staphylococcus aureus* Biofilm Formation

Next, to identify the deinococcal component responsible for the inhibition of staphylococcal biofilm formation, major cell wall components were isolated, and their effects on biofilm formation were investigated. Three major representative cell wall components, namely exopolysaccharide (DeinoPol), cell wall (DeinoWall), and cell membrane (DeinoMem), were isolated and examined for their effects on *S. aureus* biofilm formation. DeinoPol markedly inhibited staphylococcal biofilm formation, whereas DeinoWall and DeinoMem did not (**Figure 2A**). As mentioned above, we found no direct bactericidal effect of DeinoPol on *S. aureus* (**Figures 2C,D**). To investigate whether DeinoPol is a major component of the cell wall fraction that is responsible for the inhibition of biofilm formation, we compared the biofilm inhibitory effect of WT and DeinoPol knockout mutants (Δ*dra0033*) (Lin et al., 2020). As shown in **Figure 2B**, the inhibitory effect of the WT was significantly weakened in Δ*dra0033*. Confocal microscopy analysis also showed that *S. aureus* biofilm formation was inhibited by DeinoPol in a dose-dependent manner; however, dead cells were rarely detected (**Figure 2E**). We previously demonstrated that DeinoPol is composed of 89.9% polysaccharides, 8.8% proteins and 1.3% DNA (Lin et al., 2020). To confirm polysaccharides are the major components of DeinoPol inhibiting biofilm formation, *S. aureus* biofilm formation was examined in the presence of proteinase K-treated DeinoPol, DNase I-treated DeinoPol, or heat-treated DeinoPol. As shown in **Figure 2F**, proteinase K, DNase I, and heat treatment did not significantly alter the inhibitory effects of DeinoPol on biofilm formation of *S. aureus,* indicating that the polysaccharides, the main component of DeinoPol, are predominantly responsible for the anti-biofilm effect of DeinoPol against *S. aureus*. Skin tissue is the most common site for *S. aureus* colonization and infection. To investigate whether DeinoPol interferes with staphylococcal colonization of skin cells, we used HaCaT cells, immortalized human keratinocytes, in a monolayer where *S. aureus* was grown to form biofilms in the presence or absence of DeinoPol. After 8 h of incubation, the biofilm bacteria were enumerated. As shown in **Figure 2G**, DeinoPol treatment considerably decreased biofilm bacterial burdens on HaCaT cell monolayers compared with the non-treatment group. Next, to examine whether the anti-biofilm effect of DeinoPol is a common characteristic of all *D. radiodurans*, DeinoPol was purified from various *D. radiodurans* strains, such as *D. radiodurans* KCTC13953BP, KCTC13954BP, and KCTC13955BP, and its effect on *S. aureus* biofilms was investigated. As expected, all tested DeinoPol significantly inhibited *S. aureus* biofilm formation at levels similar to those of DeinoPol isolated from the R1 strain (**Figure 2H**). Biofilm formation is considered an important mechanism in the pathogenesis of methicillin-resistant *S. aureus* (MRSA). Biofilms confer drug tolerance to broad-spectrum antibiotics, which contributes to the emergence of antibiotic-resistant bacteria, such as MRSA (Mirani and Jamil, 2011; Piechota et al., 2018). To confirm whether DeinoPol also inhibits the biofilm formation of MRSA, MRSA strains such as USA300, MW2, and Mu50 were treated with DeinoPol, and biofilm formation was examined. **Figures 2I–K** show that all tested MRSA strains showed significantly lower levels of biofilm formation after treatment with DeinoPol, indicating that DeinoPol might be a broad-spectrum inhibitor for various *S. aureus* strains including MRSA.

DeinoPol Interferes With Biofilm Formation in the Early and Late Phases and Collapses Preexisting Biofilm

To determine the phase of biofilm development at which the inhibitory effect of DeinoPol occurs, *S. aureus* biofilms formed for 1, 3, 6, 12, 24, or 48 h in the presence or absence of DeinoPol were measured. The inhibitory effect of DeinoPol was observed from 3 h after biofilm formation and lasted up to 48 h (**Figure 3A**). Next, we determined whether DeinoPol has preventive or destructive effects on biofilms. First, the plates were pre-coated with DeinoPol at various concentrations, and their effects on the biofilm-forming ability of *S. aureus* were investigated. **Figure 3B** shows that biofilm formation was hampered by DeinoPol at concentrations from 8 to 200 μg/well in a dose-dependent manner, but was not inhibited by DeinoPol at 16 μg/ml. To examine the destructive effect of DeinoPol against *S. aureus* biofilms, preexisting biofilms for 24 h were treated with DeinoPol for 6 h, and the biofilm was measured. *S. aureus* biofilm was significantly destroyed by DeinoPol at 30 and 50 μg/ml, but not at 10 μg/ml (**Figure 3C**). These results suggest that DeinoPol can inhibit *S. aureus* biofilm formation at the early and late stages with prophylactic and destructive effects.

DeinoPol Regulates *ica* Gene Expression and Poly-*N*-Acetylglucosamine Production in *Staphylococcus aureus*

The intracellular adhesion (*ica*) locus is responsible for the production of poly-*N*-acetylglucosamine (PNAG), an essential component for staphylococcal biofilm formation (O'Gara, 2007). In addition, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) genes, such as *fib* (fibrinogen binding protein), *cna* (collagen binding protein), *fnbA* (fibronectin binding protein A), *fnbB* (fibronectin binding protein B), *clfA* (clumping factor A), *clfB* (clumping factor B), *ebps* (elastin-binding protein), and *eno* (laminin-binding protein), have been reported to be involved in the attachment of

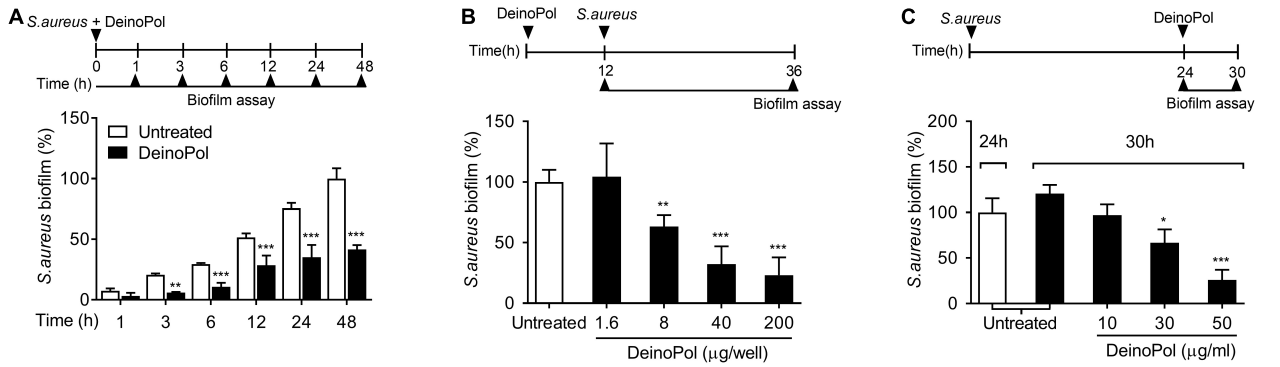

FIGURE 3 | DeinoPol interferes with biofilm formation in the early and late phases and collapses preexisting biofilm. **(A)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates at 37°C for 1, 3, 6, 12, 24, or 48 h in the presence of DeinoPol (30 μg/ml). **(B)** *S. aureus* (5×10^7 CFU/ml) was grown at 37°C for 24 h on polystyrene plates pre-coated with DeinoPol (1.6, 8, 40, or 200 μg/well) for 12 h. **(C)** Preexisting biofilm established with *S. aureus* (5×10^7 CFU/ml) at 37°C for 24 h on polystyrene plates was treated with DeinoPol (10, 30, or 50 μg/ml) and further incubated for 6 h. Biofilms were determined by crystal violet assay. Data are the mean values ± S.D. of triplicate samples. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

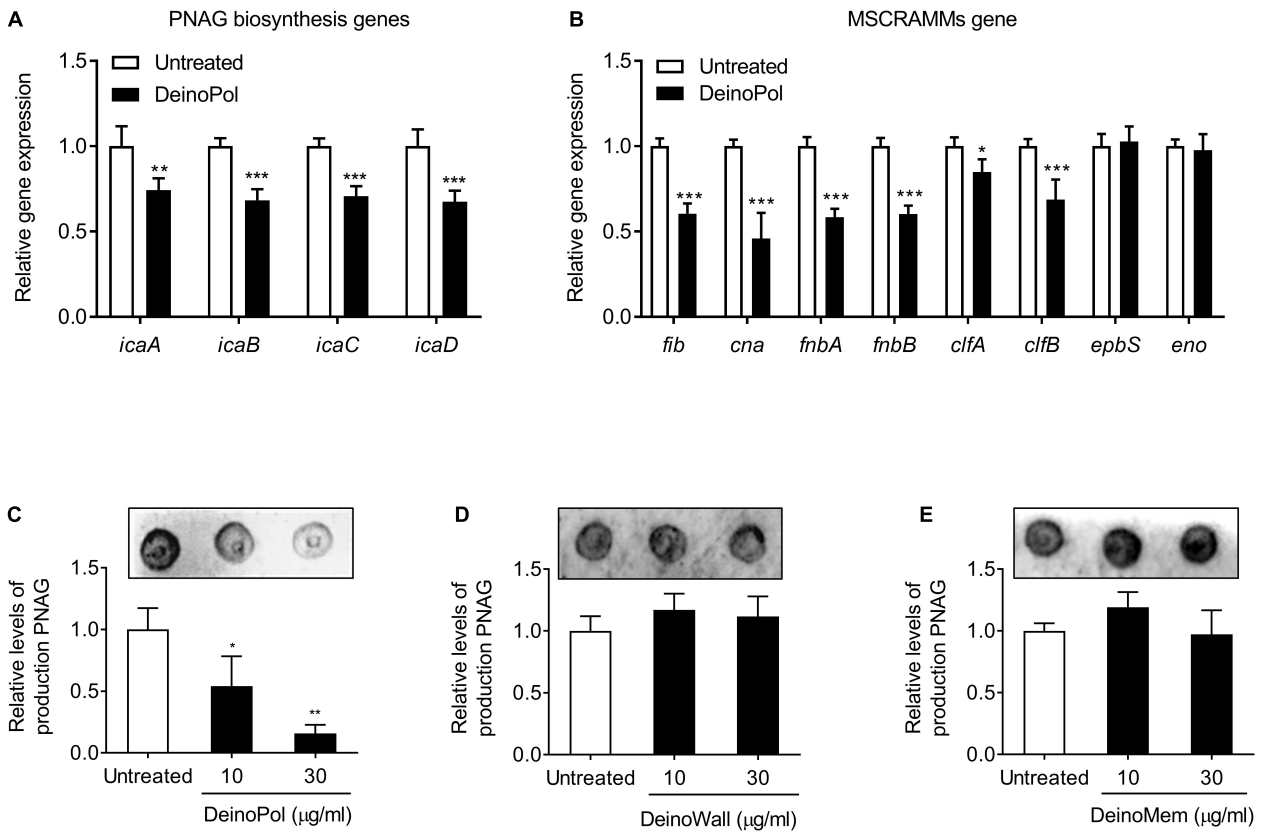

FIGURE 4 | DeinoPol regulates *ica* gene expression and poly-*N*-acetylglucosamine production in *S. aureus*. **(A,B)** *S. aureus* (1×10^8 CFU/ml) was treated with DeinoPol (50 μg/ml) for 12 h. Total RNA was isolated, and the mRNA expression levels of **(A)** *ica* genes (*icaA*, *icaB*, *icaC*, and *icaD*) and **(B)** microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) genes (*fib*, *cna*, *fnbA*, *fnbB*, *clfA*, *clfB*, *ebpS*, and *eno*) were examined by qRT-PCR analysis. The expression of *gyrB* was used for q-PCR normalization. Data are the mean values ± S.D. of triplicate samples. **(C–E)** *S. aureus* (1×10^8 CFU/ml) was treated with DeinoPol (10 or 30 μg/ml), DeinoWall (10 or 30 μg/ml), or DeinoMem (10 or 30 μg/ml) for 12 h. The crude extract of PNAG from *S. aureus* was blotted on nitrocellulose membrane and detected by WGA-biotin/HRP-streptavidin, followed by visualization with chemiluminescence detection. One of three similar results is shown. In the lower panel, the densities of PNAG were quantified by densitometry analysis. Data are the mean values ± S.D. of triplicate samples. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

staphylococci. To identify the underlying mechanisms for biofilm inhibition by DeinoPol, the regulatory effect of DeinoPol on the expression of these staphylococcal biofilm-associated genes was examined. As shown in **Figure 4A**, when *S. aureus* was incubated with DeinoPol (50 μg/ml), the mRNA expression of icaABCD was significantly downregulated. Moreover, the mRNA expression of *fib*, *cna*, *fnbA*, *fnbB*, *clfA*, and *clfB* was suppressed by DeinoPol treatment, but *ebps* and *eno* expression was not affected (**Figure 4B**). Next, the production of PNAG was measured in *S. aureus* treated with DeinoPol, DeinoWall, or DeinoMem. **Figures 4C–E** show that PNAG production was inhibited by DeinoPol in a dose-dependent manner, whereas DeinoWall and DeinoMem did not affect to PNAG. These results suggest that DenoPol inhibits *ica* gene expression and PNAG production, contributing to the inhibition of *S. aureus* biofilm formation.

DeinoPol Inhibits *Staphylococcus aureus* Burdens in Skin Wound Infection Model

The inhibition of *S. aureus* biofilm formation by DeinoPol *in vivo* was evaluated in a mouse model of burn wound biofilm. *S. aureus* was treated to the mouse dorsal burn wound in the presence or absence of DeinoPol for 48 h to allow biofilm formation and the amount of biofilm bacteria was calculated by counting colony-forming units. **Figure 5A** shows that the average bioburden on the mouse wound skin surface was 5.5×10^8 CFU/g tissue in the untreated group. However, this average of bioburden was decreased to 9.6×10^7 CFU/g and 7.6×10^7 CFU/g by treatment with 10 and 50 μg DeinoPol, respectively. Gram staining analysis of the skin tissue also showed that a dense bacterial community (deep violet) was observed below the epidermis of the skin in the *S. aureus* infection group, and it was substantially reduced in the DeinoPol treatment group (**Figure 5B**). These results suggest that DeinoPol exerts anti-biofilm effects *in vivo*, implying its potential use as a clinical treatment for infectious diseases associated with *S. aureus* biofilms.

DeinoPol Enhances the Antibacterial Susceptibility of *Staphylococcus aureus* in Biofilms

Biofilm resistance to antibiotics has been considered a public health concern owing to the improper use and overuse of antibiotics (Sharma et al., 2019). To examine if DeinoPol increases the inhibitory effect of antibiotics on *S. aureus* biofilm formation, *S. aureus* biofilm was treated with various antibiotics clinically used for *S. aureus* infection, such as penicillin, vancomycin, oxacillin, and cefazolin, in the presence or absence of DeinoPol, and then the biofilm was measured. As shown in **Figures 6A–D**, DeinoPol enhanced the capacity of all tested antibiotics to inhibit *S. aureus* biofilms. Although macrophages are major innate immune defenders against microbial infection, *S. aureus* biofilms have been reported to prevent macrophage phagocytosis and avoid immune responses (Thurlow et al., 2011). The effect of DeinoPol on macrophage function to eradicate *S. aureus* biofilms was examined. Pre-incubated *S. aureus* biofilm with DeinoPol was treated with RAW264.7 cells, and the degree of biofilm reduction and the cell viability were measured. **Figure 6E** shows that DeinoPol enhanced the removal effect of macrophages against *S. aureus* biofilm (left panel). The macrophage viability was not altered (right panel). These results indicate that DeinoPol can improve the susceptibility of *S. aureus* biofilms to antibiotics and macrophages.

DISCUSSION

D. radiodurans, an extremophile bacterium, is a normal inhabitant flora of the human body, but its interactive role in the defense against pathogen infection has not been studied. In this study, we revealed that *D. radiodurans* inhibited *S. aureus* biofilm formation. Specifically, DeinoPol acted as a major component of *D. radiodurans*, inhibiting biofilm formation without affecting bacterial growth by abrogating the expression of the *ica* genes required for PNAG production. Furthermore, DeinoPol enhanced the susceptibility of biofilms to antibiotics and macrophages. These results indicate that *D. radiodurans* functions as a commensal that prevents *S. aureus* infection, and provide a compelling evidence that DeinoPol can be a potential antimicrobial agent for controlling *S. aureus* biofilm formation.

DeinoPol, not DeinoWall and DeinoMem, was identified as a functional constituent of *D. radiodurans* to inhibit biofilm formation by *S. aureus* in this study. Moreover, this inhibitory function was not confined to DeinoPol of the type strain, but was also possessed by the DeinoPol of *D. radiodurans* KCTC13953BP, KCTC13954BP, and KCTC13955BP strains isolated from Baekrokdam, Jeju, Republic of Korea. These results are consistent with previous reports showing the inhibitory function of extracellular galactan from *Kingella kingae* against biofilm formation by *S. aureus*, *S. epidermidis*, *Candida albicans*, and *Aggregatibacter actinomycetemcomitans* (Bendaoud et al., 2011), and inhibition of enterohemorrhagic *Escherichia coli* biofilm formation by the EPS released by *Lactobacillus acidophilus* (Kim et al., 2009). In contrast, EPS composes up to 40% of the dry weight of dental plaque, and glucan is essential for biofilm formation by *Streptococcus mutans* (Koo et al., 2010). An EPS-deficient strain of *Bacillus subtilis*, Δ*eps*H, formed smooth colonies and fragile pellicles compared with the WT strain (Epstein et al., 2011). This contradictory effect of EPS on bacterial biofilm formation is due to the diverse structures and compositions of each bacterial species (Joseph and Wright, 2004; Jiang et al., 2011). We previously showed that DeinoPol is composed of xylose, galactose, fucose, glucose, arabinose, and fructose in a molar ratio of 10.6:6.1:4.2:3.8:2.6:1.0, and 14.89% of unknown sugars (Lin et al., 2020). This oligosaccharide composition is similar to that of *Lactobacillus plantarum* WLPL04 EPS, which consists of xylose, glucose, and galactose at a molar ratio of 3.4:1.8:1 and exerts an anti-biofilm activity against *Pseudomonas aeruginosa* CMCC10104, *Escherichia coli* O157:H7, *Salmonella typhimurium* ATCC13311, and *S. aureus* CMCC26003 (Liu et al., 2017). In particular, DeinoPol contains fucose, which is considered a rare sugar contained in bacterial EPS and has been reported to have an inhibitory function on the biofilm of formation several bacteria (Khodse and Bhosle, 2010). L-Fucose inhibited the biofilm formation of *Campylobacter*

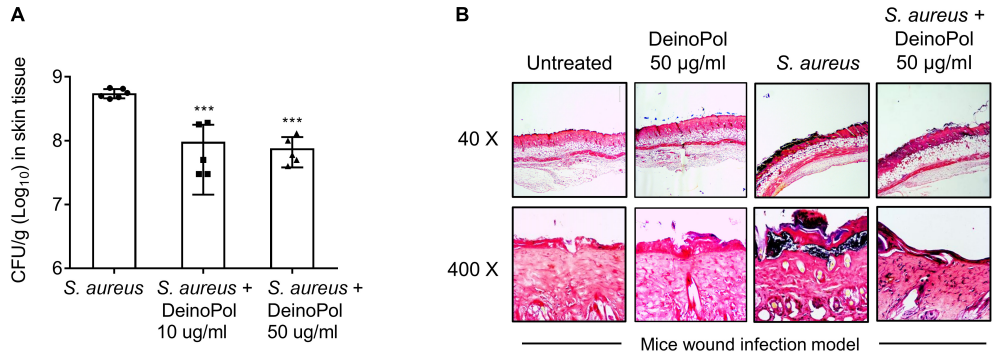

FIGURE 5 | DeinoPol inhibits *S. aureus* burdens in skin wound infection model. **(A,B)** *S. aureus* (1×10^7 CFU) was inoculated to the dorsal burn wound of mice in the presence or absence of DeinoPol (10 or 50 µg/wound) for 48 h to allow biofilm formation. **(A)** *S. aureus* infected-skin tissues were homogenized by using a 2-mm homogenizer bead, and bioburden was calculated by counting CFU. Six and five mice were used in the non-treatment and DeinoPol-treatment groups, respectively. Asterisks indicate significant induction compared with the non-treatment group (***$P < 0.001$). **(B)** The tissue sections were subjected to Gram-staining and imaged at 40 × or 400 × magnification. One of five similar results is shown.

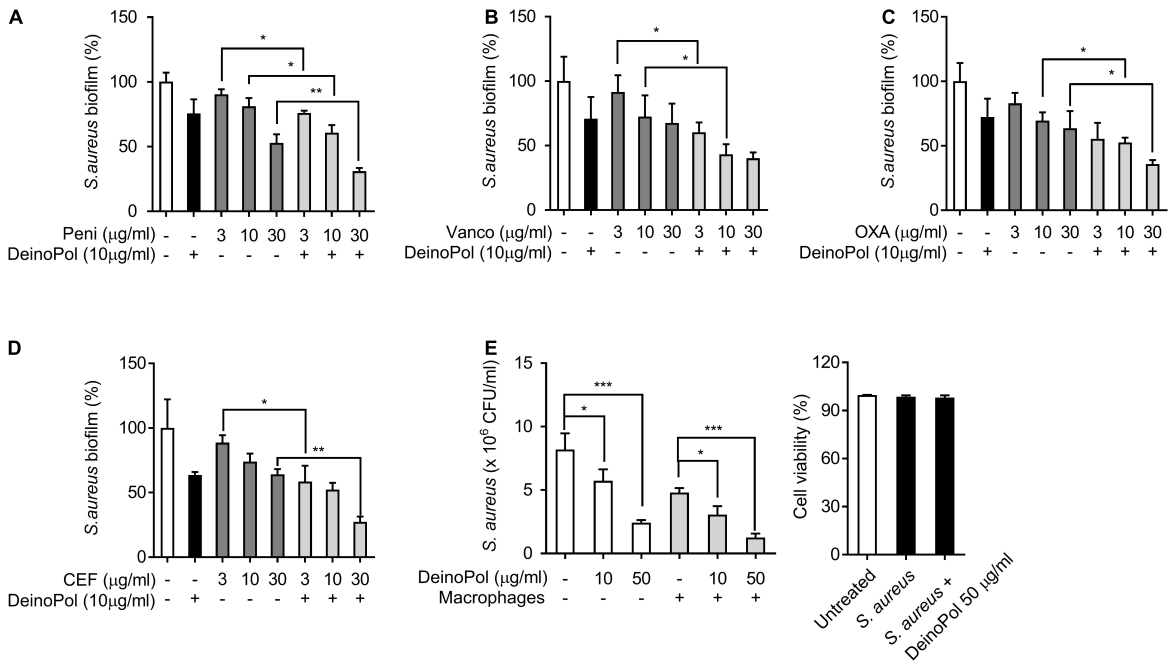

FIGURE 6 | DeinoPol enhances the antibacterial susceptibility of *S. aureus* in biofilm. **(A–D)** *S. aureus* (5×10^7 CFU/ml) was grown on polystyrene plates with DeinoPol (10 µg/ml) at 37°C for 24 h, and further incubated for 6 h in the presence or absence of **(A)** penicillin (3, 10, or 30 µg/ml), **(B)** vancomycin (3, 10, or 30 µg/ml), **(C)** oxacillin (3, 10, or 30 µg/ml), or **(D)** cefazolin (3, 10, or 30 µg/ml). Biofilms were determined by crystal violet assay. **(E)** *S. aureus* (5×10^7 CFU/ml) was grown on coverslips at 37°C for 24 h with DeinoPol (10 or 50 µg/ml) and RAW 264.7 cells (1×10^6 cells/ml) were added to the preformed biofilm and further incubated at 37°C for 2 h. The remaining biofilms were measured by counting colony forming units (left panel) and the cell viability was assessed by flow cytometry after staining with propidium iodide (right panel). Data are the mean values ± S.D. of triplicate samples. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

jejuni NCTC11168, but not that of the fucose permease mutant strain (Dwivedi et al., 2016). L-Fucose and trithiotriazine-cored L-fucose cluster inhibited biofilm formation by *P. aeruginosa* through binding with LecB, a lectin of *P. aeruginosa*, and this effect was also observed in *P. aeruginosa*-infected cystic fibrosis patients (Hauber et al., 2008; Smadhi et al., 2014; Grishin et al., 2015). These previous reports support that the unique oligosaccharide composition and fucose content may play a crucial role in the anti-biofilm activities of DeinoPol.

Here, we showed that DeinoPol inhibited the biofilm formation of *S. aureus* in the early and late phases, and even destroyed preformed biofilms. This implies that the inhibition of biofilm formation by DeinoPol might be elicited by several distinct mechanisms. Carbohydrate, the major component of

EPS, confers an anionic property to the bacterial EPS, which has been reported to interfere with the prerequisite for biofilm formation, such as cell-surface and cell-cell interactions, by electrostatic modifications (Valle et al., 2006; Jiang et al., 2011). The ability of DeinoPol to destroy the preformed biofilms is coincident with previous resports, such as dispersion of *P. aeruginosa* biofilm by A101 polysaccharide purified from *Vibrio* sp. QY101 (Jiang et al., 2011) and disruption of preformed biofilm of *S. aureus* and *S. epidermidies* by *Kingella kingae* exopolysaccharide (Bendaoud et al., 2011). However, the precise mechanisms by which the preformed biofilms are destroyed by bacterial polysaccharide are still unknown. It has been reported that bacterial polysaccharides act as a signaling molecule that can regulate gene expression of bacteria (Kim et al., 2009), which encourages speculation that DeinoPol induces the production of self-produced factors of *S. aureus* mediating biofilm disassembly, such as phenol soluble modulins (PSMs) or extracellular proteases (Boles and Horswill, 2008; Otto, 2013), *via* regulating gene expression in *S. aureus*. Further studies are required to elucidate the exact mechanisms of the potential receptor for DeinoPol and its signaling pathway modulating the expression of molecules known to degrade PNAG and *S. aureus* biofilm.

S. aureus in biofilms is 10–1,000 times more resistant to antibiotics and phagocytosis by macrophages and neutrophils than the planktonic bacteria, and this is associated with chronic inflammation (Mah, 2012). Hence, there have been recent attempts to use additives to enhance the susceptibility of bacterial biofilms to antibiotics or immune cells (Verma et al., 2009; Ferrer-Espada et al., 2020). As we found in this study, DeinoPol increased the anti-biofilm effect of antibiotics and macrophages. It is hypothesized that the biofilm matrix polymer is loosened by DeinoPol by regulating PNAG production, which eases the penetration of antibiotics. Inhibition of PNAG production by DeinoPol might be a key mechanism for enhancing the susceptibility of *S. aureus* biofilms to antibiotics, as PNAG has been reported to act as a physical barrier for *S. aureus* biofilms, conferring resistance to antibiotics and the immune system (Brooks and Jefferson, 2014). Molecules that can dissemble bacterial biofilms by degrading or regulating EPS synthesis are referred to as biofilm-dispersing agents, and they can be applied to various bacterial biofilms detected in the environment or clinics (Verderosa et al., 2019). For example, chitosan, D-amino acids, phenol-soluble modulins, and dispersin B are dispersing agents that enhance the susceptibility of biofilms to antibiotics by inducing the release of planktonic cells from biofilms (Alksne and Projan, 2000; Izano et al., 2007; Mu et al., 2014; Sanchez et al., 2014). This study confirms that DeinoPol is a potential biofilm-dispersing agent against *S. aureus*.

DeinoPol regulated the biofilm formation of *S. aureus* laboratory strains as well as MRSA isolates, suggesting that DeinoPol could be widely used to inhibit the biofilm formation of various *S. aureus* strains. Most natural biofilms are composed of multispecies bacteria (Yang et al., 2011). In particular, *S. aureus* has been reported to form a multispecies biofilm with *P. aeruginosa*, *Salmonella* spp., *Enterococcus faecalis*, *Cutibacterium acnes*, *C. albicans*, and *S. epidermidis* in various tissues (Citron et al., 2007; Pathak et al., 2012; Gannesen et al., 2018; Iniguez-Moreno et al., 2018; Trizna et al., 2020). Multispecies biofilms strengthen their protective effect against hostile environments *via* cell-cell communication by quorum sensing or diffusible signals, genetic exchanges, or physical interaction (Li and Tian, 2012; Rendueles and Ghigo, 2012; Hansen et al., 2017; Krzyzek and Gosciniak, 2018). Therefore, it is important to clarify the anti-biofilm effect of candidate molecules against single-species biofilms as well as multispecies biofilms. The regulatory effect of DeinoPol against multispecies biofilms containing *S. aureus* was not determined in this study, but should be investigated in future studies to advance the application of DeinoPol as a clinical treatment for *S. aureus* infectious diseases.

Although Δ*dra0033* strain, DeinoPol-deficient strain, was treated to *S. aureus* biofilm, approximately 30% *S. aureus* biofilm was reduced. This implies that unknown substances other than DeinoPol may be slightly involved in the residual effect of Δ*dra0033* strain on biofilm inhibition. Various substances derived from microorganism have been reported to inhibit *S. aureus* biofilm formation. For example, autoinducer-2 (AI-2), a quorum-sensing signaling molecule, is known to inhibit biofilm of *S. aureus* by down-regulating the transcription of *icaA* (Yu et al., 2012). AI-2 has also been reported as a major quorum-sensing molecule to regulate physiological functions in *D. radiodurans* (Lin et al., 2016), which may have acted as a substance responsible for residual inhibitory effect of Δ*dra0033* strain. *D. radiodurans* highly expresses carotenoids that play an important role in the antioxidant effects of the strain through resistance to ROS. Carotenoids have been reported to exhibit the anti-biofilm functions against various bacterial strains. Carotenoid zeaxanthin inhibited virulence gene expression and biofilm formation of *P. aeruginosa via* quorum sensing inhibition (Gokalsin et al., 2017). Biofilm formation of Gram-positive strains, such as *S. aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*, were also inhibited by carotenoids isolated from skull and exterior covering body parts of prawn (Jeyachandran et al., 2020). These reports lead us to speculate that Δ*dra0033* strain-derived carotenoids were involved in its residual effects of biofilm inhibition. Besides, we previously demonstrated that single mutant Δ*dra0033* strain produced 79.8% less DeinoPol than that of WT strain. However, it still expressed DeinoPol in a detectable amount, which may function, at least in part, as an inhibitor against *S. aureus* biofilm. To date, there have been few studies on the anti-biofilm substance of *D. radiodurans*, so it can only be speculated. Further studies are needed to determine the existence and mechanisms of *D. radiodurans*-produced biofilm regulating molecules other than DeinoPol.

Live beneficial bacteria, such as probiotics, generally used as foods or dietary supplements rather than drugs due to its risk and safety issues (Venugopalan et al., 2010). However, various bacterial products, such as exopolysaccharides, have been approved for medical applications in human since the mid-nineteenth century (Moscovici, 2015). For example, dextran or hyaluronic acid/hyaluronan produced by bacteria are now applicated in chronic wound healing, osteoarthritis treatment, or plasma volume expansion for controlling wounds shock

(Necas et al., 2008; Nwodo et al., 2012). DeinoPol contents and structure have already been described in our previous report (Lin et al., 2020) and its anti-biofilm efficacy *in vitro* and *in vivo* was also demonstrated in this study. Thereafter, if clinical trials demonstrate acceptable effects of DeinoPol, it could be used clinically as a standalone antimicrobial agent or an additive to antibiotics to treat biofilm-associated infections.

Taken together, the results of this study suggest that DeinoPol is a major constituent of *D. radiodurans* that inhibits *S. aureus* biofilm formation. DeinoPol can be used as a potential alternative or additive dispersing agent for the treatment of infectious diseases caused by *S. aureus* biofilms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Korea Atomic Energy Research Institute.

AUTHOR CONTRIBUTIONS

KA and HS conceived the idea and contributed to the discussion of the results followed by writing and reviewing the manuscript. KA, FC, SH, and HS designed the experiments. FC, KA, JZ, and HJ performed the experiments, and/or interpreted the data. M-KK, KK, J-IC, SH, and SL provided critical comments and contributed to the discussion of the results followed by writing and reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.712086/full#supplementary-material

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Phenolic-Rich Plant Extracts With Antimicrobial Activity: An Alternative to Food Preservatives and Biocides?

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In recent years, the search for natural plant-based antimicrobial compounds as alternatives to some synthetic food preservatives or biocides has been stimulated by sanitary, environmental, regulatory, and marketing concerns. In this context, besides their established antioxidant activity, the antimicrobial activity of many plant phenolics deserved increased attention. Indeed, industries processing agricultural plants generate considerable quantities of phenolic-rich products and by-products, which could be valuable natural sources of natural antimicrobial molecules. Plant extracts containing volatile (e.g., essential oils) and non-volatile antimicrobial molecules can be distinguished. Plant essential oils are outside the scope of this review. This review will thus provide an overview of current knowledge regarding the promises and the limits of phenolic-rich plant extracts for food preservation and biofilm control on food-contacting surfaces. After a presentation of the major groups of antimicrobial plant phenolics, of their antimicrobial activity spectrum, and of the diversity of their mechanisms of action, their most promising sources will be reviewed. Since antimicrobial activity reduction often observed when comparing *in vitro* and *in situ* activities of plant phenolics has often been reported as a limit for their application, the effects of the composition and the microstructure of the matrices in which unwanted microorganisms are present (e.g., food and/or microbial biofilms) on their activity will be discussed. Then, the different strategies of delivery of antimicrobial phenolics to promote their activity in such matrices, such as their encapsulation or their association with edible coatings or food packaging materials are presented. The possibilities offered by encapsulation or association with polymers of packaging materials or coatings to increase the stability and ease of use of plant phenolics before their application, as well as to get systems for their controlled release are presented and discussed. Finally, the necessity to consider phenolic-rich antimicrobial plant extracts in combination with other factors consistently with hurdle technology principles will be discussed. For instance, several authors recently suggested that natural phenolic-rich extracts could not only extend the shelf-life of foods by controlling bacterial contamination, but could also coexist with probiotic lactic acid bacteria in food systems to provide enhanced health benefits to human.

Keywords: phenolic-rich plant extracts, antimicrobial activity, food preservation, biocides, biofilms, delivery systems

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INTRODUCTION

Search for natural alternatives to synthetic food preservatives and disinfectants has been the subject of intensive research during the last decade. It has namely been stimulated by increasing concerns regarding their innocuity [e.g., nitrites (EFSA Panel et al., 2017), sulfites (EFSA Panel, 2016), polyhexamethylenebiguanide (PHMB), 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan; Müller and Kramer, 2008)] or their environmental impact [e.g., triclosan (Abbott et al., 2020)]. Regulatory changes [e.g., in the European Union (EU)] disinfectants are covered by the European Regulation concerning the marketing and use of biocidal products [Regulation (EU) no 528/2012, 2012] and increasing demand of consumers for organic foods (in which only a limited number of food preservatives are authorized) or for «clean label» food products (without or with a limited number of food additives such as food preservatives) are important drivers of this trend. New natural antimicrobial extracts/molecules are expected (i) to preserve raw foods (e.g., raw fish or meat) or foods minimally processed to better preserve their organoleptic and nutritional properties and (ii) to reduce food waste by extending shelf life of highly perishable foods [one of the objectives of Sustainable Development Goal (SDG) to "End hunger, achieve food security and improved nutrition and promote sustainable agriculture" of United Nations in line with EU objective to reach a 50% food waste reduction by 2030].

In this context, plant extracts are promising sources of antimicrobial molecules. Plant antimicrobial molecules are grouped into different classes based on their chemical structure and properties: essential oils, phenolics, alkaloids, saponins, and peptides (Ferdes, 2018). The present review is focused on phenolics, which are the most numerous among secondary metabolites groups of plants. Plant secondary metabolites (also called phytochemicals) are biosynthesized by plants as a result of biotic (e.g., contamination by phytopathogenic microorganisms) and abiotic factors (e.g., UV light; Harborne, 2001). Some phytochemicals are thus toxic and only edible plants will thus be considered as potential sources of alternative to food preservatives. Another trait of some plant phenols and polyphenols is their biosynthesis in response to seasonal fluctuations in UV light for plant protection (Köhler et al., 2017). This variability of phenolics content of plants as a function of climatic conditions must be kept in mind, when the objective is to produce plant extracts with a standard antimicrobial activity resulting from a given content in antimicrobial phenolics.

As stated by Cheynier et al. (2013), an ever-increasing number of different plant phenolics, representing tens of thousands of different chemical structures, have been identified. Progresses both in analytical techniques, namely mass spectrometry techniques, and in extraction and separation techniques result in a better knowledge of the structural diversity of plant phenolics and of their distribution in plant tissues, as recently reviewed by Piccolella et al. (2019). Essential oil phenolics, which are volatile molecules generally extracted from plants by steam distillation, will be out of the scope of the present review, since their potential use as alternatives to food preservatives (Falleh et al., 2020), as well as their capacity to fight against biofilms (Nuta et al., 2021), have been recently reviewed. Despite their large structural diversity, most antimicrobial plant phenolics belong to six groups: flavonoids, phenolic acids, tannins, stilbenoids, quinones, and coumarins (Bouarab-Chibane et al., 2018a).

The antimicrobial activity of most plant phenolics has been investigated *in vitro* in microbiological media with a far more simple composition and microstructure than food matrices and against planktonic bacteria. Namely due to interactions of plant phenolics with other food constituents at the expense of their interactions with microorganisms, most antimicrobial plant phenolics have a far lower activity in food than *in vitro*. A major concern for hygienic safety of food production systems is the presence of microbial biofilms on the surfaces of food production equipment and facilities. Microorganisms in biofilms are embedded in a network of exopolymeric substances. This network of exopolymeric substances limits direct contact of microorganisms with antimicrobial substances, including plant phenolics, exerting thereby a protective effect. Moreover, microorganisms in biofilms are in a particular physiological state, which also modifies their sensitivity to antimicrobial substances. Therefore, after a presentation of the structural diversity of plant phenolics and of their mechanisms of antimicrobial action, a focus on the factors limiting efficiency of antimicrobial plant phenolics in food and biofilms matrices, as well as on their specific mechanisms of action against biofilms, such as quorum sensing inhibition is proposed. Delivery vehicles promoting the antimicrobial activity of plant phenolics in food matrices or against biofilms (e.g., active coatings, films, or particles) are also presented.

STRUCTURAL DIVERSITY OF ANTIMICROBIAL PLANT PHENOLICS

Dietary Sources of Phenolics

Phenolics are a class of organic compounds that occurs in all plants as secondary metabolites in varying concentrations. Perez-Jimenez et al. (2010) exploited Phenol-Explorer database to identify the 100 richest dietary sources of polyphenols: they contain from 10 mg per 100 ml (rosé wine) to 15 g per 100 g (cloves). The richest sources are various spices and dried herbs, cocoa products, berries, some seeds, nuts, and some vegetables, including olive and globe artichoke heads. Due to their availability in sufficient amounts for a low cost, some by-products or wastes of phenolic-rich edible plants are promising sources of antimicrobial phenolics. The most studied sources in the 2007–2017 decade for extraction of antimicrobial phenolics were listed by Bouarab-Chibane et al. (2018a) and the potential of application of most of their extracts was recently reviewed by several authors: they include spent coffee (Monente et al., 2015), green tea waste (Siddiqui et al., 2016), olive pomace and olive leaf (Munekata et al., 2020; Difonzo et al., 2021), pomegranate peel (Chen et al., 2020) or aril, grape pomace or seeds (Silva et al., 2021), mango kernel (Mwaurah et al., 2020),

myrtle berries seeds (Jabria et al., 2016), dates (Kchaou et al., 2016), walnut green husk (Jahanban-Esfahlan et al., 2019), almond skin (Bolling, 2017), tomato seeds (Taveira et al., 2010; Szabo et al., 2019), buckwheat hull extract (Cabarkapa et al., 2008), pomelo peel (Liu et al., 2017b; Tocmo et al., 2020).

Moreover, for a given plant, phenolics presence and/or concentration greatly varies from a plant tissue to another one: for instance, resveratrol concentration in vine (*Vitis vinifera*) was reported to vary from less than 1.5 mg/kg of fresh berries (where it is primarily located in skin) to 37 mg/kg in stems (Mikeš et al., 2008). As underlined by Piccolella et al. (2019) in their review, "isolating or detecting phenols and polyphenols necessarily requires a good knowledge of how, when and where these substances are bio-synthesized and stored."

Antimicrobial Activity of Individual Phenolics

Examples of antimicrobial phenolics belonging to the different structural groups of phenolics (phenolic acids, flavonoids, tannins, stilbenoids, quinones, and coumarins) as well as their minimal inhibitory concentrations (MICs) against various unwanted bacteria and their food sources are presented in **Table 1**. Examples of phenolic acids belonging to hydroxybenzoic acids and hydroxycinnamic acids are listed, as well as examples of flavonoids belonging to each of its different sub-classes according to their degree of oxidation: flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanins.

Significantly different MIC values of a given phenolic against the same bacterial species were sometimes reported by different authors. For instance, Pacheco-Ordaz et al. (2017) and Skroza et al. (2019) reported 3.4 $g{\cdot}L^{-1}$ and 0.21 $g{\cdot}L^{-1}$ MIC values of gallic acid against *Escherichia coli*, respectively. Such differences are likely due to the differences between the experimental procedures applied for MIC determination: in the absence of the use of a standard MIC determination procedure, differences regarding incubation conditions (initial number of culturable bacteria in the inoculum, temperature …) and bacterial strains can explain such differences. Indeed, MIC values were determined with *E. coli* O157:H7 ATCC 43890 and ŽMJ 129 (clinical isolate) strains, respectively. A 2.5 10^6 $cfu{\cdot}ml^{-1}$ and a 10^5–10^6 $cfu{\cdot}ml^{-1}$ inoculum were added in Mueller Hinton broth, which was the incubated for 24 h at 37°C in both studies. Besides possible differences of sensitivity to gallic acid of both *E. coli* strains, a higher inoculum in the first case results thus in a significantly lower gallic acid to bacteria ratio, which might partly explain the highest MIC value reported by Pacheco-Ordaz et al. Another difference lies in the fact that in the first case MIC was detected by monitoring wells without turbidity, while in the second case absence of viable bacteria was detected following incubation with an indicator of bacterial respiratory activity (iodonitrotetrazolium chloride).

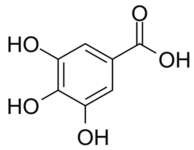
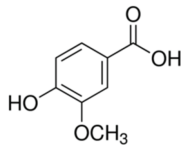
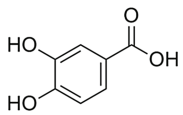
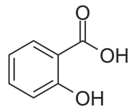
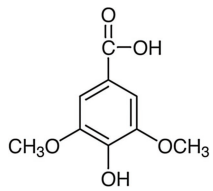
As underlined by Adamczak et al. (2020), differences can also result from the use of different solvents for the dissolution of pure phenolics although dimethylsulfoxide is generally used. However, as can be seen in **Table 1**, for most phenolics, MIC values determined by different authors against a given bacterial species are similar. The lowest MIC values reported in **Table 1** are 5 $mg{\cdot}L^{-1}$ for cranberry anthocyanins against *Staphylococcus aureus* (Gong et al., 2021) and 8 $mg{\cdot}L^{-1}$ for scopoletin against *Salmonella enteritidis* serovar Typhimurium (Mfonku et al., 2021). Since anthocyanin contents of around 50 mg anthocyanins per 100 g of cranberries from different cultivars were reported (Narwojsz et al., 2019), cranberries are a promising source of such antimicrobial phenolics.

As pointed out by Adamczak et al. (2020), compared to the number of studies regarding the antimicrobial activity of phenolic-rich plant extracts, only a few authors investigated the antimicrobial activity of individual pure phenolics (Borges et al., 2013; Pacheco-Ordaz et al., 2017; Bouarab-Chibane et al., 2019; Skroza et al., 2019). Determination of the antimicrobial activity of pure phenolics commonly present in plant extracts opens the possibility to estimate their contribution to the activity of plant extracts, in which they are present: for instance, Phadungkit and Luanratana (2006), determined the MIC against *Salmonella* of the three phenolics identified in dried fruit extracts of *Ardisia elliptica* Thunb (syringin, quercetin and a methoxylated derivative of quercetin, isorhamnetin), a fruit used as food, as well as in Thai traditional medicine. Since the antimicrobial activity of plant extracts is generally due to different individual phenolics, antimicrobial activity assays of pure phenolics alone or in combination allows to check whether they have a synergistic activity. Gutiérrez-Fernández et al. (2013) observed thus a synergistic activity against *Enterococcus faecalis* of gallic acid and octyl gallate, while Mellegard et al. (2009) reported no synergistic activity against *S. aureus* of the four dominating phenolic compounds in the leaves of *Sphagnum papillosum*. Comparison of MIC against foodborne pathogenic bacteria of pure phenolics identified with their lower concentrations in the leaves of this plant led these authors to question their potency.

However, these studies with individual pure phenolics are generally performed with synthetic phenolics commercially available. This set of commercial molecules comprises a far lower number of phenolics than the tens of thousands of plant phenolics with different structures, that have been identified. For instance, plant phenolics with high molecular weight, such as tannins, are underrepresented. Moreover, most natural plant phenolics are glycosylated, while only a limited number of commercial phenolics are glycosylated (e.g., vitoxin, orientin, naringin, **Table 1**). Several authors reported that most aglycones of plant phenolics have a higher antimicrobial activity than their glycosylated forms. This is consistent with Guo et al. (2020) observation of an increase in antibacterial activity of mulberry leaves following their solid state fermentation by edible fungi, which was correlated with an increase in kempferol and quercetin aglycones at the expense of their glycosides.

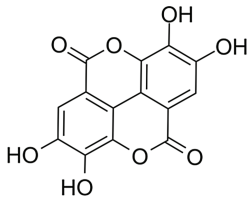
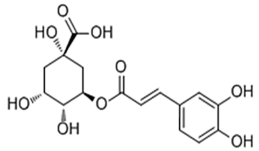
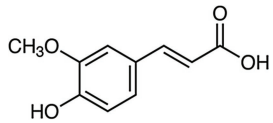
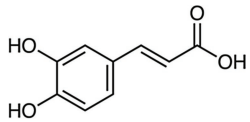
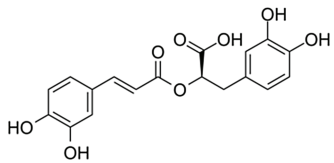
Identification of antimicrobial phenolics in a plant is necessary to guide their extraction and get extracts with a standardized antimicrobial activity. The most active phenolics of plant extracts launched with a claim for their antimicrobial activity are thus 2,6-dimethoxy-1,4-benzoquinone in moso bamboo extract (Takeguard™, Takexlabo, Osaka, Japan), hydroxytyrosol in olive extract (Hidrox 10X Liquid Concentrate, CreAgri, Inc., Hayward,

TABLE 1 | Examples of antimicrobial plant phenolics belonging to phenolic acids, flavonoids, tannins, stilbenoids, quinones, and coumarins.

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
Phenolic acids	Hydroxybenzoic acids C_6–C_1	Gallic acid		*Escherichia coli* 3.4 g·L^{-1}	Pacheco-Ordaz et al., 2017	Chestnut, clove
				Salmonella typhimurium 3.4 g·L^{-1}		
				E. coli 1.5 g·L^{-1}	Borges et al., 2013	
				Pseudomonas aeruginosa 0.5 g·L^{-1}		
				Staphylococcus aureus 1.75 g·L^{-1}		
				Listeria monocytogenes 2 g·L^{-1}		
				S. aureus 0.21 g·L^{-1}	Skroza et al., 2019	
				Bacillus cereus 0.21 g·L^{-1}		
				E. coli 0.21 g·L^{-1}		
				Salmonella Infantis 0.21 g·L^{-1}		
		Vanillic acid		*E. coli*, 3.4 g·L^{-1}	Pacheco-Ordaz et al., 2017	Açai oil, argan oil, wine, vinegar
				S. typhimurium 2.5 g·L^{-1}		
				S. aureus 0.42 g·L^{-1}	Skroza et al., 2019	
				B. cereus 0.1 g·L^{-1}		
				E. coli 0.1 g·L^{-1}		
				S. Infantis 0.21 g·L^{-1}		
		Proto-catechuic acid		*E. coli*, 3.1 g·L^{-1}	Pacheco-Ordaz et al., 2017	Bran and grain brown rice, olive oil, plums, gooseberries, white grapes, star anise, chicory, onion, almond
				S. aureus 0.38 g·L^{-1}	Skroza et al., 2019	
				B. cereus 0.19 g·L^{-1}		
				E. coli 0.38 g·L^{-1}		
				S. Infantis 0.38 g·L^{-1}		
		Salicylic acid		*S. aureus* 0.25–0.5 g·L^{-1}	Adamczak et al., 2020	Beer, coffee, tea, sweet potato, nuts, olive oil
				Enterococcus faecalis 0.5 g·L^{-1}		
				E. coli 0.25–0.5 g·L^{-1}		
				P. aeruginosa 0.5 g·L^{-1}		
				S. aureus 0.35 g·L^{-1}	Skroza et al., 2019	
				B. cereus 0.17 g·L^{-1}		
				E. coli 0.17 g·L^{-1}		
				S. Infantis 0.17 g·L^{-1}		
		Syringic acid		*S. typhimurium* 0.0625 g·L^{-1}	Phadungkit and Luanratana, 2006	Olives, dates, pumpkin
				S. aureus 0.5 g·L^{-1}	Skroza et al., 2019	
				B. cereus 0.25 g·L^{-1}		
				E. coli 0.25 g·L^{-1}		
				S. Infantis 0.25 g·L^{-1}		

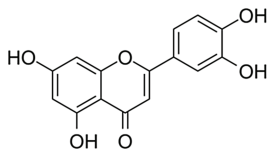
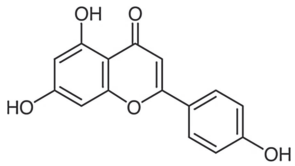
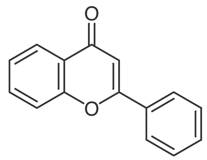
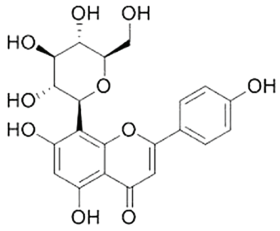
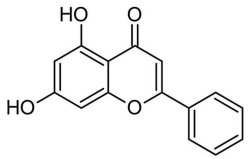
(Continued)

TABLE 1 | Continued

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
		Ellagic acid		*S. aureus* 0.25 g·L^{-1} *Bacillus subtilis* 0.5 g·L^{-1} *Enterococcus. faecium* 0.25 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1} *E. coli* 0.25 g·L^{-1}	An et al., 2021	Chestnut shells, berries, pomegranate, grape
	Hydroxycinnamic acids C_6–C_3	Chlorogenic acid		*E. coli* 0.5 g·L^{-1}	Adamczak et al., 2020	Apple, artichoke, tea
		Ferulic acid		*E. coli*, 3.9 g·L^{-1} *S. typhimurium* 3.9 g·L^{-1} *E. coli* 0.1 g·L^{-1} *P. aeruginosa* 0.1 g·L^{-1} *S. aureus* 1.1 g·L^{-1} *L. monocytogenes* 1.25 g·L^{-1} *S. aureus* 0.5 g·L^{-1} *B. cereus* 0.25 g·L^{-1} *E. coli* 0.5 g·L^{-1} *S.* Infantis 0.5 g·L^{-1}	Pacheco-Ordaz et al., 2017 Borges et al., 2013 Skroza et al., 2019	Wheat bran, rice bran, oat bran
		Caffeic acid		*E. coli* 0.25 g·L^{-1} *S. aureus* 0.22 g·L^{-1} *B. cereus* 0.22 g·L^{-1} *E. coli* 0.11 g·L^{-1} *S.* Infantis 0.11 g·L^{-1}	Matejczyk et al., 2018 Skroza et al., 2019	Sage, mint, Ceylon cinnamon, thyme
		Rosmarinic acid		*E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5–1 g·L^{-1} *S. aureus* 0.45 g·L^{-1} *B. cereus* 0.45 g·L^{-1} *E. coli* 0.45 g·L^{-1} *S.* Infantis 0.45 g·L^{-1}	Adamczak et al., 2020 Skroza et al., 2019	Rosemary, lemon balm, oregano, sage, thyme

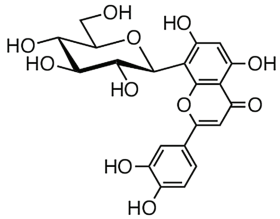
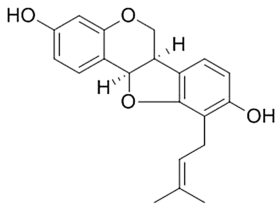
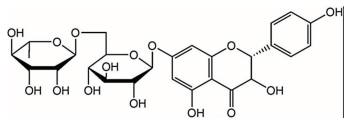
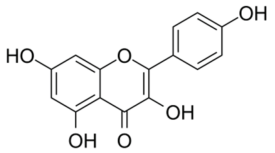
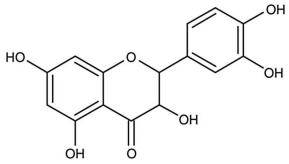
(Continued)

TABLE 1 | Continued

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
Flavonoids C_6–C_3–C_6	Flavones	Luteolin		*S. aureus* 0.5 g·L^{-1} *E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1} *S. aureus* 0.045 g·L^{-1} *B. cereus* 0.09 g·L^{-1} *E. coli* 0.09 g·L^{-1} *S.* Infantis 0.09 g·L^{-1}	Adamczak et al., 2020 Skroza et al., 2019	Radicchio, peppers, lemon, pumpkin
		Apigenin		*S. aureus* 0.5–1 g·L^{-1} *E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Parsley, chamomile, celery, artichokes
		Flavone		*E. faecalis* 0.5 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Mandarin
		Vitexin, isovitexin, and vitexin 2"-o-rhamnoside	 vitexin	*E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Flaxseed, prairie turnip, mung bean
		Chrysin	Vitexin 	*S. aureus* 0.5 g·L^{-1} *E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Honey, propolis, carrots, chamomile

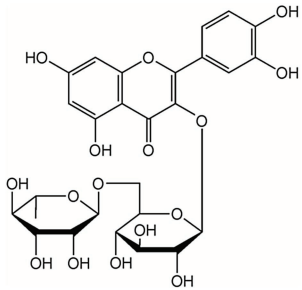
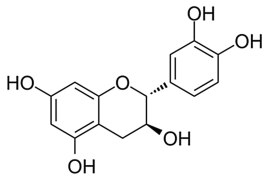
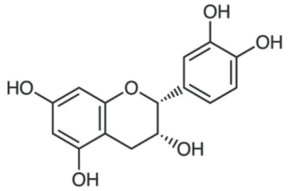
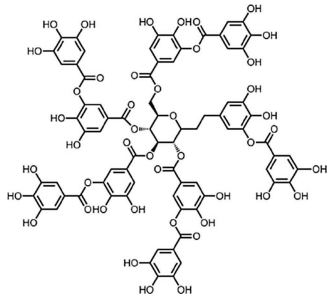
(Continued)

TABLE 1 | Continued

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
		Orientin and isoorientin		*S. aureus* 0.5 g·L^{-1} *E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Medicinal plants (e.g., bamboo leaves)
	Isoflavones	Phaseollidin		*B. cereus* 10 mg·L^{-1} *S. aureus* 10 mg·L^{-1} *E. coli* 20 mg·L^{-1} *P. aeruginosa* 20 mg·L^{-1}	Sadgrove et al., 2020	*Erythrina* medicinal plants
	Flavanones	Naringin		*E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Grapefruit, orange
	Flavonols	Kaempferol		*E. coli* 0.5 g·L^{-1} *S. aureus* 0.09 g·L^{-1} *B. cereus* 0.09 g·L^{-1} *E. coli* 0.36 g·L^{-1} *S.* Infantis 0.36 g·L^{-1} *E. faecalis* 0.1 g·L^{-1}	Adamczak et al., 2020 Skroza et al., 2019 del Valle et al., 2016	Strawberry, spinach, broccoli
		Quercetin		*E. coli* 0.5 g·L^{-1} *S. typhimurium* 15.6 mg·L^{-1}	Adamczak et al., 2020 Phadungkit and Luanratana, 2006	Capers, red onion, grapes, berries, black and green tea

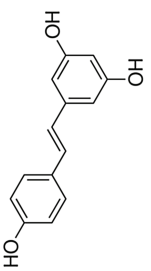
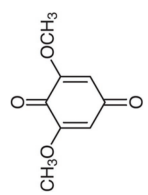
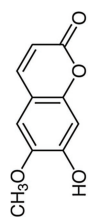
(Continued)

TABLE 1 | Continued

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
		Rutin		*S. aureus* 1 g·L^{-1} *E. faecalis* 1 g·L^{-1} *E. coli* 0.5 g·L^{-1} *P. aeruginosa* 0.5 g·L^{-1}	Adamczak et al., 2020	Carob fiber, fennel leaves, parsley
	Flavanols	Catechin		*S. aureus* 0.36 g·L^{-1} *B. cereus* 0.36 g·L^{-1} *E. coli* 0.18 g·L^{-1} *S.* Infantis 0.18 g·L^{-1}	Skroza et al., 2019	Apple, apricot, cherry, peach berries, green tea
		Epicatechin		*S. aureus* 0.72 g·L^{-1} *B. cereus* 0.36 g·L^{-1} *E. coli* 0.72 g·L^{-1} *S.* Infantis 0.72 g·L^{-1}	Skroza et al., 2019	Apple, blackberries
	Anthocyanins	Cranberry anthocyanins	n. d.	*S. aureus* 5 mg·L^{-1}	Gong et al., 2021	Cranberry
Tannins		Tannic acid		*S. aureus* 0.064 g·L^{-1}	Kırmusaoğlu, 2019	Grape, green tea, persimmon

(Continued)

TABLE 1 | Continued

Phenolics group	Phenolics subgroup	Antimicrobial phenolic	Structure	Sensitive microorganisms and corresponding MIC*	References	Source
Stilbenoids		Resveratrol		*S. aureus* 0.07 g·L^{-1} *B. cereus* 0.07 g·L^{-1} *E. coli* 0.14 g·L^{-1} *S.* Infantis 0.14 g·L^{-1}	Skroza et al., 2019	Red grape, red wine, peanut butter, dark chocolate
Quinones		2,6-dimethoxy-1,4-benzoquinone		*E. faecalis* 0.12 g·L^{-1} *S. typhimurium* 32 mg·L^{-1} *E. coli* 32 mg·L^{-1} *S. aureus* 8 mg·L^{-1} *B. cereus* 64 mg·L^{-1}	del Valle et al., 2016 Kim et al., 2010	Wheat germ
Coumarins		Scopoletin		*S. typhimurium* 8 mg·L^{-1}	Mfonku et al., 2021	Noni

*MIC, Minimal Inhibitory Concentration; n. d., not determined.

CA, United States). Nevertheless, it has been reported by many authors that extracts have generally a higher antimicrobial activity than a solution with only the most active phenolic at the same concentration. Serra et al. (2008) reported that a grape extract containing 20 mg·L^{-1} quercetin totally inhibited the growth of *Bacillus cereus* unlike a 20 mg·L^{-1} quercetin solution. Other constituents of grape extract exert thus an additive or synergistic effect with quercetin.

Antimicrobial activity assays of phenolics with different structures also allowed several authors to conduct Structure Activity Relationships (SAR) or Quantitative Structure Activity Relationships (QSAR) studies (Bouarab-Chibane et al., 2019). Since SAR mainly depends on the mechanisms of antimicrobial action on phenolics, the main conclusions of these studies are presented in the next section, after the diversity of mechanisms of actions of plant phenolics has been presented.

DIVERSITY OF ANTIMICROBIAL MECHANISM OF ACTION OF PLANT PHENOLICS

Overview of Known Antimicrobial Mechanisms of Action of Plant Phenolics

Different mechanisms of action of plant phenolics active against bacteria, yeasts or fungi have been reported. The reader interested in the mechanisms of action against fungi and more specifically against mycotoxin production can refer to da Cruz Cabral et al. (2013) review. The main mechanisms of antibacterial action of plant phenolics described in this review are summarized in **Figure 1**. As it can be observed in **Figure 1**, the most commonly reported mechanisms of action of plant phenolics are at the membrane level. Many authors reported dose-dependent alterations from microbial membranes ranging from reversible membrane permeability perturbations to membrane disruption (inducing leakage of cellular content). However, as stated by Rempe et al. (2017) in their review, while monitoring influx of fluorescent dyes (e.g., propidium iodide can only enter cells with disrupted membrane), efflux of intracellular constituents or direct microscopical observation of microbial cells allow to check, whether their membrane was disrupted following treatment with plant phenolics, this does not give any indication regarding more specific mechanisms of action leading to membrane disruption: did the phenolic compound directly interact with membrane components or alter membrane stability by interfering with intracellular processes?

Nevertheless, flow cytofluorometric analysis of microbial cells stained with different fluorescent dyes allowing to monitor membrane permeability, membrane potential, intracellular pH or intracellular enzymes activity provides information on different functions affected by phenolics at the membrane level (Léonard et al., 2016).

A common proposed mechanism of action is based on the presence of -OH groups in phenolics structure, promoting interaction of phenolics by hydrogen-binding with the microbial cells envelope. Depending on their hydrophobicity,

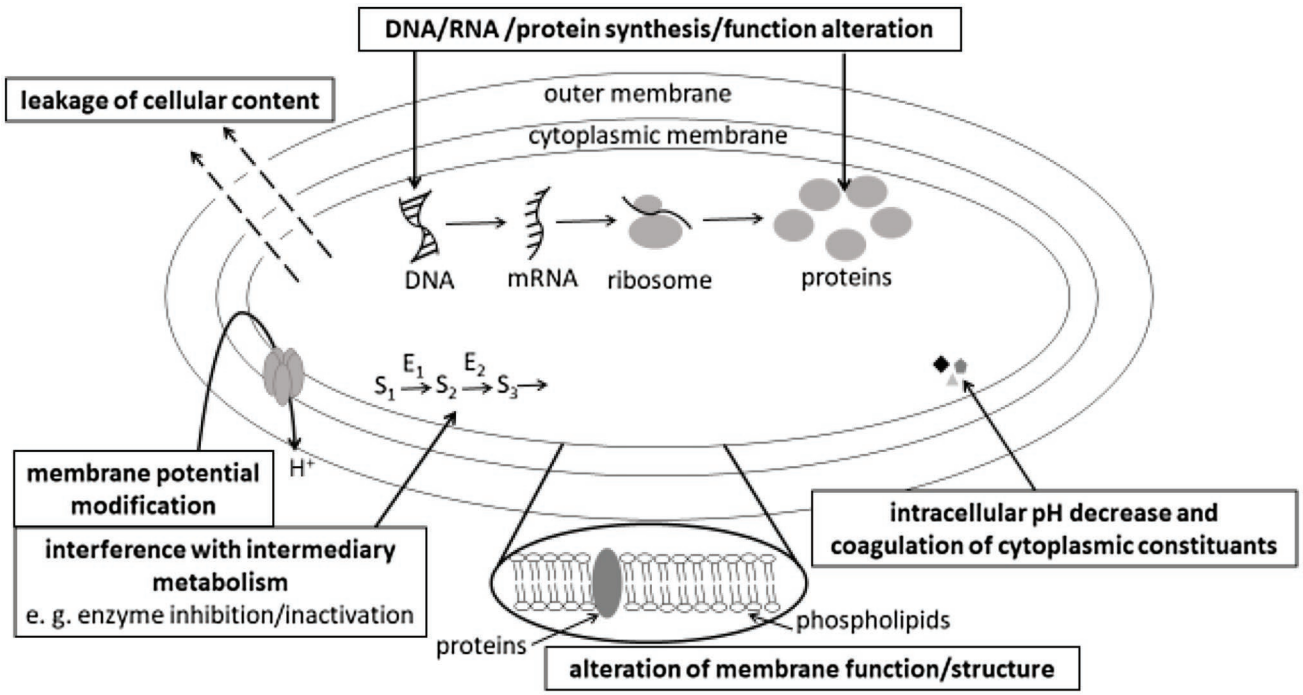


FIGURE 1 | Main mechanisms of antibacterial action of plant phenolics.

phenolics can accumulate at the surface of the cell envelope, penetrate or even cross their membrane and penetrate in the microbial cells cytoplasm, where they can interact with different cell constituents or alter intracellular pH. Gram-negative bacteria, which possess a hydrophilic cell wall, would be less sensitive to hydrophobic components of polyphenols than Gram-positive ones. Interestingly, Borges et al. (2013) compared the surface (hydrophobicity) and charge (zeta-potential) properties of two Gram-positive (*S. aureus* and *Listeria monocytogenes*) and two Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) bacterial species following their treatment with either gallic or ferulic acids. Phenolic acids application increased the electron acceptor properties for Gram-positive bacteria and decreased these properties for Gram-negative ones. The surface charge of Gram-negative bacteria was less negative in the presence of the two phenolic acids, while that of Gram-negative bacteria was unchanged. These observations and the differences of susceptibility to phenolic acids of Gram-negative and Gram-positive bacteria support the assumption, that the differences in cell membrane structure and composition play a key role in the susceptibility to plant phenolics.

Undissociated forms of phenolic acids, prevailing at pH values below their pKa values, are uncharged and can thus cross the phospholipid bilayer of bacterial membranes and decrease intracellular pH (Wen et al., 2003; Pernin et al., 2019). Reported consequences of phenolics penetration in the cytoplasm of microorganisms encompass interruption of DNA, RNA, protein synthesis or functions, interference with intermediary metabolism [namely energy (ATP)-generating system], coagulation of cytoplasmic constituents resulting from its acidification. Mora-Pale et al. (2015) observed that resveratrol-trans-dihydromer inhibited DNA gyrase activity *in vitro*, and concluded from a transcriptomic analysis, that it downregulated ABC transporters, as well as genes involved in cell division and DNA binding proteins. Its bactericidal action against *B. cereus*, *L. monocytogenes*, *S. aureus*, and *E. coli* results both from membrane potential disruption and from DNA synthesis inhibition.

Phenolics can also interact with membrane proteins involved in different functions: Duggirala et al. (2014) reported that the coumarin, scopoletin, inhibited bacterial cell division protein, FtsZ. They proposed that the increased length of *Bacillus subtilis*, in the presence of this coumarin, was probably due to the absence of septum formation, resulting from the inhibition of the first step of bacterial cell division. Examples of antimicrobial plant phenolics or phenolic-rich plant extracts with different mechanisms of action are listed in **Table 2**.

Recent Progress and Future Prospects Regarding Antimicrobial Mechanism of Action of Plant Phenolics

Despite the diversity of mechanisms of action of antimicrobial plant phenolics listed in **Table 2**, this list is biased by the fact that most investigations were performed using target-directed bioassays [e.g., membrane permeabilization is evaluated by monitoring efflux of intracellular constituents, influx of dyes such as propidium iodide, or 1-N-phenylnaphthylamine (NPN) uptake, DNA gyrase B (gyrB) inhibition assays …], which

TABLE 2 | Examples of plant phenolics or phenolic-rich plant extracts with different antimicrobial mechanisms of action.

Plant phenolic or plant extract	Microorganism	Mechanisms of action	References
Nutgall (*Quercus infectoria*) extracts and their main constituents (namely gallic and tannic acids)	*S. aureus*	– no lysis but significant loss of tolerance to low osmotic pressure and high salt concentration following treatment with ethanol extract, one ethyl acetate fraction, gallic acid and tannic acid	Chusri and Voravuthikunchai, 2011
Ferulic acid or gallic acid (1 g·L^{-1}, 30 min)	*L. monocytogenes* *E. coli* *P. aeruginosa*	– intracellular K^+ efflux: membrane permeabilization	Borges et al., 2013
Berry phenolics	*Salmonella enterica* serovar Typhimurium *S. enterica* serovar Infantis	– can reduce outer membrane permeability in a similar manner to EDTA by releasing lipopolysaccharide (LPS) and chelating divalent cations or by intercalating into the outer membrane and replacing stabilizing cations	Nohynek et al., 2006
Ethanolic and water extracts of roselle (*Hibiscus sabdariffa*), rosemary (*Rosmarinus officinalis*), clove (*Syzygium aromaticum*), and thyme (*Thymus vulgaris*)	*S. aureus* *E. coli*	– decrease in internal pH and membrane hyperpolarization following treatment with extracts suggesting bacterial membrane damage	Gonelimali et al., 2018
Satureja montana and *Origanum majorana* decoctions (1.56 g·L^{-1})	*S. aureus*	– reversible alteration of membrane permeability following the first hours of exposure to *Satureja montana* and *Origanum majorana* decoctions	Gomes et al., 2020
Kombucha polyphenolic fraction	*Vibrio cholerae*	– fraction containing mainly catechin and isorhamnetin, as well as catechin and isorhamnetin permeabilizing the inner membrane of *Vibrio cholerae*	Bhattacharya et al., 2018
Chinese wild blueberries fraction with anthocyanins	*L. monocytogenes*, *S. aureus*, *S.* Enteritidis	– leakage of nucleic acids and proteins: membrane disruption	Zhou et al., 2020
Pinosylvin	Three *S. enterica* strains	– destabilization of the outer membrane of *Salmonella* cells partially abolished by $MgCl_2$ addition indicating thus that part of its activity is related to the chelation of outer membrane stabilizing divalent cations, such as Mg^{2+}	Plumed-Ferrer et al., 2013
3-p-trans-coumaroyl-2-hydroxyquinic acid from *Cedrus deodara*	*S. aureus*	– interaction with membrane lipid and protein, damage of cytoplasmic membrane with a significant membrane hyperpolarization, a loss of membrane integrity and severe morphological changes	Wu et al., 2016
Backhousia citriodora extract	*Saccharomyces cerevisiae*	– damage of the yeast cell membrane through penetration causing swelling and lysis leading to cell death	Alderees et al., 2018
p-hydroxybenzoic, protocatechuic, gallic, chlorogenic, vanillic, p-coumaric, and ferulic acids	*L. monocytogenes*	– decrease in extracellular pH (main mechanism of action for chlorogenic and gallic acids) – penetration or accumulation in the bacterial membrane of undissociated form (caffeic acid, p-hydroxybenzoic acid, protocatechuic acid, and vanillic acids) – dissociated form that is significantly antimicrobial (p-coumaric acid and ferulic acids)	Pernin et al., 2019
Olive leaf extract	*L. monocytogenes*	– loss of flagella and reduction of motility of *L. monocytogenes* cells following their treatment by sub-inhibitory concentrations of olive leaf extract	Liu et al., 2017a
Scopoletin and daphnetin (coumarins)	*Bacillus subtilis*	– increase in length of bacteria in the presence of the coumarins probably due to the lack of septum formation, hypothesis substantiated by screening for their ability to inhibit the bacterial cell division protein *Escherichia coli* FtsZ: – scopoletin inhibited the GTPase (GTP: guanosine triphosphate) activity of FtsZ in a noncompetitive manner – molecular docking studies of interactions of coumarins with the modeled FtsZ protein indicate that they bind to T7 loop, which is different from the GTP-binding site (active site) These data support the hypothesis of the role of coumarins in halting the first step of bacterial cell division process	Duggirala et al., 2014
Quercetin	*E. coli*	– DNA gyrase inhibition either by interaction of quercetin with DNA or with ATP binding site of gyrase	Plaper et al., 2003

(Continued)

TABLE 2 | Continued

Plant phenolic or plant extract	Microorganism	Mechanisms of action	References
Chlorogenic acid	*B. subtilis*	– induction of the intracellular metabolic imbalance of the tricarboxylic acid cycle and glycolysis, leading to metabolic disorder and death	Wu et al., 2020
Cranberry concentrate	*E. coli* O157:H7	– marked downregulation of *hdeA* (cell envelope protein), *slp* (outer membrane lipoprotein) and *cfa* (cell wall phospholipid synthesis) genes	Wu et al., 2009
Hydroxytyrosol	*Lactobacillus plantarum*	– upregulation of antioxidant response involving genes from the reactive oxygen species resistome of *Lb. plantarum*, genes coding for H_2S-producing enzymes and genes involved in the response to thiol-specific oxidative stress	Reverón et al., 2020
		– upregulation of a set of genes involved in cell wall biogenesis	
Gallic acid, protocatechuic acid and vanillic acid	*Salmonella enterica* serovar Typhimurium	– membrane permeabilization by the three phenolic acids	Alvarado-Martinez et al., 2020
		– morphological defects at the polar ends of bacteria treated with gallic or protocatechuic acids	
		– treatment by vanillic acid resulting in observation of mid-division cells suggesting a perturbation of the cell division process that allows for septum formation but prevents finalization	
		– downregulation of all genes found in the Salmonella Pathogenicity Island 1 (SPI-1), which code for an assembly of proteins that aid in the attachment and subsequent invasion of host cells by gallic acid and downregulation of key regulatory genes by protocatechuic acid	
3-hydroxyphenylacetic acid (3-HPAA)	*Pseudomonas aeruginosa*	– proteomic analysis after 3-HPAA exposure of *P. aeruginosa* revealed changes in profile of proteins related to DNA replication and repair, RNA modifications, ribosomes and proteins, cell envelope, oxidative stress, as well as nutrient availability.	Ozdemir and Soyer, 2020
		3-HPAA was thus classified as a multitarget antimicrobial agent	

preclude the discovery of other or even novel mechanisms of antimicrobial action (Rempe et al., 2017).

Nevertheless, multiplication in recent years of studies by -omics approaches opens the possibility to identify new mechanisms of action or to identify several mechanisms of action acting simultaneously (which is generally the case at least for plant extracts and even for some pure plant phenolics). Transcriptomics (Alvarado-Martinez et al., 2020; Linzner et al., 2020; Reverón et al., 2020), proteomics (Ozdemir and Soyer, 2020) and metabolomics (Wu et al., 2020) approaches recently contributed to the identification of targets of antimicrobial action of some plant phenolics or phenolic-rich plant extracts. Transcriptomic (Reverón et al., 2020) and metabolomic (Wu et al., 2020) analyses allow to identify which metabolic pathways are affected by plant phenolics.

Limitations of these -omics approaches are the identification and/or detection thresholds for different technologies, the availability of annotated information for microorganisms tested, and data interpretation difficulties. However, technological evolutions [e.g., more systematic use of high-resolution mass spectrometry (HRMS) for the identification of metabolites] help to solve these limitations. Another bias in past studies lies in the fact, that mechanisms of action were often investigated by treating target microorganisms with plant extracts or phenolics concentration above their MIC or even their Minimal Bactericidal Concentration (MBC). Such conditions often result in observing membrane disruption of target cells, which is the ultimate consequence of the action of some plant phenolics, but rarely the primary cause. It is thus also relevant to investigate the effect of sub-inhibitory concentrations of plant phenolics or extracts on different functions of target microorganisms. -Omics approaches also allow to investigate the effect of phenolics sub-inhibitory concentrations on the physiology of microorganisms.

Fungi are generally more resistant to plant phenolics than bacteria. However, despite their structural diversity, no clear general rule regarding the antimicrobial activity spectrum or the mechanisms of action of a class of plant phenolics could be established to date. Nevertheless, the interested reader can refer to several reviews or articles focused on the antimicrobial activity of each phenolic class: phenolic acids (Wen et al., 2003; Cueva et al., 2010; Borges et al., 2013; Pernin et al., 2019), flavonoids (Cushnie and Lamb, 2005), tannins (Scalbert, 1991), stilbenoids (Plumed-Ferrer et al., 2013), quinones (Linzner et al., 2020), and coumarins (Duggirala et al., 2014). Several authors also presented reviews on phenolic-rich plant extracts

or plant phenolics acting on one (*L. monocytogenes*, Zamuz et al., 2021) or several (Ullah et al., 2020) foodborne pathogenic microorganisms.

Structure-Antimicrobial Activity Relationships of Plant Phenolics

With the increasing number of antimicrobial phenolics with different structures identified, several authors investigated the relationships between phenolics structure and their *in vitro* antimicrobial activity (Taguri et al., 2006; Bouarab-Chibane et al., 2019). Taguri et al. (2006) compared the antibacterial activity of 22 polyphenols against 26 species of bacteria by determining their MICs in Mueller-Hinton broth. Bouarab-Chibane et al. (2019) investigated the effect of a $1\,g{\cdot}L^{-1}$ concentration of 35 polyphenols on the growth at 37°C for 24 h of three Gram-positive bacterial strains (*B. subtilis*, *S. aureus*, *L. monocytogenes*) and three Gram-negative ones (*E. coli*, *P. aeruginosa*, and *S.* Enteritidis). In both studies, the antibacterial activity was dependent on the species of bacteria and no significant difference of susceptibility to polyphenols was observed between Gram-positive and Gram-negative bacteria.

The fact that antibacterial activity of phenolics depends on the species of bacteria motivated some authors to perform QSAR studies focused on one bacterial species. For instance, Fang et al. (2016) built reliable QSAR models for the antibacterial activity against *E. coli* of 30 flavonoids. Moreover, molecular docking study of interaction with DNA gyrase B (gyrB) of *E. coli* allowed to establish that half of flavonoids active against *E. coli* inhibit gyrB by interacting with ATP binding site of this enzyme [the mechanism of antibacterial activity against *E. coli* of quercetin already proposed by Plaper et al. (2003)]. Pernin et al. (2018) proposed a QSAR model for the antibacterial activity against *L. monocytogenes* of 21 phenolics and found a good correlation with their octanol–water partition coefficient (log $P_{o/w}$).

Taguri et al. (2006) observed that antibacterial activity of polyphenols having pyrogallol groups (i.e., with three adjacent hydroxyl groups) was higher than that of polyphenols with catechol and resorcinol rings (i.e., with two adjacent or non-adjacent hydroxyl groups, respectively). This is consistent with Phan et al. (2014) conclusion that "the higher number of hydrophilic side chains (hydroxyl, gallate, galloyl, glucoside), the more interactive the polyphenol was with the membrane" following observation of spatio-temporal real-time membrane dynamics in the presence of different polyphenols. Bouarab-Chibane et al. (2019) were not able to build reliable QSAR models predicting the effect of the set of 35 polyphenols they used on the growth of *L. monocytogenes* and *P. aeruginosa*, since they were systematically inhibited or not inhibited by polyphenols, respectively. However, reliable QSAR models, with a few independent physicochemical descriptors linked with lipophilicity and the electronic and charge properties of the polyphenols, were built for each of the four other bacterial strains. These physicochemical descriptors are consistent with the hypothesis that the main antibacterial mechanisms of action of polyphenols depend on their accumulation on the surface of bacteria, which is favored both by hydrogen binding and by their hydrophobicity.

For instance, the reduced susceptibility to polyphenols like epigallocatechin gallate of many lactic acid bacteria compared to other Gram-positive bacteria was proposed to result from exopolysaccharides production, which reduces surface hydrophobicity of lactic acid bacteria (Nakayama et al., 2015). Therefore, Bouarab-Chibane et al. (2019) investigated the surface properties of the six species of test bacteria they used by Microbial Adhesion To Solvents (MATS), following the method described by Bellon-Fontaine et al. (1996). Interestingly, they noted that bacteria, which had a similar affinity for the four solvents they used, had similar QSAR models, while the bacteria which had different affinity for these solvents also had different QSAR models. Since only four bacterial strains had reliable QSAR models, they suggested evaluating, whether this trend would also be observed with a larger number of bacterial strains. Moreover, characterization of the surface properties of bacteria should also integrate other parameters such as their zeta-potential.

Besides investigating the structure-antimicrobial activity relationships of phenolics naturally present in plants, several authors performed SAR or QSAR studies with series of epicatechin gallate groups, from which gallate group was substituted with 3-O-acyl chains of varying lengths (C_4–C_{18}; Stapleton et al., 2004), or with series of esters of phenolic acids esterified with various alkyl or aryl substituents (Andrade et al., 2015; Shi et al., 2018; Araujo et al., 2019). This allowed them both to get further insight in the physicochemical characteristics of active antimicrobial phenolics and to design new antimicrobial phenolics.

ANTIMICROBIAL ACTIVITY OF PLANT PHENOLICS IN FOODS: INTEREST, LIMITS AND FUTURE PROSPECTS

Examples of Antimicrobial Action of Plant Phenolics in Different Food Matrices

The potential of several phenolic-rich plant extracts or pure plant phenolics to inhibit the growth of unwanted microorganisms in some food matrices has been demonstrated in many studies. Some recent examples are listed in **Table 3**. As illustrated by these examples, many studies aimed at improving the preservation of meat and meat products, likely since these foods both have a high added-value and are highly perishable. Application of phenolic-rich plant extracts to meat and meat products preservation has been recently reviewed (Efenberger-Szmechtyk et al., 2021). Moreover, addition of herbs and spices in meat and meat products is a common practice to improve their sensory properties. As stated in **Table 3** (e.g., Ranucci et al., 2019), plant phenolics and phenolic-rich plant extracts addition in meat and meat products has frequently been reported to delay oxidation reactions altering organoleptic properties of such foods (e.g., color alteration due to myoglobin oxidation

TABLE 3 | Examples of phenolic-rich plant extracts or plant phenolics effectively inhibiting the growth of unwanted microorganisms in food matrices.

Plant phenolic or plant extract	Food supplemented with plant phenolic or plant extract	Effects on food quality	References
Clove extract	Raw porcine meat supplemented with clove extract [concentration of clove extract expressed in g of powdered clove used for the extraction per 100 g of meat was 0.5% (w/w)]	– clove extract decreased the growth of total viable count, *Pseudomonas*, *Enterobacteriaceae* during storage at 4°C but did not increase shelf life of pork meat	Muzolf-Panek et al., 2019
Rumex tingitanus extract with a high luteolin content	Raw bovine minced meat supplemented with 10, 20 and 30 mg of *R. tingitanus* extract/g of meat	– bactericidal effect following inoculation of *L. monocytogenes* (2.10^2 cfu/g of meat) and inhibition of growth of mesophilic and psychrotrophic bacteria from meat during storage at 4°C for 30 days	Mhalla et al., 2017
White cabbage (*Brassica oleracea* var. *capitate* f. *alba*) extract	Raw bovine meat	– white cabbage extracts addition to meat [at either a 0.5% or a 1% (w/w) concentration] decreased total viable counts, psychrotrophic bacteria, yeasts and molds over 16 days storage at 4°C	Rubab et al., 2020
Hibiscus sabdariffa L. ethanolic extract	Raw bovine meat sprayed with 0.25 g·L^{-1} to 1.25 g·L^{-1} *H. sabdariffa* ethanolic extract	– decreased growth of mesophilic and psychrophilic bacteria over 15 days storage at 4°C	Márquez-Rodríguez et al., 2020
Green tea, stinging nettle and olive leaves extracts	Frankfurter sausage	– plant extracts (0.5 g/kg of sausage) reduced the count of total viable bacteria, mold and yeast by at least 2 log cfu/g over 45 days storage at 4°C	Alirezalu et al., 2017
Pomegranate (*Punica granatum*) and *Citrus* spp. extract mix	Sausage made from pork meat, emmer wheat (*Triticum dicoccum* Schübler), almond (*Prunus dulcis* Mill.), and hazelnut (*Corylus avellana* L.)	– addition of 0.5% or 1% (w/w) mix in the sausages delayed the pH drop, oxidation, total viable count, lactic acid bacteria and psychrotrophic microbial counts and extended the estimated shelf life of vacuum_packaged cooked sausages stored at 4°C from 44 to 50 or 60 days, respectively	Ranucci et al., 2019
p-coumaric acid, caffeic acid, and rutin	Chicken soup	– for concentrations above 0.23% (w/v), each phenolic totally inhibited the growth of *S. aureus* inoculated in chicken soup at 4°C within 3 days	Stojkovic et al., 2013
Cranberry extract or oregano extract	Seafood products (cod fish fillets and shrimps)	– inhibition of growth of *Vibrio parahaemolyticus* inoculated on fish fillets or shrimps (10^3 cfu/g of seafood) during storage at 4°C for 8 days	Lin et al., 2005
Perilla frutescens leaf ethanolic extract	Surimi fish balls	– addition of 0.03% (w/w) extract into surimi fish balls decreased lipid and protein oxidation, formation of total volatile basic nitrogen and growth of *E. coli* during storage at 4°C – improved sensory properties	Zhao et al., 2019
Quercus infectoria ethanolic extract	Pasteurized bovine milk	– addition of extract to pasteurized milk resulted in lower total bacterial and yeast-mold counts and higher pH compared to control milk	Başyiğit et al., 2020
Olive mill wastewater extract	Fior di latte cheese	– addition of 250 mg/L or 500 mg/L of olive mill wastewater polyphenols in batches of Fior di latte cheese retarded *Pseudomonas fluorescens* and *Enterobacteriaceae* growth resulting in a 2 days and 4 days shelf life extension, respectively	Roila et al., 2019
Broccoli by-products extract	Fresh-filled pasta	– addition of 10 to 20% (v/w) of phenolic-rich broccoli extract in ricotta and spinach-based filling of fresh pasta resulted in a decrease in mesophilic bacteria growth during subsequent storage at 4°C thereby contributing to an extension of shelf life from 6 to 24 days	Angiolillo et al., 2019
Olive mill wastewater extract	Bread	Olive polyphenols emulsion (200 mg/kg) extended the shelf life of bread from 10 to 15 days	Galanakis et al., 2018
Olive leaf extract	Ready-to-use olive-based pâté	– addition of 0.5 or 1 g olive leaf extract.kg^{-1} of pâté resulted in a significant loss of *ca.* 0.5–1 logarithmic cycles of main microbial groups in samples added with extract, especially with 1.0 g·kg^{-1} during storage at 4°C under argon-based atmosphere for 120 days	Difonzo et al., 2019
Mulberry leaf extract	Fresh-cut cantaloupe	– spraying of fresh-cut cantaloupe with 5 g·L^{-1} mulberry leaf polyphenols resulted in a significant decrease in bacterial counts over 4 days of subsequent storage at 25°C compared to a control sprayed with sterile water	Yu and Shi, 2021

into metmyoglobin or taste or aroma alteration due to fat oxidation).

However, addition of plant phenolics or phenolic-rich plant extracts to meat and meat products and other food matrices has frequently more positive effects on retardation of oxidation phenomena than on retardation of the growth of unwanted microorganisms. For instance, Bouarab-Chibane et al. (2017) added 1% (w/w) of antimicrobial phenolic-rich plant extracts to raw bovine meat patties, enumerated bacteria and monitored different quality attributes of meat during 12 days storage at 4°C in a high-oxygen modified atmosphere. While pomegranate peel, green tea leaves, grape seed extracts, and Gaillac red wine powder reduced the increase in ThioBarbituric Acid Reactive Substances (TBARS are namely aldehydes generated following the decomposition of lipid peroxidation products) in bovine patties over 12 days by more than 93%, these extracts had no significant effect on total viable and psychrotrophic bacterial counts, despite their *in vitro* bactericidal activity against several Gram-positive and Gram-negative bacteria at a 0.1% (w/w) concentration. This apparent discrepancy can be due to the absence of susceptibility to these extracts of total viable and psychrotrophic bacterial strains, resulting from the diversity of strains in the meat microbial ecosystem. However, it could also be due to the interactions of antimicrobial plant molecules with proteins and/or fat which are the main constituents of bovine meat: these interactions could limit the quantity of "free" phenolics not interacting with these food constituents.

Nevertheless, despite their generally lower *in vitro* antimicrobial activity than in microbiological media, several authors reported plant phenolics or phenolic-rich plant extracts effectively inhibiting the growth of foodborne pathogenic microrganisms [e.g., *L. monocytogenes* (Mhalla et al., 2017), *S. aureus* (Stojkovic et al., 2013)] or of food-spoiling microorganisms [e.g., *Pseudomonas*, *Enterobacteriaceae* (Muzolf-Panek et al., 2019)] in foods such as meat products. Mhalla et al. (2017) reported that incorporation of a luteolin-rich *Rumex tingitanus* extract in raw bovine minced meat retarded the growth at 4°C of mesophilic and psyschrophilic bacteria and addition of a pomegranate and *Citrus* spp. extracts mix in the formulation of pork sausages resulted in a significant extension of their shelf life (Ranucci et al., 2019). The potential of direct addition of plant extracts in other perishable animal origin foods containing proteins and fat than meat was investigated by other authors: addition of *Perilla frutescens* leaf extract into surimi fish balls decreased growth of *E. coli* during storage at 4°C (Zhao et al., 2019) and addition of olive mill wastewater polyphenols in batches of Fior di latte cheese retarded *Pseudomonas fluorescens* and *Enterobacteriaceae* growth resulting in significant shelf life extension (Roila et al., 2019). Addition of plant extracts at concentrations exceeding 1% (w/w) are generally required to effectively inhibit the growth of unwanted microorganisms in foods. It might even be more promising to add phenolic-rich plant extracts in foods containing lower amounts of food constituents interacting with proteins and fat, such as fruits, vegetables or bread (Galanakis et al., 2018).

Interestingly, non-minced meat pieces or fish fillets can be treated by spraying plant extracts or phenolics solutions on their surface, that is precisely where microbial contaminations and growth occur during storage. For instance, Márquez-Rodríguez et al. (2020) reported that spraying raw bovine meat pieces with a $\sim 1\,g{\cdot}L^{-1}$ *Hibiscus sabdariffa* extract solution decreased the growth of mesophilic and psychrophilic bacteria over 15 days subsequent storage at 4°C. Similarly, Lin et al. (2005) voluntarily inoculated seafood with *Vibrio parahaemolyticus* and observed that spraying cranberry and oregano extracts solutions on their surface inhibited its growth for 8 days storage at 4°C. This strategy is also promising for fresh-cut fruits preservation as recently illustrated by Yu and Shi (2021) who sprayed fresh-cut cantaloupe with $5\,g{\cdot}L^{-1}$ mulberry leaf polyphenols to inhibit bacterial growth on their surface.

Limits to the Application of Plant Phenolics for Food Preservation

Effect of Interaction With Food Constituents on Plant Phenolics Antimicrobial Activity

Besides interactions of phenolics with food constituents such as proteins or fat and resulting decrease in their "free" amount available to act on target microorganisms, unwanted microorganisms could also be protected by a "layer" of food constituents limiting the direct contact of antimicrobial phenolics with their cell envelope, the most commonly described mechanism of action of antimicrobial phenolics: da Cruz Cabral et al. (2013) reported that "lipids in food could form a coating around the microorganisms, protecting them from antimicrobial agents." Boziaris et al. (2011) also reported the absence of effect on viable populations of spoilage or pathogenic bacteria were found between fish flesh or fish roe (tarama salads) treated or not with 10% (v/w) of *Filipendula ulmaria* liquid extract. For instance, *L. monocytogenes* Scott A was not affected by 10% (v/w) *F. ulmaria* extract over 12 days storage at 5°C of seafoods inoculated by this strain, while it was inhibited by the same concentration of this plant extract on solid microbiological medium also incubated at 5°C to better mimic food preservation conditions. They proposed that the loss of susceptibility of *L. monocytogenes* is due to interactions of *F. ulmaria* extract active phenolics (e.g., caffeic, p-coumaric and vanillic acids, myricetin …) with proteins and fat (the main constituents of such foods), at the expense of their interactions with unwanted microbial cells.

Indeed most studies regarding the antimicrobial activity of plant phenolics have been performed *in vitro* in microbiological media with a less complex composition and microstructure than food matrices. Miceli et al. (2014) compared the *in vitro* activity of borage (*Borago officinalis*) and Indian mustard (*Brassica juncea*) aqueous extracts against several foodborne pathogenic bacterial strains *in vitro* in Brain Heart Infusion broth and in meat, fish and vegetable broths (considered as food models). They observed that a 10-fold higher concentration than the *in vitro* bactericidal concentration was necessary to get a bacteriostatic effect in food models for both extracts. They suggested that this difference could be due to binding of the active compounds of extracts to food components.

Carbohydrates, lipids and proteins are the main components of food, and interactions between polyphenols and these components have been reported by many authors and reviewed by Jakobek (2015). Most studies regarding interactions between polyphenols and these components aimed at investigating their effect on their bioavailability following their ingestion. Interactions between phenolics and proteins are the most studied for this reason, and because interactions between compounds such as tannic and gallic acids and salivary proteins are involved in their astringency. Xu et al. (2018) compared thus the MICs against different microorganisms of epigallocatechin gallate and grape seed extract in a protein-free chemically defined medium and in the same medium supplemented with up to 0.4 g·L^{-1} of bovine serum albumin. A 64-fold increase in MIC of epigallocatechin gallate against a *Streptococcus mutans* strain was observed and a similar trend was observed with grapeseed extract, as well as against other microbial species. Such an increase in MIC was not observed for non-phenolic antimicrobial compounds, which were tested in parallel.

Besides protein content, which is far higher in most perishable foods than in microbiological media, another important difference between *in situ* antimicrobial activity assays and *in vitro* ones lies in the physiological state of microorganisms under refrigeration conditions and at optimal temperatures for microbial growth (i.e., between room temperature and 37°C), respectively. Klancnik et al. (2011) compared the activity in buffered peptone water supplemented with 50% (w/v) of meat, vegetable or dairy products to estimate the effect of the components of these foods on the activity against *L. monocytogenes* and *E. coli* of rosemary phenolic extracts. They also observed an increase in MIC against these two bacterial species for every food type added in buffered peptone water.

Therefore, Bouarab-Chibane et al. (2018b) compared the antibacterial activity of five bacteriostatic or bactericidal phenolics active against a *S. aureus* strain for 24 h at 37°C in Mueller-Hinton broth, with their activity in the same medium supplemented with up to 20% (w/w) bovine meat proteins (the protein content of bovine meat) for up to 8 days at 6°C (to mimic refrigeration conditions). While resveratrol and chrysin always lost their bacteriostatic activity in the presence of bovine meat proteins, gallocyanin kept its bactericidal activity at 37°C up to a 5% (w/w) protein content in the medium, but not at 15°C or 6°C, unlike naphthazarin, which was bactericidal at 6°C and 15°C, unlike at 37°C in the presence of bovine meat proteins. Finally, isobutyl-4-hydroxybenzoate kept its bactericidal activity under all the conditions investigated. The partition coefficient at 6°C of each phenolic between a 20% (w/w) bovine meat extract suspension and the same suspension without proteins was determined. Interestingly, the antibacterial activity reduction of phenolics in the presence of bovine meat proteins was correlated with their affinity for bovine meat proteins. Ansari et al. (2015) also reported that antimicrobial activity loss in the presence of casein or gelatin was correlated with their affinity for these proline-rich proteins.

It is thus advisable to perform at an early stage an *in vitro* screening of antimicrobial activity of phenolic-rich plant extracts in media containing proteins, if an application to foods containing proteins is foreseen (Bouarab-Chibane et al., 2018c), and preferably with the same proteins as in food. Screening at refrigeration temperatures is also preferable, if application to preservation of perishable foods is foreseen. Antimicrobial activity of plant extracts can be greatly influenced by temperature, as underlined by Hayrapetyan et al. (2012). They reported a higher inhibition of *L. monocytogenes* in meat pâté by pomegranate peel extract at 4°C than at 7°C, or 12°C. More systematic studies on the effect of food ingredients on the antimicrobial activity of phenolic-rich plant extracts or plant phenolics under conditions closer to food preservation conditions than classical *in vitro* antimicrobial activity screening assays should contribute to anticipate their *in situ* activity in real foods. While studies regarding the effect of proteins on the antimicrobial activity of plant phenolics exist, studies regarding the effects of carbohydrates or lipids on their activity are scarce and will also be necessary, since these compounds also interact with polyphenols. In the absence of such data, the efficiency of a given plant extract or phenolic to preserve a defined food can hardly be extrapolated to other foods differing in their composition and/or microstructure.

Effect of Plant Phenolics Addition on Foods Organoleptic Properties

Another important limit of many plant extracts is their effect on organoleptic properties of foods: the taste (e.g., astringency of some tannins, bitterness of green tea extract), odor and/or color (e.g., green color of green tea extract) of many plant extracts can alter the organoleptic quality of some foods (Bouarab-Chibane et al., 2017). However, in some cases, plant extracts added in concentrations effectively inhibiting the growth of unwanted microorganisms can also improve the color or the taste of some foods. Swer et al. (2019) added up to 0.2% (w/v) of anthocyanins extracted from Sohiong (*Prunus nepalensis* L.) in yogurts. Increase in anthocyanins content improved overall acceptability of yogurts by panelists. It is also possible to use plant extracts from which coloring substances have been removed. For instance, Nirmal and Benjakul (2011) added green tea ethanolic extracts without chlorophyll to Pacific white shrimps. One advantage of antimicrobial pure phenolics over plant extracts might also result from their absence of effect on taste of foods at a dose effectively inhibiting unwanted microorganisms, as underlined by Stojkovic et al. (2013) for chicken soup and porcine meat preserved by incorporation of 1.87 g·L^{-1} or spraying of 1.87 mg.10 cm^{-2} of phenolics (p-coumaric acid, caffeic acid, or rutin). However, as stated by Albuquerque et al. (2021), only the use of following pure phenolics is authorized in foods: anthocyanins [E 163, authorized as colorant by European Food Safety Authority (EFSA in the European Union) or Food and Drug Administration (FDA in the United States)] and ferulic acid (as antioxidant in Japan).

Prospects for a Broader Use of Plant Phenolics for Food Preservation

Another potential advantage of the addition of some plant extracts or phenolics lies in their health-promoting properties.

Since health-promoting properties of edible plant extracts or phenolics are not in the scope of the present review, the interested reader can refer to a recent review on this subject (Samtiya et al., 2021). As stated above, no plant extract will have the capacity to replace synthetic food preservatives, such as potassium sorbate as antifungal agent, in all their applications for the same cost. This is also namely due to the narrower antimicrobial activity spectrum of plant extracts compared to most food preservatives. A solution to have a broader antimicrobial activity spectrum is to use mixtures of plant extracts with different antimicrobial activity spectra. This is likely one of the reasons for the better preservation of raw bovine meat by mixtures of clove, cinnamon and oregano extracts rather than each of these extracts alone, or binary combinations of these extracts reported by Radha Krishnan et al. (2014). The narrow spectrum of antimicrobial activity of phenolic-rich plant extracts can also be positively exploited in some cases. For instance, the fact that lactic acid bacteria are generally less susceptible to the antibacterial plant phenolics compared to most undesirable bacteria (Pacheco-Ordaz et al., 2017; Chan et al., 2018) opens the possibility to add phenolic-rich plant extracts in foods fermented by lactic acid bacteria at sub-inhibitory concentrations of lactic acid bacteria, while effectively inhibiting the growth of unwanted microorganisms.

Another promising strategy to expand the application of phenolic-rich plant extracts is in line with hurdle technology principles application: appropriate combinations of phenolic-rich plant extracts addition, with for instance the addition of organic acids (Apostolidis et al., 2008), inoculation of food with bioprotective microorganisms (Sireswar et al., 2017), modified atmosphere or vacuum (Ranucci et al., 2019) packaging, high hydrostatic pressure (Hygreeva and Pandey, 2016) or UV-A treatment (Cossu et al., 2018) of foods have for instance been used to get a synergistic antimicrobial activity. Sireswar et al. (2017) inoculated seabuckthorn juice supplemented with malt extract with probiotic lactic acid bacteria strains. They demonstrated that growth of probiotic strains was possible thanks to malt extract addition and despite antibacterial phenolics presence in seabuckthorn juice. Moreover, they demonstrated the capacity of this food to rapidly eliminate different enteropathogenic bacteria: they proposed that the rapid elimination of these pathogens results from the synergistic action of metabolites such as lactic acid produced by probiotic bacteria and antimicrobial phenolics from seabuckthorn juice, such as isorhamnetin, myricetin, kaempferol, and quercetin.

DIVERSITY OF MECHANISMS OF ACTION AGAINST BIOFILMS OF PLANT PHENOLICS

Overview of Known Mechanisms of Action of Plant Phenolics Against Biofilms

Biofilms confer favorable growth environments to pathogens and resistance to antimicrobials and disinfectants (Oulahal et al., 2008). Biofilms formed by undesirable bacteria are a major concern for food microbial safety, due to production of virulence factors, persistence of bacterial pathogens, cross contamination causing many risks for consumers. Mishra et al. (2020) recently reviewed strategies to control biofilm-forming pathogens based on the exploitation of natural anti-biofilm agents, including phytochemicals such as plant antimicrobial phenolics. Guzzo et al. (2020) more specifically reviewed the activity against *P. aeruginosa* and *S. aureus* biofilms of plant-derived natural products and Slobodníková et al. (2016) reviewed antibiofilm activity of plant polyphenols. However, these three reviews were focused on their clinical application in the medical and healthcare sectors and not in the food sector. The reader interested in plant phenolics potential against biofilm-forming food-contaminating microorganisms can refer to the review by Takó et al. (2020).

Briefly, increased tolerance to antimicrobial compounds of microorganisms present in biofilms results from different phenomena: microbial cells in biofilms are embedded in self-secreted extracellular polymeric substances (Sivaranjani et al., 2016), that attach these cells. These extracellular polymeric substances are namely polysaccharides, proteins and extracellular DNA. They protect cells from desiccation, pH variations but also from antimicrobial molecules including plant phenolics. They act as a protective layer of the surface of cells, as viscosifying agents slowing down the penetration and the diffusion of antimicrobial molecules in biofilms structure, thereby favoring adaptation of microbial cells, which have more time to become tolerant. Besides this "physical tolerance" favored by extracellular polymeric substances, "physiological tolerance" to antimicrobial molecules of microbial cells embedded in the deepest layers of biofilms has also been reported (Mishra et al., 2020). Indeed, important decreasing gradients of nutrients or oxygen result in the downregulation of the metabolic activity of microbial cells located inside the structure of biofilms. These reversible adaptative stress responses make these cells more tolerant to antimicrobial molecules. That is why these slow-dividing cells are called persister cells. Progresses in -omics (genomics, transcriptomics and metabolomics) allow identification of molecular pathways leading to biofilm formation and will contribute to get information for the rational design of efficient strategies to control biofilm formation.

Knowing the different stages of formation and development of biofilms also guides the design of strategies to prevent biofilms formation or to favor their eradication. Indeed, biofilm formation (i) starts with the attachment of microbial cells to a surface, is followed by (ii) biofilm structure development, (iii) its maturation and finally, (iv) its dispersion. Since attachment involves cytoskeletal elements, such as flagella and lipopolysaccharides, molecules inhibiting their formation are particularly promising to prevent their formation. Many authors recently reported the inhibition of biofilm formation by phenolic-rich plant extracts/plant phenolics by different mechanisms of action. A survey of such studies illustrating the diversity of these mechanisms of action is provided in **Table 4**.

Recent Progress and Future Prospects Regarding Mechanism of Action Against Biofilms of Plant Phenolics

Staphylococcus aureus biofilms are a major concern for food safety. Interestingly, Wu et al. (2019) observed that coumaroyl-2-hydroxyquinic acid from pine needles of *Cedrus deodara*, not only inhibits *S. aureus* biofilm formation, but also the attachment phase, by inhibiting the transmembrane peptidase sortase A, SrtA. This enzyme catalyzes the covalent binding of surface proteins sharing a typical sorting signal, with a conserved C-terminal LPXTG motif, to cell wall peptidoglycan and these cell wall-anchored proteins initiate bacterial adherence by binding host surface. SrtA inhibitors are thus promising to inhibit the initial stage of biofilm formation. The same group (Liu et al., 2021b) performed combined transcriptomic and proteomic analyses in the absence and in the presence of sub-inhibitory concentrations of coumaroyl-2-hydroxyquinic acid, in order to elucidate its molecular mechanism of action against *S. aureus*. They observed a differential expression of 935 genes and 438 proteins in both situations. Downregulation of surface proteins associated with cell adhesion supports the hypothesis, that this antimicrobial plant phenolic prevents *S. aureus* biofilm formation by inhibiting adhesion of *S. aureus* cells. Bioinformatic analysis also demonstrated that coumaroyl-2-hydroxyquinic acid affects different functions of *S. aureus*, namely at the membrane level.

Recently, when investigating *Adiantum philippense* extract inhibitory activity of biofilm formation by several foodborne pathogens, Adnan et al. (2020) performed an *in silico* molecular docking study of the inhibitory activity of *S. aureus* SrtA by 28 of its identified constitutive phenolics: scutellarin, a glycosyloxyflavone, was found to have the highest affinity for SrtA. Interestingly, this extract also inhibited biofilm formation by other foodborne pathogenic bacteria, and other compounds of the extract had also a high affinity for other adhesins than SrtA. Moreover, this extract significantly inhibited exopolysaccharides production by these bacteria: this is likely another mechanism of biofilm formation inhibition.

Carraro et al. (2014) reported that an olive mill waste extract, inhibiting *E. coli* K12 biofilms formation, also inhibited swarming and swimming motility of these bacteria. Consistently with this observation, they noticed repression of genes for flagellar synthesis and other genes linked to biofilm formation in the presence of this extract. Indeed, reduction of swarming or swimming motility of bacteria by phenolic-rich plant extracts or plant phenolics inhibiting biofilm formation has been frequently reported (e.g., in **Table 4**: Carraro et al., 2014; Zhang et al., 2014; Vasavi et al., 2015; Yang et al., 2018; Kamiya et al., 2019).

A promising strategy to prevent biofilm formation is based on the identification of quorum sensing inhibitors. Quorum sensing is a mechanism of regulation of gene expression, as a function of microbial cells population density: this mechanism results in the production of extracellular compounds called autoinducers, which alter gene expression of other bacterial cells, when their concentration exceeds a minimal threshold. Since these autoinducers allow bacteria, not only to communicate within species, but also with different species, this mechanism allows bacteria to induce coordinated responses to their environment, like signaling in higher organisms. The phenotypes regulated by quorum sensing include motility, biofilm formation, and resistance to antibiotics (LaSarre and Federle, 2013; Castillo Rivera et al., 2019). Quorum sensing is also involved in the regulation of phenotypes contributing to virulence of bacteria.

Interestingly, several authors have reported the interaction of plant phenolics with homoserine lactones, which are typical auto-inducers of Gram-negative bacteria (Hossain et al., 2017). They reported that methyl gallate had anti-quorum sensing effect on *Chromobacterium violaceum* (an aquatic bacterium used to study the inhibition by diverse molecules of acyl homoserine lactone-dependent quorum sensing, since production of violacein pigment is associated with its quorum-sensing regulated gene expression) and on *P. aeruginosa*, including inhibition of biofilm formation by this bacterium, as well as of its production of exopolysaccharides, which are extracellular polymeric substances in biofilms, and of its swarming motility. Indeed, swarming has been reported to play an important role in the preliminary stage of quorum sensing-regulated bacterial biofilm formation. Since attachment and involvement of quorum sensing are critical steps for the development of biofilms, antimicrobial phenolics acting on these mechanisms are preferred strategies to inhibit biofilm formation.

Baptista et al. (2019) investigated the activity against *E. coli* biofilms of nine phenolics with a catecholic moiety. Increases in the hydrocarbon side chain and lipophilicity, as well as a contribution of hydroxyl groups, were proposed as structural traits favoring anti-biofilm activity of catecholic molecules following a SAR study. SAR and QSAR studies regarding the activity against biofilms of plant phenolics are still scarce. Multiplication of such SAR and QSAR studies should contribute to get a better insight in structural traits of plant phenolics active against biofilms.

Many plant phenolics [e.g., gallic and ferulic acid (Borges et al., 2012), vanillic, caffeic, cinnamic and ferulic acid (Ugurlu et al., 2016), tannic acid (Dong et al., 2018), morin (Sivaranjani et al., 2016), myricetin (Slobodníková et al., 2016), 4-methylcatechol, 4-tert-butylcatechol, and pyrogallol (Baptista et al., 2019), quercetin (Vazquez-Armenta et al., 2020)] and phenolic-rich plant extracts [e.g., garlic extracts (Vadekeetil et al., 2015), *Moringa oleifera* extract (Onsare and Arora, 2015), *Brassicaceae* (radish, radish sprout, red cabbage, kale) extracts (Hu et al., 2019), *Capsicum* peppers extracts (Castillo Rivera et al., 2019), phenolic-enriched extracts produced by enzyme-assisted extraction from oven-dried and lyophilized black grape, apple and yellow pitahaya (Zambrano et al., 2019), tea and turmeric extracts (Tamfu et al., 2020), anthocyanin-rich aqueous extract from purple highland barley bran (Zhang et al., 2020a), avocado peel extract fractions (Trujillo-Mayol et al., 2021)] have been reported to inhibit formation or to eradicate biofilms with undesirable food spoiling or foodborne pathogenic microorganisms.

TABLE 4 | Examples of plant phenolics or phenolic-rich plant extracts acting against biofilms by different mechanisms of action.

Plant phenolic or plant extract	Microorganism	Mechanisms of action	References
Moringa oleifera extract	*P. aeruginosa*	– growth inhibition at a 0.05 mg·ml^{-1} concentration, while biofilm formation was reduced by 88% after 24 h	Onsare and Arora, 2015
3-p-trans-coumaroyl-2-hydroxyquinic acid from pine needles of *Cedrus deodara*	*S. aureus*	– inhibits the biofilm formation of *S. aureus* by affecting the initial attachment phase of biofilm development	Wu et al., 2016, 2019
Olive mill waste (olive vegetation water) extract	*Escherichia coli* K12	– olive vegetation water extract sub-inhibitory concentrations decreased biofilm formation, swarming and swimming motility	Carraro et al., 2014
		– repression of genes for flagellar synthesis and of other genes linked to biofilm formation was observed	
Ethyl acetate fraction of *Adenanthera pavonina* ethanolic extract	*P. aeruginosa* PAO1	– 0.1 mg·ml^{-1} ethyl acetate fraction of *A. pavonina* ethanolic extract inhibits swarming motility of *P. aeruginosa* PAO1	Vasavi et al., 2015
		– 0.5 mg·ml^{-1} ethyl acetate fraction of *A. pavonina* ethanolic extract inhibited pyocyanin production by *P. aeruginosa* PAO1	
		– viability of *P. aeruginosa* PAO1 was not affected at the tested concentrations of ethyl acetate fraction of *A. pavonina* ethanolic extract as observed by cell count	
Dichloromethane fraction of ethanolic extract of *Camellia nitidissima* Chi flowers	*P. aeruginosa* PAO1	– fraction of *C. nitidissima* Chi flowers extract inhibits pyocyanin production by *P. aeruginosa* without affecting its growth	Yang et al., 2018
		– fraction of *C. nitidissima* Chi flowers extract exerts a concentration-dependent inhibitory activity of swarming and swimming motility of *P. aeruginosa*	
Allium sativum extracts: raw garlic extract, heated garlic extract and toluene extract	*P. aeruginosa* PAO1	– inhibits biofilm formation and exerts anti-quorum sensing activities against *P. aeruginosa* PAO1 at sub-inhibitory concentrations (i.e. below MIC)	Vadekeetil et al., 2015
Rosa rugosa tea polyphenolic extract	*P. aeruginosa* PAO1 *Escherichia coli* K12	– inhibits swarming motility and biofilm formation of both strains in a concentration-dependent manner	Zhang et al., 2014
		– inhibited quorum-sensing controlled violacein production in *Chromobacterium violaceum* (a quorum sensing - controlled phenotype)	
		This extract is proposed to be a quorum-sensing inhibitor and/or anti-biofilm agent	
Heracleum orphanidis methanolic and ethanolic extracts	*P. aeruginosa* PAO1	– inhibit biofilm formation at sub-inhibitory concentrations	Mileski et al., 2016
		– reduce the twitching and flagella mobility	
		– reduces pyocyanin production	
		Taken together, these observations suggest an inhibition of *P. aeruginosa* PAO1 quorum sensing by *H. orphanidis* extracts	
Aqueous *Tradescantia pallida* extract	*Pseudomonas aeruginosa*	– inhibits both bacterial growth and biofilm formation as well as swarming motility	Kamiya et al., 2019
		– biofilm treated by *T. pallida* extracts remain premature, likely because of swarming motility inhibition	

Interestingly, many authors reported that biofilm formation inhibition by plant phenolics requires lower concentration than to inhibit the growth of the corresponding microorganisms (Carraro et al., 2014; Zhang et al., 2014; Vasavi et al., 2015; Yang et al., 2018; Kamiya et al., 2019). Since biofilms are major causes of cross-contamination of foods, exploitation of antibiofilm activity of plant phenolics is promising to limit foodborne diseases, as well as food spoilage. Disinfection in food processing factories relies upon biocides, requires large amounts of water and generates huge volumes of sewage with high loads of cleaning agents and biocides. In France, 11,000 tons of biocides are used in the agri/food industry per year (data from the French Association of Detergents and Hygiene Product Industry Professionals). Biodegradability of cleaning agents and toxicity of disinfectants are both issues for public authorities, who have established regulations to protect the environment, including the currently implemented EU no 528/2012 Regulation concerning the making available on the market and use of biocidal products in the European Union. However, among cleaning agents and biocides, the most environmentally friendly products are still not widely used. Use of plant phenolics or phenolic-rich plant extracts for this purpose is still in its infancy.

Besides societal and regulatory evolutions related to environmental concerns and the necessity to limit the emergence of multidrug-resistant microorganisms, a broader use of

phenolic-rich plant extracts will require, not only a better understanding of the molecular mechanisms leading to biofilm formation prevention or their eradication, but will also benefit from progresses regarding the formulation of delivery systems improving their efficiency. Ongoing progresses in this field are presented in the next section of this review. A promising strategy presented in the next section relies on functionalization of materials with antimicrobial phenolics (e.g., curcumin as proposed by Dogra et al., 2015), in order to prevent biofilm formation on food-contacting surfaces.

DELIVERY MODES TO PROMOTE THE STABILITY AND THE ANTIMICROBIAL ACTIVITY OF PLANT PHENOLICS

Antimicrobial plant phenolics direct addition to foods may face the same limits than other antimicrobial compounds, which were listed by Fu et al. (2016) in their review:

- a limited solubility in water, which is a major constituent of perishable foods
- a limited stability, oxidation or heat treatments may affect the activity of some plant phenolics
- uncontrolled release
- alteration of organoleptic properties [alteration of colour or taste (e.g., astrincency of many plant polyphenols)]

Design of food-grade systems to deliver antimicrobial plant phenolics is promising to circumvent these limits. The two main goals of such systems are (i) protection and (ii) sustained release of plant phenolics. Indeed, delivery systems with diverse structures can be prepared with different food-grade components, such as lipids, proteins, carbohydrates, surfactants, and minerals (Zhang et al., 2020b). Controlled release of phenolics from such systems can result from physical entrapment in structures with different geometries (e.g., gels with differing porosity, tortuosity, coated microcapsules …), from weak interactions (hydrophobic or ionic interactions, hydrogen-bonding) between phenolics and delivery systems components or from a mixture of both phenomena. Delivery systems can also be classified according to their dimensions, ranging from molecular inclusion of phenolics in cyclodextrins (Pinho et al., 2015) and the rapidly growing field of nanosystems of delivery [nanoemulsions (Saini et al., 2019; McClements et al., 2021) and nanoparticles (Hu et al., 2017; Hosseini and Jafari, 2020; Vidallon and Teo, 2020; Spizzirri et al., 2021)] to edible coatings or food packagings incorporated with antimicrobial plant phenolics (Mir et al., 2018; Zhu, 2021), and including microemulsions and other microencapsulation systems (Hosseini and Jafari, 2020). Most of systems for controlled delivery of active molecules are issued from the important effort of research for pharmaceutical applications. For controlled delivery of plant phenolics, the goal is often to improve the bioavailability of dietary polyphenols, as reviewed by Hu et al. (2017). Several examples of application to antimicrobial plant phenolics of each of these delivery systems are listed in **Table 5**.

Molecular Inclusion in Cyclodextrins

Molecular inclusion in cyclodextrins of several plant phenolics has increased their solubility in water and their stability before use, while preserving (Zhao et al., 2010) or even in some cases enhancing their antimicrobial activity (Pinho et al., 2015). However, most of antimicrobial activity assays were performed *in vitro* and efficiency of plant phenolics-cyclodextrin complexes antimicrobial activity in foods has still to be demonstrated. This is likely one of the reasons why, to our knowledge, there is still no commercial application of plant phenolics-cyclodextrin complexes to date unlike for natamycin-cyclodextrin complexes for sliced bread preservation in the United States. Progress is also expected to result from a better understanding of the structural and thermodynamic determinants of interactions between phenolics and cyclodextrins: interestingly, Andreadelis et al. (2021) recently investigated the interactions between two antimicrobial phenolics (caffeic acid and rosmarinic acid) and hydroxypropyl-β-cyclodextrin with either 4, or 10 hydroxypropyl moieties by combining experimental methods, such as isothermal calorimetry and calculatory methods, such as molecular dynamics simulation. Further research in this direction should provide data for the rational design of cyclodextrins with optimized structures for the complexation of a given phenolic.

Nanoemulsion-Based Delivery Systems

Nanoemulsion-based systems to deliver plant antimicrobial compounds in foods have been recently reviewed by McClements et al. (2021). Emulsions are colloidal dispersions of two immiscible fluids. In nanoemulsions, one of them is dispersed in the other one, as small particles with a less than 200 nm diameter. Nanoemulsions can be elaborated with low- or high-energy methods (Saini et al., 2019).

Low-energy methods are based on spontaneous emulsification, phase inversion point, or membrane emulsification. However, they necessitate a high surfactant to oil ratio. Therefore, due to the cost of food-grade surfactants, high-energy methods such as high-pressure homogenization, microfluidization, and sonication are generally preferred for the elaboration of nanoemulsions intended for application in food sector. As illustrated in **Table 5**, oil-in-water (o/w) nanoemulsions (e.g., antimicrobial phenolics of mangosteen peel soluble in virgin coconut oil; Sungpund et al., 2020) as well as water-in-oil (w/o) nanoemulsions (e.g., water-soluble antimicrobial phenolics of cactus pear; Medina-Pérez et al., 2019) can be prepared and have a protective effect against oxidation of phenolics and/or a positive effect on their antimicrobial activity, by enhancing their dispersion. Although thermodynamically unstable, most of nanoemulsions can be designed to be kinetically stable for sufficient time for commercial applications (McClements et al., 2021), which is advantageous compared to microemulsions. Addition of emulsifiers, which are amphiphilic molecules both facilitating emulsion formation and improving their stability, is necessary. Emulsifiers used can be food ingredients such as proteins, or food additives such as soya lecithin (a phospholipid), modified starch or synthetic food-grade surfactants, such as Tween® 20 [polyoxyethylene (20) sorbitan monolaurate, E 432], which is a non-ionic surfactant.

Besides emulsifiers, the addition of other stabilizers of antimicrobial nanoemulsions, such as thickening agents (e.g., carboxymethyl cellulose, pectin), Ostwald ripening inhibitors (e.g., corn or sunflower oil), weighting agents of the oil phase to inhibit gravitational separation (e.g., dammar gum) is often required to enhance the stability of nano- and micro-emulsions, as briefly reviewed by McClements et al. (2021). As illustrated by Sungpund et al. (2020) in their study on extraction by virgin coconut oil of antimicrobial phenolics from mangosteen peel and subsequent (o/w) nanoemulsion preparation, many other authors explored the possibility to valorize fruits and vegetables wastes through "green" extraction of bioactive compounds, including antimicrobial phenolics and nanoemulsions-based delivery systems, as recently reviewed by Saini et al. (2019). The elaboration of nanoemulsion-based delivery systems is till now better established for essential oils and their components, which are not or poorly soluble in water: essential oil nanoemulsions resulted thus in a better dispersion of their antimicrobial constituents in aqueous systems, often resulting in a higher antimicrobial activity. This approach is more recent for antimicrobial polyphenols and promising for dispersion of poorly soluble in water ones. Future research will not only consider simple (o/w) or (w/o) nanoemulsions of antimicrobial plant phenolics but also the possibilities offered by double (w/o/w) or (o/w/o) nanoemulsions as well as by pickering emulsions to improve their stability and/or their sustained release in food systems. (w_1/o/w_2) double emulsions make possible the controlled release of water-soluble compounds trapped within the internal aqueous phase. Pickering emulsions are stabilized by solid colloidal particles. The increased stability of pickering emulsions compared to conventional ones is ascribed to the irreversible adsorption of colloidal particles at the interface and the formation of a thicker rigid layer around the dispersed phase (Sabaghi et al., 2021).

Nano- or Micro-Encapsulation

Nano- or micro-encapsulation of ingredients extending the shelf life of foods, including some antimicrobial plant phenolics or phenolic-rich plant extracts, have recently been reviewed by Hosseini and Jafari (2020). Nano- or micro-encapsulation of antimicrobial plant phenolics consists in their incorporation into another(other) compound(s), acting as wall material(s) protecting them from factors affecting their stability and/or activity, such as light or oxygen, before and/or during their use and/or controlling their release once used. Most frequently used wall materials are proteins, polysaccharides or their combinations. Their interactions with antimicrobial phenolics, as well as their intrinsic physicochemical properties condition important parameters, such as encapsulation efficiency or kinetics of release of active phenolics. The choice of wall materials also depends on the technique used to encapsulate antimicrobial plant phenolics. Hosseini and Jafari (2020) classified encapsulation techniques in chemical procedures (e.g., interfacial polymerization), physicochemical procedures (e.g., complex coacervation, entrapment in liposomes, ionic gelation of alginate …), physical procedures (e.g., co-extrusion, freeze- or spray-drying …) and emerging encapsulation procedure (e.g., electrospinning/electrospraying).

Several authors compared the *in vitro* and the *in situ* antimicrobial activity of nanoencapsulated antimicrobial plant extracts or phenolics and their free counterparts (**Table 5**). Interestingly, quercetin-loaded polycaprolactone nanoparticles inhibited for a longer period than free quercetin the *in vitro* growth of foodborne pathogenic bacteria, likely due to its sustained release from nanoparticles (Dinesh Kumar et al., 2016). However, Pinilla et al. (2019) reported that fungal alteration of sliced bread was not better prevented by the addition of garlic extract-loaded nanoliposomes in dough, than by the addition of the same amount of free garlic extract. More recently, Li et al. (2018) prepared chitosan-based nanoparticles simultaneously loaded with catechin and quercetin by ionic gelation of chitosan by sodium tripolyphosphate followed by crosslinking with genipin. The 180 nm size nanoparticles had a significantly lower MIC against *E. coli*, *S. aureus* and *B. subtilis* than catechin, quercetin and chitosan. These lower MIC values might result both from sustained release of catechin and quercetin and from the electrostatic attraction by the negatively charged surface of bacteria of positively charged chitosan nanoparticles.

Incorporation in Food Contact or Packaging Materials or in Edible Coatings

Incorporation of antimicrobial plant phenolics or extracts in the formulation of food contact materials, deposition of coatings embedding active phenolics or grafting of active phenolics on their surface have been proposed by several authors as a way for controlling microbial growth, cross-contamination, and biofilm formation on food-contacting surfaces (Pei et al., 2014; Dogra et al., 2015; Shlar et al., 2018). Primary food packaging films and trays are also food-contacting surfaces. Moreover, many microbial contaminations of high-added value perishable foods, such as meat pieces or fish fillets, occur in the superficial zone of foods. Therefore, several authors investigated the possibility to functionalize the inner surface of packaging materials with antimicrobial compounds to extend the shelf-life and or improve the microbial safety of perishable foods. Functionalization of food contact materials can rely upon grafting of antimicrobial phenolics resulting from their covalent binding with constitutive polymers or upon their incorporation in the formulation of coatings or packaging materials. In this latter case, coatings or packaging materials act as a reservoir of antimicrobial phenolics, which will reach the surface of food mainly through diffusion. The release of antimicrobial phenolics is controlled by their partition equilibrium between reservoir material and food matrix in direct contact and kinetics of migration of plant phenolics in food contact material and in food, respectively. The release rate of plant phenolics over time should maintain a sufficient concentration to inhibit growth of unwanted microorganisms for a sufficient time to extend the shelf life of food in contact.

In the European Union, the possibility to launch active food packaging systems is authorized since 2009 [Regulation

TABLE 5 | Examples illustrating the diversity of systems to deliver antimicrobial plant extracts/phenolics to extend the shelf life or improve the microbial safety of foods or to remove microbial biofilms.

Delivery system component(s)	Antimicrobial plant phenolic/extract	Elaboration method	Antimicrobial activity	References
Molecular inclusion				
β-cyclodextrin	Chlorogenic acid	9 h stirring at 50°C followed by drying with a rotary evaporator	– antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* of chlorogenic acid-cyclodextrin complexes was similar, but chlorogenic acid stability against oxidation was improved	Zhao et al., 2010
β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin	Caffeic acid	Ultrasounds bath for 30 min followed by 24 h at 25°C under constant stirring in the absence of light	– higher *in vitro* antibacterial activity against *Staphylococcus aureus* ATCC 6538 of caffeic acid inclusion complexes with both β-cyclodextrins than caffeic acid alone	Pinho et al., 2015
Methyl-β-D-cyclodextrin	Curcumin	mixing of equal volumes of a 20 mmol·L^{-1} curcumin ethanolic solution and a 20 mmol·L-1 methyl-β-cyclodextrin aqueous solution for 2 h at room temperature followed by ethanol evaporation with a rotary evaporator	– 0.4 mmol·L^{-1} minimal inhibitory concentration against *Escherichia coli* of curcumin-methyl-β-cyclodextrin complexes	Dogra et al., 2015
Cyclodextrin	Propolis	mixing of propolis with cyclodextrin for "green" extraction of phenolic substances, terpenoids, and flavonoids	– the complexes obtained had an antifungal activity suggesting they could be a natural alternative to potassium sorbate	Stagkos-Georgiadis et al., 2021
Nanoemulsions				
Aqueous phase with Span® 20 and Tween® 20 emulsifiers to prepare an oil in water (o/w) nanoemulsion	Mangostins extracted from mangosteen peel extract with virgin coconut oil	successive mixing of mangosteen extract in virgin coconut oil and an aqueous phase with 10% (w/v) emulsifiers with an homogenizer and an ultrasonic processor	– nanoemulsions had a higher antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* than the extract (a twice lower minimal inhibitory concentration)	Sungpud et al., 2020
Orange essential oil [70% (v/v)], liquid soya lecithin [20% (v/v)], and cactus pear fruit aqueous extract were mixed to prepare a water in oil (w/o) nanoemulsion	Cactus pear fruit aqueous extract	(w/o) emulsion components were stirred with an ultrasonic processor	– incorporation of 0.4% (v/v) of cactus pear extract (w/o) nanomemulsion in starch film-forming suspensions resulted in films with an *in vitro* antibacterial activity against *Escherichia coli* and *Salmonella typhimurium*	Medina-Pérez et al., 2019
Nanoencapsulation				
Chitosan/poly(ethylene oxide; PEO) nanofibers	Pomegranate peel extract	Active chitosan/PEO nanofibers with pomegranate peel extract were prepared by electrospinning of a 4% (w/v) chitosan/PEO blend and 20 g·L^{-1} pomegranate peel extract solution	– addition of active nanofibers in aluminium foil used to wrap raw bovine meat pieces artificially contaminated with *Escherichia coli* O157:H7 resulted in a 2.96 and 5.80 log CFU/g total viable counts reduction compared to meat wrapped in control aluminium foil after 10 days of storage at 4 and 25°C, respectively	Surendhiran et al., 2020
Polycaprolactone (PCL)	Quercetin	Nanoparticles were elaborated by nanoprecipitation followed by freeze-drying [PCL and quercetin acetone solution was added to an aqueous phase with a hydrophilic surfactant (Pluronic F-127) under moderate stirring]. The acetone and water were then removed by vacuum evaporation. The nanosuspension was then ultracentrifuged. The resulting pellets were re-suspended in distilled water and frozen. Frozen nanoparticles were freeze dried.	– quercetin nanoparticles had a higher growth inhibitory effect of *B. subtilis*, *E. coli*, *S. aureus*, and *S. typhimurium* than the same amount of free quercetin for 48 h at 37°C. This difference can be ascribed to sustained release of quercetin from nanoparticles, while free quercetin lost its antibacterial effect against all test bacteria after 2–9 h. The growth of bacteria can then restart.	Dinesh Kumar et al., 2016

(Continued)

TABLE 5 | Continued

Delivery system component(s)	Antimicrobial plant phenolic/extract	Elaboration method	Antimicrobial activity	References
Phosphatidylcholine and oleic acid nanoliposomes	Garlic extract	Nanoliposomes were prepared by the thin film hydration method	– garlic extract-loaded nanoliposomes and free garlic extract had a similar *in vitro* antifungal activity against *Penicillium expansum*, *Penicillium herquei*, *Fusarium graminearum*, *Aspergillus flavus*, and *Aspergillus niger* – addition of 0.65 ml of free garlic extract or the same amount of garlic extract-loaded nanoliposomes in bread dough prevented fungal spoilage of sliced bread for 5 days	Pinilla et al., 2019
Microencapsulation				
Calcium alginate beads	Onion scale	Ionic gelation by calcium ions of a sodium alginate and onion scale extract solution	– more than 2 log cycle reduction in the growth of *Staphylococcus aureus* and *Pseudomonas fluorescens* in minced chicken meat during chilled storage when 6% (w/v) onion-scale extract encapsulated beads were incorporated	Kanatt et al., 2018
Chitosan and sodium tripolyphosphate	Green tea extract with epigallocatechin gallate as main constituent	Chitosan cross-linked particles loaded with green tea extract were prepared by dropwise addition of a sodium tripolyphosphate solution in a chitosan solution with green tea extract followed by ultrasonication for 3 min	– Total mesophilic aerobic count, coliform bacteria, and yeasts and moulds were monitored for 8 days storage at 4°C of hamburger patties incorporated with green tea extract or green tea-loaded chitosan particles: significant differences in microbial counts in favor of green tea microparticles were observed	Özvural et al., 2016
Maltodextrin, gum arabic	Sugar cane bagasse extract	After solubilization, mixing with a homogenizer and freezing of sugar cane bagasse extract, maltodextrin and gum arabic, microparticles were obtained by freeze-drying	– sugarcane bagasse extract exerted antibacterial activity against *E. coli*, *B. cereus* and *S. aureus* – microencapsulation of sugar bagasse extracts resulted in an improved thermal stability of sugarcane bagasse extract phenolics	Velazquez-Martinez et al., 2021
Edible coatings or films				
Sodium alginate	Gallnut ethanolic extract	Preparation of cast films following drying of sodium alginate, gallnut extract and glycerol (as a plasticizer) film forming solutions	– edibles films showed a good *in vitro* antibacterial activity against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11775	Aloui et al., 2021
Sodium alginate	Pomegranate peel extract (PPE)	PPE or alginate nanospheres containing PPE prepared through water in oil (w/o) emulsification and external gelation with calcium chloride nanoparticles and incorporation in sodium alginate	– fresh chicken breast coated with alginate with PPE nanospheres was less susceptible to microbial growth over 14 days at 4°C than that coated with alginate with "free" PPE – the difference can be ascribed to a more sustained release of PPE antimicrobial compounds from PPE nanospheres	Rahnemoon et al., 2021
Gelatin	*Arbutus unedo* L. fruit methanolic extract	Preparation of films by solvent casting of a gelatin film suspension with glycerol as a plasticizer and *Arbutus unedo* L. fruit extract	– fresh sardine fillets artificially inoculated with *Staphylococcus aureus*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* were stored at 4°C for 12 days either alone, after covering with *Arbutus unedo* L. fruit extract, or wrapped in gelatin film alone or with *Arbutus unedo* L. fruit extract – interestingly, wrapping of fillets with gelatin-film with *Arbutus unedo* L. fruit extract resulted in the highest reduction of the three inoculated foodborne pathogenic or food spoiling bacterial strains	Bouhanna et al., 2021

(Continued)

TABLE 5 | Continued

Delivery system component(s)	Antimicrobial plant phenolic/extract	Elaboration method	Antimicrobial activity	References
Chitosan	Phenolic acids (p-coumaric acid, ferulic acid, gallic acid, vanillic acid, and salicylic acid)	Preparation of chitosan-phenolic acid composite films by solvent casting	Chitosan-ferulic acid composite films better preserved shrimps than other chitosan-phenolic acid films (reduction of total bacterial count and total basic volatile nitrogen over 6 days of storage at 4°C)	Liu et al., 2021a
Chitosan	Cinnamic acids (p-coumaric acid, caffeic acid and ferulic acid)	Carbodiimide-mediated grafting of cinnamic acids with chitosan and subsequent use of grafted chitosan to prepare films by solvent casting	– caffeic acid-grafted chitosan films presenting the highest *in vitro* antibacterial properties were used to wrap pork meat and effectively extended its shelf life to 10 days at 4°C	Yong et al., 2021
Active packaging				
Polyethylene terephthalate/ polypropylene (PET/PP) films impregnated with olive leaf extract	Olive leaf extract	Supercritical solvent impregnation of PET/PP films with olive leaf extract	Impregnated films inhibited *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* growth and extended cherry tomatoes shelf life by 20 days	Cejudo Bastante et al., 2019
Multilayer polyelectrolyte coating embedding curcumin-cyclodextrin complexes on polyethylene terephthalate (PET) films as a support matrix	Curcumin - carboxymethyl-cyclodextrin complexes	– after a hydrolysis pre-treatment to provide sufficient electric charge to the PET surface, it was electrostatically coated with repeated multilayers comprising alternately deposited positively-charged poly-L-lysine and negatively-charged poly-L-glutamic acid and carboxymethyl-cyclodextrin complexes – carboxymethyl-cyclodextrin molecules were either covalently cross-linked using carbodiimide or left unbound	– coatings with uncrosslinked curcumin carboxymethyl-cyclodextrin complexes were active against *Escherichia coli* both in the dark and when illuminated, while coatings with cross-linked complexes were only active when illuminated due to photodynamic properties of curcumin. – interestingly, cross-linked coatings kept their antibacterial activity following soaking in phosphate buffer saline (PBS) unlike uncrosslinked ones	Shlar et al., 2018
Polyethylene	Pomegranate peel extract	Melt blending of pomegranate peel extract with polyethylene	– 3 days shelf-life extension of pork meat packaged in films with pomegranate peel extract	Hu et al., 2016

(EC) no 450/2009, 2009]. However, only molecules with a food additive or a food ingredient status might be released from such food packaging systems to foods. This could be limiting for the commercial application of packaging materials releasing phenolic-rich antimicrobial plant extract, since not all molecules transferred from film to food would be characterized. The use of pure phenolics with a food additive [e.g., curcumin (E 100) anthocyanins (E 163) as colorants] or a food ingredient status (e.g., naringin, tannic acid, or hesperetin as food flavourings) would thus be preferred.

Up to now, most of food packaging materials are plastic-based materials made of polyolefins, such as polyethylene (PE), polyethylene terephthalate (PET) or polypropylene (PP) and are elaborated by extrusion-injection for rigid materials such as trays or by extrusion-blowing for films. If antimicrobial compounds are incorporated in their formulation before their elaboration, this requires that antimicrobial activity of phenolics is not lost due to the high temperature conditions (which can exceed 200°C for the extrusion of polypropylene for instance) and the shear stress prevailing during extrusion process. Interestingly, Hu et al. (2016) blended 15 g·kg^{-1} of pomegranate peel extract with PE by twin-screw extrusion at 160–190°C to elaborate active films and reported a decrease in total volatile basic nitrogen (TVB-N) during refrigerated storage of pork meat packaged in such film and an extension of 3 days of its shelf life. This suggests that pomegranate peel extract kept its antimicrobial activity following extrusion of films. Similarly, Cottaz et al. (2019) incorporated a synthetic antimicrobial phenolic, isobutyl-4-hydroxybenzoate in poly(ethylene-co-vinyl acetate; EVA), linear low density polyethylene (LLDPE), and PP by melt-blending to prepare pellets, which were subsequently used to prepare heat-pressed films: all films had an antibacterial activity, demonstrating thus the preservation of isobutyl-4-hydroxybenzoate antibacterial activity after melt processing. Antimicrobial polyphenols, which are less volatile than antimicrobial essential oil components, and which are not denatured by temperatures exceeding 140–150°C like antimicrobial proteins, such as lysozyme, or inactivated by heat, such as antimicrobial peptides like nisin, are thus promising compounds for incorporation in packaging materials elaborated by extrusion.

However, for regulatory reasons, it might be more interesting to incorporate food-grade phenolic-rich extracts of plants in edible films or coatings: the only condition is that their formulation only contains food ingredients, additives or processing aids. When developing active packaging films or edible films or coatings

releasing antimicrobial plant phenolics in the superficial zone of foods (e.g., Zam and Ali, 2018), one must keep in mind that such delivery systems are advantageous, only if a more sustained release of phenolics over time is observed, compared to dipping foods in a plant phenolic solution or spraying a plant phenolic solution on their surface before packaging. Interestingly, Bouhanna et al. (2021) reported that the growth of bacteria contaminating the surface of sardine fillets was better inhibited, when fillets were wrapped with a gelatin film incorporated with *Arbutus unedo* L. fruit extract, than when they were covered with a layer of *A. unedo* L. fruit extract. This difference is likely due to the sustained release of *A. unedo* L. fruit extract antibacterial constituents, resulting from their entrapment in a gelatin-based network and/or to the weak interactions between gelatin and these constituents. Recently, Rahnemoon et al. (2021) compared the preservation of fresh chicken breast meat in alginate coatings with either free pomegranate peel extract or alginate nanospheres loaded with pomegranate peel extract: fresh chicken meat had a longer shelf life when coated with pomegranate peel extract nanospheres. This difference was attributed to the more sustained release of active phenolics from pomegranate peel extract nanospheres. Protein and polysaccharides are the most frequently used biopolymers for the preparation of edible coatings or films. Interestingly, some polysaccharides, such as chitosan, have an intrinsic antimicrobial activity, which can act synergistically with antimicrobial plant phenolics. Yong et al. (2021) recently grafted cinnamic acids with chitosan and observed that films elaborated with caffeic acid-chitosan conjugates had the highest antimicrobial activity. Many other authors recently investigated the effect of the addition of polyphenols to polysaccharides for food packaging applications, as recently reviewed by Zhu (2021).

Further commercial development of antimicrobial plant phenolics delivery systems requires that more studies include their application to a real perishable food to evaluate their potential for shelf life extension. Moreover, there is still a too limited number of studies including an application of such controlled delivery systems to real foods, without including a comparison with the effect of direct addition of the same amount of antimicrobial plant phenolic or extract, which is necessary to estimate the added-value of controlled delivery systems, from which formulation and elaboration represent an additional cost. As suggested by McClements (2018) in his review proposing a "delivery by design" approach for the design of efficacious nanoparticle- and microparticle-based systems to deliver active agents, the development of such systems has to be inspired by quality by design approach used in industrial sector.

CONCLUSION AND PERSPECTIVES

Compared to the diversity of antimicrobial plant phenolics and phenolic-rich plant extracts and the current use of biocides for disinfection of food production facilities and of food preservatives, their application is still in its infancy. However, more and more formulations containing phenolic-rich plant extracts with antimicrobial activity are proposed. For instance, the interested reader can refer to Sima et al. (2018), who reported that a commercial formulation with namely citrus, grape seed and oregano extracts in addition to lactic and citric acids effectively reduced *in vitro* and *in vivo* pathogenicity of T6SS positive *Campylobacter jejuni* and *Campylobacter coli* chicken isolates. T6SS positive highly virulent *Campylobacter* spp. are positive for the Type VI secretion system (T6SS), which have an increased ability to invade the host gastrointestinal epithelium are highly prevalent in poultry. Therefore, their observations are of particular importance, since preventing *Campylobacter* spp. infections in humans is considered a public health priority.

Research on antimicrobial activity of edible phenolic-rich plant extracts or plant phenolics expands rapidly, not only benefitting from progress in -omics to better describe their molecular mechanisms of action, but is also being stimulated by (i) the "clean label" marketing trend, which stimulates the search of natural ingredients, as alternatives to synthetic food preservatives, (ii) the necessity to develop more sustainable food systems, favoring the valorization of antimicrobial phenolics-rich food by-products or wastes, by following the principles of circular economy, (iii) the environmental and microbial safety considerations to limit the emergence of multi-drug resistant microorganisms, and (iv) the search for alternatives to plastic food packagings [as illustrated by the "single use plastics" directive in the European Union (EU 2019/904 Directive, 2019)]. However, despite this scientific and societal context favorable to the expansion of the use of antimicrobial phenolic-rich plant extracts or plant phenolics in the food sector, present limitations, such as (i) high cost for extraction of antimicrobial plant phenolics from plants resulting from climate variability and long growth cycle of plants, the requirement of solvents and/or high energy consuming extraction methods or (ii) the necessity to perform more in-depth studies regarding the toxicity of some plant phenolics, which can be pro-oxidant or mutagenic at high dosage and to check that their broad use will not promote the emergence of resistant microbial strains. Some of these challenges were recently reviewed and discussed by Ofosu et al. (2020). The issue of the cost can partly be addressed by applying hurdle technology principles to combine antimicrobial plant phenolics application with other antimicrobial factors (processes, antimicrobial compounds) acting synergistically.

AUTHOR CONTRIBUTIONS

NO and PD conceptualized, searched initial bibliography, wrote the first manuscript draft, and revised the manuscript. All authors have read and approved the final version of the manuscript.

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