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Research article

Potential application of prickly pear vinegar as a surface disinfectant: in vitro and molecular docking studies

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Abstract:

The surface disinfectant property of a homemade prickly pear vinegar (PPV) is investigated throughout this study, using in vitro testes assisted by a molecular docking study, against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Salmonella enterica*) strains. Results show that PPV exhibited growth-inhibitory effect for all tested bacterial strains at MIC values ranging between 1.56, and 3.12% (v/v). Obtained results showed that PPV was more effective to inhibit initial cell attachment (86.33, 54.15, and 72.19% at 2×MIC) compared to 24h-preformed biofilms (67.64, 42.69 and 39.94% at 2×MIC). Treatment of abiotic surface with PPV at 2×MIC resulted in a significant reduction ($p < 0.05$) of bacterial metabolic activity (72.10, 52.92 and 56.96%, respectively). The anti-biofilm activity of PPV was also evaluated on two additional abiotic surfaces such as glass and stainless steel and results showed that treatment of biofilms with PPV at 2×MIC (% v/v) resulted in a log reduction of the initial biomass. Molecular docking analysis predicted the ability of phenolic compounds present in PPV, particularly, rutin to interact with SarA protein involved in S. aureus biofilm formation.

Keywords: Prickly pear vinegar; Antimicrobial activity; Anti-biofilm activity; Abiotic surfaces; Molecular docking

Introduction

Nowadays, one of the most worldwide public concerns facing various food industries is the microbial food-borne disease, particularly those attributed to biofilms that account for up to 80% of microbial infections (Epstein et al. 2011). Biofilms pose a serious challenge in food industries such as brewing, seafood, dairy, poultry, and meat processing. These microbial communities protect their inhabitants, making them resistant to antimicrobial agents and cleaning processes, thus complicating sanitation and food safety efforts (Srey et al. 2013).

In fact, pathogen contamination is inevitable since it could be realized in any step of food processing, from farm to fork (Srey et al. 2013). Also, in food industry, pathogenic microorganisms tend to contaminate food equipment and attach onto surfaces, forming organized communities called biofilms (Al-shabib et al. 2017), resulting in a great economic loss to society. *Salmonella enteritidis* has been shown to form biofilms on food contact surfaces, significantly increasing the risk of cross-contamination (Galié et al. 2018). Therefore, control methods with improved efficacy against planktonic cells and biofilms in food industry are required.

Biofilms are sessile communities of embedded microorganisms firmly attached to each other or attached to a substratum. The architectures of biofilms are comprised either of single species or multiple species bacteria and are constituted by the extracellular polymeric substances of exopolysaccharides (EPS), proteins and DNA secreted by microorganisms (Xu et al. 2019). In addition, it was reported that most pathogens mostly exist in biofilm matrix instead of in planktonic form, and pathogens in biofilm matrix are more resistant than in planktonic form when encountering antimicrobial treatment, which presents a new challenge in food industry (Cui et al. 2018). Hence, more efficient sanitizing and disinfecting strategies are required to deal with such a problem.

Regarding biofilm prevention or removal, numerous approaches have been used for many years such as, flushing, chlorination, and ultraviolet disinfection (Srinivasan et al. 2008). However, these physicochemical methods lack both effectiveness and safety. Therefore, the development of new and natural anti-biofilm agents seems to be interesting, given that the emergence of antibiotic-resistant bacterial strains as well as the consumer's attitude towards the consumption of chemically treated food products have encouraged researchers to find effective alternatives and develop natural antimicrobial agents.

Even several natural compounds such as plant extracts, essential oils and honey have been known for their anti-biofilm properties (Sadekuzzaman et al. 2015), vinegar was scarcely described in the literature as an anti-biofilm agent. Recently, Shay (2016) has demonstrated that vinegar could effectively remove bacteria from dentures, which will not cause oral mucosal damage even if residual vinegar remains on the denture. In the same vein, Liu and Hannig (2020) showed that using vinegar for mouth rinsing during 5 s could be a potential approach for inhibition of biofilm formation in the oral cavity.

It has been reported that prickly pear vinegar (PPV) presented a richness with phenolic compounds such as quinic, protocatchuic, syringic, *p*-coumaric, transferulic acids, quercetin, naringin and rutin among others (Ben Hammouda et al. 2021). These bioactive molecules could have antibiofilm activities and interference with quorum sensing regulatory systems. In fact, these systems are essential for defining bacterial activity since they are implicated in several cellular functions, namely, sporulation, biofilm formation, virulence gene expression, among others (Oliveira et al. 2016). Also, it's worthier mentioning that virulence factors could be inhibited in pathogenic microorganisms through the utilization of quorum sensing inhibitors (QSI) (Kalia 2013). In this context, it has been demonstrated that plants are considered to be one of the main sources of natural QSI, including medicinal plants, vegetables and edible fruits (Santos et al. 2021). Recently, it has been shown that phenolic compounds can act as QSI (Liseth et al. 2019; Santos et al. 2020). However, additional studies are required in order to identify the mode of action of the phenolic compounds in inhibiting biofilm formation.

The goal of the present study was to investigate the effect of traditional homemade Tunisian PPV on inhibiting initial cell attachment as well as eradicating 24-h mature biofilms. *Staphylococcus aureus* (CIP 4.83) (Gram-positive), *Escherichia. coli* (CIP 54127) and *Salmonella enterica* (CIP 8297) (Gram-negative) biofilm-forming bacterial strains were considered as the most pathogens encountered in food industry and thus selected in this work. Moreover, its inhibitory effect was investigated not only in polystyrene micro-titer plates but also on two additional abiotic surfaces commonly encountered in food processing environments namely, glass and stainless-steel surfaces. Adding to that a molecular docking study with SarA QS regulator of *S. aureus* has been performed to assess the binding energies and interactions of PPV's phenolic compound with SarA, as a target protein involved in *Staphylococcus aureus* biofilm formation.

Materials and Methods

Chemicals and reagents

Luria-Bertani broth (LB) and Luria-Bertani agar (LB agar) were used for bacteria culture media, containing per liter: Tryptone, 10 g; NaCl, 10 g; and yeast extract, 5 g. For LB-agar, 20 g of agar were added. Muller Hinton (MH) was used as a culture medium in the determination of the MI. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) and crystal violet were purchased from Sigma-Aldrich. All other chemicals and reagents such as glacial acetic acid, phosphate buffered saline (PBS), pure ethanol, and potassium hydroxide (KOH) were of analytical grade. Stainless steel (SS) (304L, Equinox) (50 × 25 mm) and glass (G) (2×2 cm) surfaces were purchased from France.

Vinegar sample

Traditional homemade prickly pear vinegar (PPV) produced at lab-scale by spontaneous fermentation of yellow-skinned variety of *Opuntia ficus indica* collected from Sfax-Tunisia (August 2020) was used for this study. Its physicochemical characterization was summarized in Table 1.

Table 1: Physicochemical composition of traditional prickly pear vinegar (*Opuntia ficus-indica* yellow-skinned variety)

Parameter	Value
pH	3.44 ± 0.11
TA (% Acetic acid)	7.05 ± 0.10
DM (%)	7.08 ± 0.12
Ash (%)	0.59 ± 0.11
Alcohol content (%)	0.18 ± 0.01
Protein content (mg/L)	357.00 ± 0.75
TPC (mg GAE/L)	1005.59 ± 45.25

TA: Total Acidity, DM: Dry Matter, Ash, TPC: Total Phenolic Content

Data were expressed as mean ± standard deviation (n = 3).

Bacterial strains

Bacterial strains, namely *S. aureus* (CIP 4.83), *E. coli* (CIP 54127) and *S. enterica* (CIP 8297) were kindly provided by Pr. Nour-Eddine Chihib (University of Lille, France). They were sub-cultured by streaking on LB agar plates and incubated at 37 °C for 24 h. Then, single colonies from the plate were inoculated into sterile LB broth and incubated in a shaking incubator at 37 °C for a period of 18 to 24 h to ensure that the bacteria were in the log phase. The bacterial suspensions were adjusted to an approximate concentration of 10^8 CFU/mL by diluting the bacterial suspensions with fresh sterile LB broth to obtain an absorbance at 600 nm (Abs_{600}) of 0.1 for all tested bacteria using a spectrophotometer (SHIMADZU, Kyoto, Japan).

Determination of MIC and MBC concentrations of traditional PPV

The antibacterial activity of the traditional PPV sample was investigated by the determination of its minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations, against Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *S. enterica*) bacterial strains.

The MIC value of homemade PPV was determined in MH broth using the broth micro-dilution method in 96-well round-bottomed polystyrene microtitre plates (Sarstedt, Germany), as described by Javdha et al. (2013) with some modifications. Briefly, to each well of sterile 96-well microplates,100 µL of MH broth was added. Then 100 µL of filtered PPV sample (100%) was placed in the first well and two-fold serially diluted in sterile MH to obtain final concentration range of 50% (v/v) - 0.1% (v/v). The inhibition of bacterial growth in the wells containing PPV was assessed by comparing it with growth in blank control wells containing sterile distilled water instead of PPV sample. After the dilution of the samples, 20 µL of the inoculum were added to each well and incubated for 24 h at 37 °C. Positive growth controls (inoculum + MH) were also included in the plate. Following incubation, 25 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H tetrazolium bromide) (0.5 mg/mL) was added to each well and incubated at room temperature for a further 1-2 h. Bacterial growth was assessed by the pink-red coloration of the wells. The well of the lowest sample concentration where bacterial growth was inhibited, viz. no pink-red coloration was observed, corresponds to the MIC value.

After determining the MIC values, 50 µL from all the wells where no color change was observed, were streaked on LB agar plates and incubated for 24 h at 37 °C. These plates were afterwards checked for colony formation, sample with the lowest concentration of PPV that showed no growth on LB agar was recorded as the MBC.

Determination of biofilm inhibition activity

Inhibition of initial cell attachment

The effect of traditional PPV on biofilm formation was evaluated as described by Sandasi et al. (2010). Briefly, solutions of PPV equivalent to MIC, and 2×MIC concentrations were prepared in LB broth. Aliquots (100µL) of each solution were added to individual wells of sterile flat-bottomed 96-well polystyrene microtitre plates (Sarstedt, Germany). An equal volume of sterile distilled water was added as negative control. 100 µL of the bacterial cultures were then added to the wells to yield a final volume of 200 µL in each well. The cultures were added into the wells in quadruplicate and sterile LB broth was also added as an additional control to confirm the sterility of the medium. The plates were sealed and incubated for 24 h at 37 °C to allow cell attachment. Biofilm formation was assessed using the crystal violet assay and the metabolic activity of bacterial cells incubated with PPV was investigated using the MTT assay (assays described in paragraph 2.5.4).

Effect of PPV on biofilm formation and development

The effect of PPV on 24h-old biofilms was evaluated in 96-well polystyrene microplates as described by Bai et al. (2019) with minor modifications. Briefly, bacterial suspension with density of 10^8 CFU/mL without PPV sample was incubated in 96-well polystyrene microplates at 37 °C for 24 h. After the

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incubation time, planktonic cells were discarded from the wells which were gently washed with sterile PBS (0.01 M, pH 7.4). Following the washing procedure, the preformed biofilms of *S. aureus*, *E. coli*, and *S. enterica* were treated with PPV at MIC and 2×MIC values (%, v/v). Microplates were then incubated at 37 °C for 24 h. Afterwards, wells were performed for biofilm biomass through crystal violet staining assay.

Crystal violet assay

Indirect assessment of cell attachment for *S. aureus*, *E. coli*, and *S.enterica* strains was evaluated using the crystal violet assay described by Jardak et al. (2021) with minor modifications. Following the incubation time, culture medium from each well was gently removed and the plates were washed twice with sterile PBS (0.01 M, pH 7.4) to remove any loosely attached bacterial cells. The plates were then air-dried and further oven-dried at 60 °C for 45 min. Cells in the biofilm were then stained with 150 µL of crystal violet (0.4 %, v/v) and incubated at room temperature for 15 min. After that, wells were washed three times with sterile distilled water to remove the excess of stain. A volume of 200 µL of glacial acetic acid (33%, v/v) was then added to all wells to destain the wells, followed by 1 hour of incubation at room temperature for biofilm solubilization. Finally, the content of each well (125 µL) was then transferred to a new sterile microplate, and the amount of biofilm was quantified by measuring the absorbance at 595 nm using a microplate reader (Multiskan FC, Thermo Scientific, version 10106). The mean absorbance (Abs_{595}) was used for determining the inhibition percentage of the biomass formation for each concentration of the sample according to the following equation:

$$\text{Inhibition percentage (\%)} = [(Abs_{\text{Negative control}} - Abs_{\text{Sample}}) / Abs_{\text{Negative control}}] \times 100$$

Biofilm metabolic activity assay

The metabolic activity of the biofilms formed by the tested bacterial strains was assessed using modified 3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide reduction assay (MTT) according to Schillaci et al. (2008). MTT is a tetrazolium salt which will be reduced into a blue-colored product in the presence of metabolically active cells, that can be measured using a spectrophotometer at a wavelength of 570 nm, thus serving as a respiratory indicator of live cells (Krom et al. 2007). Briefly, MTT salt was dissolved in phosphate-buffered saline (PBS) to give a final concentration of 0.5 mg/mL. For the plates containing cells incubated with MIC and 2×MIC concentrations (%, v/v) of PPV sample, following the incubation period, for both initial cell attachment and 24-h mature biofilms, culture medium was gently removed, and the plates were air-dried. One hundred microliters of PBS and 25 µL of MTT solution were pipetted under sterile conditions into each well and incubated for 2 h at 37 °C. The absorbance was then measured at 570 nm using the microplate reader (Multiskan FC, Thermo Scientific, version 10106).

Inhibition of biofilm formation on glass and stainless-steel surfaces

Surface preparation

Stainless steel (SS) (304L) slides (50 × 25 mm) and glass slides (GS) (2×2 cm) were cleaned according to the method described by Akbas and Cag (2016) with minor modifications. Briefly, SS coupons were soaked in pure ethanol solution (95°) for 24 h to remove grease, after that, they were rinsed with distilled water and soaked again in an alkaline solution (KOH, 5%, w/v) for 30 min. SS coupons were thoroughly rinsed five times, for 1 min, in 500 mL of distilled water at room temperature to eliminate detergent residues and three times in ultrapure water. SS slides were air-dried, then oven-dried for 45 min and finally sterilized by autoclaving at 121 °C for 15 min. Regarding G slides, they were first soaked in pure ethanol solution (95°) overnight and then sterilized in pure ethanol (95°) for 10 min.

Biofilm inhibitory activity of traditional PPV against SS and G surfaces

The biofilm inhibitory effect of traditional PPV on initial cell attachment and mature biofilms of the studied pathogens was further investigated on two additional abiotic surfaces viz., SS and G slides.

- Inhibition of initial cell attachment: Biofilm inhibitory activity of traditional PPV on SS and GS surfaces was performed as described by Javdha et al. (2013) with some modifications. Briefly, both sterile surfaces were placed in horizontal position in sterile Petri dishes. The upper face was covered by 500 µL of the standardized cell suspension (108 CFU/mL) together with 500 µL of PPV sample dissolved in sterile LB medium at MIC and 2×MIC concentrations (%, v/v) and were finally incubated at 37 °C for 24 h. Similar treatment was given to the control plates where sterile distilled water has been used instead of PPV sample. After incubation, SS coupons and GS were gently rinsed with PBS to remove loosely-attached cells before anti-biofilm quantification assays.

Regarding the quantification of bacterial biofilm biomass, both surfaces were immersed into 100 mL sterile sample pot containing 10 mL of physiological water (0.85%, w/v) and were then incubated for 5 min in a mechanical shaker at room temperature to remove bounded cells. Serial dilutions were realized and 100 µL of the samples were spread onto LB agar plates and incubated at 37 °C for 24 h. After the incubation time, the number of viable and cultivable cells on the plates was counted and results are expressed in log CFU/cm^2. This experiment was realized in duplicates.

Inhibition of preformed biofilm: 500 µL of bacterial suspensions containing 10^8 CFU/mL were deposited on each surface in a sterile Petri dish and were incubated for 24 h at 37 °C. Bacterial suspension was then removed and surfaces were washed twice with PBS. After that, 500 µL of PPV sample dissolved in sterile LB medium at MIC and 2×MIC concentrations were added and Petri dishes were incubated for a further 24 h at 37 °C. After the incubation period, surfaces were gently rinsed with PBS and immersed with 10 mL of physiological water and used for the anti-biofilm assay as described above.

Microscopic visualization of biofilm: In situ visualization of biofilm inhibition by traditional PPV (initial cell attachment and inhibition of 24-h old biofilms against GS) was obtained using light microscopic analysis as described by Bakir et al. (2017). Briefly, biofilms were allowed to grow on GS with and without (control) PPV at MIC and 2×MIC concentrations and incubated for 24 h at 37 °C. After incubation, GS surfaces were washed with PBS and stained with crystal violet (0.4%, v/v) and rinsed with sterile distilled water. The stained slides were visualized and captured with a light microscope (EVOS XL, AMME3300, USA) equipped with a digital camera at 1000× magnification.

2.8 Molecular docking study

A molecular docking study used SarA protein, a transcription factor implicated in the regulation of biofilm formation, as a target to which phenolic compounds could bind and indirectly inhibit *S.aureus* biofilm formation, has been conducted. The phenolic compounds profile of PPV sample, that was used in the current study, has been described previously (Ben Hammouda et al. 2021). In this regard, molecular docking was performed to assess the binding energies and interactions of the different phenolic compounds in PPV with SarA. The 3D structures of SarA (PDB ID: 2FNP) from *S. aureus* were retrieved from the Protein Data Bank, and the chemical structures of the different molecules were obtained from PubChem (NCBI; pubchem.ncbi.nlm.nih.gov) (Table 2). A previously reported ligand for SarA was used as positive control: hesperidin (PubChem ID: 10,621). Molecular docking analysis was performed using MOE 2015.10 and AutoDock Tools v1.5.6,65 and the 3D and 2D structures were visualized through the BIOVIA Discovery Studio visualizer 2016 v16.1.0.15350.

Table 2: Molecular docking analysis reveals phenolic compounds binding efficacy with SarA protein as receptor of *S. aureus*

	ΔG	Rmsd refine	E_conf	PUBCHEM CID	Non- bonds Interactions
o-Coumaric acid	-6.47	0.90	78.34	5280804	Asn158; Lys163; Gln164; Arg190
Quercetin	-4.96	2.35	2.81	5280343	Pro165; Val168; Arg190

Caffeic acid	-5.07	1.29	-52.11	689043	Phe153; Lys154; Ile157; Arg190
Quercetin-3-glucoside	-4.69	0.68	-39.94	637540	Phe153; Lys154; Val168; Val192
Ferulic acid	-4.94	1.22	-40.05	445858	Lys154; Ile157; Arg184; Arg190; Val192
(E)-Cinnamic acid	-4.56	0.56	-37.56	444539	Lys154; Val168; Arg190
Naringenin	-5.05	2.64	-39.11	439246	Lys154; Ile157; Arg190; Val192
Epicatechin	-5.36	1.51	-7.12	72276	Lys154;Ile157;Val168;Arg184;Glu189; Arg190;Val192
Syringic acid	-5.04	0.92	-9.64	10742	Val192
Tyrosol	-4.46	0.69	-17.74	10393	Ile157; Arg190; Val192
Catechin	-5.29	1.04	-7.18	9064	Lys154; Ile157; Val168; Arg184; Glu189; Arg190; Val192
Protocatechualdehyde	-4.34	1.08	-1.50	8768	Phe153; Lys154; Ile157; Arg190
Vanillic acid	-4.73	1.11	-15.26	8468	Ile157; Gln164; Arg190; Val192
Quinic acid	-3.90	0.74	15.55	6508	Asn158; Tyr162
Gallic acid	-4.52	2.08	-32.22	370	Gln164; Val168; Arg184; Arg190
p-Hydroxybenzoic acid	-4.23	0.98	-26.03	135	Ile157; Gln164; Arg190; Val192
Kaempferol-3- glucoside	-6.03	2.26	80.13	5282102	Asn161; Gln164
Rutin	-7.40	0.89	136.05	5280805	Lys154; Ile157; Asn158; Tyr162; Gln164; Arg190
hespiridin	-9.54	2.23	193.37	10621	Pro165; Arg184; Asp188; Glu189; Arg190

Statistical analysis

All experiments were performed in triplicate and results were expressed as the mean values ± standard deviations. Statistical analyses were carried out using SPSS software for Windows, version 20.0 (Statistical Package for the Social Sciences, The Predictive Analytics Company, Chicago, IL, USA). The analysis of variance (ANOVA) was performed using Duncan test to determine significant differences between samples ($p \leq 0.05$).

Results and discussion

Determination of MIC and MBC of traditional homemade PPV

The antimicrobial activity of PPV sample was investigated against Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *S. enetrica*) bacterial strains by the determination of both MIC and MBC values. MIC was defined as the lowest concentration of antimicrobial agent that yielded in no visible growth and MBC as the lowest concentration that will prevent the growth of an organism after subculture onto antibiotic-free media (Chemsa et al. 2018).

Results presented in Table 3 showed that PPV sample exhibited growth-inhibitory effect for all tested bacterial strains at MIC concentrations (v/v), of (3.12%) for *E. coli* and *S. enetrica* being more resistant than *S. aureus* (1.56%). Higher resistance of Gram-negative bacteria compared to Gram positive ones is mainly due to their cell wall structure. In fact, Gram-negative bacteria have an effective permeability barrier, formed by a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the studied antimicrobial agent. MBCs of PPV were determined as 6.25, 12.5, and 12.5% (v/v), for *S. aureus, E. coli*, and *S. enetrica*, respectively. To the best of our knowledge, MIC and MBC of vinegar from prickly pear fruits were not yet described in the literature. Sengun et al.(2020] found MIC values of a variety of fruit vinegars ranging from 6.25 to 12.5 % (v/v) for *S. aureus* and from 3.12 to 12.5 % (v/v) for *E.coli*. On the other hand, our results indicate the high potential of using traditional PPV as a natural antimicrobial agent mainly due to its low pH (3.44) and also to its high content in phenolic compounds (1005.59 mg GAE/L). Indeed, it was demonstrated that the presence of phenolic compounds in vinegar samples is not only responsible for its antioxidant activity, but also enhances its antimicrobial activity (Bakir et al. 2017). Campos et al. (2016) reported that phenolic compounds inhibit bacterial growth either through permeabilization or disruption of the cell membrane, or through the inhibition of the extracellular microbial enzymes.

Table 3. Minimal inhibitory and bactericidal concentrations of prickly pear vinegar against *S. aureus*, *E. coli* and *S. enterica*

Bacterial strains	**Prickly pear vinegar**	
	MIC (%, v/v)	**MBC (%, v/v)**
E. coli	3.12 ± 0.00	12.50 ± 0.00
S. enterica	3.12 ± 0.00	12.50 ± 0.00
S. aureus	1.56 ± 0.00	6.25 ± 0.00

Data were expressed as mean ± standard deviation (n = 3).

Inhibitive effect of PPV on biofilms

Data regarding the effect of vinegar in general and PPV particularly in planktonic and biofilm cultures is scarcely described in literature. The anti-biofilm activity of traditional PPV was assessed at its MIC and 2×MIC concentrations, on biofilm cultures (inhibition of initial cell attachment and effect on mature biofilms) of *S. aureus, E. coli* and *S. enetrica*.

3.2.1. Inhibition of initial cell attachment

Numerous indirect approaches were tested in order to quantify the bacterial adhesion such as biomass determination, cultivability, viability, metabolic activity, or other cell properties, most of these were determined in micro-titer plates. According to the targeted parameters, these different methods will provide different data (Klančnik et al. 2020).

In this study, the crystal violet assay, one of the most commonly-used method in the determination of the anti-adhesion and anti-biofilm activities, was performed. In fact, crystal violet has been used as an indicator of attached biomass in a biofilm and stains both viable and non-viable cells that may be attached (Kouidhi et al. 2010). As it can be seen, in Fig.1. the effect of traditional PPV on initial cell attachment was dose dependent ($p < 0.05$). Indeed, the inhibition percentages of the tested biofilm forming bacteria were recorded as 64.05, 40.03, and 63.30% occurred at MIC levels and as 68.33, 54.15, and 72.19% when treated with 2×MIC concentration for *S. aureus, E. coli,* and *S. enterica* strains, respectively. However, complete inhibition of initial cell attachment was not achieved even using 2×MIC concentration of PPV.

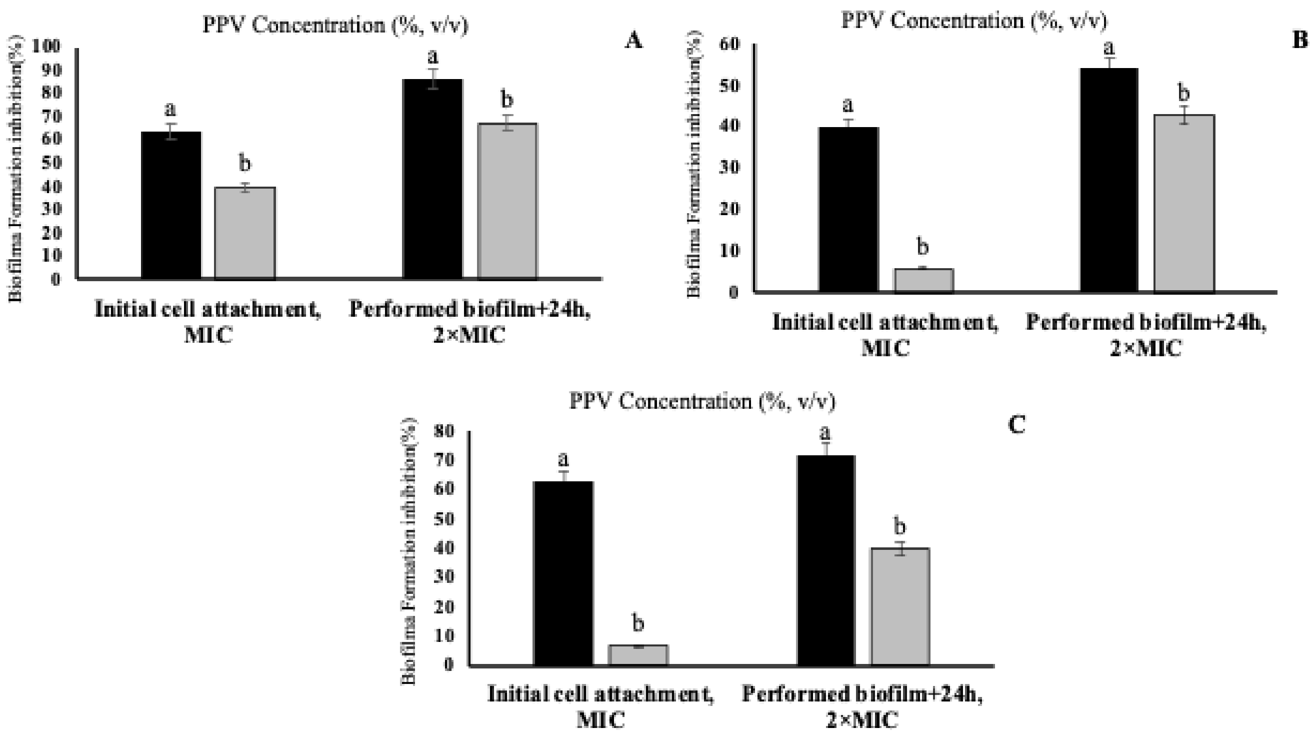

Fig.1: Effect of traditional PPV on biofilm formation (expressed as inhibition percentage). (■):Initial cell attachment inhibition, and (■): effect on 24-h preformed biofilms of: (A) *S. aureus*, (B) *E. coli*, and (C) *S. enetrica*. Different letters on data bars indicate a significant difference according to Duncan's multiple range test ($p< 0.05$).

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Effect of PPV on preformed biofilms

When MIC and 2×MIC values of traditional PPV were tested against 24-h preformed biofilms, the results demonstrated that PPV eradicated established biofilms of *S. aureus, E. coli,* and *S. enterica* (Fig. 1). In fact, the highest biofilm eradication percentage (86.33%) of *S. aureus* was observed at 2×MIC of PPV, whereas this latter showed a lower biofilm eradication capacity against *E. coli,* and *S. enterica* strains with eradication percentages of 42.69, and 39.94% obtained at 2×MIC, respectively. Herein, our findings emphasize again the greater resistance of biofilm forming sessile cells compared to planktonic cells since PPV inhibitory effect was greatly reduced compared to initial cell attachment. This was expected when compared to planktonic growth inhibition percentages since it is known that biofilms are more resistant to antimicrobial agents than planktonic cells, due to various factors such as (*i*) the presence of negatively charged, anionic, and extracellular polysaccharide serving as physical barriers for the diffusion of antimicrobials into the cells organized in biofilms (Javdha et al. 2013), and (*ii*) most of the antimicrobial compounds are more effective against actively growing cells than attached cells in biofilms exhibiting poor growth rate due to the lack of nutrients and oxygen (Sandasi et al. 2010).

Effect of traditional PPV on metabolic activity of bacterial pathogens

Regarding the effect of PPV on the metabolic activity, MTT assay was used as an indicator of attached viable cells. Results of the MTT assay confirm that PPV significantly inhibited the metabolic activity of biofilms formed by *S. aureus, E. coli,* and *S. enterica* ($p < 0.05$) at both MIC and 2×MIC values (Fig. 2).

Indeed, the addition of PPV to prevent the initial cell attachment did not only reduce the biomass (as indicated by crystal violet assay) but also decreased the cell metabolic activity, which resulted in 65.88, 45.84, and 54.96% inhibition, respectively, for *S. aureus, E. coli,* and *S. enterica*, when treated at MIC value. These values were increased up to 72.10, 52.92, and 56.96% at 2×MIC of PPV. However, in the case of preformed biofilms, the inhibition percentages decreased in comparison with those of initial cell attachment. This inhibitive effect could be due to the richness of PPV in phenolic compounds. These compounds are protocatchuic, syringic, *p*-coumaric, and transfrulic acids (Ben hammouda et al. 2021). In this regard, Chen et al. (2020) demonstrated that *p*-coumaric acid has an inhibitory effect on quorum sensing and thus enhancing meat preservation by preventing biofilm formation.

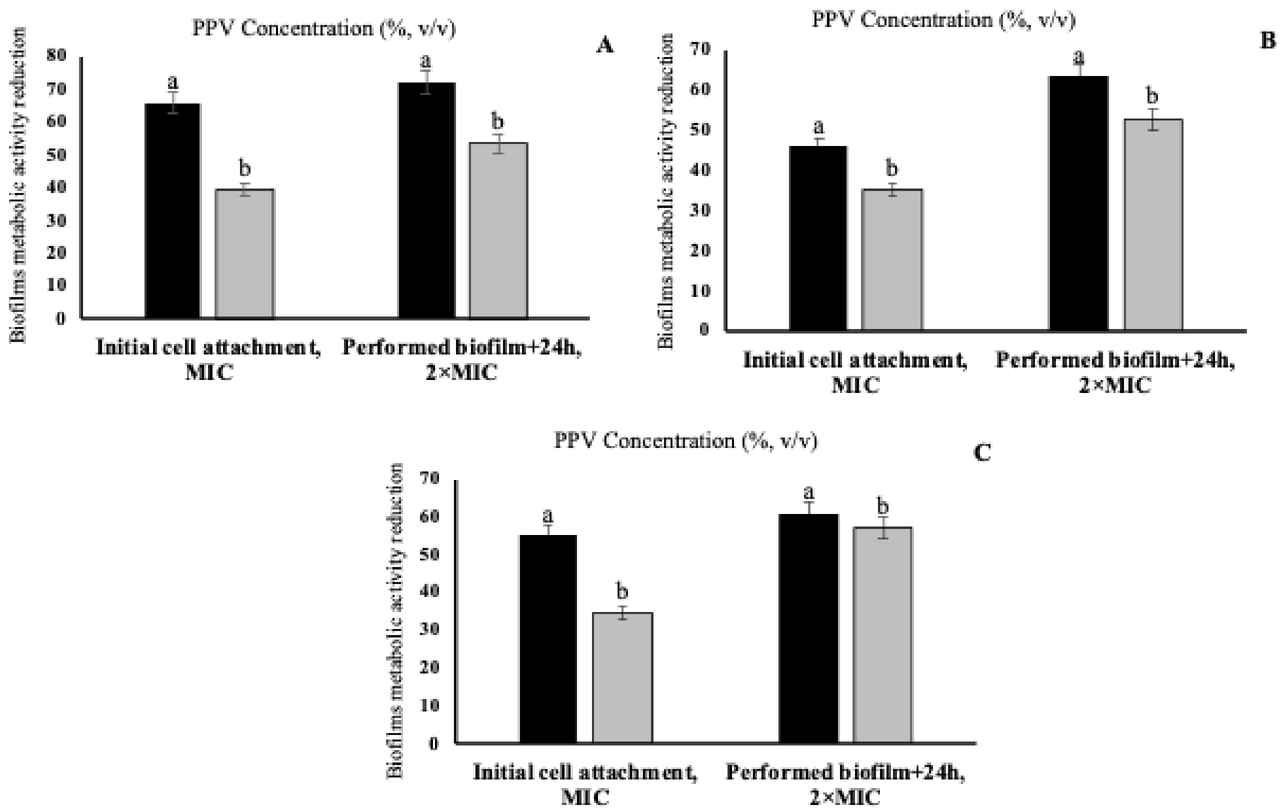

Fig. 2: Effect of traditional PPV on the metabolic activity of: (A) *S. aureus*, (B) *E. coli*, and (C) *S. enterica*.(■): Co-incubated with PPV sample (inhibition of initial cell attachment), (■): effect on 24-h preformed biofilms. Different letters on data bars indicate asignificant difference according to Duncan's multiple range test (p< 0.05).

Effect of PPV on the inhibition of biofilms formed on glass and stainless-steel abiotic surfaces

Since the effect of traditional PPV resulted in a significant inhibition of initial cell attachment on polystyrene surface for all tested isolates, further experiment was performed to investigate its effect on two additional abiotic surfaces commonly used in food industries viz., glass (G) and stainless steel (SS) surfaces. Results presented in Fig.3 and Fig.4 indicate that PPV was successfully able to reduce both the initial cell attachment and the 24-h preformed biofilms of *S. aureus, E. coli,* and *S. enterica* formed in either SS or GS surfaces. Additionally, as it can be seen in Fig.3 and Fig.4, the inhibition of *S. aureus, E. coli,* and *S. enterica* biofilms formation was highest at 2×MIC concentration, which confirm that the effect of PPV on bacterial biofilms varies in a dose dependent manner. Noticeably, the effect of PPV was more efficient in inhibiting initial cell attachment than on eradicating preformed biofilms, which could be explained by the fact that once established, biofilms are very difficult to eradicate (Hathroubi et al. 2017). Srey et al. (2013) indicated that the formation of a biofilm follows mainly two distinct phases: an initial reversible (weak) attachment phase mediated by weak long-range forces of attraction, followed by an irreversible (strong) attachment phase resulting from covalent and hydrogen bonds that create strong short-range forces. Consequently, inhibiting the first step of biofilm formation (initial cell attachment) is easier than disrupting preformed biofilms. The effect of PPV was more prominent on GS surface as it led to a greater logarithmic reduction in the attached cell number compared to the SS coupons. This could be explained by the fact that bacterial attachment to a surface and the biofilm formation are influenced by surface topography (Cheng et al. 2019). Overall, PPV treatment was effective to prevent and, in some case, to remove biofilms formed on GS and SS surfaces. This inhibitive effect could be mainly due to its richness in both phenolic and volatile compounds, namely acetic acid, major volatile compound in vinegar samples. In this context, Akbas & Cag (2016) demonstrated that organic acids such as citric, malic, and gallic acids, were able to prevent and remove *Bacillus subtilis* biofilms on food contact surfaces. Moreover, they showed that citric acid treatments were as powerful as chlorine treatments for prevention and removal of biofilms.

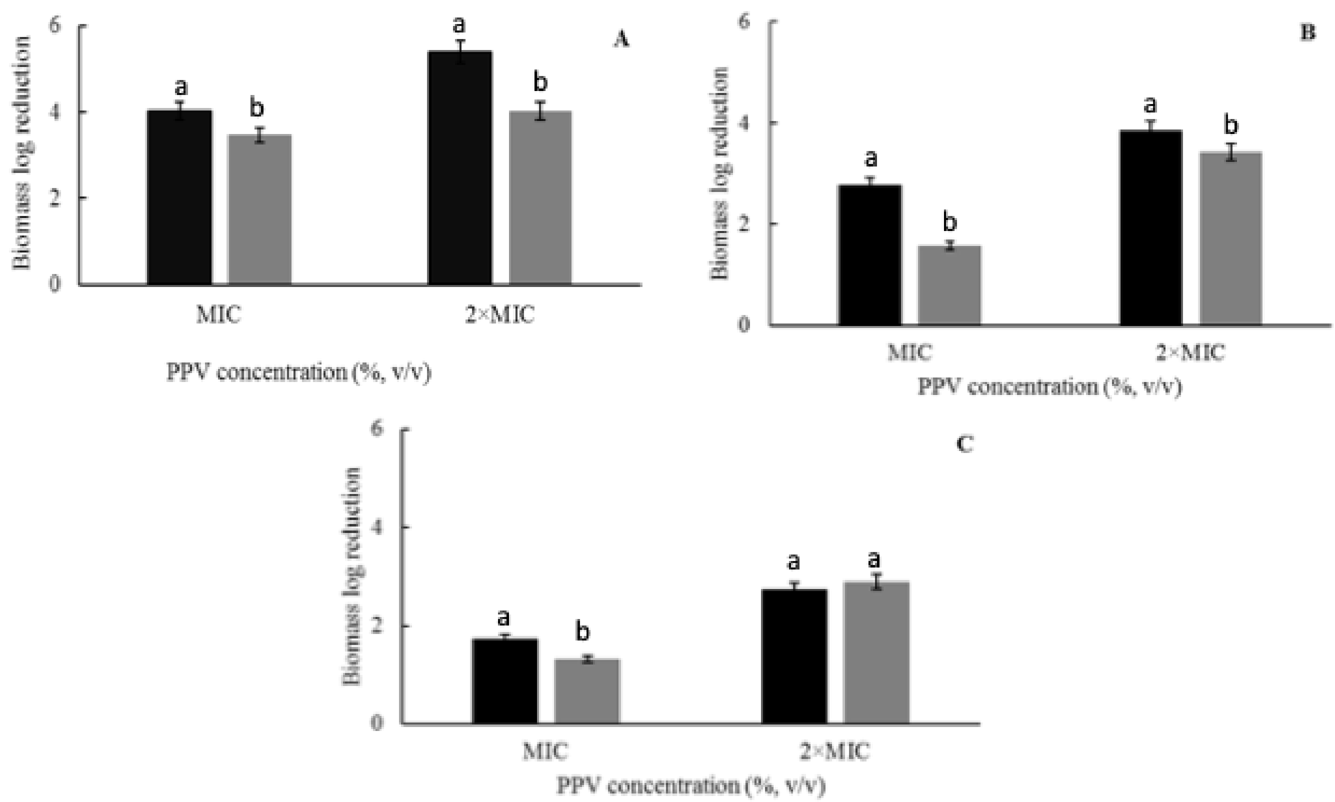

Fig. 3: Effect of PPV on the inhibition of initial cell attachment of biofilm tested strains to (■): stainless steel (SS) and (■): glass (G) surfaces: (A) *S. aureus*, (B) *E. coli*, and (C) *S. enterica*. Data represent the mean of the recovered viable and cultivable cells count (log CFU/cm^2) ± standard deviation (n=3) and different letters on data bars indicate a significant difference according to Duncan's multiple range test (p< 0.05).

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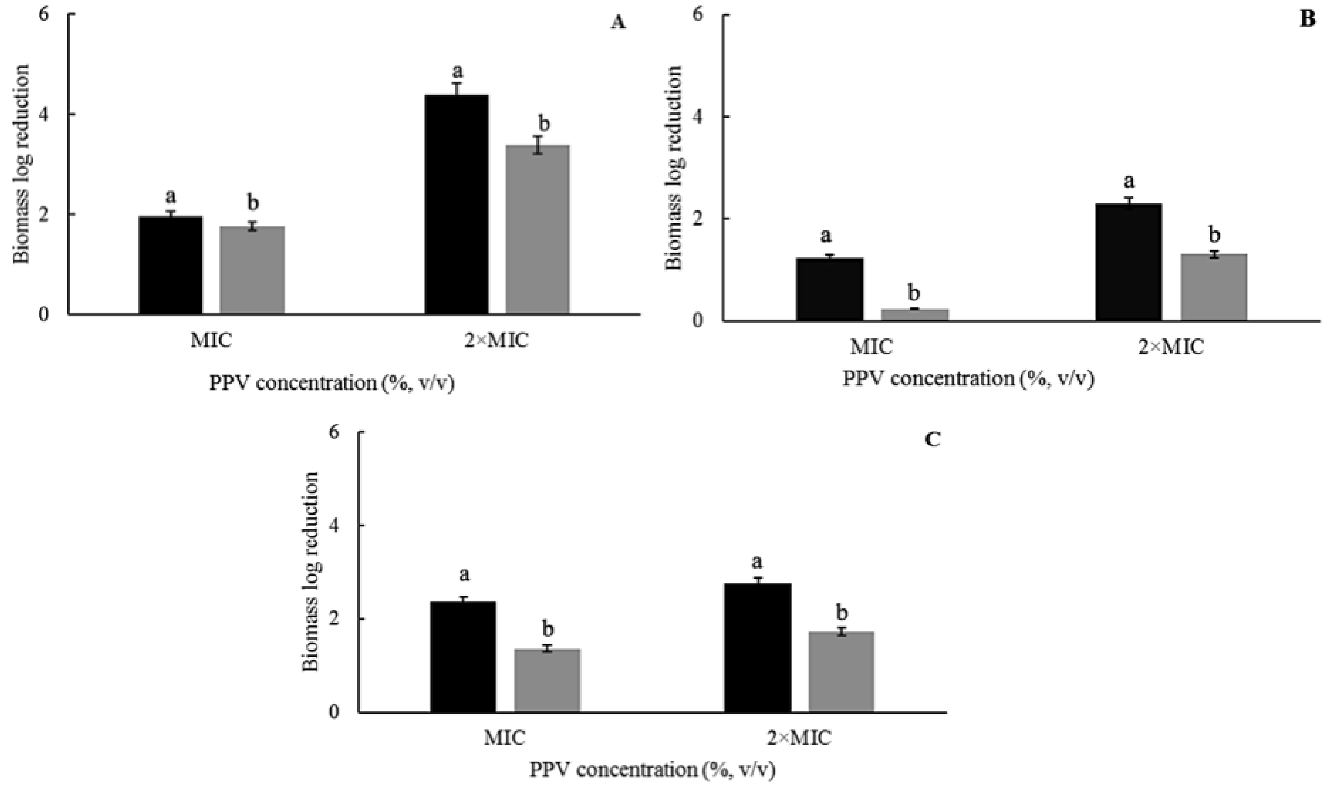

Fig. 4: Effect of traditional PPV on 24-h mature biofilms preformed on (■):SS and (■): glass surfaces: (A) *S. aureus*, (B) *E. coli*, and (C) *S. enterica*. Data represent the mean of the recovered viable and cultivable cells count (log CFU/cm^2) ± standard deviation (n=3) and different letters on data bars indicate a significant difference according to Duncan's multiple range test (p< 0.05).

Microscopic visualization of biofilm formation

Efficacy of traditional PPV, to inhibit the initial cell attachment and to disrupt *S. aureus, E. coli, and S. enterica* biofilms on glass surface (GS), was confirmed using light microscopy after crystal violet staining of bacterial biofilms (Fig.5). Untreated slides (Negative controls) showed a clumping of complex biofilm, whereas a remarkable reduction in biofilms was observed in PPV-treated slides. Moreover, the biomass of biofilm in the presence of traditional PPV were significantly reduced in a dose dependent manner. These photomicrographs suggested that traditional PPV possessed a capacity to reduce the biofilm formation and adhesion, which confirmed the results above. Untreated control biofilms were observed to adhere on the surface of G slides and form thick aggregates, while the biofilms exposed to traditional PPV gradually decreased. Besides, cell suspensions, that were treated with 2×MIC of PPV, showed dispersed colonies of fewer cells and attached loosely on the G slides. However, in the case of 24-h preformed biofilms (Fig. 5: D1 to F3), the number of dispersed colonies was higher in comparison with those obtained in the case of initial cell attachment (Fig. 5: A1 to C3). All these studies clearly indicated that the biofilm formed by *S. aureus* was the most sensitive to treatment with PPV.

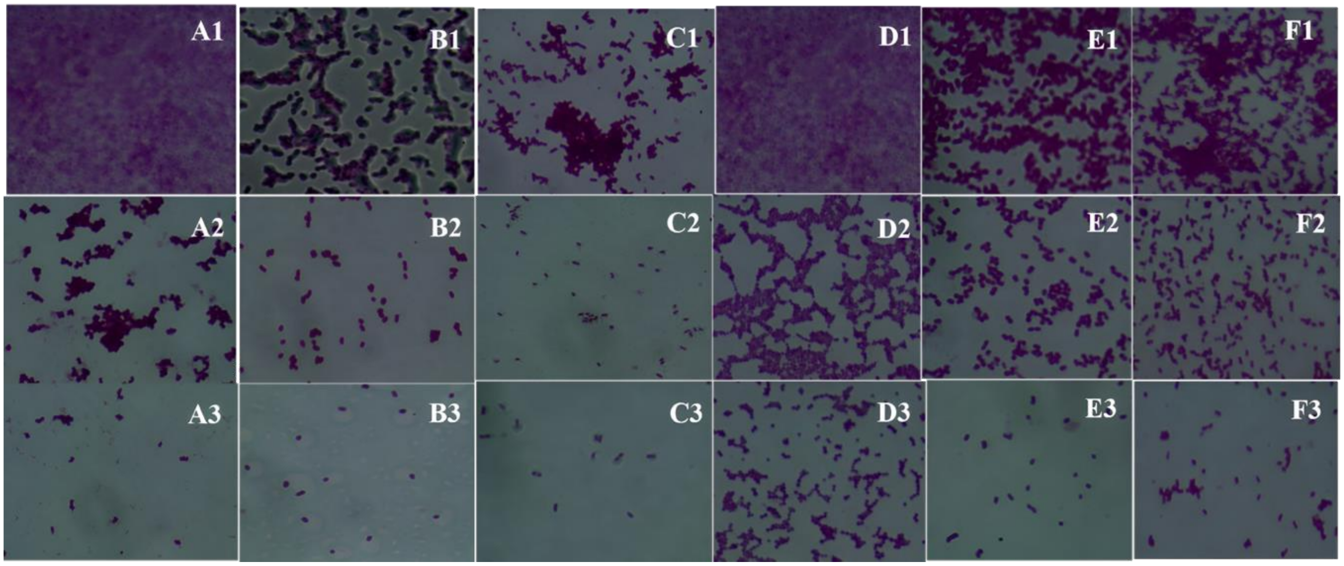

Fig. 5. Light Microscopy assay: Effect of traditional PPV on the inhibition of biofilm formation on glass surface (initial cell attachment and 24-h mature biofilms) was as follows (from A1 to C3: initial cell attachment inhibition; from D1 to F3: inhibition of 24-h preformed biofilms): **A1**:Negative control of *S. aureus* (non-treated slides), **A2**: *S. aureus* supplemented with PPV at MIC, **A3**: *S. aureus* supplemented with PPV at 2×MIC, **B1**: Negative control of *E.coli*, **B2**: *E. coli* supplemented with PPV at MIC, **B3**: *E. coli* supplemented with PPV at 2×MIC, **C1**: Negative control of *S.enterica*, **C2**: *S.enterica* supplemented with PPV at MIC, **C3**: *S.enterica* supplemented with PPV at 2×MIC, **D1**:Negative control of *S. aureus* (non-treated slides), **D2**: *S. aureus* supplemented with PPV at MIC, **D3**: *S. aureus* supplemented with PPV at 2×MIC, **E1**: Negative control of *E.coli*, **E2**: *E. coli* supplemented with PPV at MIC, **E3**: *E. coli* supplemented with PPV at 2×MIC, **F1**: Negative control of *S.enterica*, **F2**: *S.enterica* supplemented with PPV at MIC, **F3**: *S.enterica* supplemented with PPV at 2×MIC.

SarA-dependent antibiofilm efficacy of phenolic compounds from PPV

Results of molecular docking analysis revealed the ability of the phenolic compounds present in PPV to interact with SarA proteins of *Staphylococcus aureus*. Obtained data were compared with the positive control hesperidin (Vijayakumar et al. 2022). Obtained data showed that most of the phenolic compounds interact with the DNA-binding site of the SarA protein, and these interactions block or interfere with the attachment of SarA to DNA. Table 2 lists the binding energies and the different types of bonds for the most significant compounds in the PPV. As it is shown in table 2, *o*-Coumaric acid and rutin presented the greatest interaction energy (-6.47, -7.40, kcal/mol, respectively) comparable to that obtained with positive control hesperidin (-7.92). Furthermore, obtained results revealed that rutin strongly interacts with the active sites of SarA with a binding energy of − 7.40 kcal/mol and exhibited six hydrogen bonding interactions (Lys154; Ile157; Asn158; Tyr162; Gln164; Arg19). These results suggest that the binding of rutin to the target sites of SarA, as a target protein to which phenolic compounds could bind and indirectly inhibit *S. aureus* biofilm formation, could be a suitable mechanism responsible for the inhibition of *S. aureus* biofilm formation. In fact, it has been reported that several phenolic compounds have shown a wide range of biological activities and are being utilized in the healthcare system thanks to their safety and cost-effectiveness (Dehelean et al. 2021). Among these compounds, rutin, a polyphenolic natural flavonoid and known as quercetin-3-O-rutinoside and vitamin P (Negahdari et al. 2021). Moreover, it's worthier mentioning that rutin showed antibacterial and antiboflm properties against a wide range of pathogenic strains, namely *P. aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii,* and *Escherichia coli* (Negahdari et al. 2021). It was reported that the mechanism of action of rutin in inhibiting biofilm formation was either through inhibiting bacterial DNA gyrase, resulting thus in the inhibition of DNA replication, or through preventing cytoplasmic membrane function and so inhibiting bacterial energy metabolism (Negahdari et al. 2021).

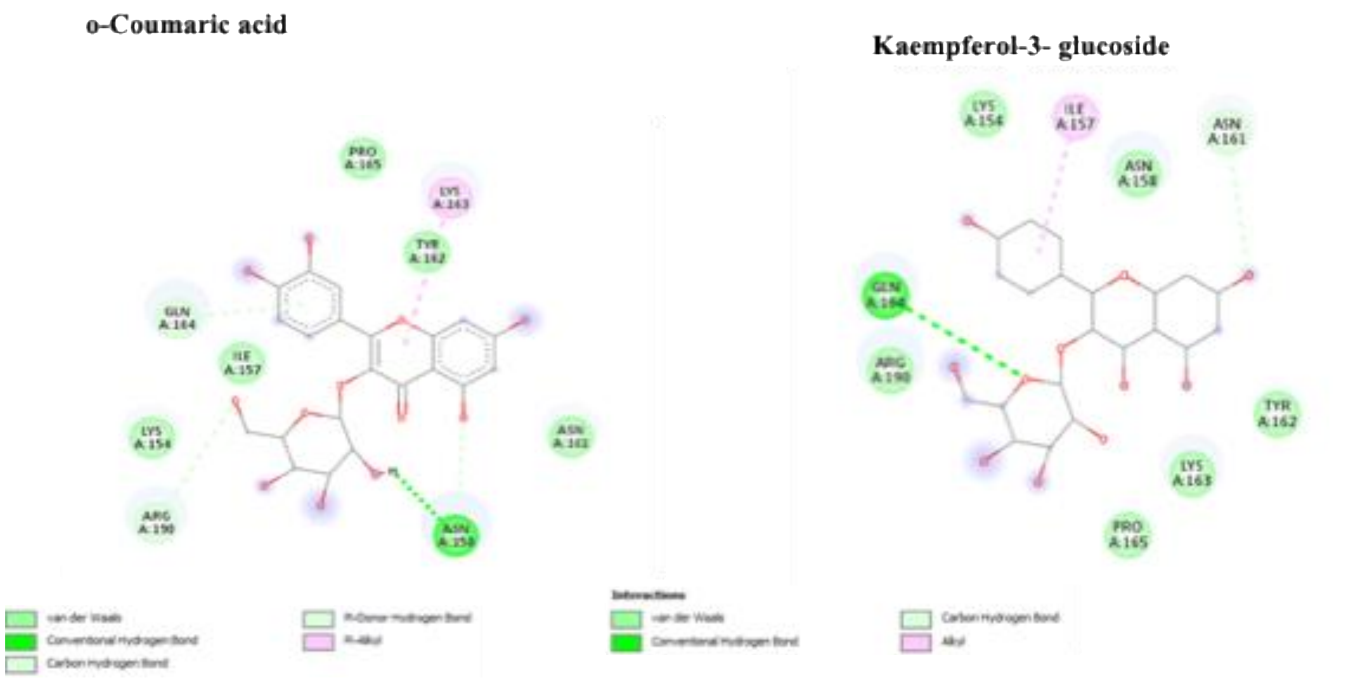

Fig. 6. Molecular docking analysis: two-dimensional (2D) representation of interaction patterns of rutin, Kaempferol-3-glucoside, *o*-Coumaric acid and positive control (Hesperidin) with SarA protein

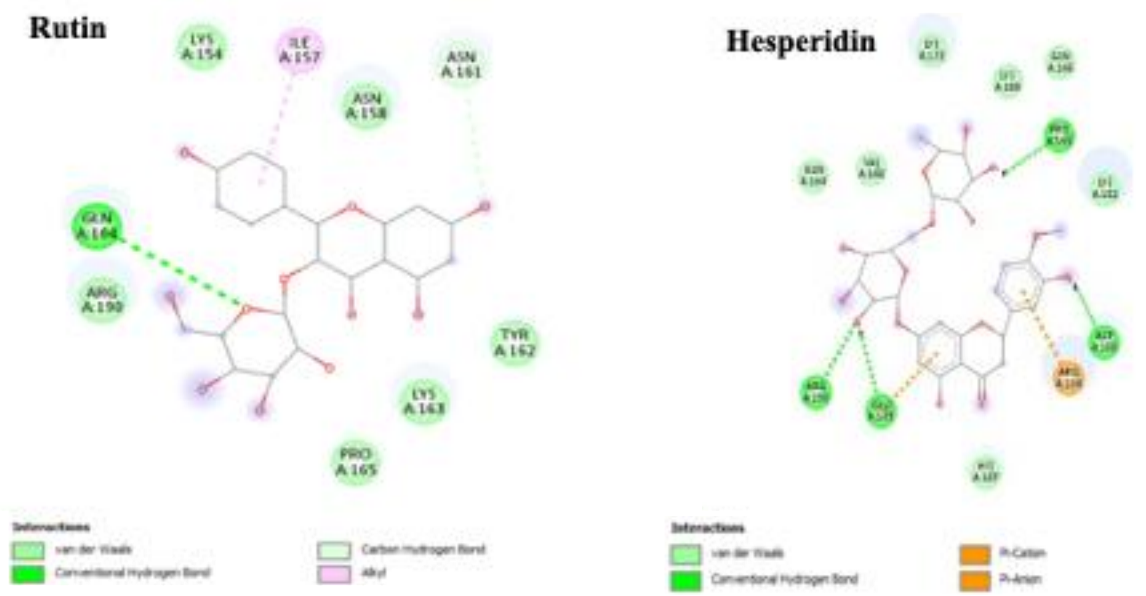

Fig. 6. Molecular docking analysis: two-dimensional (2D) representation of interaction patterns of rutin, Kaempferol-3-glucoside, *o*-Coumaric acid and positive control (Hesperidin) with SarA protein

Conclusion

In the present study, the anti-biofilm activity of traditional homemade PPV against three biofilm-forming strains viz., *S. aureus*, *E. coli*, and *S. enterica* on microtiter plate, glass, and stainless-steel abiotic surfaces have been evaluated for the first time. Overall, homemade PPV showed an interesting antimicrobial and anti-biofilm activity against the tested bacterial strains. Indeed, PPV significantly inhibited the initial cell attachment of the tested cells on polystyrene, stainless steel and glass surfaces but was less inhibitory towards 24-h preformed biofilms. Moreover, the metabolic activity of *S. aureus*, *E. coli*, and *S. enterica* biofilms considerably decreased after incubation with PPV. Microscopic analysis on glass surface confirmed PPV inhibitive effect, where a reduction in the dispersed cells was observed. Finally, *in silico* analysis depicted a high binding affinity of phenolic compounds for biofilm and quorum-sensing associated proteins in *S. aureus*, with rutin showing the strongest affinity for SarA (- 7.40 kcal/mol).

To sum up, this study demonstrates that homemade PPV has the potential to be applied as a natural antimicrobial and anti-biofilm agent that could be used to inhibit biofilm formation in concerned sectors such as food industries to reduce the risk of some microbial foodborne diseases.

Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by **Mouna BEN HAMMOUDA.** The molecular docking study has been performed by **Pr. Sami MNIF and Fares ELGHALI.** The first draft of the manuscript was written by **Mouna BEN HAMMOUDA.** The manuscript was critically revised by **Dr. Samia AZABOU**, **Dr. Mariam SIALA** and **Pr. Sami MNIF.** The study conception and design was performed by **Dr. S. AZABOU** and **Pr. Hamadi ATTIA,** and he approved the version to be published.

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