Effect of Spices on Biofilm Forming Capacity of Bacteria

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Abstract—Spices, which are plant substances used to enhance flavor are at the same time, the most commonly used natural antimicrobial agents in food. Besides this they have shown to effect the biofilm forming capacity of bacteria at different concentrations. In our study we tested the antibacterial effect of different w/V solutions of commercially available spices: cinnamon, curcuma and ginger and investigated their effect on biofilm formation of Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 19433. The results of our study indicate that cinnamon had an antibacterial effect on gram positive bacteria, ginger only on E.coli and curcuma did not exhibit any antibacterial properties. Results of the effect of different w/V solutions of spices on biofilm formation of the tested bacteria indicate that the spices had different effects on the tested bacteria and that the applied spice w/V solution did modify biofilm formation of bacteria. Hereby it is evident that the finding of novel antimicrobial compounds should be accompanied by biofilm formation studies since biofilms represent the natural state of bacteria and as such must be taken into consideration.

Keywords—Spices, Antimicrobial Effect, Antibiofilm Effect

I. INTRODUCTION

Biofilms are sessile communities of micro-organisms where microbes are embedded in an extracellular polymeric substance [1] and as such show elevated resistance rates to antibiotics and antimicrobials [2, 3, 4]. Nearly all (99.9%) micro-organisms have the ability to form biofilms on a wide range of biological and nonbiological surfaces [1]. Due to their elevated resistance rates towards antimicrobials [2, 3, 4] and the fact that antibiotics can act as signaling molecules and effect biofilm formation [6,7,8,9], today biofilms represent a major problem in public health, medicine, industry and everyday life [3, 4].

Due to the inducing effect some antibiotics have on biofilm formation, many studies were directed towards investigating the antimicrobial and antibiofilm effect of different natural substances [10, 11, 12, 13, 14] to solve this issue. The existence of many small molecules within natural substances makes them good candidates as quorum quenching molecules in biofilm studies.

Since biofilms have a profound influence in the food industry [15, 16, 17] the effect of commonly used spices on biofilm forming capacity of microbes is of great significance. Spices, which are plant substances used to enhance flavor [18] are at the same time, the most commonly used natural antimicrobial agents in food [5].

Ginger, a famous spice used in the regular diet in many Asian countries, consists of more than 400 different compounds. Many studies confirmed its antimicrobial and antibiofilm properties [19, 20, 21, 22].

Cinnamon, a spice commonly used in culinary and a health promoting agent for many diseases [23, 24, 25] has also been confirmed as a antimicrobial and antibiofilm agent [26, 27, 28].

Curcumin, the major constituent of Curcuma longa L. possess a broad range of pharmacological properties including antimicrobial, anti-diabetic, anti-inflammatory, anticancer, and antioxidant effect [29, 30, 31]. Its antibiofilms forming effect was also confirmed on some bacteria [30] while in the case of K.pneumoniae it had no antibiofilm effect [32].

The main aim of our study was to test the antimicrobial effect of three commonly used commercially available spices and the effect these spices have on biofilm formation of referent strains of the following pathogenic microorganisms found in food: Staphylococcus aureus, Escherichia coli and Enterococcus faecalis.

II. MATERIALS AND METHODS

A. Bacterial Strains

The tested organisms included cultures of: Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 19433 in Lauria Bertani (LB) Broth supplemented with 50% glycerol and kept at -80ºC. These bacterial strains were recovered from glycerol stocks by plating on Blood Agar Base and incubated overnight at 37ºC followed by overnight incubation in Tryptic soy broth (TSB) supplemented with 1% glucose.

B. Source of Spices

The used spices included commercially available Ginger, Cinnamon and Curcumin. The used spices were aseptically diluted in TSB supplemented with 1% glucose in the following (w/V) solutions: 75%, 50%, 25%.

The 25% (w/V) solutions were further used for the creation of the following serial dilutions: 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.195%, 0.098%.

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C. Antibacterial Testing
The antibacterial testing of the tested spices (ginger, cinnamon, curcumin) was performed using agar well method on Mueller Hinton Agar for the following (w/v) solutions: 75%, 50% and 25%. Zones of inhibition after overnight incubation were recorded in mm.

D. Biofilm Inhibition Efficiency of Spices
The effect of ginger, cinnamon and curcumin were tested to determine their effect on biofilm forming capacity of the tested bacteria. Different w/V solutions of the tested spices were added to TSB in 96 well plates inoculated with the desired bacterial suspension to a density of McFarland 0.5 where the absorbance was quantified spectrophotometrically at 595 nm. After 48h incubation at 37°C the plates were stained with 0.1% crystal violet.

Uninoculated media was used as negative control and bacteria in TSB as positive control.

E. Examination of Biofilm Forming Capacity
The biofilm formation was classified as strong, moderate, weak or non-adherent according to predefined formulas using the cut-off OD (ODc) (Table I).

TABLE I. 
CUT OFF OD VALUES (AT 595 NM) AND CLASSIFICATION OF BACTERIAL ADHERENCE BY SPECTROPHOTOMETRIC ASSAY.

| Formula          | Biofilm Formation |
|------------------|-------------------|
| ODc ≤ ODc        | Not adherent      |
| ODc < ODc ≤ 2 x ODc | Weak              |
| 2 x ODc < ODc ≤ 4 x ODc | Moderate       |
| 4 x ODc < ODc      | Strong            |

F. Statistical Analysis
All experiments were carried out in triplicates and the data were obtained by ANOVA. The statistical analysis was carried out in IBM SPSS.

III. RESULTS

A. Antibacterial Effect of Different Spices w/V solutions on Bacteria
The results of the effect of different spice w/V solutions on the three tested referent bacterial strains is shown in Table 2. Cinnamon had an antibacterial effect on *E. faecalis* and *S.aureus*, where the highest zone of inhibition for *E. faecalis* and *S.aureus* was recorded at the 50% w/V solution. Ginger had an antibacterial effect only on gram negative *E. coli*, while no zones of inhibition were recorded for *E. faecalis* and *S.aureus*. The highest zone of inhibition around *E. coli* appeared around the ginger 75% w/V solution. Curcuma did not have any antibacterial effect on all the tested bacterial strains.

TABLE II. EFFECT OF DIFFERENT SPICE W/V SOLUTIONS ON TESTED REFERENT BACTERIAL STRAINS

| Spices       | E.coli Zone of Inhibition | E.faecalis Zone of Inhibition | S.aureus Zone of Inhibition |
|--------------|---------------------------|------------------------------|----------------------------|
| Cinnamon     | 0,21 mm                   | 2,0 mm                       | 1,7 mm                     |
| Ginger       | 0,2 mm                    | 0,0 mm                       | 0,0 mm                     |
| Curcuma      | 0,0 mm                    | 0,0 mm                       | 0,0 mm                     |

Since the 25% w/V solution for all the tested spices was not inhibitory for bacterial growth, serial dilutions for further testing were made from it.

B. Effect of Different Spicy w/V solutions on Biofilm Forming Capacity of Bacteria

1) Effect of Different w/V solutions of Cinnamon on Biofilm formation of *E.coli, E.faecalis* and *S.aureus*

The calculated ODc for cinnamon in the case of *E.coli* was 0,0929, for *E.faecalis* 0,0853, and for *S.aureus* 0,0867. For *E.coli* a change in biofilm category, compared to the positive control, was registered at the 25%, 12,5% and 6,25 w/V cinnamon solution. In the case of *E. faecalis*, a change in category occurred at the following w/V cinnamon solutions: from 25% to 1,56%. In the case of *S. aureus* w/V cinnamon solution caused changes in biofilm formation ranging in category from weak to strong compared to the positive control (Table III).

TABLE III. EFFECT OF DIFFERENT W/V SOLUTIONS OF CINNAMON ON BIOFILM FORMATION OF *E.COLI, E.FAECALIS* AND *S.AUREUS*.

| OD% Solution | *E. coli* Mean OD | *E. faecalis* Mean OD | *S. aureus* Mean OD |
|--------------|-------------------|----------------------|---------------------|
| (+) Control  | 0,0640            | 0,0593               | 0,0778              |
| (+) Control  | 0,0698            | 0,0665               | 0,3175              |
| Only space   | 0,0745            | 0,0745               | 0,0908              |
| 0,098%       | 0,0658            | 0,0808               | 0,1035              |
| 0,195%       | 0,0660            | 0,0795               | 0,2720              |
| 0,390%       | 0,0670            | 0,0808               | 0,2275              |
| 0,780%       | 0,0770            | 0,0783               | 0,1960              |
| 1,560%       | 0,1180            | 0,0878               | 0,1928              |
| 3,125%       | 0,1015            | 0,0950               | 0,3180              |
| 6,25%        | 0,2343            | 0,1135               | 0,5968              |
| 12,5%        | 0,2028            | 0,2663               | 0,4950              |
| 25%          | 0,4150            | 0,2863               | 0,2080              |
2) Effect of Different w/V solutions of Curcuma on Biofilm formation of E.coli, E.faecalis and S.aureus

The calculated ODc for cinnamon in the case of E. coli was 0.0843, for E. faecalis 0.0853 and for S. aureus 0.0920. Curcuma had no effect on biofilm formation in E.coli. A change in biofilm forming category was registered for E. faecalis at the w/V curcuma solution of 6,25%, 1,56%, 0,39% and 0,39%. In the case of S. aureus curcuma had an inhibitory effect on biofilm formation (Table IV).

| Table IV. Effect of Different w/V Solutions of Curcuma on Biofilm Formation of E.coli, E.faecalis and S.aureus |
|---------------------------------------------------------------|
| w/V solution used    | Escherichia coli | Enterococcus faecalis | Staphylococcus aureus |
|                     | Mean Abs. Biofilm | Mean Abs. Biofilm | Mean Abs. Biofilm |
| Min. daily dose      | Non adherent     | Non adherent     | Non adherent |
| (-) Control          | 0.0593           | 0.0593           | 0.0783 |
| (+) Control          | 0.0658           | 0.0650           | 0.3750 Moderate |
| Only spice           | 0.0700           | 0.0685           | 0.1310 Weak |
| 0,098%               | 0.0610           | 0.0675           | 0.1293 Weak |
| 0,195%               | 0.0625           | 0.0693           | 0.1000 Weak |
| 0,390%               | 0.0653           | 0.1870           | 1.178 Weak |
| 0,780%               | 0.0638           | 0.1193           | 0.0933 Weak |
| 1,560%               | 0.0635           | 0.1388           | 0.0920 Weak |
| 3,125%               | 0.0680           | 0.0773           | 0.0918 Non adherent |
| 6,25%                | 0.0627           | 0.1785           | 0.1003 Weak |
| 12,5%                | 0.0660           | 0.0678           | 0.0930 Weak |
| 25%                  | 0.0763           | 0.0730           | 0.1073 Weak |

C. General Comparison of All Results

1) Comparison of the Effect of Different Spices on S.aureus

S. aureus in the presence of ginger expressed the highest biofilm forming capacity at the 0,0975% w/V solution and the lowest biofilm forming capacity was measured at the 0,78% w/V solution. In the presence of cinnamon S. aureus expressed the highest biofilm forming capacity at the 6,25% w/V solution and the lowest biofilm forming capacity was measured at the 0,0975% w/V solution. S. aureus in the presence of curcuma expressed the highest biofilm forming capacity at the 0,0975% w/V solution and the lowest biofilm forming capacity was measured at the 3,125% w/V solution.

Fig. 1. Comparison of the effect of different spices on biofilm formation of S.aureus ATCC 25923

2) Comparison of the Effect of Different Spices on E.coli

D. coli in the presence of ginger expressed the highest biofilm forming capacity at the 25% w/V solution and the lowest biofilm forming capacity was measured at the 12,5% w/V solution. In the presence of cinnamon E. coli expressed the highest biofilm forming capacity at the 25% w/V solution and the lowest biofilm forming capacity was measured at the 0,0975% w/V solution. E. coli in the presence of curcuma expressed the highest biofilm forming capacity at the 25% w/V solution and the lowest biofilm forming capacity was measured at the 0,0975% w/V solution.
E. faecalis in the presence of ginger expressed the highest biofilm forming capacity at the 6.25% w/V solution and the lowest biofilm forming capacity was measured at the 0.195% w/V solution. In the presence of cinnamon E. faecalis expressed the highest biofilm forming capacity at the 25% w/V solution and the lowest biofilm forming capacity was measured at the 0.78% w/V solution. E. faecalis in the presence of curcuma expressed the highest biofilm forming capacity at the 0.390% w/V solution and the lowest biofilm forming capacity was measured at the 0.0975% w/V solution.

1) Comparison of the Effect of Different Spices on E. faecalis

Antibiotic resistance worldwide represents a major clinical and public health issue. The misuse and overuse of antibiotics, which have transformed medicine and saved millions of lives, has led to the emergence of resistant bacteria with the propagation of progeny no longer susceptible to them [34-38]. The fact that 99% have the ability to form biofilms - in which the antibiotic resistance rates are multiple times elevated - only makes this issue more complicated [4]. Another concern is the increasing of antibiotic resistance of pathogens that are the causative agents of food born illnesses [40]. Until now there have been many attempts to find effective and nontoxic antimicrobial and antibiofilm compounds, other than antibiotics, that can be used on a regular basis - as in example in food. A good group of compounds that can respond to all these requirements are herbal spices. Many studies were directed towards testing the antibacterial properties of herbal spices, some also focusing on the antibiofilm forming properties of spices [10-14;18-33] and other substances [34]. However, in small doses molecules within spices can act as signals for quorum sensing in bacteria and affect bacterial biofilm forming capabilities. Considering that spices are multiple times diluted in food - identifying the effect of small doses of commercially available spices on different food pathogens would aid in the adequate choice of spices as well as its dose for food preservation. In our study we tested the antimicrobial effect of different spice (w/v) solutions using agar well method and subsequently serial dilutions of the 25% w/V solutions of spices were tested for their effect on biofilm formation in 96 well plates. This study showed significant variations in biofilm formation of Staphylococcus aureus, Escherichia coli and Enterococcus faecalis in the presence of different spices and their w/V solution (cinnamon, curcumin and ginger). Results of other studies showed that the effect the tested substances had on biofilm formation in different concentrations was dose dependent [9], which was in accordance with our results.

Namely, biofilm formation of E. coli, where the bacteria expressed no biofilm formation in positive control, in the presence of cinnamon increased and ranged from non adherent at the 3,125% w/V solution to moderate at the 6.25% w/V solution to strong at the 15% w/V solution. Similar results were obtained for Enterococcus faecalis incubated with cinnamon w/V solutions where at the 25% and 12.5% w/V solution biofilm formation was moderate as opposed to the positive control where no biofilm formation was registered. In the case of Staphylococcus aureus significant changes in the biofilm forming category were registered using different cinnamon w/V solutions (weak biofilm formation at the 0.098% w/V solution and strong biofilm formation at the 6,25% w/V solution and 12.5% w/V solution, as opposed to the positive control which had moderate biofilm formation). Unlike that in the case of testing biofilm formation of all the tested bacteria in the presence of curcumin, changes in biofilm forming category were registered but they were not as significant as in the case of cinnamon. Curcumin enhanced biofilm formation in E. faecalis where a change in biofilm forming category was registered (from non adherent in positive control to weak at the 0.39% w/V solution). For E. coli no change in biofilm forming category occurred when comparing positive control and all tested spice concentrations.

On the other hand curcumin reduced the biofilm forming capacity of S. aureus from moderate (positive control) to weak and non adherent and proved to have antibiofilm forming properties on the tested S. aureus strain. The results obtained for curcumin were in accordance with results from another study where curcumin nanoparticles expressed had an antibiofilm forming effect on S.aureus [33]. Similar results were also obtained in the case of ginger. Ginger slightly enhanced biofilm formation in E.faecalis (change in category from non-adherent in positive control to weak to certain spice w/V solutions) and did not have any effect on the biofilm formation of E.coli. Unlike that in the case of S. aureus ginger significantly reduced the biofilm forming capacity from moderate to weak and non adherent at higher
spice w/V solutions. This was in accordance with the results of other studies where ginger expressed significant antibiofilm forming effect on bacteria [20, 21, 22]. The difference on the biofilm forming capability effect of different spices on the tested bacteria, (where certain spices enhanced, certain inhibited while others had no effect on the biofilm formation of tested bacteria) could potentially be explained by the difference in the spice molecular composition. Namely, recent literature even suggests adding small molecules into the central dogma of molecular biology because of the profound effect they have on gene expression [41]. The fact that different genes and gene clusters are responsible for biofilm formation in Escherichia coli, Staphylococcus aureus and Enterococcus faecalis could explain the fact that different spices have different effect on the tested bacteria.

On the other hand, the different effect the same spice has on the biofilm forming capacity of the same bacteria could be explained by the fact that biofilm formation is controlled by quorum sensing and is population density dependent. Since bacteria communicate through a chemical language that is registered by the amount of certain signaling molecules in their surrounding the effect of different spice concentrations could, through quorum sensing, alter collective group behavior including biofilm formation. From this it is apparent that the testing the effect not only of different spices but also their concentration on the biofilm formation of bacteria is crucial for further contribution to the field.

The limitation of our study was the number of the tested bacterial strains, as clinical strains that have a more resistant profile could yield more applicable results compared to the results obtained from referent strains. Statistical analysis was performed in IBM SPSS and values obtained with ANOVA test - which both showed no correlation between the applied spice w/V solution and biofilm formation. This is in accordance with literature findings and supports the hypothesis that different concentrations of the same substance have a different effect on biofilm formation in bacteria [9].

On the whole, this study has opened a novel frontier and has showed that despite the fact that certain compounds can act as microbicidal substances - once diluted, they can have a profound effect on the expression of genes responsible for biofilm formation. Hence, the finding of novel antimicrobial compounds should be accompanied by biofilm formation studies since biofilms represent the natural state of bacteria and as such must be dealt with.

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