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Effect of traditional food preservatives on biofilm formation by food pathogens

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Aims: This study investigated the effect of food preservatives on biofilm formation by food pathogens *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.*
Methodology and results: Foodborne pathogens isolated from spices mix were analyzed for their resistance and biofilm formation in presence of certain traditional food preservatives. Sensitivity of pathogens against traditional preservative were tested by agar well diffusion method and agar dilution method. Biofilms growth were measured by crystal violet staining and distaining followed by absorbance at 563 nm. Biofilms were observed under electron microscope. *P. aeruginosa* was found to grow in Brain Heart Infusion broth supplemented with 7% ginger; similarly, *E. coli* and *S. aureus* also exhibited resistance to high level of garlic and sodium chloride, respectively. Although these pathogens belong to different classes e.g. *S. aureus* is a Gram-positive whereas *E. coli* and *P. aeruginosa* are Gram-negative. However, they employed similar strategy to nullify the toxic effect of food preservatives. During survival, these pathogens were found to change their living pattern from planktonic to biofilm mode of growth.
Conclusion, significance and impact study: This study revealed that presence of resistant pathogens in food could nullify the impact of traditional food preservatives. The biofilm formation by pathogens could be potential hazard in traditionally preserved foods.

Keywords: Food preservative, biofilm, food pathogens

INTRODUCTION

Biofilm is a surface-associated community of microbes penetrated within a matrix of extracellular polymeric materials. Free-floating bacterial cells endure a sundry of phenotypic and genotypic transformations during biofilm generation, including reversible adhesion, irreversible attachment, maturation, and detachment (Dewanti and Wong, 1995). Despite from planktonic bacterial cells, sessile bacterial cells have great adoptive ability in unfavorable challenging conditions e.g. chemicals used for disinfections and antimicrobial compounds (Anaissie *et al.*, 1995; Costerton *et al.*, 1995; James *et al.*, 1995; Jass *et al.*, 1995; Kumar and Anand, 1998). It has been reported that inside the food manufacturing and processing units, foodborne pathogens, in the form of biofilm, are mostly accountable for infectivity of the utensils and cross contamination, directing to food quality retrogression and food-borne illnesses (Chmielewski and Frank, 2004; Latorre *et al.*, 2010). Therefore, a diverse range of preservatives has been used to control the bacterial growth and maintain the quality thus increasing the shelf life of food products. In Asian countries, natural preservatives e.g. ginger, garlic and different salts are very popular for long-term preservation of food products. The uses of traditional food preservatives were preferred by manufacturer due to consumers' demands for organic food products without any artificial treatments; these ingredients not only fulfil the requirement of preservation but also provide unique taste and flavor. A salted product provides desire flavor, color, texture and evidently prolonged shelf life. High salt concentration decreases the water activity (a_w) of the product together with temperature and pH. These are the major reason of bacterial cell death in salted food products. The harmful effect of salt concentration is due the fact that a low a_w effect on turgor pressure in a cell intracellular a_w and the a_w in the surrounding medium. A process known as plasmolysis, describes how hyperosmotic shock causes an instantaneous efflux of water, accompanied by a decrease in the cytoplasmatic volume of the cell, which leads to the cease of cell process and ultimate cell death. The active ingredient in garlic is called allicin, volatile oil-containing sulphur which is responsible for garlic pungent odor. The antimicrobial activity of garlic is known to be due to allicin. Antimicrobial activity of garlic could be explained by a process by which allicin obstructs certain groups of enzymes such as cysteine proteinases and alcohol

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dehydrogenases. These groups of enzymes are found in a wide variety of infectious organisms such as bacteria, fungi and viruses, which provides a scientific basis for broad-spectrum antimicrobial activity of garlic. Unlike garlic and sodium chloride, the antibacterial action of ginger is poorly defined. Ginger has strong antibacterial properties and it inhibits the colon bacteria. Ginger inhibited the growth of *E. coli*, *Proteus* sp., Staphylococci, Streptococci and *Salmonella* (Gugnani and Ezenwanze, 1985). Hence, ginger should have affect on the growth of *P. aeruginosa*, which is a major foodborne pathogen.

This study was designed to evaluate the survival tactics by mean of biofilm formation by pathogenic strains *P. aeruginosa*, *E. coli* and *S. aureus* in the presence of ginger, garlic and NaCl, respectively. Food pathogens *P. aeruginosa*, *E. coli* and *S. aureus* were isolated from three spice mix containing major ingredients of ginger, garlic and NaCl, respectively. This study will help to understand the growth of pathogenic strains in the presence of traditional food preservatives.

MATERIALS AND METHODS

Strains

All three strains were obtained from analytical centre microbiology of Pakistan Council of Scientific and Industrial Research, which were previously isolated from spices mix. Pure cultures of the isolates were preserved at –80 °C in Mueller Hinton broth supplemented with 20% glycerol.

Sensitivity test by agar diffusion method

Antimicrobial activity of ginger, garlic was tested by agar diffusion method. Plates containing Mueller–Hinton agar were spread with 0.1 mL of the inoculum. Wells (8 mm in diameter) were cut from agar plates with a sterile stainless steel borer. The wells were filled with 1% to 10% of ginger or garlic paste and incubated at 35 °C for 24 h. The zones were measured and the experiment was repeated three times. The concentration showing a clear zone of more than 12 mm was considered to be effective.

Sensitivity by agar dilution method

Stock solution was made by mixing 10 g of ginger or garlic paste in 100 mL (w/v) of sterile water and allowed to stand for 72 h. Different concentrations ranging from 4 µg/mL to 1000 µg/mL from this stock solution were added to Mueller-Hinton broth and inoculated with overnight culture of subject strain, *E. coli* and *P. aeruginosa*, and incubated at 35 °C for 48 h.

Biofilm growth environment

Biofilms growth environment was set as described before (McAuliffe *et al.*, 2006) with few amendments. Overnight in brain heart infusion broth (BHIB, Oxoid) bacterial cells were harvested. Biofilms were allowed to form in 96-well plates and on glass slide. Sterile glass slides were placed perpendicularly in 50 mL flask with 100 mL pre-warmed BHIB supplemented with 2% to 9% NaCl for *S. aureus*, 1% to 7% garlic paste for *E. coli* and 1% to 7% ginger paste for *P. aeruginosa*. Glass slides were inoculated with 100 µL 0.8 McFarland standard suspension of a 24 h old culture. Cultures were allowed to grow on slides for different periods of time (48 h, 72 h) at 37 °C. Ninety six well plates with BHIB in all wells, inoculated with 100 µL 0.8 McFarland standards from a 24 h culture. Plates were allowed to grow bacteria aerobically at 37 °C for different intervals of time up to 72 h.

Biofilm visualization

Crystal violet was used to satin biofilms as illustrate by O'Toole and Kolter (1998) with slight adjustment. The biofilms were washed carefully with phosphate buffered saline (PBS, Sigma) to remove non-adherent bacteria. Biofilms were then stained with 0.5% crystal violet solution for 30 min, washed five times with distilled water and left to dry at room temperature for 30 min. The crystal violet was then solubilized by the addition of 95% ethanol (200 µL). The absorbency was recorded with Spectrophotometer (Nicollet Evolution 300 BB) at 563 nm using 100 µL aliquots of the liberated crystal violet. Each experiment was repeated four times.

Electron microscopy

Scanning electron microscopy of biofilm materials was performed cutting 4 mm sections of glass slides having biofilm material, which were washed thoroughly with phosphate-buffered saline (PBS, Sigma) to remove non-adherent cells. Biofilms were then negatively stained with 0.2% uranyl acetate solution for 1 min. Electron Microscope (JEOL-JEM-1200EX1I) was used to observe the biofilms.

Statistical analysis

All experiments were performed in 3 replicates; results are presented as mean values ± standard deviation of triplicates. The ANOVA was calculated with SPSS 17.0 software (Chicago, IL, USA).

RESULTS

Biofilm formation

All three isolates used were assessed for their ability to form a biofilms on glass during growth in various concentrations of ginger for *P. aeruginosa*, garlic for *E. coli*, and NaCl for *S. aureus*. *P. aeruginosa* showed highest biofilm formation in 7% ginger, *E. coli* produced thickest film on glass slide in 7% garlic paste and *S. aureus* showed stronger biofilms in 9% NaCl in BHIB after 72 h of incubation at 37 °C (Table 1). When the Glass slides were stained with crystal violet, there was a clear air and liquid interface 1–7 mm wide showing biofilm. All

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Table 1: Biofilm quantification in terms of optical density taken after 72 h of incubation at 37 °C

P. aeruginosa		*E. coli*		*S. aureus*	
Ginger (%)	OD of Biofilm (mean ± SD)	Garlic (%)	OD of Biofilm (mean ± SD)	NaCl (%)	OD of Biofilm (mean ± SD)
1.00	0.010 ± 0.001[a]	1.00	0.011 ± 0.001[a]	1.00	0.011 ± 0.001[a]
2.00	0.011 ± 0.001[a]	2.00	0.012 ± 0.003[a]	2.00	0.012 ± 0.002[a]
3.00	0.011 ± 0.001[a]	3.00	0.203 ± 0.025[b]	3.00	0.013 ± 0.003[a]
4.00	0.373 ± 0.025[b]	4.00	0.227 ± 0.025[b]	4.00	0.013 ± 0.003[a]
5.00	0.420 ± 0.020[b]	5.00	0.630 ± 0.036[c]	5.00	0.413 ± 0.038[b]
6.00	0.917 ± 0.076[c]	6.00	0.653 ± 0.021[c]	6.00	0.580 ± 0.036[b]
7.00	1.083 ± 0.076[c]	7.00	0.970 ± 0.020[d]	7.00	0.850 ± 0.036[c]
				8.00	1.113 ± 0.015[d]
				9.00	1.150 ± 0.030[d]

OD Values are mean ± standard deviation of triplicate experiments; Superscript with different alphabets within column indicates significant difference (p<0.01)

the three isolates showed intense staining below the line. This demonstrated that the organism can actually form a biofilm due to the toxic effect of natural food preservatives. This was confirmed by control without addition of any preservative under similar conditions either on glass slide or 96-well plate. Clear indications for biofilm formation were noticed after 48 h of incubation i.e. increase in optical density and ring formation at water and air interface. The ring formation develops into more prominent form after 72 h of incubation (Table 2).

Scanning electron microscopy

All three isolates exhibited similar characters during biofilm formation process, though these were recovered from different food samples and belonged with different genera. After exposure to natural preservatives all three isolates *S. aureus*, *E. coli* and *P. aeruginosa* showed surface roughness. This character was observed after 24 h of incubation at 37 °C before visible biofilms or adhesion on glass slides. The second phenomenal observation was the production of extra-cellular matrix material after 36 h of incubation at 37 °C in the presence of 9% NaCl for *S. aureus* (Figure 1a and d), 7% garlic paste for *E. coli* (Figure 1b) and 7% ginger paste for *P. aeruginosa* (Figure 1c). The maximum extra-cellular matrix material production was noticed after 72 h at 37 °C.

DISCUSSIONS

This study demonstrates the biofilm formation process in selected food-borne pathogens induced by ginger, garlic and sodium chloride in growth medium, and evaluates the effect of cell surface characteristics on biofilm mode of growth as analyzed by scanning electron microscopy. The results demonstrate a clear pattern of the effects of ginger, garlic and NaCl on phenotypic characters of three major isolates *E. coli*, *P. aeruginosa*, and *S. aureus.* These isolates isolated from spices mix and exhibited resistance to certain classical food additives e.g. *P. aeruginosa* was found to grow in BHI broth supplemented with 7% ginger; similarly, *E. coli* and *S. aureus* also exhibited resistance to high level of garlic and sodium chloride, respectively. These pathogens seem to adapt this resistance pattern from environment containing sub-inhibitory concentrations of ginger, garlic and sodium chloride. During the course of survival, these pathogens were found to change their living pattern from planktonic to biofilm mode of growth. Although, all three isolates used in present study belong to different genera, they employed similar strategy to nullify the toxic effect of food preservatives and changed their behavior from planktonic to biofilm mode of growth. It has been described that whether biofilms consist of one or a mixture of a range of species, they display similarities with respect to architectural features and behavioral responses during biofilm mode of growth (Charlton *et al.*, 2000; Hentzer *et al.*, 2002; Webb, 2003; Webb *et al.*, 2003; Labbate *et al.*, 2004; Christensen *et al.*, 2007; Matz *et al.*, 2008) . This was further confirmed by the results of present study, where three different isolates i.e. *E. coli*, *P. aeruginosa* and *S. aureus* were exposed to three different food preservatives of diverse nature and mode of action.

Interestingly, they showed similarities in terms of biofilm architectural features and behavioral responses. The surface properties of a microorganism largely determine its interactions with the environment, other microbes and host organisms. Surface properties also influence organism's pathogenicity, exchange of nutrients and waste products, and resistance to external stresses as caused by mechanical, chemical, thermal, and osmotic

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Table 2: Biofilm quantification in terms of optical density taken after different interval of incubation at 37 °C.

Time (h)	*P. aeruginosa*	*E. coli*	*S. aureus*
	OD of biofilm in the presence of 7% ginger	OD of biofilm in the presence of 7% garlic	OD of biofilm in the presence of 9% NaCl
4	0.010 ±0.0010[a]	0.010 ±0.0010[a]	0.011 ±0.0020[a]
8	0.011 ±0.0015[a]	0.013 ±0.0025[a]	0.012 ±0.0026[a]
12	0.020 ±0.0015[a]	0.021 ±0.0010[a]	0.012 ±0.0021[a]
24	0.072 ±0.0082[b]	0.041 ±0.0036[a]	0.032 ±0.0050[a]
36	0.085 ±0.0050[b]	0.063 ±0.0067[a]	0.035 ±0.0010[a]
48	0.427 ±0.0252[bc]	0.390 ±0.0200[b]	0.480 ±0.0265[b]
60	0.763 ±0.0569[bc]	0.540 ±0.0361[bc]	0.950 ±0.0458[bc]
72	1.113 ±0.0153[c]	0.963 ±0.0306[c]	1.267 ±0.2082[c]

OD values are mean ± standard deviation of triplicate experiments; Superscript with different alphabets within column indicates significant difference (p<0.01).

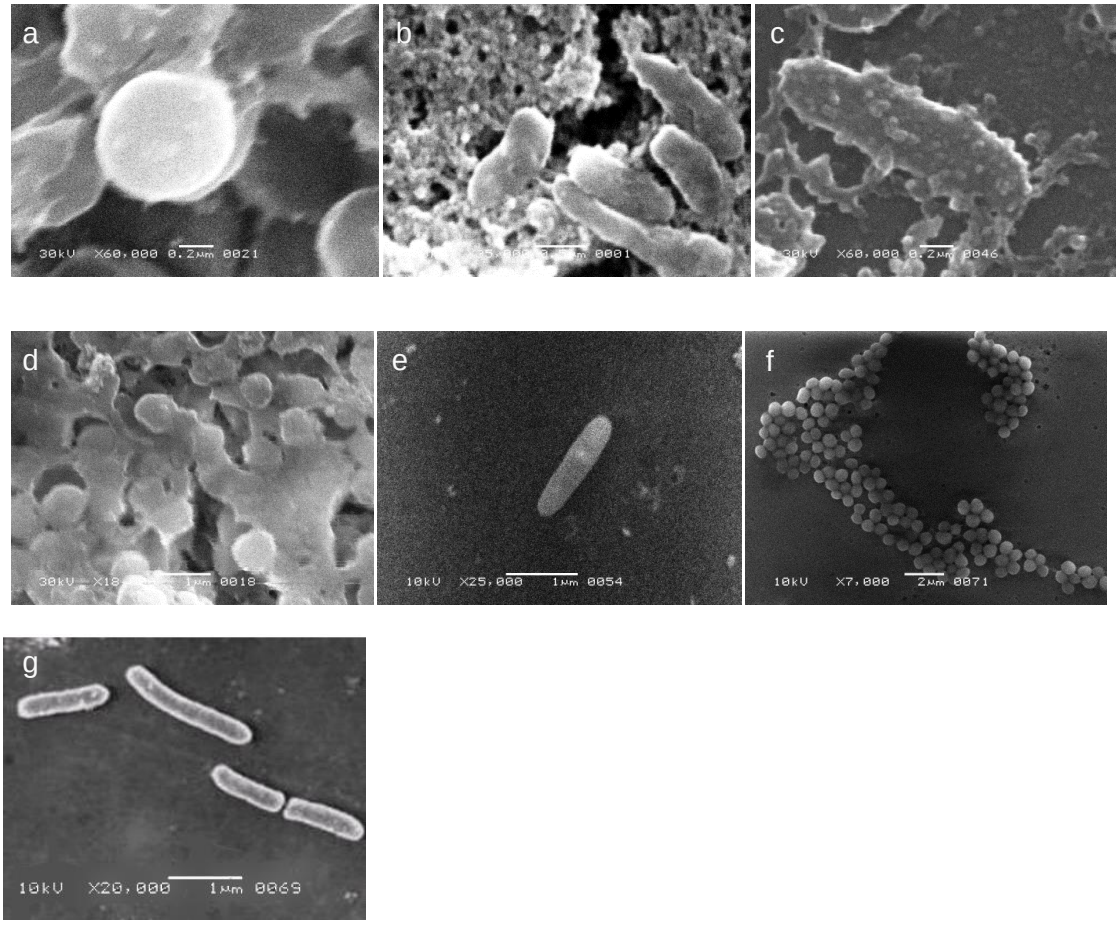

Figure 1: a) *S. aureus* in a biofilm state of growth surrounded by extracellular matrix material induced by 9% NaCl. b) Biofilm positive *E. coli* showing rough surface surrounded by extracellular matrix material after exposure to 7% garlic. c) *P. aeruginosa* adapted biofilm mode of growth during the growth under 7% ginger. d) *S. aureus* in multi-cellular aggregates surrounded by biofilm matrix material. e) Biofilm negative control of *E. coli* without garlic. f) Biofilm negative control of *S. aureus* without NaCl. g) Biofilm negative control of *P. aeruginosa* without ginger.

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factors. The surface structure and architecture of *E. coli*, *P. aeruginosa*, and *S. aureus* was found to have changed from smooth to rough after exposure to 7% garlic, 7% ginger and 9% NaCl, respectively. Cell morphology on scanning electron micrographs verified that the magnitude of surface roughness to *E. coli* cells was significantly greater than that of *S. aureus* cells. However, *E. coli* did not show multi-cellular aggregates. *P. aeruginosa* also showed similar characters like *E. coli* after exposure to 7% ginger. Normally, *E. coli*, *P. aeruginosa* and *S. aureus* possess smooth surfaces. However, they have tendency to change their surface properties according to environmental conditions e.g. production of exo-polysaccharides and other extracellular products that increase the surface roughness and induce bacterial adhesion and biofilm formation. Synthesis of cellulose by enteric-bacteria such as *Salmonella* spp. and *E. coli* has been associated with the ability to form biofilms on abiotic surfaces, such as glass and polystyrene (Zogaj *et al.*, 2001; Garcia *et al.*, 2004; Pizarro *et al.*, 2004). High-level production of cellulose by these organisms resulted in wrinkly or rough colonies on agar plates. Lee *et al.* (2011) described that garlic extract seems to contain biological materials that promote glycosyl-transferase expression responsible for stress response and biofilm formation in bacteria. According to Lundstrom *et al.* (2010) the glycosyl-transferases are responsible for making cellulose that forms the rosette terminal complexes seen in plasma membranes. Possibly, ginger and garlic induce glycosyl-transferase resulting in cellulose production which increases bacterial surfaces roughness. Iwabuchi *et al.* (2003) described that rough strains adhere well to various materials and form cell aggregates, while their mucoidal derivatives did so very poorly, if at all. Cell surfaces of the rough strains were more hydrophobic than those of the corresponding mucoidal strains. It has been noticed that under stress environment, exerted by natural food preservatives (ginger, garlic and sodium chloride), resistant isolates of *E. coli*, *P. aeruginosa* and *S. aureus* change their colony morphology type such as from smooth to rough. As a result of this change in cell surface, hydrophobicity also increases and isolates with rough surface exhibit greater net negative surface charges (Dickson *et al.*, 1992). All these factors i.e. cell surface roughness, hydrophobicity and negative surface charges contribute to initial bacterial adhesion, colony establishment and biofilm formation. After conversion from smooth to rough surface and adhesion to the surface, the production of extra-cellular matrix material was observed after 36 h of incubation at 37 °C in 9% NaCl for *S. aureus*, 7% garlic paste for *E. coli* and 7% ginger paste for *P. aeruginosa.* Harmsen *et al.* (2010) described that the formation and maintenance of structured microbial communities critically depends on extra-cellular substances that constitute cell-to-cell interconnecting matrices. Naturally, ginger, garlic and NaCl are being used as anti-bacterial food additives to reduce the bacterial growth and increase the shelf life of food items. In a study, Vattem *et al.* (2007) described that dietary phytochemicals e.g. ginger, garlic and NaCl from plants, target quorum-sensing system and inhibit food spoilage and biofilm formation in food-related bacteria. Scanning electron microscopic observations of *S. aureus* under NaCl stress conditions revealed large, multi-cellular aggregates. Extensive literature has suggested that NaCl is responsible for induction of biofilms in *S. aureus* (Rachid *et al.*, 2000; Conlon *et al.*, 2002; Lim *et al.*, 2004). Salts generate high osmotic pressure which may damage the bacterial cell wall. Mirani and Jamil (2011) described that cell wall deformity and the state of cell wall deficiency encourages the biofilm formation in bacteria and protects it from toxic effect of antibacterial agents. This study shows that bacterial isolates belonging with different genera exhibited similar changes in terms of phenotypic characters.

CONCLUSION

In present study, the strains of *S. aureus, E. coli* and *P. aeruginosa* were isolated from different food samples. These isolates exhibited resistance to commonly used natural food preservatives e.g., *S. aureus* was able to grow in 9% NaCl, *E. coli* in 7% garlic, and *P. aeruginosa* in 7% ginger. These are the traditional preservatives used for food preservation at home as well as industry while making pickles, jams, jellies and juices etc. All three isolates were found to use similar strategy to overcome the toxic effect of these natural food preservatives i.e., by adapting biofilm mode of growth. The presence of food preservative resistive strains could be hazardous for traditionally preserved foods.

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