Biofilms on Fresh Produce and Difficulties in Decontamination

BY **BASSAM A. ANNOUS, ETHAN B. SOLOMON** AND **BRENDAN A. NIEMIRA**

➤ INTRODUCTION

The incidence of foodborne illness linked to fresh fruits and vegetables has increased significantly during the past three decades[15]. *Escherichia coli O157:H7* and *Salmonella*, both previously regarded as pathogens linked to foods of animal origin, have emerged as common agents of produce-related outbreaks. Initial research into the safety of fresh produce focused primarily on surveys to determine the prevalence of pathogens, as well as the efficacy of various sanitation methods to remove or inactivate bacteria on produce post-harvest. Results indicated that although the incidence of pathogens was low, none of the treatments proposed was completely effective. The rise in the number of produce-related outbreaks, coupled with the lack of an effective intervention, has given rise to an intense research effort into the ecology of human pathogens in the growing environment. Contrary to earlier theory, pathogens have been found to survive for long periods of time in water, animal manure and a variety of agricultural soils.

Recently, the intimate interactions between human pathogens and plant tissues have begun to be characterized. Bacteria have been found to be capable of attaching to and colonizing the surfaces of growing plants. It is now becoming clear that once attached, human pathogens are capable of forming biofilms on plant tissues. This formation of a biofilm was reported to be one of the main factors in failure of washing treatments to remove or inactivate human pathogens on produce surfaces [2-4].

Biofilm Basics

A biofilm is generally defined as "an assemblage of microorganisms adherent to each other and/or to a surface and embedded in a matrix of exopolymers"[8]. In food-processing settings, biofilms are found on food-processing surfaces such as stainless steel and glass. The presence of biofilms in processing environments increases the opportunity for contamination of finished product. Biofilms form in a step-wise process of preconditioning, initial reversible attachment, irreversible attachment, formation of microcolonies and maturation of the biofilm. All of these steps are dependent on a variety of factors including nutrient availability, temperature, substratum topography, agitation, flow rate and inoculum density. Bacterial cells embedded in a biofilm are far more resistant to inactivation using chemical sanitizers than their planktonic (free-floating) counterparts. A large number of reports demonstrate resistance of biofilm-associated cells to all major sanitizers common in the food industry such as chlorine, hydrogen peroxide and quaternary ammonium compounds[7].

Microscopic studies indicate that plant-associated epiphytic bacteria form biofilms on surfaces of a wide variety of plants[9, 11]. Between 30 and 80 percent of bacteria on plant surfaces exist within biofilms[10]. The formation of biofilms by bacterial cells on plant surfaces is likely a survival strategy for these cells to withstand the harsh environment of the plant surface (wide temperature changes, desiccation, UV, oxidative stress). Similar to biofilms on food-processing surfaces, bacteria embedded within biofilms on plant tissue are more difficult to remove and more resistant to inactivation than their planktonic counterparts. Three commodities that have been repeatedly linked to outbreaks are cantaloupes, apples (unpasteurized juice or cider), and parsley, each associated with a different human pathogen. Research into the interactions between *Salmonella*, *E. coli*, and *Shigella* and these three vehicles are discussed below.

Cantaloupe

Cantaloupe melons have been implicated in at least six multistate outbreaks of salmonellosis since 1990. FDA surveys conducted in response to a 1997 outbreak of *Salmonella* enterica serovar Saphra indicated that approximately 5 percent of imported cantaloupes tested positive for *Salmonella*[17]. Three successive outbreaks (2000-2002) linked to the consumption of melons imported from Mexico prompted the FDA to issue an import alert detain-

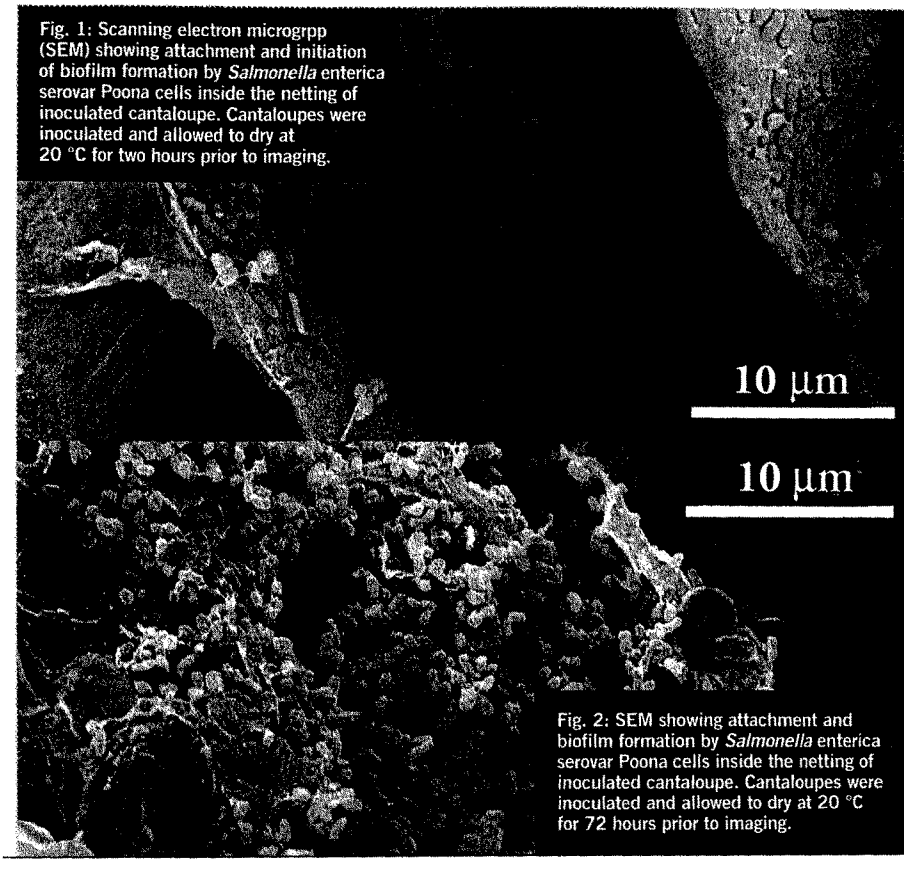

Fig. 1: Scanning electron microgrpp (SEM) showing attachment and initiation of biofilm formation by *Salmonella* enterica serovar Poona cells inside the netting of inoculated cantaloupe. Cantaloupes were inoculated and allowed to dry at 20 °C for two hours prior to imaging.

Fig. 2: SEM showing attachment and biofilm formation by *Salmonella* enterica serovar Poona cells inside the netting of inoculated cantaloupe. Cantaloupes were inoculated and allowed to dry at 20 °C for 72 hours prior to imaging.

ing all cantaloupes from Mexico offered for entry at U.S. ports[18].

Research in our laboratory has documented the inability of a variety of sanitizers to remove or inactivate *Salmonella* on cantaloupes [4, 14, 16]. Furthermore, the efficacy of sanitizers applied to cantaloupes decreased significantly when the organism was allowed to reside on the rind surface for more than 24 hours. These results led us to speculate that increased residence time allowed for the formation of a biofilm prior to application of the sanitizer[3]. We recently investigated the ability of *Salmonella* to produce biofilms on whole cantaloupes[3, 5]. Scanning electron microscopy (SEM) demonstrated that biofilm formation occurred rapidly following introduction of cells onto the rind. Fibrillar material could be seen after just two hours following inoculation and drying at 20 °C (Figure 1). Once attached, *Salmonella* cells developed biofilms by growth and excretion of extracellular material following 24 hours of storage at either 20 or 10 °C [3, 5].

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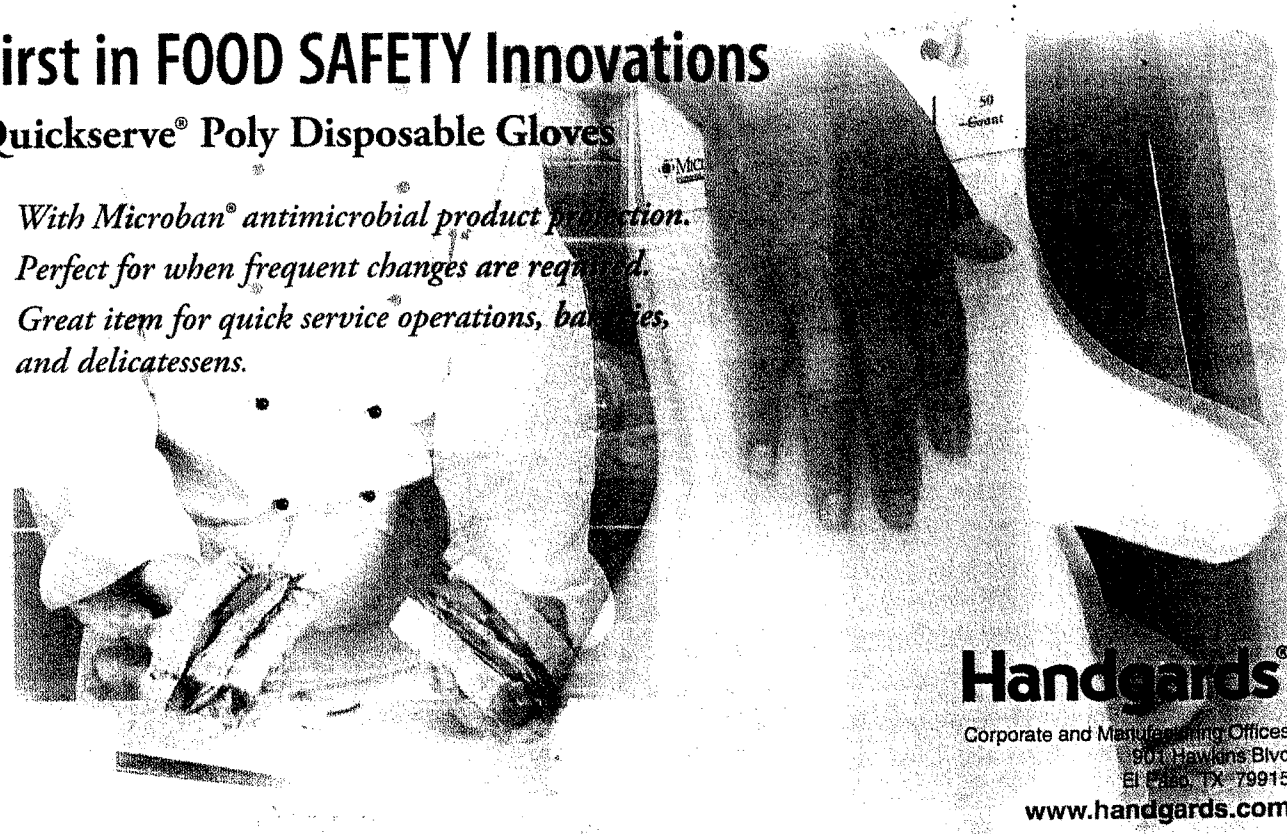

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Figure 2 shows attachment and biofilm formation by *Salmonella* enterica serovar Poona inside the netting of an inoculated cantaloupe. Cell attachment to inaccessible sites (netting) of the rind along with biofilm formation may be responsible for their resistance against aqueous sanitizers.

Apples

Seven outbreaks of *E. coli O157:H7* associated with apple juice or cider occurred between 1982 and 2002 (13). As a result, the FDA imposed a mandatory HACCP program for juice processors requiring a process that results in a 5-log reduction in the level of the target pathogen. Sanitizers such as chlorine and hydrogen peroxide allow significant populations of *E. coli O157:H7* to remain attached to apples following treatment. Burnett et al.[6] demonstrated that cells attached to subsurface structures were protected against inactivation using chlorine. Our laboratory has found that the majority of cells present fol-

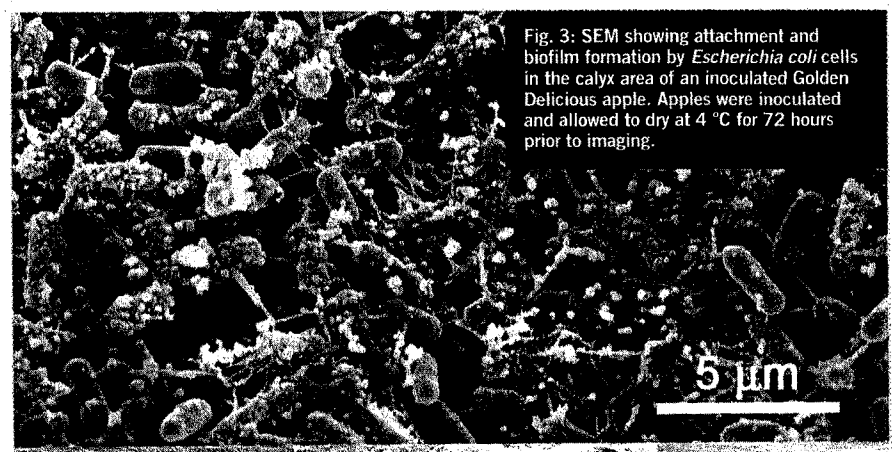

Fig. 3: SEM showing attachment and biofilm formation by *Escherichia coli* cells in the calyx area of an inoculated Golden Delicious apple. Apples were inoculated and allowed to dry at 4 °C for 72 hours prior to imaging.

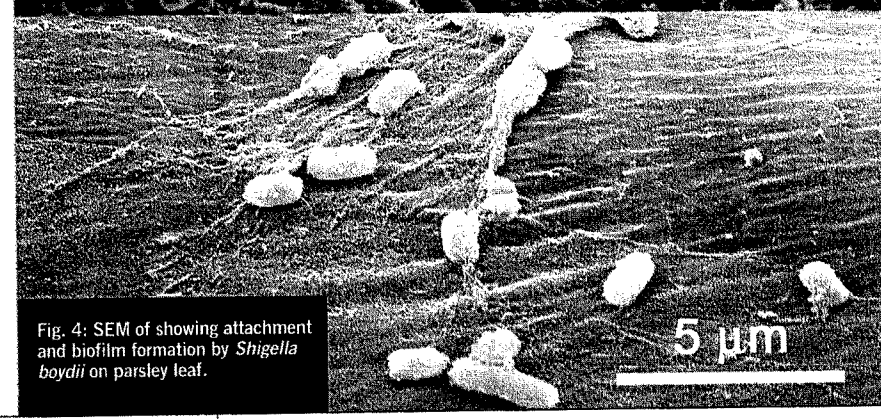

Fig. 4: SEM of showing attachment and biofilm formation by *Shigella boydii* on parsley leaf.

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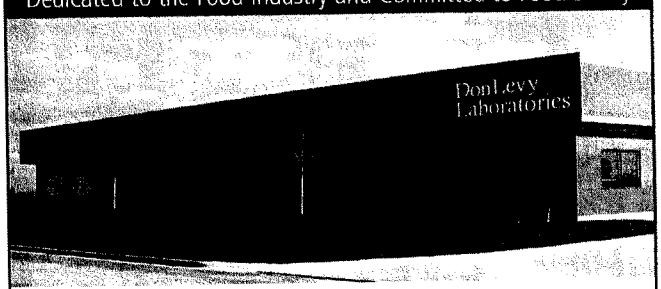

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lowing washing are located in the stem and calyx areas[2]. An SEM study of these areas demonstrated that *E. coli* cells were able to penetrate into the core of the apple and were able to form biofilm within the calyx region (Figure 3).

Parsley

In August of 1998, eight separate outbreaks of *Shigella sonnei* occurred in four states and two Canadian provinces[12]. All of the outbreaks, while geographically dispersed, were linked to fresh parsley from a supplier in Mexico. In March of 1999, an outbreak of *Sh. boydii* linked to bean salad prompted an investigation into the ability of *Sh.* to persist and form biofilms on the surface of parsley plants[1]. *Sh. boydii* was found to survive well on parsley for more than 20 days when stored at refrigeration temperatures. SEMs confirmed the ability of the organism to produce and become entrapped within a matrix of extracellular polymeric material on the surface of a parsley leaf (Figure 4).

Conclusion

Recent investigations into the interactions between enteric pathogens and plant tissues have begun to document the ability of these pathogens to form biofilms. It is likely that this phenomenon is responsible for the consistent finding that aqueous sanitizers are ineffective at inactivating bacteria on plant tissues. New strategies which apply sanitizers, and physical processes, singly and in combination, are being investigated for their ability to overcome the protective ability of the biofilm habitat. ■

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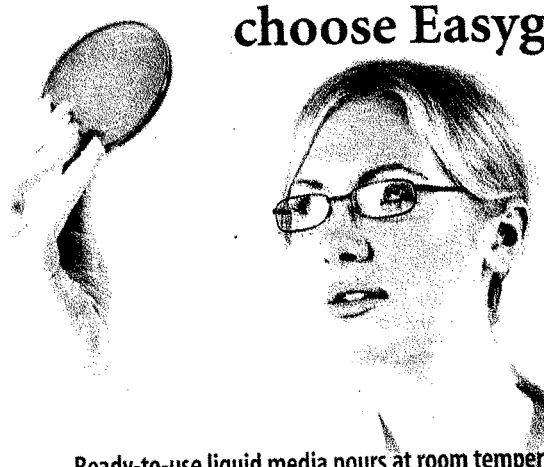

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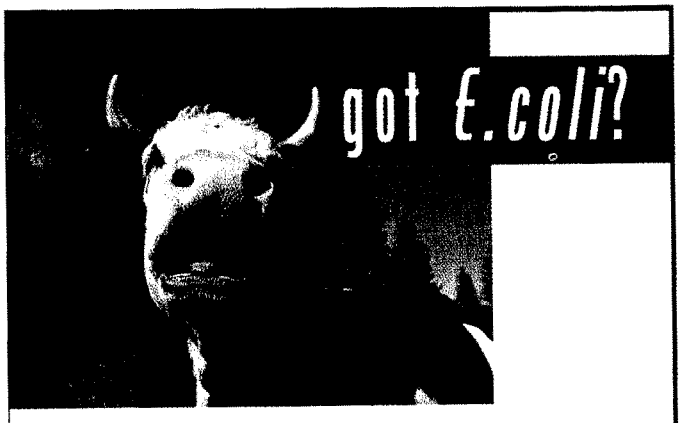
Bassam A. Annous and **Brendan Nimiera** are research microbiologist and plant pathologist respectively, for USDA-ERRC (Wyndmoore, Pa.). Reach them at 215-233-6406 or bannous@errc.ars.usda.gov, and 215-836-3784 or bniemira@errc.ars.usda.gov. **Ethan B. Solomon** is a research scientist for DuPont Chemical Solutions Enterprise (Wilmington, Del.) and can be reached at 302-695-6715 or ethan.b.solomon@usa.dupont.com.



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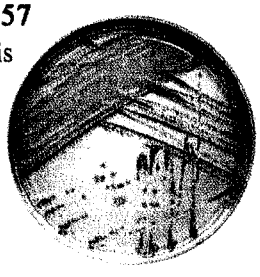

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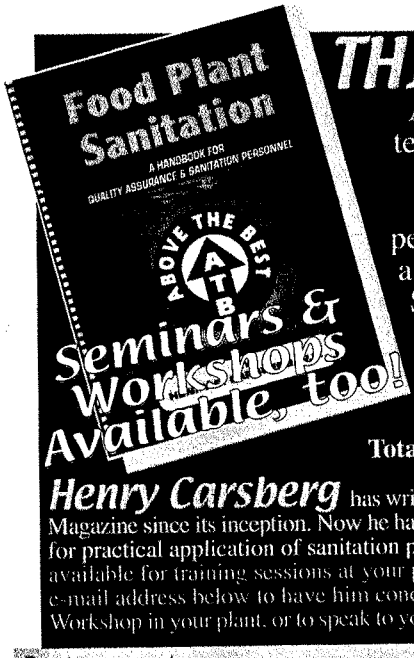

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