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A REVIEW OF MICROBIAL SAFETY AND BACTERIAL BIOFILM FORMATION OF FRESH VEGETABLES

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Abstract: Fresh vegetables are usually eaten raw and do not receive any treatment to ensure adequate removal or inactivation of harmful microorganisms prior to eating. Foodborne diseases associated with the consumption of fresh vegetables appear to be rising. Farming practices, post-harvest processing, and storage conditions affect microbial communities for fresh vegetables. *Salmonella* spp, *Escherichia coli (E. coli), Staphylococcus aureus,* and *Listeria* are common foodborne pathogenic bacteria associated with the consumption of fresh vegetables. Biofilm is characterised as a population of bacteria firmly adhered to a surface, and these communities provide more prolonged survival and resistance to adverse conditions such as the presence of disinfectants and antibiotics. The attachment of bacteria to the plant surface or biofilm formation is the initial step toward contaminating fresh vegetables. This review emphasises pre- and post-harvest contaminants in the safety of fresh vegetables, bacterial biofilm formation, and food safety strategies to reduce risk and the foodborne disease outbreaks linked to fresh produce.

Keywords: Microbial contamination, biofilm, fresh produce, food safety.

Introduction

To maintain good health, vegetables have high nutritional value, such as vitamin C, dietary fibre, carotenoids, and antioxidants. Most countries have their dietary recommendations, which include fruits and vegetables. Although fresh vegetables are deemed vital components of a healthy diet, previous research has reported them as possible health risks to humans when consumed raw and receive minimal treatment (Mritunjay & Kumar, 2015b). Note that foodborne outbreaks associated with the consumption of fresh vegetables have been increasing and alarming worldwide. Microbial contamination could be a health problem and cause a substantial economic loss.

According to Buck *et al*. (2003), the growth of bacteria in raw vegetables is caused by bacterial biofilm formation. When biofilm forms on fresh produce surfaces, it not only causes cross-contamination to the equipment surfaces in the industry but can also pose health hazards to consumers. The structural barriers and persistent cells within the biofilm play a main role in antibiotic resistance. Other than that, biofilms are also a general survival mechanism used by pathogenic and non-pathogenic microorganisms that involve surface binding and the growth of heterologous cells. Some biofilm strategies have been validated in certain microbiomes and target different life cycle stages. The different stages of biofilm formation are bacterial attachment to a surface, microcolony formation, biofilm maturation, and detachment. According to Sewell and Farber (2001), most of the problems with the survival strategy of biofilm formation in fresh vegetables are the increased consumption of vegetables, which can also increase Salmonellosis outbreaks related to fresh produce. Besides, the formation of bacterial biofilm provides a protective environment for pathogens and resistance and reduces the effectiveness of disinfection and cleaning agents (Buck *et al*., 2003).

Thus, this review aims to highlight the main sources of contamination of fresh vegetables, biofilm formation as a survival strategy of bacteria, and explain food safety strategies for preventing bacterial contamination.

Microbial contamination prevention and control must be based on documented risk assessments. Hence, raw materials must be evaluated, as well as their treatment. The use of non-registered or registered biocides exceeding registration limitations is prohibited to avoid microbiological growth.

The employed search strategy for this review encompassed prominent databases such as PubMed, Web of Science (WoS), and Scopus, complemented by including pertinent scientific literature sourced from online repositories and previously published reviews. Consequently, the search terms and strategy used for the study selection focused on microbial contamination, bacterial biofilm, and fresh produce.

Sources of Contamination for Fresh Vegetables

Fresh vegetables can be contaminated at any time in their production chain. Contamination sources can be grouped into two larger groups: Pre-harvest and post-harvest contamination. Sources of contamination before harvesting are irrigation water, contaminated soil and groundwater, water used for fungicides and insecticides, dust, organic fertilisers, and faecal contamination, affecting the safety and quality of fresh vegetables.

Post-harvest contamination is mainly identified in watering, washing, handling, and storage. Containers used to wash vegetables by farmers mostly have not been washed after use, and even when they are rinsed, the water is still used for multiple cycles, allowing for contamination. Therefore, washers should be disinfected before and after use to ensure their safety and prevent microbial contamination.

Irrigation Water

Irrigation water is one of the risk factors for the contamination of vegetables, and poor-quality water can cause fresh vegetables to be contaminated with foodborne pathogens. Aside from that, groundwater is common. It has acceptable microbial quality unless it has been contaminated by surface runoff. Nevertheless, human wastewater has poor microbial quality and requires considerable treatment before irrigating crops safely. Paramithiotis *et al.* (2013) presented that *Clostridium botulinum* and *Listeria monocytogenes* are the most common human pathogens associated with fresh soil-related products. Besides that, *Salmonella Typhimurium*, *E coli* 0157:H7, parasites, *Shigella* spp. Hepatitis A Virus, and Norovirus are microorganisms associated with excrement.

Pre-harvest contamination of bacteria *Escherichia coli* (*E. coli*) may be due to the roots of the plant and the use of unclean water in direct contact with plant leaves because bacteria have the ability to attack internal tissues via the open stomata and lesions (Paramithiotis *et al*., 2017).

Contaminated Soil

Bacterial contamination of fresh vegetables is also caused by contaminated soil during cultivation. The soil used for crop production is always mixed with treated or untreated animal manure or human biosolids that are applied as fertilisers and can harbour pathogenic microorganisms. Consequently, microorganisms can transmit from contaminated soil to fresh vegetables, even from water droplets. Rain and water from irrigation planting have indicated that they can carry soil particles to the surface of plants, which can easily be exposed to bacterial contamination.

Mishandling During Processing

There are several steps in the processing method involving fresh vegetables, such as washing, disinfecting, cutting, and trimming (Jideani *et al*., 2017). Due to the soft exterior skin nature of vegetables, any mishandling in these steps leads to mechanical injury. According to Yahaya and Mardiyya (2019), that mechanical injuries such as cracking of fresh vegetables and bruising cause inner tissues and cells to rupture and make them more likely to be exposed to microorganisms. Correspondingly, Riordan *et al*. (2000) proved that *E. coli* O157:H7 might survive in wounds on apple surfaces, and interestingly, the number of *E. coli* O157:H7 increased in the presence of plant-pathogen *Glomerella cingulata*. This is due to the increased pH level of the affected site on the apple surface.

Storage and Transportation

Food contamination in vegetables may also occur during storage and transportation due to the cross-contamination in the vehicle used for transportation. Hence, cross-contamination can pose a significant threat to vegetables. The damaged vegetables should not be stored together and must be placed separately from fresh vegetables before transporting them. Table 1 presents the pre-harvest and post-harvest contamination of fresh produce.

Table 1: Sources of pathogenic microorganisms during pre-harvest and post-harvest on fresh produce

Pre-harvest	Post-harvest
Faeces	Faeces
Soil	Air (dust)
Air (dust)	Ice
Irrigation water	Insects
Insects	Improper display temperature
Human handling	Harvesting equipment
Wild and domestic animals (including fowl and reptiles)	Transport vehicles
Water used to apply fungicides, insecticides	Wash and rinse water
Green or inadequately composted manure	Improper packaging (including new packaging)
	Transport containers (field to packing shed)
	Improper storage (temperature, physical environment)
	Cross-contamination (other foods in storage, preparation, and display areas)
	Sorting, packing, cutting, and further processing equipment
	Improper handling after wholesale or retail purchase
	Wild and domestic animals (including fowl and reptiles)

Source: Beuchat and Ryu, (1997).

Types of Bacterial Lead to Contamination of Fresh Vegetables

Salmonella spp.

Salmonella spp. is the most commonly identified aetiological agent associated with fresh produce-related infection and is also isolated in 48% of cases (Sivaplasingham *et al*., 2004). Research conducted by Denis *et al*. (2016) revealed a global outbreak of *Salmonella* infection associated with contaminated basil imported from Israel in 2007. This outbreak has impacted at least 51 people across England, Scotland, Wales, Denmark, the Netherlands, and the USA. The present study conducted by Pérez-Lavalle *et al*. (2023) reported that in 2003, there was a Salmonellosis outbreak in California, which was traced to contaminated strawberries. Hence, this strengthens the literature. Strawberries are one of the examples of fresh produce linked to *Salmonella* infections.

Escherichia Coli (E. Coli)

Leafy vegetables are most commonly infected by *E. coli*, which is a Gram-negative bacterium with a rod shape and is anaerobic (Sharma *et al*., 2016). Nevertheless, the significance of fresh produce as a potential vector for *E. coli* O157:H7 was not widely studied until the mid-1990s. During this period, a sequence of outbreaks connected to raw or minimally processed horticultural items indicated that contamination could take place through indirect routes (Delaquis *et al*., 2007). According to Pérez-Lavalle *et al*. (2023), the consumption of raw strawberries was linked to an outbreak of *E. coli* O157:H7. The same case is related to the *E. coli* O157:H7 outbreak was reported in the United States, where 2006 infections were attributed to leafy vegetables. This was associated with the consumption of bagged spinach (Denis *et al*., 2016). *E. coli* can form biofilms and are identified as the primary causative agent in many cases related to intestinal infections (Sharma *et al*., 2016).

Listeria Monocytogenes

Listeria monocytogenes is a member of the genus *Listeria* and can naturally exist in agricultural settings like manure, soil, and water. The scientific evidence also discussed that this microbe can survive in the conditions and machinery used in food processing and production packing. Hence, this pathogenic bacteria is also linked to listeriosis, a rare but severe infection (Zhu *et al*., 2017). Listeriosis is a serious systemic illness caused by *L. monocytogenes* and can affect primarily the elderly, pregnant women, infants, and people who have impaired immune systems (Fulano *et al*., 2023). Other than linking to that severe illness, the *Listeria* contamination also included ready-to-eat meat, seafood, poultry, and some dairy products, and tainted rock melons, celery, packed salads, caramelised apples, bean sprouts, and frozen vegetables have caused the major outbreaks for the past few years.

Campylobacter

According to Mohammadpour *et al*. (2018), *Campylobacter* spp. is approximated as the third most ubiquitous microbes that cause foodborne illness, yet only a small number of outbreaks have been recorded recently. Thus, in 2011, *Campylobacter* was reported for 220 cases related to foodborne disease outbreaks in the European Union (EU) (Mohammadpour *et al*., 2018). *Campylobacter* infection involving salad vegetables is known to be the second highest risk factor, followed by consumption and preparation of chicken (Verhoeff-Bakkenes *et al*., 2011).

Aeromonas

Aeromonas spp. are waterborne microbes isolated from animal and plant-based food products and are normally implicated in developing some severe clinical diseases (Hincapié *et al*., 2023). These bacteria are also foodborne pathogens due to their potential to spread virulence factors such as aerolysin, haemolysin, and cytotoxic enterotoxins (Lee *et al*., 2023). Consuming seafood such as oysters, salads, and drinking water contaminated with *Aeromonas hydrophile* can be the source of foodborne outbreaks.

Bacterial Biofilm on Fresh Vegetables

According to Li *et al*. (2023), biofilms are defined as structures made mostly from autogenous Extracellular Polymeric Substances (EPS), which serve as a scaffold to wrap the bacteria on a surface to protect them from external stresses and prevent phagocytosis. Bacterial biofilms comprise polysaccharides, proteins, lipids, and extracellular DNA (eDNA) that stick together and cling to a surface. Note that biofilms differ from planktonic microorganisms in several ways. The process of biofilm formation is initiated and controlled by numerous factors such as quorum sensing, environmental conditions, nutrient availability, signalling pathways, secondary messengers hydrodynamics and cell communication. Most microbes employ biofilms to adapt to their surroundings. Biofilms can be discovered in food, food processing equipment, water pipes, industrial pipes, fans, medical equipment, and even damaged human tissues and organs. Note that biofilms are bacterial growths that stick to a surface and are composed of various microorganisms.

Biofilm production is influenced by environmental factors such as temperature and pH. These factors may help bacteria to stick together. The four basic biofilm formation processes are bacterial adhesion, microcolony formation, bacterial biofilm maturation, and diffusion (Juhlin *et al*., 2017). Wet surfaces included the surfaces of food and pipes. The earliest traces of build-up can be visible on the surface of fruits and food processing equipment. The combination of environmental changes and bacterial signalling regulation determines the first phase of biofilm growth.

Roughness and hydrophobic interactions on the surface have a significant impact on adhesion. The maturation phase of biofilms, as well as the structure, are influenced by signals produced by various bacteria. Biofilms are also made more enduring by adhesion factors such as Accumulation-related proteins (Aap), eDNA, and intercellular polysaccharide adhesion (Henning, 2007; Juhlin *et al*., 2017). The last step of separation is typically triggered by changes in the external environment. Various enzymes can be used to break down Extracellular Polymer (EPS). In a biofilm evolution model, changes in microcolony generation, maturation, and dissemination over 48 hours. Note that bacterial cells cling to the water's surface. After adhering to the surface, the bacteria group together to form microcolonies. The extracellular polymer matrix is seen as an orange boundary around the microcolony. After that, the biofilm grows into a three-dimensional structure. Finally, adhesion factors provide the biofilm with a high level of stability, which is later destroyed by proteases and other enzymes, resulting in the formation of free cells.

Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Atomic Force Microscopy (AFM) images are used to track the growth of biofilm at various stages of development after it is cultivated at 37°C for up to 48 hours. According to reports, biofilms can build in as little as 6 hours and settle in as little as 24 hours.

Some pathogenic microbes such as *Listeria monocytogenes, E. coli, Salmonella enterica, Campylobacter jejuni, Pseudomonas aeruginosa,* and toxigenic bacteria (*Bacillus cereus, Staphylococcus aureus*) have the potential to create biofilms. Consequently, it may also expose the fresh produce business to the persistence of these microbes. The persistence of such bacteria in fresh vegetables is due to biofilms.

E. coli O157:H7 can generate biofilms due to its ability to bind and colonise on various surface types. The biofilms can be formed by *E. coli* O157:H7 on biotic or abiotic surfaces, such as Chinese cabbage, celery, spinach, lettuce, leek, basil, and parsley. Alternatively, Stepanovic *et al*. (2004) highlighted that *Salmonella enterica* is a dominant biofilm producer and is recognised for its potential to develop biofilm on plant surfaces. Bahri *et al*. (2020) also examined the biofilm formation of *E. coli* and *Salmonella* isolates from *ulam* in Terengganu, Malaysia. Bahri *et al*. (2020) reported *that E. coli* and *Salmonella* were identified as strong biofilm and moderate biofilm producers in the sample of *ulam* (*salad kampung*, *kangkung*, *daun sup*, and *ketumbar*) stored at 4-8^{o}C. This report highlights the concern about foodborne pathogens present in fresh vegetables in Malaysia.

Salmonella can adhere to and form biofilms in various vegetable products, including melon, lettuce, cucumber, parsley, and tomatoes (Pérez-Lavalle *et al*., 2023). It produces coiled extracellular fibre proteins that participate in cell-surface contact and promote community behaviour and host colonisation. Note that cellulose in the biofilm matrix helps cells resist mechanical forces and adhere to abiotic surfaces (Giaouris *et al*., 2015).

According to Van Houdt and Michiels (2005), *E. coli* also uses flagella, fimbriae, and membrane proteins to initiate binding to inanimate surfaces. After adhesion, the flagella disappears, and the bacteria produce EPS, making the bacteria more resistant to disinfectants (such as hypochlorite).

Campylobacter jejuni is an anaerobic bacteria that can form biofilms under microaerobic (5% O_2 and 10% CO_2) and aerobic (20% O_2) conditions. Cells incorporated in the biofilm matrix are more resistant to oxygen and can persist for several days in food processing conditions. *Pseudomonas* spp. serve as a model bacterium for research on biofilms. The bacterial species are commonly found in various habitats such as water, soil, plant, and animal tissues. It is a Gram-negative, aerobic, motile, and popular opportunistic human pathogen that leads to most severe acute life-threatening infections (Li *et al*., 2023).

Food Safety Strategies to Reduce Contamination

Researchers, food scientists, and legislators closely monitor food and waterborne viruses since they are one of the primary etiological agents in most foodborne diseases (Leite *et al*., 2023). Some unprocessed, uncooked, or minimally cooked foods such as shellfish, fruits, and fresh vegetables have frequently been blamed for viral outbreaks of foodborne illness. Thus, several actions have been suggested prior to this matter, including the uses of antiviral films and coatings in food packaging systems that might be possible (Leite *et al*., 2023).

Some have been provided to reduce the potential contamination of fresh vegetables. All employees, including those involved in fresh produce production, such as equipment operators, potential purchasers, and shippers, must follow good hygiene measures. Besides, the operators should consider good hygienic practices. Hence, the first step is to design a training program that ensures that all workers and employees are well-versed in hygiene standards. Depending on the sort of activity, duties, and responsibilities allocated, the necessary understanding level will vary. Each manufacturer should implement a hygiene training program for their personnel, as well as proper hand washing techniques. Workers with other communicable disease symptoms should avoid handling fresh produce or sorting and packing items in a packaging facility. The manager should be familiar with the signs and symptoms of communicable diseases. If symptoms appear, they can take the necessary precautions to avoid exposure.

Subsequently, practising good hygiene also includes protection from injury. A lesion containing pus that may come in contact with the product or equipment being harvested, sorted, or packaged can also cause a hazard that will increase the contamination of fresh produce. If a worker's injury cannot be effectively covered to avoid contact with fresh produce or related equipment, the employee should not work with the produce under any circumstances. Surfaces of raw items, utensils, or equipment that come into contact with food. Correspondingly, good hygiene practices should also be considered instead. In some situations, disposable gloves can be an important and effective hygiene technique when used with hand washing.

In addition, washing and rinsing fresh vegetables under running water has been found to improve their shelf life by decreasing or eradicating bacteria on their surfaces. However, only a few dangerous bacteria may respond to this treatment technique, while many would survive. Disinfection is another treatment approach that can increase the efficacy of germ eradication by a factor of a hundred over the previous method.

Professionals and other people working in the food production industry, including farmers and market women, should be made aware of the potential risks of certain procedures and the possibility of contamination. They should be educated to understand the origins of the etiological agents that cause contamination and the diseases that emerge from them.

Conclusions

The chain from the grower to the consumer has a role to play in ensuring the safety and quality of the vegetable supply. Therefore, growers and shippers should be aware of the various physical properties of fresh produce, the practices that can affect possible sources of microbial contamination, and the most effective management strategies for them.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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