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# Viable bacterial population and persistence of foodborne pathogens on the pear carpoplane

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## Abstract

### BACKGROUND

Knowledge on the culturable bacteria and foodborne pathogen presence on pears is important for understanding the impact of postharvest practices on food safety assurance. Pear fruit bacteria were investigated from the point of harvest, following chlorine drenching and after controlled atmosphere (CA) storage to assess the impact on natural bacterial populations and potential foodborne pathogens.

### RESULTS

*Salmonella* spp. and *Listeria monocytogenes* were detected on freshly harvested fruit in season one. During season one, chemical drenching and CA storage did not have a significant effect on the bacterial load of orchard pears, except for two farms where the populations were lower 'after CA storage'. During season two, bacterial populations of orchard pears from three of the four farms increased significantly following drenching; however, the bacterial load decreased 'after CA storage'. Bacteria isolated following enumeration included Enterobacteriaceae, Microbacteriaceae, Pseudomonadaceae and Bacillaceae, with richness decreasing 'after drench' and 'after CA storage'.

### CONCLUSION

*Salmonella* spp. and *L. monocytogenes* were not detected after postharvest practices. Postharvest practices resulted in decreased bacterial species richness. Understanding how postharvest practices have an impact on the viable bacterial populations of pear fruit will contribute to the development of crop-specific management systems for food safety assurance. 

## INTRODUCTION

Pome fruit (apples and pears) are the most widely consumed fruit type in the European Union and the second most important in the USA.[1] Currently, there has been a global drive to increase the consumption of pome fruit in terms of whole fruit, fresh cut fruit and as a convenience food product. Fruit is sourced globally to meet local demands, resulting in complex and lengthy supply chains requiring more advanced technologies to retain shelf-life, quality and safety. The fruit itself is thus exposed to changing environmental conditions and treatments that have an impact on the resident microflora. In general, microbial populations of plants are stable in composition compared to the environments in which they proliferate.[2] Erlacher *et al.*[3] recently demonstrated a shift in Enterobacteriaceae on lettuce under biotic stresses and also showed that the presence of phytopathogenic microorganisms induced a shift in the microbiome to an increase in species richness.[3] As far as we can determine, there has been no comprehensive report in the literature about the natural bacterial populations of pear fruit surfaces (carpoplane) and the presence of foodborne pathogens.[4]

The carpoplane epiphytic microbial population consists of a variety of organisms co-existing within a characteristic community, representing an ecological balance.[5] Epiphytic microorganisms may provide some protection against pathogenic microorganisms that cause decay or food spoilage[6] or contribute to food safety concerns. Interventions such as washing and/or chemical applications and storage disrupt the natural microbial balance, causing a population shift that benefits opportunistic organisms associated with food spoilage, decay or food safety.[7] Human health-relevant foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. e*nterica* serovar Typhimurium and *Staphylococcus aureus* have been shown to attach and colonize fruit surfaces.[8, 9] Population shifts as a result of more favourable environmental conditions and/or the wounding of fruit skins have been found to favour foodborne pathogens such as *E. coli* O157:H7.[10]

The present study aimed to determine the presence and persistence of bacterial foodborne pathogens *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium on freshly harvested pear fruit, assess the impact of commercial postharvest practices on epiphytic bacteria and determine whether any of these foodborne bacteria form part of the natural pear carpoplane microbiome.

# MATERIALS AND METHODS

## Sites selected and process flow

The sites selected for the present study included four farms supplying fruit to one communal packhouse (within a radius of 30 km). Temperature and relative humidity for the area was obtained from World Weather Online.[11] Fruit were harvested at commercial optimal maturity, transported in crates and drenched in chlorine-water (75 ppm) upon arrival at the packhouse. Thereafter, crates were moved into controlled atmosphere (CA) storage (1.5% $O_2$, 1.5% $CO_2$ and at −0.5 °C, in accordance with industry standards) for 12 weeks. All farms and the packhouse were certified to Global-GAP Integrated Farm Assurance. The pH of the drench bath was monitored multiple times throughout the day. The chlorine drench water was managed in accordance with standard commercial practices (pome fruit postharvest guidelines) and the pH was adjusted to 6.5–7.7 as required to provide high concentrations of microbicidal hypochlorous acid.[12]

## Sampling strategy

Pear (*Pyrus communis* L. cv. Packham's Triumph) fruit samples were collected 'at harvest' at the time of commercial harvesting during two consecutive seasons (Farms 1 and 2: 18 February 2013 and 18 February 2014; Farms 3 and 4: 26 February 2013 and 18 February 2014). Fruit were collected using a random selection strategy, one fruit was collected from five different trees from one row, with four rows per orchard selected (the four rows were treated as replicates). The same block and rows were revisited at a similar time in the consecutive season. Pear fruit samples (five fruit each) corresponding to the specific orchard were also collected from the communal packhouse 'after drench' from four random crates (crates were treated as replicates). Following the 12-week CA storage ('after CA storage'), samples (five fruit from four random crates originating from the same farm) were collected (crates were treated as replicates). Therefore, for each treatment ('at harvest', 'after drench' and 'after CA storage'), four replicates were analyzed from each farm and the experiment was repeated. Following sampling, the pear fruit were kept in cold storage (±5 °C) and transported to the laboratory for analysis within 48 h.

## Microbial analysis

The isolation strategy is shown in Fig. 1 and consisted of an approach to (1) selectively detect the presence of foodborne pathogens using cultural isolation and molecular tools for confirmation; (2) determine the bacterial species present on the pear carpoplane following enumeration; and (3) assess the presence of foodborne pathogens within the culturable microbial population (Fig. 1). Each replicate consisted of five fruit, which were washed individually in quarter strength Ringer's solution (Merck, Johannesburg, South Africa) (500 mL) amended with 0.2 mL $L^{-1}$ Tween 80 in an ultrasonic bath for 5 min following the recording of volume displacement. Volume displacement was converted to surface area.[9] The wash water was concentrated through a sterile nitrocellulose membrane (pore size 0.45 µm) and the filter was then placed into 9 mL of buffered peptone water (3 M BPW) (3 M Food Safety, St Paul, MN, USA) and vortexed. Total viable aerobic bacterial populations were determined by plating a ten-fold dilution series onto standard 1 nutrient agar (STD1) (Merck). The STD1 agar plates were incubated at 25 °C for 48 h to enumerate the naturally occurring bacterial population on the pear fruit. Counts were recorded and data were converted to $\log_{10}(x + 1)$ CFU $cm^{-2}$

**Figure 1**
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Experimental workflow.

Two to five bacterial isolates were then selected from STD1 plates per replicate dilution used for enumeration. Isolates were selected randomly, based on phenotypic characteristics and numerical prevalence.[13] Bacterial cultures were purified and preserved aseptically in glycerol (32.5%) and stored at −70 °C. For identification of the isolates, each was separately re-cultured on STD1 agar and one colony was used to inoculate tryptone soy broth and incubated for 24 h at 37 °C. Biomass was then used for genomic DNA extraction using the Quick-gDNA miniprep kit (Zymo Research, Irvine, CA, USA). The DNA concentration of each isolate was determined with the Qubit 2.0 Fluorometer (Life Technology, Johannesburg, South Africa) and then subjected to 16S rDNA amplification as described by Brosius *et*

*al.*14 using the F-27 (5′-GAGTTTGATCCTGGCTCAG-3′) and R-1492 (5′-TACGGYTACCTTGTTACGACTT-3′) universal primers. Amplicons were visualized in a 0.2 g $L^{-1}$ agarose gel. The amplified polymerase chain reaction (PCR) products were purified from the agarose gel using a Zymoclean Gel DNA Recovery kit (Zymo Research) and sequenced using BigDye Terminator v3.1 cycle sequencing on an ABI 3500XL sequencer in forward and reverse directions (InquabaBiotec, Johannesburg, South Africa). Sequences were analyzed through BLAST nucleotide identification. Phylogenetic alignment analyses were conducted using MEGA, version 6.15 Microbial phylogenetic trees were created with MEGA using the distance Neighbour-joining statistical algorithm.16 Corrected nucleotide substitutions were calculated using the Tamura–Nei model.

Each of the filtered pear samples was enriched for determining the presence of *E. coli* (including *E. coli* O157:H7) and *Salmonella* spp. by incubation of the 9 mL of 3 M BPW containing the filter membranes at 37 °C for 24 h. Additionally, 1 mL of incubated 3 M BPW broth was transferred into 9 mL of 3 M *Listeria* selective broth (3 M Food Safety) for enrichment purposes and incubated at 37 °C for 24 h. One loopful of each of the samples was streaked onto Eosin methylene blue differential medium (Merck) for detection of *E. coli*, Salmonella brilliance medium (Oxoid, Johannesburg, South Africa) for detection of *Salmonella* spp. and Oxford-Listeria selective medium for detection of *Listeria* spp. Typical colony morphology was used as presumptive isolation criteria. All isolates were purified and preserved aseptically in glycerol (32.5%) and stored at −70 °C. Presumptive colonies were re-cultured in tryptone soy broth, incubated for 24 h at 37 °C and then genomic DNA was extracted using the Quick-gDNA miniprep kit (Zymo Research). The DNA concentration of each isolate was determined with the Qubit 2.0 Fluorometer (Life Technology). Following DNA extraction, a foodborne pathogen-specific multiplex PCR for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium was conducted as described by Standing *et al.*17 using a T100™ Thermal Cycler (Bio-Rad Laboratories Ltd, Johannesburg, South Africa). The PCR mixtures contained 25 ng of genomic DNA, 0.5 µmol $L^{-1}$ of each primer (IDT, WhiteSci, Cape Town, South Africa), 200 µmol $L^{-1}$ of each deoxynucleotidetriphoshate and 1 U of My Taq polymerase (both supplied by Bioline, Celtic Molecular Diagnostics, Cape Town, South Africa) in a total reaction volume of 25 µL. The PCR conditions were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min. The PCR amplicons were visualized in a 0.2 g $L^{-1}$ agarose gel.

The 24 h 3 M BPW and 3 M *Listeria* specific enrichment broths were additionally used to determine the presence/absence of *E. coli* O157 (including H7), *Salmonella* spp. and *Listeria* spp. using the respective 3 M Molecular Detection System (3 M-MDS) kits in accordance with the manufacturer's instructions: 3 M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3 M Molecular Detection Assay *E. coli* O157 (including H7) (AOAC RI Certificate 071202, July 2012) and 3 M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

During the first season, after the identification of all presumptive positive isolates from selective chromogenic media, many of these isolates were determined not to be the target organism of interest using multiplex PCR analysis. Subsequently, in the second season, the experimental approach was changed to initially screen the samples for the presence of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium with the 3 M-MDS. Samples that tested positive using the 3 M-MDS were then subjected to traditional viable plating on

selective chromogenic media. The identities of the presumptive positive isolates were confirmed using the species-specific multiplex PCR as described previously.

### Statistical analysis

Data were subjected to an appropriate analysis of variance (ANOVA) using the farms as main-plot factor (Farms 1 to 4), the stages (‘at harvest’, ‘after drench’ and ‘after CA storage’) as a subplot factor and the repeated measurements over the two seasons as a sub-sub-plot factor.[18] The Shapiro–Wilk's test was performed on the standardized residuals to test for deviations from normality.[19] In cases where there was significant deviation from normality as a result of skewness, outliers were removed until the residuals had a normal or symmetric distribution.[20] Student's *t*-test (least significant difference) was calculated at a 5% significance level aiming to compare means of significant source effects. All of the data analyses were performed using SAS, version 9.3.[21]

# RESULTS

### Climatic conditions

Season one had an average day temperature of 23 °C with maximum temperatures of 26, 28 and 26 °C recorded at 11.00, 14.00 and 17.00 h, respectively.[11] The average relative humidity was 68.4% during the day. Average night-time temperatures of 18.7 °C were recorded, with a maximum of 22 °C, and an average relative humidity of 92.7% was recorded.[11] During season two, an average day temperature of 18.8 °C was recorded, with maximums reaching 21, 27 and 24 at 11.00, 14.00 and 17.00 h, respectively.[11] The average relative humidity recorded during the day was 57.4%, with 68% recorded at night.[11] Night-time temperatures were considerably lower, with an average of 10 °C.[11]

### Viable bacterial population

The ANOVA results indicated that there was a significant interaction between season, farm and sampling stage ($P < 0.0001$). Bacterial populations on fruit ‘at harvest’ collected in the orchards within season one from all four farms were not significantly different, nor were the bacterial populations on fruit collected from all four farms ‘at harvest’ within season two. Therefore, fruit taken ‘at harvest’ per season from all four farms had similar bacterial loads irrespective of the farm from which they were collected. However, fruit collected ‘at harvest’ in season one had significantly higher populations compared to fruit in season two (Fig. 2). In addition, as a result of climatic differences between the two seasons, the bacterial species present, isolated and identified from pear fruit ‘at harvest’ in season one ($n = 6$) were less than in season two ($n = 9$) (Fig. 3). *Salmonella* Typhimurium and *L. monocytogenes* were detected on pear fruit collected ‘at harvest’ in the orchard in season one. *Salmonella* Typhimurium was detected from fruit ‘at harvest’ from Farm 2 and two fruit samples collected ‘at harvest’ from Farms 2 and 3 were positive for *L. monocytogenes.* No *E. coli* O157 (including H7) was detected in any of the fruit samples. None of the foodborne pathogens tested for could be detected after postharvest handling. No foodborne pathogens were detected in season two.

**Figure 2**
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Total bacterial population after three production processing practices (i.e. ‘at harvest’, ‘after drench’ and ‘after CA storage’) on the pear carpoplane. Error bars on bar graphs indicate the SD. Graph bars with the same lowercase letter represent no significant difference at the 0.05 significance level. $LSD_{P=0.05}$ bar represents the least significant difference.

**Figure 3**
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Bacterial species grouped per stage (‘at harvest’, ‘after drench’ and ‘after CA storage’). A hash (#) indicates presence in season one; an asterisk (*) indicates presence in season two; x/x/x, indicates presence in all three stages in different seasons, with a hash (#) and an asterisk (*) indicating presence in the specific season.

During season one, the bacterial populations on fruit collected from Farms 1, 2 and 3 demonstrated no significant difference between ‘at harvest’, ‘after drench’ and ‘after CA storage’. However, in season two, the bacterial loads on the fruit were significantly higher ‘after drench’ but were subsequently significantly lower ‘after CA storage’ (Fig. 2). Bacterial populations on Farm 4 demonstrated no significant difference ‘at harvest’ and ‘after drench’, although there was a significant decrease ‘after CA storage’ in season one. The reverse trend was seen ‘after CA storage’ in season two (Fig. 2). During season two, fruit from Farm 4 were the only fruit with bacterial populations that did not increase ‘after drench’ and the only fruit that demonstrated a significant increase ‘after CA storage’ (Fig. 2). ‘After drench’, the number of different bacterial species, observed and isolated following enumeration, decreased from 14 ‘at harvest’ to 5 ‘after drench’
(Fig. 3). *Curtobacterium* spp., *Pantoea* spp., *Pseudomonas* spp., *Frigoribacterium* spp. and *Erwinia billingiae* were present ‘after drench’
(Fig. 3). *Curtobacterium* spp., *Pantoea* spp., *Pseudomonas* spp., *Erwinia billingiae*, *Bacillus* spp., *Arhtorbacter oxydans* and *Sanguibacter* spp. were present ‘after CA storage’. In addition, *Listeria* spp. were detected on fruit from Farm 4 in season one ‘after CA storage’. However, the species was confirmed to not be ‘human pathogenic’ *L. monocytogenes* by species specific multiplex PCR.9

*Curtobacterium* spp., *Pseudomonas* spp., *Pantoea* spp. and *Erwinia billingiae* were found, following enumeration, on fruit throughout all stages of sampling
(Fig. 3). *Curtobacterium* was the only genus present after every stage in both seasons
(Fig. 3). *Frigoribacterium* spp. were found present ‘at harvest’ and ‘after drench’ (Fig. 3).

## Phlyogenetic analysis

Figure 4 represents the 74 bacterial isolates from the pear carpoplane. The phylums separated at a single node into Actinobacteria (including Firmicutes) and Proteobacteria. Actinobacteria accounted for 62% of bacteria and Proteobacteria accounted for 38% identified. Multiple similar genera have been found ‘at harvest’ and ‘after drench’, as well as ‘after CA storage’. Clustering of other phylogeny groups included 13% Bacillaceae, 26% Enterobacteriaceae, 9% Pseudomonadaceae and 6% other families. *Lactobacillus plantarum* (Lactobacillaceae), *Streptomyces thermocarboxydus* (Streptomycetaceae) and *Chromobacterium* sp. (Neisseriaceae) clustered separately. *Lactobacillus plantarum* aligned under Firmicutes grouping, closely related to *S. thermocarboxydus* that

allocated under Actinobacteria, whereas *Serratia marcescens* and *Chromobacterium* sp. aligned under the Proteobacteria grouping.

**Figure 4**
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Phylogenetic tree based on bacterial isolates' 16S rDNA sequences, constructed using the Neighbour-joining methodology with allocated bootstraps. An asterisk (*) indicates microbes isolated ‘after CA storage’.

# DISCUSSION

Apples and pears are often cultivated on the same farms because of the similarity in growing conditions and postharvest practices. Pears have previously not been implicated in foodborne disease outbreaks. The first *L. monocytogenes* outbreak linked to apples occurred in 2014 and, prior to that, a three multistate *Salmonella* spp. outbreak occurred in 1999.[22] In the present study, *Salmonella* Typhimurium and *L. monocytogenes* were found on the pear carpoplane ‘at harvest’ in season one but not in season two. Fruit at the point of harvest can become contaminated by handling.[22] However, in the present study, fruit collected ‘at harvest’ were aseptically removed from the trees and therefore contamination must have occurred in the growing environment. Orchard contamination of fruit can occur in a number of ways, including the use of poor quality water used for spray cooling fruit in summer or for foliar treatments or pesticide sprays, as well as improperly composted manure and animal feces, including birds.[23-25] Previous studies in the USA[26] and South Africa[27] have demonstrated the presence of *Salmonella* spp. in ground water used for irrigation. Gemmel and Schmidt[27] found that *Salmonella* spp. were detected 9 months of the year from river water used for domestic, agricultural and recreational purposes. Irrigation water sources on Farm 2, a farm with contaminated fruit, were found to be contaminated with *Salmonella* Typhimurium (Duvenage FJ *et al.*, unpublished data). Islam *et al.*[28] demonstrated the presence of *Salmonella* Typhimurium on lettuce and parsley growing in fields following irrigation with water artificially contaminated with the pathogen. *Listeria monocytogenes* is commonly found in the growing environment in soil and water.[29] Despite the presence of *L. monocytogenes* and *Salmonella* Typhimurium on fruit in the growing environment, fruit exiting the CA storage and intended for the retail market were found to be pathogen free in the present study.

On-farm and postharvest practices such as chlorine washing and CA storage do not selectively limit the growth of human health-relevant foodborne pathogens[13, 30, 31] but, instead, collectively act as a hurdle effect. The present study has shown that foodborne pathogens may be present on fruit growing within the field but could not be detected as part of the natural resident population of the pear carpoplane. It has been previously shown that CA storage does not affect the growth of *L. monocytogenes*,[30] nor *Salmonella* spp.,[31] and pathogens are able to survive under cold storage conditions similar to that of commercial pear fruit storage.[9]

*Serratia marcenscens* was detected on orchard pears as part of the natural carpoplane bacterial population. This pathogen is known to infect the urinary tract and open wounds in humans.[32] Although this pathogen is not typically associated with foodborne outbreaks, environmental *S. marcenscens* strains have been reported to be similar to animal and human

pathogenic strains as far as virulence, phenotypic and molecular characteristics are concerned.33 *Serratia marcenscens* was neither isolated following chlorine drenching, nor after CA storage.

The clustering of the bacterial species resulted in three phyla. These include Firmicutes, Actinobacteria and Proteobacteria (divided into the Beta- and Gamaproteobacteria classes). The viable classes that dominated the carpoplane throughout the three stages of sampling were Actinobacteria (family: Microbacteriaceae, Micrococcaeae and Streptomycetaceae), Gammaproteobacteria (family: Enterobacteriaceae and Pseudomonadaceae), Betaproteobacteria (Neisseriaceae) and Bacilli (family: Paenibacillaceae, Bacilliaceae and Lactobacilliaceae). Janisiewicz and Buyer34 similarly found that Actinobacteria and Gammaproteobacteria dominated on the nectarine fruit surface. By contrast, Ottesen *et al.*35 reported that Alphaproteobacteria and Betaproteobactiera were the most dominant on the apple carpoplane. When the atmospheric or micro-environmental temperature is favorable for growth, organisms are able to multiply.34 Our research has demonstrated that the viable bacterial community was more rich and diverse 'at harvest' compared to 'after drench' and 'after CA storage'. *Curtobacterium* was the only genus that was able to remain present through the different postharvest stages sampled during both seasons, and therefore can be classified as a 'true resident'. Similar to our findings, *Curtobacterium* spp. were shown to be dominant on nectarine fruit.34

In addition, *Pantoea* spp. were found to be present on fruit 'at harvest' and 'after drench', as well as 'after CA storage' in both seasons. Lopez-Velasco *et al.*36 found that *Pantoea* spp. proliferated after 15 days of refrigerated storage and therefore showed pschryotrophic growth. This resonates with our findings.

Psychrotrophs, such as *Erwinia* spp. and *Pseudomonas* spp., can survive under cold storage conditions because they are able to proliferate at low temperatures. These organisms were isolated from fruit 'at harvest' and 'after drench' and were able to remain present throughout the 12-week cold storage period under CA conditions. Extreme conditions can cause a shift in the population density and species diversity.7 Controlled atmosphere storage represents an extreme environment that contributes to a reduced respiration rate of fruit and thereby increasing fruit shelf-life.

Upon investigation of the uniqueness of the viable bacterial species in season one 'at harvest', it was found that *Pseudomonas* spp. were not present 'at harvest' in season one but were detected 'at harvest' in season two. *Pseudomonas* spp. was similarly present 'after drench' and 'after CA storage' in both seasons. *Pseudomonas syringae* was found to prevent *E. coli* O157:H7 growth in apple wounds.37 *Pseudomonas graminis* (CPA-7) has also been shown to reduce loads of *Salmonella* spp. and *L. monocytogenes* in fresh cut melons38 and apples39 and to reduce loads of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* on fresh cut apples and peaches.40 Additional studies need to focus on the correlation between presence of *Pseudomonas* spp. and the presence or absence of foodborne pathogens to determine whether these species could act as a natural protective barrier for food safety systems.

Various studies have investigated the effect of farming practices,4, 35 as well as climatic34 and storage conditions,4, 36 on the microbial community's composition and abundance. The carpoplane is a valuable source of nutrients and moisture for epiphytic microbes,41 with the species and dominance changing up to the point of a resident

community on mature and ripe fruit.[34, 42] In the present study, the temperature and relative humidity of season one was higher than in season two, which corresponded to the higher bacterial load and lower richness demonstrated in season one. Leff and Fierer[4] and Ottesen *et al.*[35] demonstrated that bacterial loads and diversity fluctuated as a result of climatic conditions and farming and processing practices, which is in agreement with the findings of the present study on the culturable bacterial population. However, after assessing various fruit and vegetables purchased from a retailer, Leff and Fierer[4] concluded that there is 'no 'typical' produce-associated community' because environmental factors, pH and moisture availability shape the community. Based on the findings of the present study, the culturable and viable bacterial species can differ from season to season and can be affected by postharvest practices.

In conclusion, *L. monocytogenes* and *Salmonella* Typhimurium were detected on fruit 'at harvest' but not 'after drench' or 'after CA storage'. Therefore, no foodborne pathogens, forming part of the natural pear carpoplane population, were able to persist throughout commercial postharvest practices. The changes in bacterial load and species richness throughout the postharvest practices suggest that external environmental conditions and practices influence the survivability of culturable aerobic bacteria on the pear carpoplane. Future studies should focus on assessing the relationship between naturally occurring epiphytes and their role in countering foodborne pathogens, as well as on determining the microbiome of different cultivars using culture-independent analysis of the pear carpoplane.

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# Citing Literature