Bacterial dynamics and the prevalence of foodborne pathogens associated with avocado fruit *Persea americana* Mill.

C Coetzee, E du Plessis, S Duvenage and L Korsten*

* Corresponding author: Department of Plant and Soil Sciences,
University of Pretoria, SOUTH AFRICA
E-mail: lise.korsten@up.ac.za

ABSTRACT

The global demand for fresh produce has increased significantly due to healthier life styles and a growing demand for ready-to-eat food. In this context, an increase of roughly 38% has been reported between 2003 and 2013 in fresh produce consumption. However, the fresh produce industries have also received negative exposure due to associated foodborne related disease outbreaks linking on farm or packhouse practices with contamination. Avocados are a versatile fruit and has become one of the most sought after traded commodities in the world due to its highly nutritious value, excellent taste, robust skin texture and extended shelf-life under controlled export conditions. Overseas ripening rooms have also become a new marketing strategy to ripen the fruit post transport and sell it at premium prices as ready-to-eat, thus making food safety assurance along the supply chain even more important. This study was aimed at achieving a better understanding of the microbial dynamics on the avocado fruit surface (carpoplane) and -pulp in the context of micro populations and foodborne pathogens. Comparing the microbial dynamics of in-season and stored out-of-season fruit and, determining the efficiency of fruit washings on reducing the microbial load on the carpoplane and in the fruit pulp were investigated in this study. Conventional culture-based and molecular methods were used for the isolation and detection of selected foodborne pathogens. The 3M™ molecular detection system was used for the detection of *Salmonella* spp. and *Listeria* spp. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry was used for bacterial identification. In season, a decrease in viable microbial load on the carpoplane after fruit washing and an increase in the microbial concentration in the fruit pulp after washing were noted. An increase in the coliform concentrations on both the carpoplane and pulp following pre-processing fruit washings were also observed. The presence of *Escherichia coli* decreased on both the carpoplane and fruit pulp following pre-washing of the fruit, with *E. coli* isolated from 14.1% of the total number of fruit sampled (n=170). *Salmonella* spp. presence accounted for 2.4% of the total number of samples processed while no *Listeria monocytogenes* were isolated.

Keywords: *Persea americana*; avocado; microbial dynamics; *Salmonella* spp.; *Escherichia coli*

INTRODUCTION

Avocado is cultivated throughout the world with Mexico being the main producer (Affleck, 1986; Galindo-Tovar *et al.*, 2008). To put this in context, in 2014 Mexico produced an annual average of 1.5 million tonnes of avocado representing 55% of the global volumes but only export 38% of this to mainly the USA (FAO, 2017). South Africa is a small player in the global arena producing only 103 000 tonnes in 2014. However, production has since increased by 37% with the average tonnes being 38 000 tonnes in 1993 compared to the 103 000 tonnes produced during the 2014 season (Department of Agriculture, Forestry and Fisheries, 2015). Of importance is that the majority of the total crop is exported (51%) to mainly the European market (FAO, 2017). Although the South African avocado industry is primarily export-oriented, the local demand has consistently increased over the last few decades (Department of Agriculture, Forestry and Fisheries, 2015). Given the total value of avocado exports are at R978 million, a significant proportion is still processed in to high value guacamole and oil (10%) (Department of Agriculture, Forestry and Fisheries, 2015).

Over the years, the avocado has become one of the most economically important tropical fruit due to its favourable taste and nutritional composition (Donkin, 2007; Ernst, 2015). Thus, the avocado is

known for its health benefits and is recognised as a fruit that is high in monosaturated fats, has high levels of vitamins and minerals, high concentrations of dietary fibres and anti-oxidants, all of which plays an essential part of a healthy, balanced diet (Donkin, 2007). Due to healthy lifestyles, society has become more aware of fresh, healthy, convenient and additive free foods, leading to increased growth in the demand for fresh produce (Boxstael *et al.,* 2013). Over the last few decades an increasing number of foodborne disease outbreaks have been recorded that has been linked to fresh produce consumption. Fresh produce has since been described as potential transmission vehicles for foodborne pathogens (Beuchat, 2002; Gomba *et al.,* 2016; Kotzekidou, 2013).

Foodborne pathogens are a major public health concern in both developed and developing countries due to the severity of resultant food related disease outbreaks, illnesses and high mortality rates associated with the consumption of contaminated food (Boxstael *et al.,* 2013). Food safety has therefore been identified at domestic, regional and international levels as a public health priority and potential trade barrier. Key global food safety concerns include the spread of microbiological hazards (including bacteria such as *Salmonella* spp. *Listeria* spp. or *Escherichia coli*) and a voluntary implementation of strict food safety systems is thus required in most countries to ensure safe food.

Microbial biomes represent a complex ecosystem that reflect the dynamic interactions of microorganisms in different habitats being influenced by environmental conditions (International Society for Microbial Ecology, 2013). Determining the microbiome of fresh produce will therefore contribute towards improved understanding of the persistence of human pathogens within the agricultural environment (Telias *et al.,* 2011; Ottesen *et al.,* 2013). The microbial ecology of fresh avocado fruit thus remains poorly understood (Ottesen *et al.,* 2013).

Several studies indicated that enteric pathogens have been detected on fresh produce (Beuchat, 2002; Lindow and Brandl, 2003). *Salmonella enterica*, *E. coli* and *Pseudomonas syringe* have the ability to colonise a wide variety of fresh produce and is not limited to a specific plant type (Beuchat, 2002; Brandl and Mandrell, 2002; Lindow and Brandl, 2003). Duvenage *et al. (*2016) isolated several bacterial families from the pear carpoplane, which included Enterobacteriaceae, Microbacteriaceae, Pseudomonadaceae and Bacillaceae and Gomba *et al.* (2016) reported Proteobacteria, Actinobacteria, Bacteroidetes and Deinoccoccus phyla on the citrus carpoplane. According to our knowledge, we are not aware of any studies that described the natural microbiota of the avocado carpoplane.

The objective of this study was to identify the natural microbiota of the washed and unwashed avocado fruit and determine the prevalence of foodborne pathogens associated with the avocado fruit, *Persea americana* Mill.

MATERIALS AND METHODS

Sampling site

Avocado fruit were collected from a Food Safety System Certification (FSSC) 22000 certified processing facility in Gauteng, South Africa.

Sample collection

Hass and Fuerte avocado fruit were collected at receival and again after the oxiacid washing at the processing facility for two consecutive seasons (2016 and 2017).

Food safety sample processing

Avocado fruit surface

Individual avocado samples (two avocados each) were submerged in 500 mL Ringer's solution containing 0.02% Tween 80 (Associated Chemical Enterprises, Johannesburg, South Africa). The samples were sonicated for 5 min using an ultrasonic bath (Eumax, Labotec, Johannesburg, South Africa), this process assists with the detachment of all microbes from the avocado fruit surfaces. Volume displacement (vd) was documented for each of the samples and converted to area (cm^2) using the following equation ($A = 4.84\ [(vd)\ 1/3]^2$ (De Jager, 1999; Collignon and Korsten, 2010). After sonication, the liquid from the samples were filtered using a 2 µm sterile nitrocellulose membrane (Sartorius Stedim, Biotech, Germany). The filters were aseptically cut into smaller pieces, added to a 9 mL sterilised peptone buffered water solution (Biolab Diagnostics, Johannesburg). Tenfold serial dilutions were prepared from the samples in PBW and total bacterial counts, Enterobacteriaceae and hygiene indicator bacteria counts were determined using standard microbiological analysis. The samples in BPW were enriched by incubating the samples at 37°C for 24 hrs. *E. coli* was isolated by streaking a loopful of each of the samples onto Eosin Methylene Blue (EMB) agar. Bacterial counts were recorded and data was converted to log (x+1) cfu/g for avocado pulp samples and log cfu/cm^2 for avocado surface samples.

Avocado pulp

Twenty-five grams of the avocado pulp was weighed in a sterile stomacher bag, mixed with 225 mL of 3M™ Peptone Buffered Water (PBW) and homogenized for 30 seconds using a stomacher (400 Circulator, Steward Stomacher, England). Tenfold serial dilutions were prepared from the homogenized samples in PBW and bacterial counts determined as described above. The homogenized samples in BPW were enriched by incubating the samples at 37°C for 24 hrs. *E. coli* was isolated by streaking a loopful of each of the samples onto Eosin Methylene Blue (EMB) agar. Bacterial counts were recorded and data was converted to log (x+1) cfu/g for avocado pulp samples and log cfu/cm^2 for avocado surface samples.

3M™ MDS *Salmonella* spp.

The 3M™ molecular detection system (3M, Johannesburg) was used for the detection of *Salmonella* spp.

in the enriched PBW samples as per manufacturer's instructions and described by Abirami *et al*. (2016). *Salmonella* was isolated from samples that tested positive by streaking a loopful of the enriched PBW onto selective chromogenic agar.

3M™ MDS *Listeria* spp.
After 24 ± 2h incubation in a 3M™ PBW pre-enrichments, 1ml were transferred to a supplemented 3M™ modified *Listeria* recovery broth and incubated for an additional 24 ± 2 h at 37°C. The 3M™ MDS system was used for detection of *Listeria* spp. in the samples as per manufacturer's instructions. The positive samples were cultured using selective chromogenic media for purification and bacterial identification purposes.

Bacterial identification
Bacterial isolates
A single pure bacterial colony was placed on a MALDI-TOF MTX 96 target plate (Bruker Daltonik GmbH); all isolates were done in duplicate.

Mass spectrometry
Measurements were performed using the Microflex mass spectrometer (Bruker Daltonik) equipped with Bruker's smart beam solid-state lifetime laser technology.

Criteria for isolate identification
MALDI-TOF analysis, modified score values were used as proposed by the manufacturer: a score higher than 1.9 indicated species identification, a score of between 1.7– 1.9 indicated genus identification, and a score below 1.7 indicated no identification. An isolate was considered correctly identified by MALDI-TOF mass spectrometry if the score was above 2, a score of above 1.9 indicated species identification and a score above 1.7 indicated genus identification.

Bacterial characterisation
DNA was extracted from each of the bacterial isolates using QuickgDNA™ MiniPrep DNA isolation kit following the manufacturer's instructions. Bacterial characterisation will further be subjected to a multiplex Polymerase Chain Reaction (m-PCR) as described by Omar and Barnard (2010) and multilocus sequencing typing (MLST).

Statistical Analysis
Statistical analysis was performed on log CFU/cm^2 avocado surface counts and log CFU/g avocado pulp counts. Data were subjected to an appropriate analysis of variance (ANOVA).

RESULTS
The ANOVA results indicated that there was a significant interaction between season and treatment types for total bacterial counts on the carpoplane; however, Enterobacteriaceae and coliform counts were only significant with the season illustrated in figure 1. The results obtained for the avocado pulp indicated that there was no significance between seasons and treatment types. However, the Enterobacteriaceae and coliform results for the pulp indicated a significant interaction between seasons and treatment types, respectively. The results obtained during the avocado season indicated a decrease in microbial load on the carpoplane after washing of the fruit, although an increase in the microbial load was observed in the fruit pulp after washing illustrated in figure 1. Total bacterial counts on surface were significantly higher out of season when compared to in season for washed and unwashed avocado. Total bacteria counts on the surface were significantly higher on unwashed fruit when compared to washed fruit. Total bacterial counts out of season were significantly higher than counts in season on washed fruit, which were significantly higher, than in season unwashed. These results indicate that the wash water might potentially contribute to the increased microbial load. Previous studies reported an increase in bacterial populations on the pear carpoplane following drenching (Duvenage *et al*., 2016).

Detection of *E. coli* decreased in both the carpoplane and fruit pulp after washing of the fruit. *Escherichia coli* isolated accounted for 14.1%

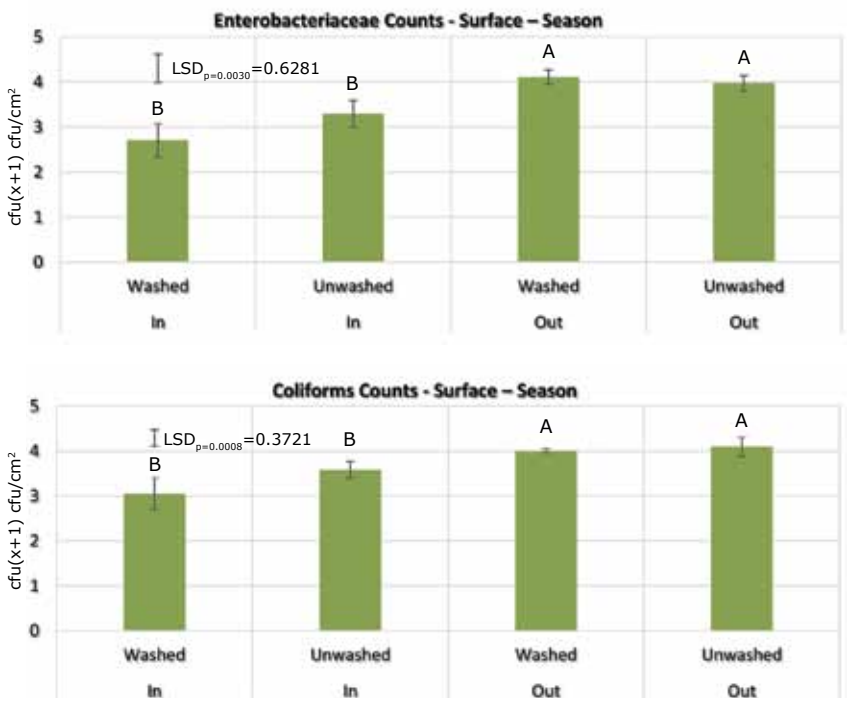

Figure 1. Total bacterial populations on the avocado carpoplane. Error bars on bar graphs indicate the SD. Graph bars with the same letter represent no significant difference at the 0.0030 significance level. $LSD_{P=0.0030}$ bar represents the least significant difference.

(n=170) of total number of samples, equally isolated from both surface and pulp samples. *Escherichia coli* were more frequently isolated from samples before washing than after washing and accounted for 7.6% of the total 14.1%. A study on ready-to-eat salads, containing avocado, reported contamination with faecal coliforms and *E. coli* (Castro-Rosas *et al.*, 2013). *Salmonella* spp. detected accounted for 2.4% of the total number of samples (n=170). *Salmonella* spp.were isolated from the avocado pulp, with avocado pulp before washing resulting in 1.2% of the total 2.4% isolated. A study by Gomba *et al*. (2016) indicated that *Salmonella* is part of the natural microbiota of the orange carpoplane. *Listeria monocytogenes* was not isolated from any of the samples tested.

DISCUSSION

A decrease in microbial load on the washed avocado surface was observed during the season, in contrast the microbial load in the fruit pulp decreased after washing. Thus far, pathogen detection indicated the presence of *Salmonella* spp. and *E. coli* associated with the avocado fruit. Future work will include avocado microbiome analysis, characterisation of *E. coli* isolates using multiplex PCR in order to determine the prevalence of virulence genes (*stx1, stx2* and *eaeA*). (Omar and Barnard, 2010) and *Salmonella* characterisation using multilocus sequence typing technology.

In conclusion, in season a decrease in microbial load on the cultural carpoplane after washing of the fruit, and an increase in the microbial concentration was observed in the fruit pulp after pre-processing washing. An increase in the coliform concentrations on both the carpoplane and pulp following pre-processing fruit washing was observed. The presence of *E. coli* decreased in both the carpoplane and fruit pulp following pre-washing of the fruit, with *E. coli* was isolated from 14.1% of total number of samples. *Salmonella* spp. presence accounted for 2.4% of the total number of samples. No *Listeria monocytogenes* were isolated.

Acknowledgements

We thank Ms. L. Morey from the Agricultural Research Council, South Africa, Biometry Unit, for statistical support. This work formed part of a poster presented at the International Postharvest Pathology Symposium in the Kruger National Park, South Africa, 28 May – 3 June 2017.

REFERENCES

ABIRAMI, N., NIDAULLAH, H., CHUAH, L., SHAMILA-SYAHADA, A.K., CHANDRAPRASAD, S.R., HUDA, N., HASMAIZAL, H. & RUSUL, G. (2016). Evaluation of commercial loop-mediated isothermal amplification based kit and ready-to-use plating system for detection of Salmonella in naturally contaminated poultry and their processing environment. *Food Control*. 70: 74-78.

AFFLECK, M. (1986). The international avocado industry: A global perspective. California Avocado Society. 70: 51-55.

BEUCHAT, L.R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruit and vegetables. *Microbes and Infection.* 4: 413–423.

BOXSTAEL, S.V., HABIB, I., JACXSENS, L., DE VOCHT, M., BAERT, L., VAN DE PERRE, E., RAJKOVIC, A., LOPEZ-GALVEZ, F., SAMPERS, I., SPANOGHE, P., DE MEULENAER, B. & UYTTENDAELE, M. (2013). Food safety issues in fresh produce: Bacterial pathogens, viruses and pesticide residues indicated as major concerns by stakeholders in the fresh produce chain. *Food Control*. 32: 190-197.

BRANDL, M.T. & MANDRELL, R.E. (2002). Fitness of Salmonella enterica serovar Thompson in the cilantro phyllosphere. *Applied Environmental Microbiology*. 68: 3614–3621.

CASTRO-ROSAS, J., CERNA-CORTÉS, J. F., MÉNDEZ-REYES, E., LOPEZ-HERNANDEZ, D., GÓMEZ-ALDAPA, C.A. & ESTRADA-GARCIA, T. (2013). Presence of faecal coliforms, Escherichia coli and diarrheagenic E. coli pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *International Journal of Food Microbiology*. 156: 176-180.

COLLIGNON, S. & KORSTEN, L. (2010). Attachment and Colonization by Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica subsp. enterica serovar Typhimurium, and Staphylococcus aureus on Stone Fruit Surfaces and Survival through a Simulated Commercial Export Chain. *Journal of Food Protection*. 73 (7): 1247–1256.

DE JAGER, E.S. (1999). Microbial ecology of the mango flower, fruit and leaf surfaces. M.S. thesis. University of Pretoria, Pretoria, South Africa.

DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES. (2015). A profile the South African Avocado Market Value Chain. Available: < http://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Commodity%20Profiles/FRUITS%20AND%20VEGETABLES/Avocado%20market%20value%20chain%20profile%202015.pdf> [Accessed 24/07/2016].

DONKIN, D.J. (2007). Avo Bytes. Available from: <http://www.avocado.co.za/avo-bytes/> [Accessed 23/11/2015].

DUVENAGE, F.J., DUVENAGE, S., DU PLESSIS, E.M., VOLSCHENK, Q. & KORSTEN, L. (2016). Viable bacterial population and persistence of foodborne pathogens on the pear carpoplane. Journal of Science and Food Agriculture. DOI: 10.1002/jsfa.7847.

ERNST, A. A. (2015). Establishing a New Generation Cultivar. Presentation.

FAVARO, M., SARMATI, L., SANCESARIO, G. & FONTANA, C. (2014). First case of Listeria innocua meningitis in a patient on steroids and eternecept. *JMM Case Reports*. 1-5.

FOOD AND AGRICULTURAL ORGANIZATION OF THE UNITED NATIONS. (2017). Avocado production. Available: < http://www.fao.org/faostat/en/#compare > [Accessed 26/02/2017].

GALINDO-TOVAR, M.E., OGATA-AGUILAR, N. & ARZATE-FERNANDEZ, A.M. (2008). Some aspects of avocado (Persea americana Mill.) diversity and domestication in Mesoamerica. *Genetic Resources and Crop Evolution*. 55: 441-450.

GOMBA, A., CHIDAMBA, L. & KORSTEN, L. (2016). Prevalence and serovar diversity of Salmonella spp. in primary horticultural fruit production environments. *Food Control*. 69: 13-16.

INTERNATIONAL SOCIETY FOR MICROBIAL ECOLOGY. (2013). What is Microbial Ecology?. Available from: <http://www.isme-microbes.org/whatis> [Accessed 12/01/2017].

KOTZEKIDOU, P. (2013). Survey of Listeria monocytogenes, Salmonella spp. and Escherichia coli 0157:H7 in raw ingredients and ready-to-eat products by commercial real-time PCR kits. *Food Microbiology*. 35: 86-91.

LINDOW, S.E. & BRANDL, M.T. (2003). Microbiology of Phyllosphere. *Applied and Environmental Microbiology*. 69 (4): 1875-1883.

OMAR, K.B. & BARNARD, T.G. (2010). The occurrence of pathogenic Escherichia coli in South African wastewater treatment plants as detected by multiplex PCR. *Water SA Young Water Professionals Special Edition 2010*. 36 (2): 172-176.

OTT, E. M., MULLER, T., MULLER, M., FRANZ, C.M., ULRICH, A., GABEL, M. & SEYFARTH, W. (2001). Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *Journal of Applied Microbiology*. 91: 54–66.

OTTESEN, A.R., PEÑA, A.G., WHITE, J.R., PETTENGILL, J.B., LI, C., ALLARD, S., RIDEOUT, S., ALLARD, M., HILL, T., EVANS, P., STRAIN, E., MUSSER, S., KNIGHT, R. & BROWN, E. (2013). Baseline survey of the anatomical microbial ecology of an important food plant: Solanum lycopersicum (tomato). *BMC Microbiology*. 13: 114.

TELIAS, A., WHITE, J.R., PAHL, D.M., OTTESEN, A.R. & WALSH, C.S. (2011). Bacterial community diversity and variation in spray water sources and the tomato fruit surface. *BMC Microbiology*. 11: 81.