Effect of postharvest practices on the culturable fungi and yeast microflora associated with the pear carpoplane

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Highlights

- Yeast and fungal biome changes on postharvest pear fruit surfaces were determined.
- Biocontrol potential within the yeast microbiota were determined.
- Postharvest processing stages affect the yeast and fungal biome dynamics.
- Potential known biocontrol organisms were isolated.
- Management of antagonistic populations may lead to effective disease control.

ABSTRACT

The bacterial microbiome on plant surfaces represents a climax community at the point of harvest and fruit maturity. How commercial postharvest interventions affect these populations are not known particularly in terms of the presence of postharvest pathogens and potential biocontrol agents. Information regarding the fungi and yeast microflora on pear surfaces (carpoplane) is limited when compared to other fruits such as grapes and apples. The objective of this study was to determine the influence of commercial postharvest practices (drenching and modified atmosphere storage) on the dominant yeast and fungal populations associated with the pear carpoplane and to identify postharvest pathogens and yeasts with biocontrol potential that can survive postharvest treatments. During season one the fungal counts on pears were significantly lower after CA storage

when compared to counts of orchard pears, however during season two, the opposite trend was observed. The yeast counts were either significantly higher or similar after CA storage compared to the orchard pear counts during both seasons. Commercial drenching led to either an increase or reduction in the fungi and yeasts, however a definite trend could not be observed. A total of 16 dominant yeast and 24 dominant fungal species were isolated. Overall a 76% dominance of Ascomycetes was observed. Known postharvest pathogens *Penicilium commune* and *P. crysogenum* was still present after CA storage. Potential known biocontrol organisms included *Aureobasidium pullulans, Cryptococcus* sp. and *Sporobolomyces roseus*. The postharvest processing stages decreased the number of dominant yeast and fungal species. By understanding bacterial population changes due to postharvest practices a more effective crop specific management system can be developed for quality control purposes.

Keywords: microbial biome, controlled atmosphere storage, plant pathogens, biocontrol

INTRODUCTION

Pear fruit has an external protective “epicarp” with a wax-coated protective covering which functions as a barrier against the entry of plant pathogenic microbes (Kalia and Gupta, 2006). Overall the fruit epicarp (carpoplane) represents a rather hostile environment for microbes that must not only be able to survive but also compete with other inhabitants. However naturally occurring microorganisms have the ability to attach, survive, multiply and grow on these surfaces forming part of the natural fruit microbiota (Hanklin and Lacy, 1992; Lima et al., 1992). The nutrient base for microbial growth is mainly provided by exudates and in the case of wounding, wound exudates. Any wounding that appears during the postharvest processing operations will allow the entry of residing microbes and colonisation of the less protected internal soft tissue (Hui, 2006). This would ultimately contribute to a change in the microbe diversity and -composition and would contribute to final fruit decay if there is postharvest pathogenic species present on the carpoplane (van Deventer, 2011). In the case of a large antagonistic population the reverse may be true due to their protective ability against plant pathogens (Janisiewicz et al., 2014).

The carpoplane microflora orginates from inoculum introduced from soil, insects, the air, animals, rain (Beuchat and Ryu, 1997; Beuchat, 2002), or the environment within the tree canopy or the orchard, or later in the packhouse, or during interventions such as harvesting,

transportation, washing or chemical treatment or through equipment or contact surfaces (Thompson, 2008). Generally all these sources or processes have a direct effect on the microbial diversity as some microbes are better adapted to survive the adverse environmental conditions presented to them throughout the postharvest system (Kubo et al., 2012).

Normal fungal microflora on pear fruit surfaces includes species such as *Rhizopus*, *Aspergillus*, *Penicillium*, *Eurotium*, and *Wallemia*, while the predominant yeasts include species such as *Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora*, *Candida*, *Debaryomyces* and *Pichia* (Hui, 2006). Within the postharvest environment *Penicillium* spp. remains the dominant postharvest pathogen on pome fruit (Andersen et al., 2004; Louw and Korsten, 2014). On pear fruit *Penicillium expansum* Link, *P. crustosum* Thom, and *P. solitum* Westling have been described as the most important pathogens causing decay (Louw and Korsten, 2014).

Knowledge of the biodiversity and ecological function of yeasts is limited compared to those of other microorganisms (Herrera and Pozo, 2010). The known ecological functions of yeasts include: the production of antifungal metabolites that kill or inhibit pathogenic fungi, the production of extracellular polysaccharides, which aids and enhances their survivability and restrict the growth of pathogens and their ability to utilize nutrients rapidly and proliferate at a faster rate than pathogens (Little and Currie, 2008).

Within specific habitats yeast species face numerous challenges such as extreme environmental conditions (Lachance, 1990; Sui et al., 2015). These factors will eventually determine the inclusion, persistence and relative abundance of a particular yeast species in that habitat. To persist and become a local resident on the fruit carpoplane the organism must be able to be deposited, attach, be capable of growing, colonising and successfully compete and survive to ensure further dispersal (Kurtzman et al., 2011; Sui et al., 2015).

The aim of this study was to determine the effect of key commercial postharvest processing stages on the yeast and fungal microflora on pears to determine the biocontrol or decay potential within this microbiota. This information will provide some baseline data for future disease control strategies.

MATERIALS AND METHODS

Sites and process flow

Pears were collected from four Global G.A.P. accredited commercial pear production farms near Grabouw in the Western Cape, South Africa. After harvesting in the orchards the pears are transported in crates on trucks to a central packhouse (within a 30 km radius). At the packhouse the pears are drenched once with water containing 75 ppm chlorine. The chlorine drench water is managed according to standard commercial practices according to pome fruit postharvest guidelines and the pH adjusted to 6.5-7.7 as required in order to provide high concentrations of microbicidal hypochlorous acid. After drenching the pears are stored in controlled atmosphere (CA) for 12 weeks, at oxygen levels set at 2–5 kPa, carbon dioxide levels at 2–5 kPa and temperature set at −0.5 °C (with variation at front and back of the room 0.2 °C to −0.7 °C), and subsequently exported.

Sample Collection

Pyrus communis cv Packhams Triumph pears were collected at harvest in the orchards of the four farms, after harvest following chlorine drenching at the communal packhouse as well as after CA storage for two consecutive seasons during 2013 and 2014. During sampling environmental conditions (i.e. temperature and humidity) were recorded in the orchards. Twenty fruit in total were collected in a random selection strategy from five trees in four rows (regarded as replicates) per orchard for each of the four farms. After chlorine drenching at the packhouse five pears each were sampled randomly from four crates (five pears, four replicates, n = 20) for each farm. Similarly, following CA storage five pears each were sampled randomly from four bulk bins (five pears each, 4 replicates, n = 20) for each farm. Sampled pear fruit were placed in labelled brown paper bags and kept in cold storage (±5 °C) until shipment for laboratory analysis within 24 h.

Sample processing

Individual pear samples (five pears each) were placed in 500 mL of 0.25× Ringer's solution amended with 0.02% Tween 80 (Sigma, Johannesburg) and sonicated in an ultrasonic bath (EUmax®, Labotec., Johannesburg) for 5 min to facilitate detachment from the pear fruit surfaces. Volume displacement (vd) was also recorded for each of the fruit and converted to area (cm^2) using the following equation ($A = 4.84 [(vd)^{1/3}]^2$ (De Jager, 1999 ; Collignon and Korsten, 2010). Wash liquid from the sonicated samples were then filtered through a sterile nitrocellulose membrane with a pore size of 0.45 μm (Sartorius Stedim, Biotech, Germany). The filters were aseptically cut into smaller pieces, added to 9 mL sterilized peptone buffered water (Biolab Diagnostics, Johannesburg) and vortexed to remove the organisms from the filter membrane. Ten-fold serial dilutions of each sample was prepared, a 100 μL of each dilution plated in duplicate onto malt extract agar (MEA) (Merck, Johannesburg) and incubated at 25 °C for five days. Filametous fungal and yeast population counts were recorded and data was converted to $\log_{10}(x + 1)$ CFU/cm^2.

Isolation of filamentous fungi and yeasts from pear fruit surfaces

Filamentous fungi and yeasts colonies were isolated randomly from MEA culture plates based on different phenotypic characteristics and prevalence as described by Janisiewicz et al. (2014). Isolated colonies were purified by triple streaking, preserved in 15% (yeasts) and 10% (filamentous fungi) glycerol (Merck, Johannesburg) and stored at −70 °C.

Molecular identification of filamentous fungal and yeast isolates

The purified filamentous fungi and yeast isolates were cultured on MEA plates and the DNA extracted using the ZR fungi/bacterial DNA miniprep™ kit (Zymo Research, USA) according to the manufacturer's protocol. The DNA concentration of individual isolates was determined using the Qubit® 2.0 fluorometer (Invitrogen, Life Technologies, USA). Each PCR reaction contained 15–20 ng/μL template DNA, 10 μM of each primer, 5U/μL MyTaq™ DNA Polymerase (Bioline, USA), 5× MyTaq™ reaction buffer (Bioline, USA, containing dNTPs, $MgCl_2$, stabilizers and enhancers), and nuclease free water (Thermo scientific) in a total volume of 25 μL. For fungal and yeast isolate identification, universal primers Internal Transcribed Spacer (ITS) 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for PCR amplification (Fell et al.,

2000). The PCR analysis was performed using a BioRAD T100™ thermal cycler (BioRAD). The PCR conditions were as follows: an initial denaturation of 95 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 57 °C, and 90 s at 72 °C, with a final extension period of 7 min at 72 °C. The amplified PCR products were purified from a 2% (w/v) agarose gel using a Geneclean kit (Zymo Research, California, USA—manufacturer's protocol). The PCR products were directly sequenced with both the forward (ITS1) and reverse (ITS 4) primers using BigDye Terminator v3.1 cycle sequencing on an ABI 3500XL sequencer (Inqaba Biotec, Pretoria, South Africa). The sequences were aligned with CodonCode Aligner 4.2 software and subjected to NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search analysis to confirm isolate identities (≥95% threshold limit). ITS gene sequences were deposited in Genbank and accession numbers were assigned to all isolates.

Phylogenetic analysis of dominant fungal and yeast cultures

Alignments of partial ITS gene sequences of the dominant fungal and yeast isolates were performed using ClustalX2 software (Thompson et al., 1997). Sequences were refined using MEGA6 software to maximize homology (Tamura et al., 2013). Primer sequences and unknowns were excluded and ends were trimmed to create an exact cognate region. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973).

Statistical analysis

Statistical analysis was performed using the log CFU/cm^2 filamentous fungi and yeast counts on pear fruit from the orchards, after drenching at the communal packhouse and after CA storage. The data were subjected to an appropriate analysis of variance (ANOVA) using the farms as main-plot factor, the stages as a sub-plot factor and the repeated measurements over the two seasons as a sub-sub-plot factor (Little and Hills, 1972). The Wilk's test was performed on the standardized residuals to test for variance heterogeneity (Shapiro and Wilk 1965). In cases where there was significant variance heterogeneity, outliers were removed until the residuals had a normal or symmetric distribution (Glass et al., 1972). Student's *t*-LSD (Least significant difference) was calculated at a 5% significance level to compare means of significant source effects. All the above data analysis was performed with SAS version 9.3 statistical software (SAS, 1999).

RESULTS

Temperatures and relative humidity

Climatic conditions differed considerably between season one and two. During season one the temperatures ranged from 26°C to 28°C from 11:00 to 17:00 with an average sunshine temperature of 23°C and an average relative humidity of 68.4%. Night time temperatures averaged at 20.3°C with an average relative humidity of 92.7%. During season two the temperatures ranged from 21°C to 27°C from 11:00 to 17:00 with an average sunshine temperature of 18.8°C and an average relative humidity of 57.4%. Night time temperatures averaged at 10°C with an average relative humidity of 68%.

Viable fungal and yeast counts on pear surfaces

Fungal and yeast counts (Log CFU/cm^2) at the three sampling stages (orchard, after drench and after CA) over two seasons for four farms are presented in Figure 1 and 2. Viable fungal counts ranged between 0.4 log CFU/cm^2 to 3.6 log CFU/cm^2 for all three processing stages during season one. However during season two, the counts were lower ranging from 0 CFU/cm^2 to 1.25 log CFU/cm^2. During season one, the fungal counts on pears were significantly lower after CA storage when compared to counts of orchard pears, however during season two the opposite trend was observed. Yeast counts ranged between 0 and 3.25 log CFU/cm^2 during season one and 0 to 2.9 log CFU/cm^2 during season two. On two of the farms the yeast counts were significantly higher after CA storage compared to the orchard pear counts, while the counts for pears from the remaining two farms were similar during season one. During season two there was a significant increase in counts after CA storage on one of the farms and the other three were similar to counts on the orchard pear surfaces. Drenching led to either an increase or reduction in the fungi and yeast counts, however a definite trend could not be observed.

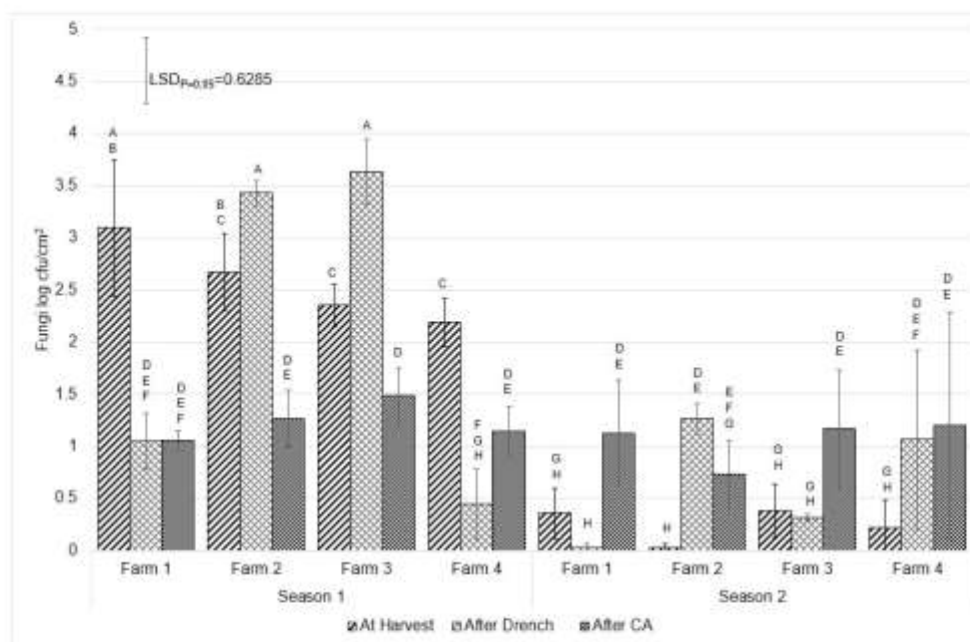

Figure 1. The average Log CFU/cm^2 fungal count on the pear carpoplane at harvest, after drench and after controlled atmosphere storage (twelve weeks, 1.5% O_2; 1.5% CO_2 and -0.5°C) for two seasons. Comparisons were made between treatments from a farm in the same season. Different letters on bars indicate a significant difference at the 0.05 significance level.

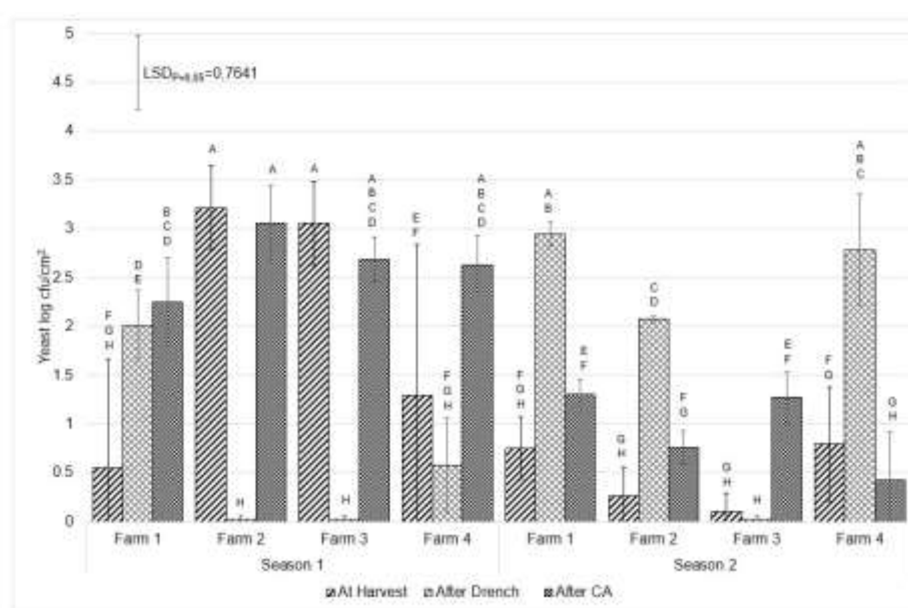

Figure 2. The average Log CFU/cm^2 yeast count on the pear carpoplane at harvest, after drenching and after controlled atmosphere storage (twelve weeks, 1.5% O_2; 1.5% CO_2 and -0.5°C) for two seasons. Comparisons were made between treatments from a farm in the same season. Different letters on graphbars indicate a significant difference at the 0.05 significance level.

Identification of dominant surface isolates

Dominant fungal isolates identified at harvest (orchard), after chemical drench (chlorine - 75 mg L^{-1}) and after CA from the four farms are summarised in Figure 3 and 4. The isolates included plant pathogens and biocontrol organisms (previously described). Some of the pear fruit associated plant pathogens included; *Penicillium brevicompactum* Dierckx, *Penicillium commune* Thom, *Penicillium chrysogenum* Thom and *Penicillium expansum* and were found before and after CA storage (Figure 3). Some organisms survived through chemical drenching and were still present as dominant microorganisms after CA storage, which included potential biocontrol organisms *Aureobasidium pullulans, Cryptococcus* sp. and *Sporobolomyces roseus* (Figure 4). A decrease in species diversity was observed when comparing the dominant fungal and yeast isolates obtained within the orchard, after chemical drench and after CA storage.

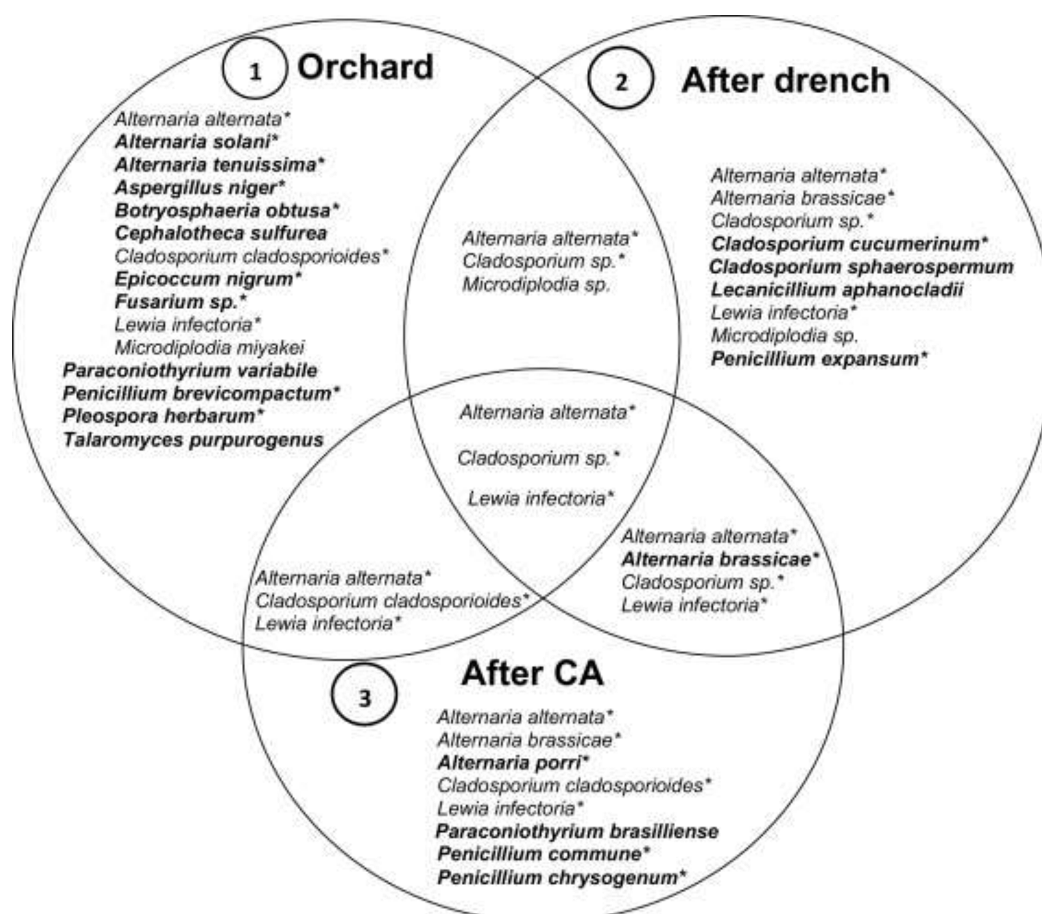

Figure 3. Dominant fungal isolates identified at harvest (orchard), after chemical drench (chlorine - 75 mg L^{-1}) and after CA storage (1.5% O_2, 1.5% CO_2 and -0.5°C) (2013-2014 season). Overlaps between processes are indicated by the arrangements of the circles.

** Host and non-host specific plant pathogenic microbes*

Bold *indicates isolates unique to the corresponding sampling stage*

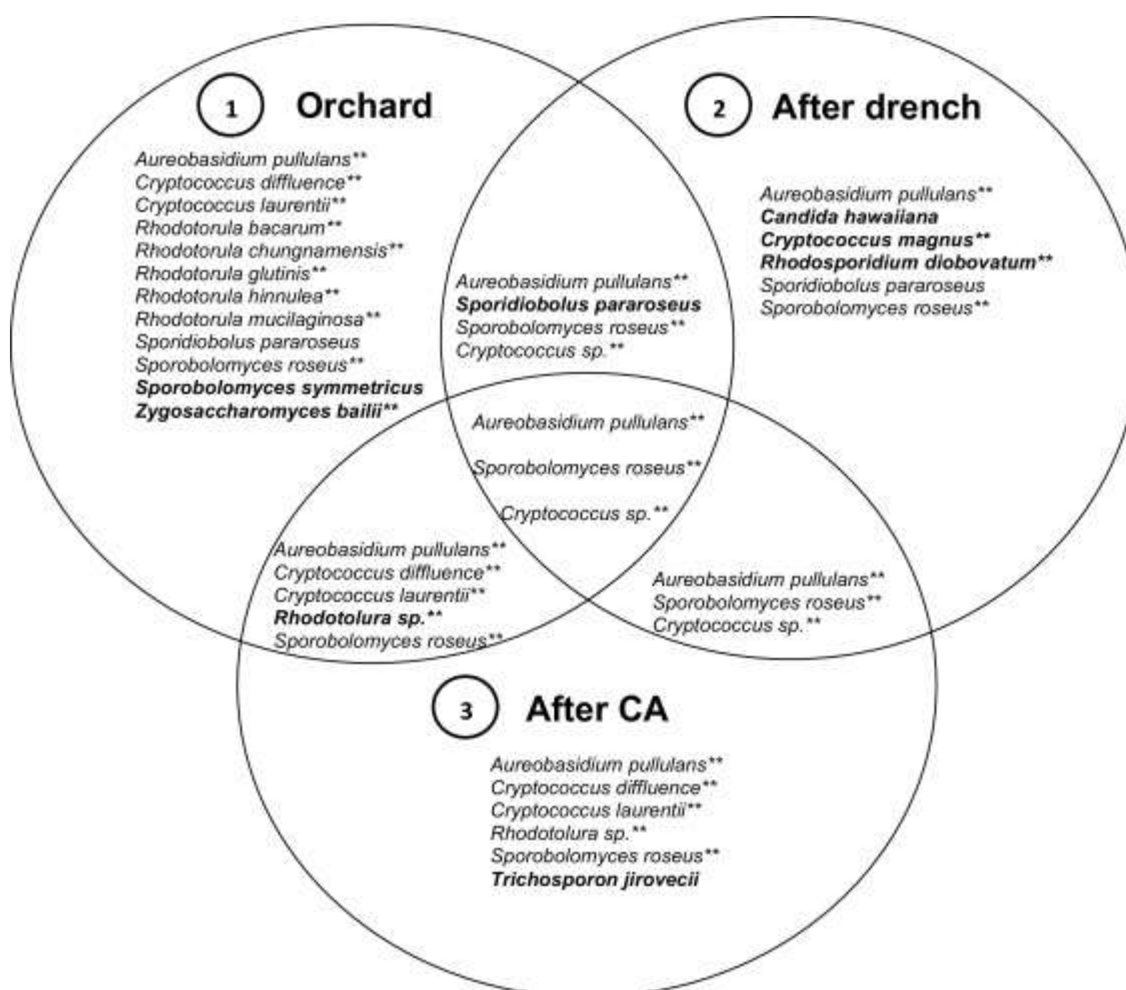

Figure 4. Dominant yeast isolates identified at harvest (orchard), after chemical drench (chlorine - 75 mg L^{-1}) and after CA storage (1.5% O_2, 1.5% CO_2 and -0.5°C) (2013-2014 season). Overlaps between processes are indicated by the arrangements of the circles.

*** Microbes previously identified as a biocontrol organisms*

***Bold** indicates isolates unique to the corresponding sampling stage*

Phylogenetic analysis of dominant isolates

Overall a 76% dominance of Ascomycetes and a 24% dominance of Basidiomycetes at isolate level were observed. The epiphytic fungal isolates grouped into Ascomycota (A), subphylum Pezizomycotina within three classes: Dithideomycetes (B – 77%), Eurotiomycetes (C – 19%) and Sordariomycetes (D – 4%) (Figure 5).

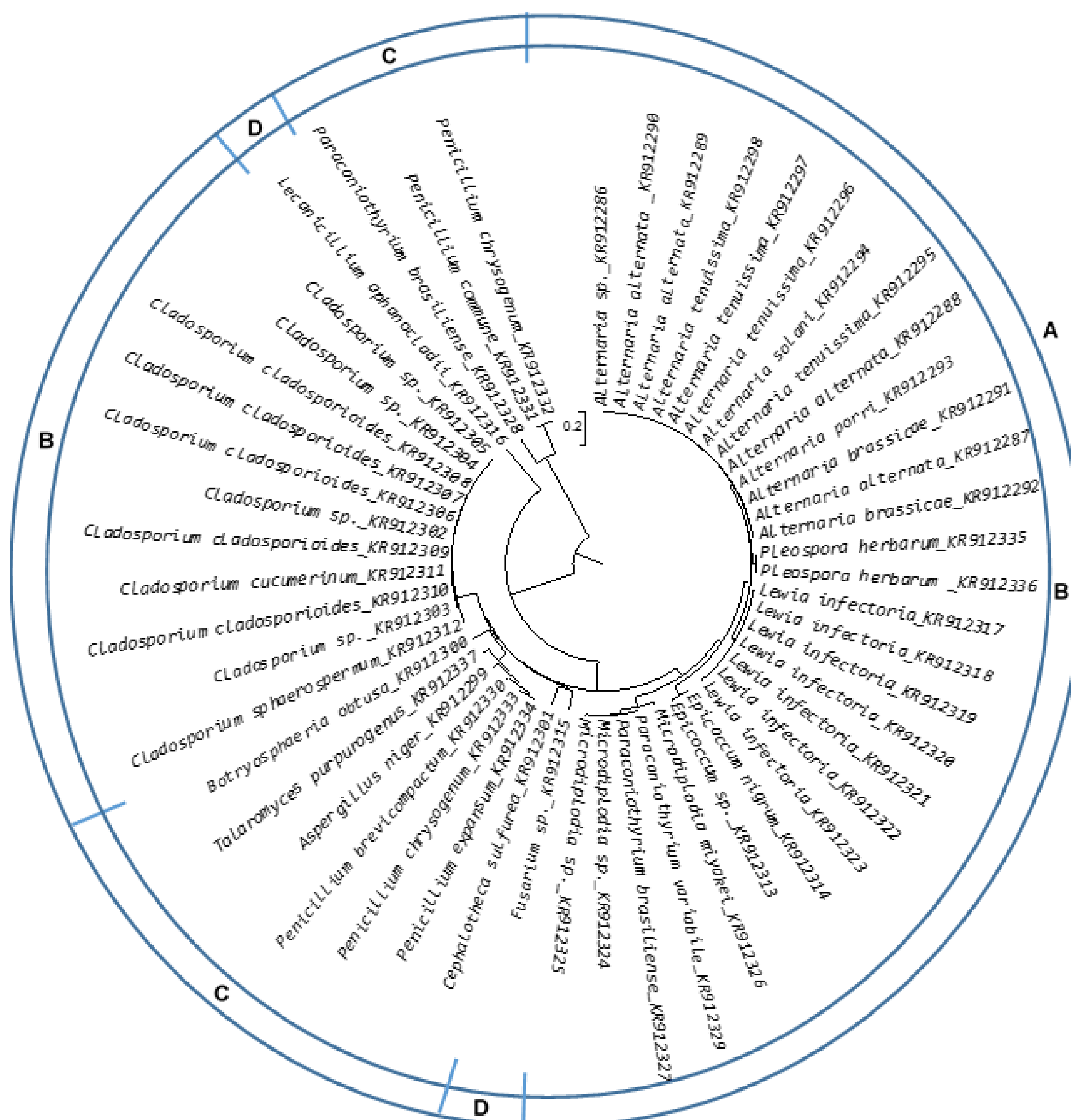

Figure 5. Evolutionary relationships of fungal taxa

*__Ascomycota__ (A) Dithideomycetes (B), Eurotiomycetes (C) and Sordariomycetes (D)

***Accession numbers indicated after isolate name*

The epiphytic yeasts grouped into two phylums; Ascomycota (A), consisting of two subphylums, Euascomycotina (C – 44%) and Saccaromycotina (D – 4%) within two classes: Dothideomycete, Saccharomycetes and Basidiomycota (B), consisting of two subphylums, Agaricomycotina (E – 14%) and Picciniomycotina (F – 38%) within four classes: Microbotryomycete, Tremellomycetes, Urediniomycetes, Ustilaginomycetes (Figure 6).

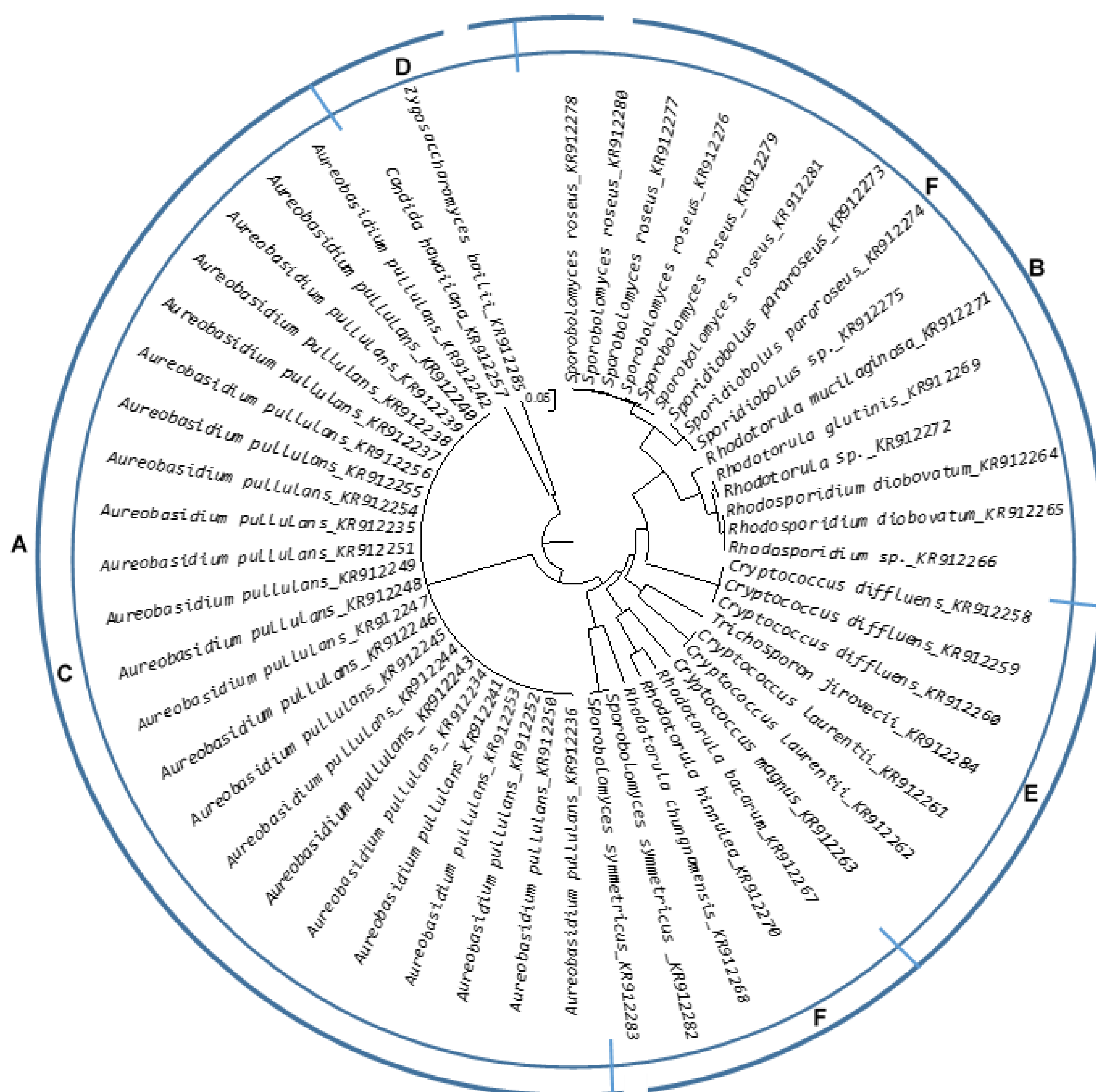

Figure 6. Evolutionary relationships of yeast taxa

***Ascomycota** (A), Euascomycotina (C), Saccaromycotina (D), **Basidiomycota** (B), Agaricomycotina (E) and Picciniomycotina (F)

***Accession numbers indicated after isolate name*

DISCUSSION

The effect of postharvest practices on the diversity and dominance of fungi and yeasts in the pear carpoplane was evident in our study. Other studies reported unique fungal (Buck et al., 2003; Camatti-Sartori et al., 2005; Kalia and Gupta, 2006) and yeast (Janisiewicz et al., 2010; Janisiewicz et al., 2014; Kalia and Gupta, 2006) microflora associated with different fruit types (nectarines, plums, grapes, apples). Most of the dominant fungal and

yeast species isolated within this study have been previously reported on fruit (Janisiewicz et al., 2014; Liu et al., 2013; Louw and Korsten, 2014; Salomão et al., 2014) except for *Cephalotheca sulfurea* and *Paraconiothyrium* spp. These fungi have been isolated from wood and soil, suggesting that this is their primary habitat. Exposure to fruit could thus have happened via wind, dust or vectors (Damm et al., 2008; Hamayun et al., 2012).

Within this study 16 dominant yeast and 24 dominant fungal species were isolated from the pear carpoplane. A 76% dominance of Ascomycetes on pear fruit was found throughout the postharvest processes. Fernandez et al. (2012) observed the same trend in a study done on yeast and yeast-like fungi associated with dry indehiscent fruits. Within their study 17 species of yeasts and yeast-like fungi were isolated from *N. nervosa* fruits, with Ascomycetes reflecting 78% dominance at isolate level and 61% dominance at species level. Their results were attributed to the fact that Ascomycetes are more stress resistant compared to Basidiomycetes, and that Ascomycetous yeast and yeast-like fungi were better adapted to low water availability and desiccation than the latter.

A total yeast and fungal viable count were found to be in the order of 1-5 log CFU/cm^2 which is comparable with the current microbiological guidelines in South Africa for ready-to-eat fresh fruit and vegetables. The Department of Health (DoH) specifies in its guideline that fresh fruit and vegetables (considered raw) should not exceed a 5 log CFU/cm^2 for yeasts and moulds (Department of Health, 2000). In this study pear fruit from all four farms over two seasons did not exceed the maximum limit specified. However, it is important to note that the DoH guideline provide specifications only for ready- to- eat fresh produce. These values should therefore not be directly applied to freshly harvested or packed pears. However it provides some comparative context of what constitutes a healthy pear. Pears are further climacteric fruit and are commercially harvested at the start of the climacteric rise and are considered ripe at the end of this period (Kader, 2002). This concept is the bases of harvesting at the correct maturity stage and for commercial CA storage, with postharvest "ripening" taking place before the product is considered ready-for-consumption. As a separate outcome of this study, we therefore provide scientific evidence that a natural yeast and fungal load of pear fruit at the point of harvest or at the packed stage can be in the order of 1-4 log CFU/cm^2 which is comparable with the ready-to-eat values specified in the national guidelines.

In this study it was found that the yeast and fungal CFU/cm^2 either decreased or increased after drenching, while Spadaro and Gullino (2004) found that the number of isolates obtained from pome fruit were less after washing or storage (1°C for 3 months). The disinfectant calcium hypochloride are usually applied within process water to effectively reduce the microbial load (Gil et al., 2009). Pirovani et al. (2000) indicated that calcium hypochlorite exert a 1 to 2 log population reduction when used in spinach process water. Typically, chlorine is applied at a concentration of no greater than 200 mg L^{-1}, and solutions are adjusted to a pH of 6.5 – 7.7 to provide high concentrations of microbicidal hypochlorous acid (Sapers, 2003). The impact of chlorine washing is evident in this study as the fungal and yeast counts decreased in most of the samples tested.

Comparing the dominant yeast and fungal cultures isolated before and after CA storage a decrease in diversity was evident. A study done by Ahmadi et al. (1999) indicated a similar trend in the reduction of dominant populations after CA storage. Also the dominant cultures which survived CA were found to be less diverse at genus level than those isolated before CA storage. The reason for this could be attributed to the effect of specific storage conditions (low O_2, CO_2 and temperatures) used during CA storage (Ahmadi et al., 1999). Controlled atmosphere storage conditions have been developed to extend the shelf-life of pear fruit. Packhams Triumph pears are stored under optimum conditions for the fruit type which is currently commercially applied at -0.5°C, a CO_2 concentration below 1% and an O_2 concentration of 2%. In this process the impact of altered temperatures on the carpoplane microflora can be significant as reported by Corbo et al. (2004) in studies conducted with cactus pear fruit. Tian et al. (2001) found that the growth of the fungal pathogen *Monilinia fructicola* decreased significantly when grown at a high CO_2 (30%) concentration on sweet cherry fruit. Of the two gases modified in CA storage, CO_2 is the most important one. It has a significant and direct antimicrobial activity due its ability to alter microbial cell membrane functioning (Das et al., 2006).

Aureobasidium pullulans, Cryptococcus spp *and Sporobolomyces roseus* were the dominant species commonly found on fruit from all three sampling stages over two seasons. These yeasts have been isolated from different substrates (phylloplane, carpoplane) and have previously been described as biocontrol agents (Fonseca and Inacio, 2006; Janisiewicz et al., 2010; Castoria et al., 2001; Fan and Tian, 2001; Janisiewicz et al., 1994). As reported for apples *A. pullulans*, *C. laurentii* and *S. roseus* were also found to be dominant on the carpoplane (Castoria et al., 2001; Fan and Tian, 2001; Janisiewicz et al.,

1994). In agreement to our study all three species were isolated and identified as dominant at the time of harvest, before CA as well as after CA. The presence of such epiphytic yeasts on the carpoplane may form a competitive barrier which could prevent the pathogen from colonizing the outer surface of the fruit (Barth et al., 2010; Mandrell et al., 2006).

Known plant pathogens commonly associated with pears have been isolated from the fruit at the time of harvest, after drench and after CA storage. These pathogens included better known organisms such as *P. expansum* and lesser known pathogens such as *P. brevicompactum*, *P. commune* and *P. crysogenum (*Louw and Korsten, 2014). Louw and Korsten (2014) found that Packhams Triumph pears are one of the most susceptible cultivars when inoculated with the more aggressive *P. expansum. Penicillium* species are highly adaptable and is known to survive low temperature conditions such -1°C (Louw and Korsten, 2014).

The presence of postharvest pathogens within the pear processing chain indicates that each step in postharvest handling (drenching or CA storage) can favour the dominance profile of organisms better adapted to survive. An increased understanding of the yeast microbiome and how various postharvest processes affect the dominant populations and the natural biocontrol biome may be targeted to achieve more effective disease control. Future studies should include the evaluation of the antagonistic activities of the yeast biome and compare carpoplane population dynamics of different cultivars.

CONCLUSION

This study describes the occurrence of yeast and fungi present on pears (cultivar Packhams Triumph) and the microbial dynamics of the carpoplane during postharvest handling. This study provides a new perspective on the biocontrol potential of the pear carpoplane in terms of the dominance and ability to survive commercial postharvest practices. The management of antagonistic populations within the postharvest environment may lead to future development of more effective disease control strategies. *Aureobasidium pullulans, Cryptococcus* sp. and *S. roseus* are known dominant antagonistic yeasts and were found to survive CA storage. Known postharvest pathogens *P. commune* and *P. crysogenum* could be detected after CA storage reflecting the persistence of these pathogens under these conditions and the postharvest decay potential in extended supply chains. Future studies

should therefore focus on the antagonistic population- interaction with pathogens in the postharvest environment.

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