Bioaerosol Composition and Associated Hazards in a Prominent Fruit Beverage Production Facility

by

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Declaration

I, Shirleen Mari Theisinger, Identity Number: , (Student Number:), hereby declare that this research project, submitted to the Central University of Technology, Free State, for the Degree Doctor of Philosophy in Environmental Health, is my own independent work. This work complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State and has not been submitted before to any institution by myself or any other person for the attainment of qualification.

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2020

I certify that the above statement is correct.

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Doctor O de Smidt (Promotor)

Dedication

I dedicate this thesis to my remarkable husband. Thank you for all your love, prayers and support.

I am blessed with you in my life.

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Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists	OPS	off-peak season
Act	Legislation	OTU	operational taxonomic unit
BDL	Butanediol	PCA	plate count agar
bp	Base pairs	PCB	polychlorinated biphenyl
BPA	Baird-Parker agar	PCR	polymerase chain reaction
BLAST	Basic Local Alignment Search Tool	PS	peak season
B. cereus	*Bacillus cereus*	PTFE	polytetrafluoroethylene filters
CA	Chloramphenicol Agar	R	reverse
DGGE	Denaturing gradient gel electrophoresis	RBC	Rose Bengal Chloramphenicol agar
DNA	Deoxyribonucleic acid	rRNA	ribosomal Ribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate	SA	South Africa
E. coli	*Escherichia coli*	SANS	System Administration Networking and Security
e.g.	or example	*S. aureus*	*Staphylococcus aureus*
EDTA	Ethylenediaminetetraacetic acid	sp.	specie
etc.	and other similar things	spp.	species
EMBL-EBI	European Bioinformatics Institute	SOP	Standard operating procedure
et al.	and others	TAE	Tris base, Acetic acid and EDTA
F	Forward	TLV	threshold limit value
FAO	Food and Agriculture Organization	TNTC	too numerous to count
FDA	Food and Drug Administration	TPC	total plate count
HCD	Health Centre Data	UPGMA	unweighted pair group method with arithmetic mean
i.e.	in other words	USA	United States of America
IgE-mediated	Immunoglobulin E-mediated	UV	ultraviolet
LOEL	Lowest observed effect level	VRB	Violet Red Bile agar with MUG (4-Methylumbelliferyl-β-D-Glucuronide)
LPS	Lipopolysaccharide	Vs	versus
NA	not applicable	WBGT	wet bulb globe temperature
No.	Number	WHO	World Health Organization
OELs	Occupational exposure limits	XLD	Xylose Lysine Deoxycholate agar

Symbols

°C	Celsius
cells.m^{-3}	cells per square meter
CFU	colony forming unit
Cfu/area	colony forming unit per area
CFU.m^{-3}	colony forming units per cubic meter
CFU.ml^{-1}	colony forming units per millilitre
cu ft. min-1	cubic feet per minute
d	Day
&	And
gDNA	genomic DNA
h	hours
H	Shannon-Weaver diversity index
KCl	Potassium Chloride
ℓ.min^{-1}	litre per minute
M	molar
mM	millimolar
m	metre
mg.m^{-3}	milligram per cubic metre
$MgSO_4$	Magnesium sulphate
m.s^{-1}	metre per second
min	minutes
mℓ	millilitre
mm	millimetre
$(NH_4)_2SO_4$	Ammonium sulphate
ng.m^{-3}	nanogram per cubic metre
P	Pearson's Correlation
®	Trademark symbol
r	Pearson's correlation coefficient
R^2	R squared
Rr	Range weighted richness
s	second/s
SOP	standard operating procedure
Tris-HCL	Tris hydrochloride
™	Trademark symbol
µl	microlitres
µM	micromolar
µm	micrometre
V	Volt
v/v	volume/volume
w/v	weight/volume
@	At
%	percentage
>	more than
~	about
≈	almost equal to

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Abstract

Bioaerosols are defined as aerosols that comprise particles of biological origin or activity that may affect living organisms through infectivity, allergenicity, toxicity, or through pharmacological or other processes. Interest in bioaerosol exposure has increased over the last few decades. This is mainly due to the association of bioaerosols with a wide variety of adverse health effects that have a major public health impact such as contagious infectious diseases, acute toxic effects, allergies, and cancer. Exposure to bioaerosols may cause three major problems in the food industry, namely: (i) contamination of food (spoilage); (ii) allergic reactions in individual consumers; or (iii) infection by means of pathogenic microorganisms present in the aerosol. Unfortunately, there is limited information available with regards to the specific organisms/biological agents involved in these processes and how exactly these processes occur. This deficiency in knowledge can be attributed to a lack of research on these processes, possibly because the importance of bioaerosols has not been considered. Furthermore, international standards on acceptable maximum bioaerosol loads are not uniform, which creates confusion as to what the acceptable limit of microorganisms in bioaerosols should be. There is also a lack of standardised methods for the collection and analysis of bacterial and fungal bioaerosols, making it difficult to compare the data released by various researchers.

According to the literature, controversy exists regarding: (i) the effect of the environment and season on bioaerosols; (ii) types of sampling procedures; (iii) whether the detection of the culturable fraction of bioaerosols is efficient; and (iv) whether these bioaerosols can in actual fact affect the product or cause occupational health problems. The aim of this study was therefore to address the above-mentioned questions by means of an investigation in a selected fruit juice production facility. The culturable and non-culturable fraction of bioaerosols were collected in this facility by active sampling using SAMPL'AIR LITE (AES Chemunex), a standard bioaerosol sampler, in different areas of the facility during the peak and off-peak seasons.

It is noteworthy that the microbial counts observed during this study were high, with high total microbial counts detected during both sampling periods. This indicates that the air in the selected facility created ideal conditions for all types of bioaerosols. Traces of presumptive positive pathogens as well as yeast and mould were observed in the samples collected from five designated areas. Several environmental factors were analysed, but temperature was the only concrete environmental factor observed in the facility during this study. However, statistical analyses indicated that temperature had no statistically significant effect on the presence of bioaerosols in the facility. More than 380 bioaerosols were detected during culturable identification, with 92 different species confirmed. A unique group of

controversial bioaerosols was identified, ranging from highly probable pathogens such as *Bacillus* spp., *Staphylococcus* spp. and even *Candida* spp. Not only were harmful microorganisms identified in the bioaerosols, but microorganisms that are capable of bio-diesel production, that possess anti-tumour activities and that are capable of post-harvest control were also detected. Data obtained by PCR-DGGE analysis were used to determine the similarity, richness and diversity of the bacterial composition in the different areas of the facility during the two sampling seasons. The highest microbial diversity and richness was obtained in the air of the area where the bottles were filled with the final product and where a large number of personnel was present.

Data obtained during this study indicated high microbial counts and species diversity in the air of this specific production facility. Even though this does raise concern, it is important to note that the dose-relationship of microorganisms, even for pathogenic agents, has not yet been established. This is mainly due to a lack of valid methods to qualitatively assess exposure. It is therefore clear that there is still a need for the development of an environment/sample/facility sampler for bioaerosols in order to facilitate the immediate evaluation of the specific health risks associated with a specific industry. The information obtained by means of this study will be useful to address this gap in knowledge and will aid the fruit juice industry to better understand and control bioaerosols in their facilities. This may also relate to other industries where it is necessary for more specific, valid risk assessments and control of bioaerosols in order to ensure product and occupational health safety.

1.1. Background and Rationale

Food safety is and will remain one of the most important factors to consider in the food industry. In light of the recent outbreak of Listeriosis in the food industry, public perception of food safety will continue to grow in importance, making this an aspect that will influence the food industry on all levels − from reputation to profitability. Food safety, by definition, refers to the assurance that food will not cause harm (chemically, biologically or physically) to the consumer when prepared, used or consumed according to its intended use. Although there are various food industries in South Africa, the fruit juice production and distribution industry is a key economic booster because the best fruits and fruit juices are destined for export (Falquera & Ibarz, 2014). In 2015, South Africa exported more than 133 000 tons of fruit juice. Despite the recorded increase in volume and value, consumers have not been compromising on product quality and demand safe food (South African Revenue Service, 2016).

In recent years, focus has shifted towards the presence of bioaerosols in food production facilities; however, there has unfortunately been a paucity of research on this topic. Aerobiology is one field that has received ample attention. Aerobiology studies the identity, behaviour, movement and survival of airborne organic particles that are passively transported in the atmosphere. Aerobiology seeks to understand interactions between biological aerosols and the atmosphere and includes the role of weather and climate in what has been described as the aerobiology pathway. The impact of aerobiology is especially notable in diverse basic applied sciences such as biological pollution, biodiversity studies, ecology, plant pathology, microbiology, indoor air quality, biological weathering, and industrial aerobiology (Beggs *et al*., 2017; Despres *et al*., 2012).

Bioaerosols are defined as aerosols that contain particles of biological origin or activity that may affect living organisms through infectivity, allergenicity, toxicity, and pharmacological or other processes (Hirst, 1995; Shale & Lues, 2007). Epidemiological and toxicological studies have shown a close association between exposure to bioaerosols and many adverse health effects, such as infectious diseases, acute toxic effects, allergies and cancer (Li *et al*., 2017; Wang *et al*., 2015). Interest in bioaerosol exposure has increased over the last few decades and this can mainly be attributed to the association of exposure with a wide range of adverse health effects and major public health impacts. Therefore, exposure to bioaerosols is a crucial occupational and environmental health issue that warrants closer attention. Current research suggests that exposure to bioaerosols may cause three major problems in the food industry, namely: (i) contaminating food (spoilage); (ii) causing allergic reactions in individual consumers; and/or (iii) causing infection by means of pathogenic microorganisms present in the aerosol (Kim *et al*., 2018; Yoo *et al*., 2017). To date, studies performed on bioaerosols

have primarily focused on the chemical and biological composition of these compounds (Adams *et al*., 2015).

It is speculated that the role of microbes in atmospheric processes is species-specific and potentially depends on cell viability (Yoo *et al*., 2017), but little is known about the composition of bioaerosols in the food industry and how it varies between locations and/or climate conditions. Airborne microorganisms are very difficult to assess accurately in the field due to factors such as the collection efficiency of the selected sampler, variations in the robustness of different species of microorganisms, and the difficulty of differentiating strains of the same species (Adhikari *et al*., 2010). Limited knowledge pertaining to the specific organisms involved in these processes is available. This gap can be attributed to non-uniform international standards on acceptable maximum bioaerosol loads and the lack of standardised methods for the collection and analysis of bacterial and fungal bioaerosols. Bioaerosol monitoring is an area of interest that is rapidly emerging in industrial hygiene. Research that has focused on the composition of bioaerosols in various industries, including the food industry, and that has determined the hazards associated with these compounds, was of particular interest in the current study.

1.2. Problem Statement

Food production facilities often devote ample resources towards ensuring and monitoring the microbial safety of their products through on-site testing of the product and the production environment. The specific fruit juice production facility, that was the study site, strives to produce a product that is 100% contamination, spoilage and allergy free. It is therefore of the utmost importance for this facility to detect all possible origins of possible contamination and to eliminate them. However, a few layout and design weaknesses may influence their success rate. For example, the facility has no barriers between clean/unclean areas and no air flow in accordance with the product flow, and these may possibly contribute to the spreading of bioaerosols. All three production lines in the facility produce different types of products; however, the lines are located in the same area and this implies that the contamination of one product could affect another. Furthermore, this plant is not a closed and controlled facility. The temperature inside the facility is not regulated and the origin of bioaerosols may vary, rendering the control thereof problematic. Prior to the actual study, it was discovered that, other than heterotrophic plate counts using a passive sampling method, no information existed about the composition of bioaerosols in this facility.

At the conception of this study, cognisance was taken of the fact that controversy exists regarding: (i) the effect of the environment and season on bioaerosols; (ii) types of sampling procedures; (iii) whether the detection method of the culturable fraction of bioaerosols is efficient (or whether there is a clear need for the detection of the non-culturable fraction); and (iv) whether these bioaerosols really could affect the product or impact the occupational health of personnel.

1.3. Aims and Objectives

The aim of this study was to address some of the above-mentioned questions in the selected fruit juice production facility by monitoring and comprehensively characterising the composition of bioaerosols sampled in different production areas. In order to achieve this aim, the objectives of the study were to: (i) conduct a bioaerosol survey of the culturable fraction of the bioaerosols during peak and off-peak seasons; (ii) determine whether the environment affected the growth of organisms in the bioaerosols in this specific manufacturing industry using statistical analyses; (iii) characterise the culturable and non-culturable fraction of the bioaerosols by using 16S and 26S rDNA PCR-sequencing and PCR-DGGE analysis respectively; (iv) categorise the culturable bioaerosols as harmful, innocuous or even useful; and, where possible, (v) compare the data obtained for culturable and non-culturable bioaerosols.

1.4. Chapter Layout

Chapter 1: Background to the study

This chapter provides a brief background to the study.

Chapter 2: Review of Related Literature

The importance of food safety in the fruit juice industry and the prevalence of related disease outbreaks are discussed. Laws and legislation pertaining to food safety in the South African fruit juice industry are elucidated, and literature related to bioaerosol investigations in the past four decades is reviewed. The relevance of bioaerosols to the food industry is presented in relation to product contamination and risk to food handler health. A comprehensive review of different sampling approaches, methods and complications was conducted to illustrate the lack of standardised methods for collection and analysis of bioaerosol samples. Sections of this chapter were published as a book chapter entitled: “Bioaerosols in the food and beverage industry” in: Ideas and applications toward sample preparation for food and beverage analysis (http://dx.doi.org/10.5772/65587).

Chapter 3: Bioaerosols and related environmental parameters in a prominent fruit juice manufacturing facility

The selected fruit juice industry devotes ample resources towards monitoring and ensuring microbial safety of their products, with on-site testing of these products and the processing environment. The mission of this specific industry is to strive towards a product that is 100% contamination, spoilage and allergen free. During this study most of the culturable fraction of bioaerosols observed were outside specifications for most of the facility during both seasons. Puzzling counts of presumptive coliforms, namely *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. were detected. These are microorganisms that are all capable of developing biofilms on food processing surfaces and they have been associated with foodborne disease outbreaks before. As no temperature control was observed in the facility, it is argued that the recorded temperature was ideal for bioaerosol growth. Furthermore, almost no airflow or ventilation systems were observed, and this may have had either a positive or negative impact on the products produced in the facility.

Chapter 4: Enumeration, classification and categorisation of culturable bioaerosols in the fruit juice manufacturing plant

The effect of bioaerosols on products and food handlers in the food industry remains controversial. The prevalence of organisms in the air depends on the nature of the industry, the facility, the capacity of the facility, as well as the season and the external environment (such as the location of nearby facilities). Unfortunately, information regarding the types of bioaerosols and their effects is not abundant. Based on the data that were obtained, the culturable fraction of the bioaerosols that were identified could be characterised into three main groups, namely: 27 innocuous, 26 useful, and 39 harmful bioaerosols. This study demonstrated that all types of culturable airborne microorganisms occurred ubiquitously and were naturally part of the air environment of this facility.

Chapter 5: Culture independent analyses of fruit juice bioaerosol microbiome

Microorganisms may lose the ability to grow (i.e., to be cultured) during the sampling process due to the damaging of cells during sampling, microbial competition, and unfavourable growth conditions. There is a risk that the inability of microorganisms to grow (or to be cultured) may be incorrectly attributed to underperforming bioaerosol samples, and this may result in underestimating their efficiency or impact. Culture-independent analysis enables the examination of culturable as well as non-culturable bioaerosols, viable and dead cells, and plant and animal fragments. Against this background, the bacterial community structure was analysed using the PCR-DGGE method. Cluster, OTU, range

weighted richness and the Shannon-Weaver diversity index were used to determine the richness and diversity of the bioaerosols. The PCR-DGGE results indicated that the diversity of the detected bacteria was moderately distributed. Three samples were significant: (i) Area 4 during peak season; (ii) Area 4 during off-peak season; and (ii) Area 5 during off-peak season. The data that are discussed in this chapter indicate a clear need to establish the relationship between culture-dependent and culture-independent approaches when studying bacterial diversity in bioaerosols.

Chapter 6: Conclusion

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CHAPTER 2

A SYNOPSIS OF MICROBIAL HAZARDS IN A PROMINENT SOUTH AFRICAN FRUIT JUICE MANUFACTURING FACILITY: A REVIEW

This chapter was partially published with the title: Ideas and applications towards sample preparation for food and beverage analysis. Available at: http://dx.doi.org/10.5772/65587

2.1. Introduction

Microbes are ubiquitous in the environment and play key functional roles in nearly all ecosystems (Jaenicke, 2005). Environmental bacteria, fungi and viruses are part of our natural environment as they co-evolved with other living organisms, including humans. Airborne dissemination is a natural and necessary part of the life cycle of many microbes (Morris *et al*., 2008). Bioaerosols originate from all types of environments such as the atmosphere, soil, freshwater sources, and oceans. Their dispersal into the air is temporarily and spatially variable. Recently, the prevalence of bioaerosols has emerged as they are important yet poorly understood agents in atmospheric processes. Research on bioaerosols has experienced, and continues to experience, stellar growth (Basinas *et al*., 2014).

In 1861, the first measurement of airborne microbes was reported by Louis Pasteur in the journal *Annales des Sciences Naturelles* (Pasteur, 1861). A century later, research into the role of bioaerosols in occupation-related diseases mainly focused on non-infectious diseases. Pepys and co-workers (1963) first demonstrated that patients with existing diseases were more likely to suffer attacks of farmer's lung when inhaling spores from thermophilic Actinomycetes. Byssinosis among cotton workers was also an important research topic during the 1970-80s. The most likely causative agents for this disease were Gram-negative bacteria and the endotoxins located in their outer cell wall (Rylander, 1981). Interest in bioaerosol exposure has increased over the last few decades, and this is largely due to the direct association of bioaerosols with a wide range of adverse health effects (Kim *et al*., 2018). These effects can have major public health impacts such as contagious infectious diseases, acute toxic effects, allergies, and cancer (Yoo *et al*., 2017). Furthermore, bioaerosols could potentially settle on surfaces and equipment and contribute to safety or spoilage risks where food is prepared, processed or packaged (Sutton, 2004).

Due to the presence of excessive quantities of organic matter, the release of bioaerosols can be very high in certain industrial sectors such as in agriculture, all types of food industries, waste management facilities, and textile and wood industries. Each bioaerosol sample is unique as the composition varies in time and space (e.g., abundance and diversity of species and quantity of pro-inflammatory components). This often leads not only to a high variation among samples from the same workplace (which can be due to external factors), but also to the dynamic evolution of the colonised substrate and the fast multiplication rate of many microbes.

This literature review elucidates bioaerosol composition, the relevance of bioaerosols to food processing facilities (especially in the fruit juice industry), approaches to and complications associated with the detection of bioaerosols, and approaches to the sampling of bioaerosols in industrial settings.

2.2. The Fruit Juice Industry

2.2.1. The history of fruit juice manufacturing

According to Wolf *et al.* (2007), the first 'modern' fruit juice that was mass produced was lemonade. This process was devised in the Middle East and products were imported to Italy during the sixteenth century. In the eighteenth century, James Lind discovered that citrus fruit was useful for the prevention of scurvy. More than a hundred years later, the Merchant Shipping Act of 1867 made it compulsory for British vessels to carry citrus juice on ocean voyages. However, the journey of the industrial-scale production of fruit juice only started in 1869 when Thomas Welch began bottling unfermented Concord grape juice in Vineland (New Jersey) by applying the principle of heat sterilization (Brown *et al.,* 1993). Aseptic processing was introduced and commercialised on a large scale only in the early 1970s, which was an essential breakthrough that allowed the fruit juice market to expand worldwide, thereby ensuring the safety of juices and reducing the production and marketing expenses thereof (Morris, 2010).

The Codex Alimentarius (FAO/WHO, 2005) defines fruit juice as "the unfermented, but fermentable liquid obtained from the edible part of sound, appropriately mature and fresh fruit or of fruit maintained in sound conditions by suitable means". In addition, the Codex also states that juice must be prepared by suitable processes "that maintain the essential physical, chemical, organoleptic and nutritional properties of the juice of the fruit from which it comes" (FAO/WHO, 2005).

2.2.2. Foodborne diseases associated with fruit juice

Between 1974 and 2015, numerous outbreaks of illnesses associated with unpasteurised fruit juice and cider were reported worldwide (Table 2.1) (Callejon *et al*., 2015; Danyluk *et al*., 2010; Mihajlovic *et al*., 2013; Park *et al*., 2017). However, no such outbreaks were reported between 2015 and 2019. Eighteen of the reported outbreaks were associated with apple juice or cider, fifteen implicated orange juice, three were thought to be from mixed fruit juice, and the remainder implicated other types of fruit such as watermelon, mamey, sugarcane, and guava.

Table 2.1: Recorded outbreaks of foodborne diseases in humans due to microorganisms traced to fruit juice during the period 1974 to 2019

Year	Pathogen	No. of cases	Vehicle	Location	Comments
1974	*Salmonella typhimurium*	296	Apple cider	New Jersey, USA	Manure used as fertiliser; drop apples
1980	Most likely *Escherichia coli* O157:H7	14	Apple cider	Toronto, Ontario, Canada	Not reported
1989	*Salmonella typhi*	69	Orange juice	USA	Hotel
1991	*Escherichia coli* O157:H7	23	Apple cider	Massachusetts, USA	Drop apples; no washing; cattle raised in vicinity
1991	Norwalk-like virus	3053	Orange juice	Australia	Food served in flight by an airline
1992	Enterotoxigenic *Escherichia coli*	6	Orange juice	India	Roadside vendors selling freshly squeezed orange juice
1993	*Cryptosporidium* spp.	160	Apple cider	Maine, USA	Drop apples
1993	*Salmonella* spp.	18	Water-melon juice	Florida, USA	Homemade watermelon juice
1995	*Salmonella hartford, Salmonella gaminara* and *Salmonella rubislaw*	63	Orange juice	Florida theme park, USA	Local processing plant production for large Florida theme park; inadequately sanitised processing equipment; unclean facility

Year	Pathogen	No. of cases	Vehicle	Location	Comments
1995	*Shigella flexneri*	14	Orange juice	South Africa	Contamination of the hands of staff squeezing the oranges to make juice
1996	*Escherichia coli* O157:H7	14	Apple cider	Connecticut, USA	Drop apples
1996	*Escherichia coli* O157:H7	6	Apple cider	Washington State, USA	Juice for local church event from local orchard; apples were washed in a chloride solution
1996	*Cryptosporidium parvum*	20 confirmed, 11 suspected	Apple cider	New York, HCD	Drop apples; orchard adjacent to dairy farm
1996	*Escherichia coli* O157:H7	70	Apple cider	Western USA; British Columbia, Canada	Drop apples; improper use of sanitizers; deer and cattle in close proximity; distribution through fresh juice shakers and energy bars
1996	Virus suspected	2	Orange juice	USA	Food service
1997	*Escherichia coli* O157:H7	6	Apple cider	Indiana State, USA	All cases visited a local apple orchard and cider pressing operation
1998	*Escherichia coli* O157:H7	14	Apple cider	Southwestern Ontario, Canada	Origin four trees, some in a cattle pasture; drop apples used; apples not washed; distribution to family and friends
1999	*Salmonella typhimurium*	500	Orange juice	South Australia	Oranges source of contamination

Year	Pathogen	No. of cases	Vehicle	Location	Comments
1999	*Salmonella muenchen*	200	Orange juice	14 states in USA and two provinces in Canada (British Columbia and Alberta)	Juice distributed in frozen and liquid form for commercial use in restaurants and hotels; products included 'smoothies'; detected in samples taken from blenders and dispensers.
1999	*Salmonella anatum*	4	Orange juice	Sarasota County, Florida, USA	Contamination most likely occurred during the manufacturing process
1999	*Escherichia coli* O157:H7	7	Apple cider	Tulsa, Oklahoma, USA	Contamination most likely occurred at apple orchard or cider-pressing operation
1999	*Salmonella typhimurium*	16	Mamey frozen puree	Florida, USA	Imported from Guatemala and Honduras
2000	*Salmonella spp.*	14	Orange juice	Colorado, California, Nevada, USA	Unpasteurised citrus products produced by a juice company in California
2000	*Salmonella enteritidis*	88	Orange juice	USA (6 states)	Distributed through retail and food services
2002	*Shigella sonnei*	78	Mixed fruit	Canada, USA, UK, British West Indies	Holiday Resort
2003	*Cryptosporidium parvum*	144	Apple cider	Ohio, USA	Ozone treatment was insufficient to inactivate pathogens
2004	*Escherichia coli* O111 and *Cryptosporidium parvum*	213	Apple cider	New York, USA	Retail establishment

Year	Pathogen	No. of cases	Vehicle	Location	Comments
2004	Hepatitis A	351	Orange juice	Egypt	Juice contaminated during manufacturing process
2005	*Escherichia coli* O157:H7	4	Apple cider	Ontario, Canada	Juice produced and sold at a small local retail outlet
2005	*Trypanosoma cruzi*	25	Sugarcane juice	Brazil	Juice sold at a roadside kiosk; infected triatomine bugs and opossums were found in and around the kiosk
2005	*Trypanosoma cruzi*	27	Apple juice	Brazil	All cases consumed juice from a single sales outlet
2005	*Salmonella typhimurium* and *Salmonella saintpaul*	157	Orange juice	Multistate, USA	'Freshly squeezed' orange juice; outbreak was identified in 24 states
2006	*Trypanosoma cruzi*	94	Mixed fruit	Brazil	Not reported
2007	*Trypanosoma cruzi*	103	Guava juice	Venezuela	Outbreaks occurred at a school in Caracas; juice may have become contaminated with triatomine bugs during overnight storage outside
2007	*Escherichia coli* O157:H7	9	Apple cider	Massachusetts, USA	Not reported
2007	Hepatitis A	3	Mixed fruit	USA	Not reported

Year	Pathogen	No. of cases	Vehicle	Location	Comments
2008	*Salmonella panama*	15	Orange juice	The Netherlands	The causative *Salmonella* strain was able to survive under low pH conditions, such as those in the human stomach
2008	*Escherichia coli* O157:H7	7	Apple cider	Iowa, USA	Fair/festival; cider purchased from a temporary booth
2009	*Norovirus*	10	Orange juice	Connecticut, USA	Not reported
2009	*Norovirus*	189	Lemonade	Illinois, USA	Not reported
2010	*Escherichia coli* O157:H7	37	Fruit juice compote	Winnipeg, Canada	Not reported
2010	*Escherichia coli* O157:H7	7	Apple cider	Maryland, USA	Retail establishment
2010	*Salmonella typhimurium*	9	Mamey, frozen pulp	USA	Retail
2011	*Norovirus*	207	Juice	Georgia, USA	Not reported
2011	*Norovirus*	18	Orange juice	California, USA	Not reported

Year	Pathogen	No. of cases	Vehicle	Location	Comments
2011	*Norovirus*	80	Lemonade	Georgia, USA	Not reported
2011	Unknown	3	Apple cider	Ohio, USA	Not reported
2011	*Cryptosporidium parvum, Escherichia coli* O111:NM	14	Apple cider	Minnesota, USA	Not reported
2011	*Cryptosporidium parvum, Escherichia coli* O111:NM	4	Apple cider	Ohio, USA	Not reported
2011	*Norovirus*	14	Fruit punch	Wisconsin, USA	Not reported
2012	*Escherichia coli* O157:NM (H-)	3	Apple cider	Michigan, USA	Not reported
2013	*Cryptosporidium*	11	Apple cider	Iowa, USA	Not reported
2014	*Escherichia coli* O157	3	Apple cider	Ontario, Canada	Not reported
2015	*Cryptosporidium* and pathogenic *Escherichia coli* (suspected)	30	Apple cider	Illinois, USA	Not reported

Year	Pathogen	No. of cases	Vehicle	Location	Comments
2015	*Escherichia coli* O111	7	Apple cider	California, USA	Not reported

2.2.3. Consumer law and legislation associated with South African fruit juice industries

For a long time, the global food industry focused on the growing demand for food, often regardless of the threat of pathogenic contamination. Nowadays, the global food industry is experiencing a progressive shift towards a more complex system in which quality rather than quantity has become the leading concept. Quality issues may be classified into six groups: nutritive value, organoleptic properties, market trends, effects on health, impact on society, and impact on the environment (Falguera *et al.,* 2012). Emphasis has also shifted to the non-traditional attributes of food as consumers have become concerned about the impact of their decisions on their own health and on the environment (Falguera *et al.,* 2012). Juice manufacturing processes now have to meet the new trends in consumer demands that have led to technical, social, economic, and environmental changes. Fruit juice industries are now obligated to use the best raw materials without (or with the minimum amount of) pesticides and inorganic fertilizers, and therefore they have to develop new processing technologies to maintain the original nutritive and organoleptic value of the fruit (Falquera & Ibarz, 2014). It is important for all fruit juice industries to produce a product that is safe yet can be enjoyed by the consumer. Moreover, the new consumer law encourages microbial and analytic testing of the production facility as well as the fruit juice products they manufacture (South Africa, 2008). Suppliers and distributors of fruit juice therefore spend an enormous amount of money on safety testing which includes testing of equipment, surface swabs, hand swabs, air plates, water, and product testing (South Africa, 2008).

Considering that South Africa's best fruits and fruit juices are distributed to other countries, the fruit juice industry constitutes a major economic boost for the country. However, South African fruit juice manufacturers must constantly be aware of ever-changing national and international regulations, especially those concerning the type of ingredients that may be added as well as labelling specifications (Falquera & Ibarz, 2014). South African fruit juice manufacturers must also constantly challenge themselves to develop new processing technologies that ensure the safety and freshness of the juice whilst adhering to rigorous quality requirements (Falquera & Ibarz, 2014). In this context, food-handling organisations must abide by relevant legal requirements and adhere to both national and international standards for the safe handling of food. For example, these standards provide guidelines for the hygienic design of buildings by describing the necessary requirements for all food industries, including the processing of fruit juices. They also guide the production of safe and wholesome food from specialised raw materials by outlining programmes that address food processing in terms of effective pest and waste control (SANS, 10049).

In industrialised nations and with reference to international trade agreements, every step of juice manufacturing (from the cultivation of the raw materials to the marketing of the end product) is subject to some form of regulatory control. Although some regulations can be onerous, burdensome and may even be seen as unnecessary, there is a definite need to control commercial food items (Bates *et al.,* 2001). According to the regulations in the Foodstuffs, Cosmetics and Disinfectant Act No. 54 of 1972 (R692), no person in South Africa is allowed to sell fruit juice for consumption that has a total viable colony count of more than 10^4 colony forming units (CFU) per 1.0 ml of the product. Further microbial limits include a coliform organism count of $<10^2$ CFU.ml^{-1}, and yeast and mould of $<10^3$ CFU.ml^{-1} with no detectable *E. coli*/1 ml and *Salmonella*/25 ml (South Africa, 1972).

2.3. The Composition of Bioaerosols

An aerosol is a two-phase system of a gaseous phase (air) and particulate matter (dust, pathogens), thus making it an important microbial vehicle. Bioaerosols are defined as “aerosols comprising of particles of biological origin or activity which may affect living things through infectivity, allergenicity, toxicity, [and] pharmacological or other processes” (Hirst, 1995; Shale & Lues, 2007). Bioaerosols are a diverse collection of small pieces of material emitted directly from the biosphere into the atmosphere (Oppliger, 2014).

Bioaerosols are globally ever-present and, in some cases, can dominate suspended particle concentrations. They can be comprised of a diverse selection of particle types, including whole organisms (bacteria, mould, fungi, yeast and algae), reproductive entities (pollen, spores from fungi, bacteria, ferns, and mosses), biopolymers (DNA, chitin, cellulose and other polysaccharides), plant debris, insect parts, and decaying biomass (Kim *et al*., 2018). The components of bioaerosols range in size; e.g., pollens from anemophilous plants have a typical diameter of 17-58 µm, fungal spores are typically 1-30 µm in diameter, bacteria are typically 0.25-8 µm in diameter, and viruses are typically less than 0.3 µm in diameter. Fragments of plants and animals may vary in size. Apart from the fact that bioaerosol particles can span several orders of magnitude in diameter, bacteria may also occur as clusters of cells or may be dispersed into the air on plants or animal fragments, on soil particles, on pollen, or on spores that have become airborne (Shaffer & Lighthart, 1997). All these characteristics contribute to making accurate analyses of bioaerosols very challenging.

2.3.1. Microbial components

Microbes are ubiquitous in nature and are also present in the air as living cells that are able to infect or contaminate the surface or tissue they settle in or upon. These airborne bacterial and fungal cells can

reach concentrations of 10^3 and 10^5 cells.m^{-3} respectively (Yoo *et al*., 2017). Table 2.2 and Table 2.3 list different bacterial and mould genera that have been detected as bioaerosol components in food industries by noteworthy research since 2003. Although only a few results are available for yeast, it is sampled and classified as yeast (Brandl *et al*., 2013; Hameed *et al*., 2010; Sutton, 2004; Zacharski *et al*., 2018). The tables depict only data from food-related industries where microbial components were detected and identified to at least genus level. The tabled research focused on viability testing only (i.e., total plate counts, total mould counts).

Despite the wide diversity that has been detected, not all microbial components have been directly indicated as spoilers or contaminants of food or of being the causative agents of disease due to bioaerosol exposure. Furthermore, not all species in a genus are necessarily harmful (which emphasises the need for using appropriate sampling techniques and identification methods to suit the objective for bioaerosol testing). Although all the microbes present in the air may not be harmful, pathogens in their vegetative state, their spores, toxins, endospores, lipopolysaccharide (LPS) layer and other constituents have been linked to disease and could pose a considerable risk to human health.

Table 2.2: Different bacterial genera detected as bioaerosol components in food processing environments

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
Actinomycetales	Brevibacteriaceae	*Brevibacterium*	Milk Processing	Morris *et al*., 2008
			Abattoir (Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
	Cellulomonadaceae	*Cellulomonas*	Abattoir (Pork)	Sutton, 2004
	Microbacteriaceae	*Curtobacterium*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
		Frigoribacterium	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
		Kocuria	Milk Processing	Morris *et al*., 2008
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
		Microbacterium	Milk Processing	Morris *et al*., 2008
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
	Micrococcaceae	*Arthrobacter*	Milk Processing	Morris *et al*., 2008

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
			Milk Processing	Brandl *et al*., 2013
		Micrococcus	Noodle manufacturing	Jaenicke, 2005
			Milk Processing	Morris *et al*., 2008
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
		Nesterenkonia	Abattoir (Beef/Pork)	Sutton, 2004
	Nocardiaceae	*Rhodococcus*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*	Milk Processing	Morris *et al*., 2008
			Abattoir (Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
		Wautersiella	Milk Processing	Morris *et al*., 2008

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
			Milk Processing	Brandl *et al*., 2013
Bacillales	Bacillaceae	*Bacillus*	Milk Processing	Morris *et al*., 2008
			Food warehouse (storing of rice grains)	Pasteur, 1861
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
		Lysinibacillus	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
	Listeriaceae	*Brochothrix*	Abattoir (Pork)	Sutton, 2004
	Paenibacillaceae	*Paenibacillus*	Abattoir (Beef)	Sutton, 2004
	Staphylococcaceae	*Macrococcus*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
		Staphylococcus	Noodle manufacturing	Jaenicke, 2005
			Milk Processing	Morris *et al*., 2008
			Broiler Chicken Barn	Basinas *et al*., 2014
			Food warehouse (storing of rice grains)	Pasteur, 1861

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
Lactobacillales	Leuconostocaceae	*Leuconostoc*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
	Streptococcaceae	*Streptococcus*	Food warehouse (storing of rice grains)	Pasteur, 1861
Burkholderiales	Oxalobacteraceae	*Massilia*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Caulobacterales	Caulobacteraceae	*Brevundimonas*	Milk Processing	Morris *et al*., 2008
Enterobacteriales	Enterobacteriaceae	*Cedecea*	Abattoir (Beef/Pork)	Sutton, 2004
		Citrobacter	Abattoir (Beef)	Sutton, 2004
		Enterobacter	Food warehouse (storing of rice grains)	Pasteur, 1861
			Abattoir (Beef/Pork)	Sutton, 2004
		Escherichia	Food warehouse (storing of rice grains)	Pasteur, 1861
			Abattoir (Beef/Pork)	Sutton, 2004
		Klebsiella	Abattoir (Pork)	Sutton, 2004

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
		Kluyvera	Abattoir (Beef/Pork)	Sutton, 2004
		Leclercia	Abattoir (Pork)	Sutton, 2004
		Morganella	Abattoir (Beef/Pork)	Sutton, 2004
		Rahnella	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
		Salmonella	Abattoir (Beef/Pork)	Sutton, 2004
		Shigella	Abattoir (Beef/Pork)	Sutton, 2004
	Erwiniaceae	*Pantoea*	Abattoir (Beef/Pork)	Sutton, 2004
	Morganellaceae	*Proteus*	Food warehouse (storing of rice grains)	Pasteur, 1861
	Yersinaceae	*Serratia*	Abattoir (Pork)	Sutton, 2004
Rhodospirillales	Acetobacteraceae	*Roseomonas*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Pseudomonadales	Moraxellaceae	*Acinetobacter*	Milk Processing	Morris *et al*., 2008
			Abattoir (Beef)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013

Bacteria				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
		Moraxella	Milk Processing	Morris *et al*., 2008
			Abattoir (Beef)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
	Pseudomonadaceae	*Chryseomonas*	Abattoir (Beef/pork)	Sutton, 2004
		Flavimonas	Abattoir (Beef/Pork)	Sutton, 2004
		Pseudomonas	Milk Processing	Morris *et al*., 2008
			Food warehouse (storing of rice grains)	Pasteur, 1861
			Abattoir (Beef/Pork)	Sutton, 2004
			Milk Processing	Brandl *et al*., 2013
		Novosphingobium	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	Milk Processing	Morris *et al*., 2008
			Milk Processing	Brandl *et al*., 2013
Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	Abattoir (Beef/Pork)	Sutton, 2004

Table 2.3: Different mould genera detected as bioaerosol components in food processing environments

Mould				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
Capnodiales	Davidiellaceae	*Cladosporium*	Noodle manufacturing	Jaenicke, 2005
			Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017
			Fruit and vegetable processing facility	Bulski *et al*., 2017
	Mycosphaerellaceae	*Cercospora*	Food warehouse (storing of rice grains)	Pasteur, 1861
Eurotiales	Trichocomaceae	*Aspergillus*	Noodle manufacturing	Jaenicke, 2005
			Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017
			Fruit and vegetable processing facility	Bulski *et al*., 2017
		Eurotium	Wheat flour mill	Pepys *et al*., 1963
		Penicillium	Noodle manufacturing	Jaenicke, 2005

Mould				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
			Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017
			Fruit and vegetable processing facility	Bulski *et al*., 2017
Glomerellales	Glomerellaceae	*Colletotrichum*	Food warehouse (storing of rice grains)	Pasteur, 1861
Helotiales	Sclerotiniaceae	*Botrytis*	Food warehouse (storing of rice grains)	Pasteur, 1861
Hypocreales	Hypocreaceae	*Trichoderma*	Food warehouse (storing of rice grains)	Pasteur, 1861
			Cake factory	Yoo *et al*., 2017
	Nectriaceae	*Fusarium*	Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
	Stachybotryaceae	*Stachybotrys*	Food warehouse (storing of rice grains)	Pasteur, 1861
Incertae sedis	Incertae sedis	*Cephalosporium*	Food warehouse (storing of rice grains)	Pasteur, 1861
	Plectosphaerellaceae	*Verticillium*	Food warehouse (storing of rice grains)	Pasteur, 1861
Pleosporales	Pleosporaceae	*Alternaria*	Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017

Mould				
Order	**Family**	**Genus**	**Food Processing Environment**	**Reference**
		Curvularia	Food warehouse (storing of rice grains)	Pasteur, 1861
		Epicoccum	Wheat flour mill	Pepys *et al*., 1963
		Helminthosporium	Food warehouse (storing of rice grains)	Pasteur, 1861
Mortierellales	Mortierellaceae	*Mortierella*	Food warehouse (storing of rice grains)	Pasteur, 1861
Mucorales	Cunninghamellaceae	*Absidia*	Wheat flour mill	Pepys *et al*., 1963
	Mucoraceae	*Mucor*	Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017
		Rhizopus	Food warehouse (storing of rice grains)	Pasteur, 1861
			Wheat flour mill	Pepys *et al*., 1963
			Cake factory	Yoo *et al*., 2017

2.3.1.1. Spores

Bioaerosols generally contain spores that are tougher, metabolically less active and often better adapted to dispersal than other bioaerosol compounds. Spores are single or multicellular units surrounded by a rigid cell wall and each spore is capable of reproducing the entire organism. Certain bacteria can survive adverse environmental conditions for prolonged periods by producing a thick-walled spore structure called an endospore. Endospores function to protect the bacterial DNA against the conditions or substances in the environment that would lead to the destruction of non-endospore forming bacteria (Agranovski, 2011). *Bacillus cereus* is one such spore-forming bacterium that naturally occurs in many foods. *B. cereus* forms spores that are resistant to heating and dehydration and, when food containing *B. cereus* spores are in the 'temperature danger zone', the spores germinate and the bacteria grow and produce toxins that cause illness in humans. *B. cereus* can cause vomiting or diarrhoea and even both, depending on the type of toxin it produces (Zukiewicz-Sobczak, 2013).

Mould spores are somewhat resistant to destruction and they are not usually pathogenic to humans. Epidemiological and experimental studies have supported the fact that *Aspergillus* spp., for example, are highly allergenic moulds. These moulds and their spores are known to cause two allergic diseases of the respiratory system namely bronchial asthma and allergic rhinitis. Spore concentrations of above 50 CFU.m^{-3} have been associated with a high prevalence of 'sick building syndrome' (Kobayashi *et al*., 2009; Mandal & Brandl, 2011).

2.3.1.2. Toxins

Endotoxins are composed of lipopolysaccharides and lipo-oligosaccharides associated with proteins and lipids and are part of the exterior cell membrane of Gram-negative bacteria. Endotoxins consist of components such as a core polysaccharide chain, O-specific polysaccharide side chains (O-antigen), and a lipid component referred to as Lipid A, which is responsible for toxic effects (Kim *et al*., 2018). Endotoxins are either present in the fragments of the cell wall or in the bacterial cell and are released during bacterial lysis. Endotoxins are non-allergenic with strong pro-inflammatory properties. They are present in many occupational environments, ambient air, and house dust (Ruzer & Harley, 2012). Induction of airway inflammation and dysfunction can be attributed to the inhalation of endotoxins (Piriel *et al*., 2003). Endotoxin exposure has been associated with the occurrence of respiratory disorders, including asthma-like symptoms, chronic airway obstruction, bronchitis, increased airway responsiveness, and byssinosis (Madsen, 2006). Unlike moulds, endotoxins have also been recognised as a causative factor in the ethnology of occupational lung diseases, including non-allergic asthma and organic dust toxic syndromes (Douwes *et al*., 2003).

Fungi are also responsible for toxin production. During the nutrient degradation process, fungi release secondary metabolites called mycotoxins. Mycotoxins are toxic fungal metabolites produced by moulds in vegetal matrices and could potentially be detected in bioaerosols due to their adsorption into spores and dust particles (Kim *et al*., 2018; Sorensen *et al*., 1984; Straumfors *et al*., 2010). Mycotoxins are non-volatile compounds and will be found in the air only if the environment in which they are produced is disturbed. These molecules act as a defence mechanism against other microbes, including other fungi. A given fungal species may produce different toxins depending on the substrate and local environmental factors. However, mycotoxins and their associated health effects through respiratory exposure are not well known. One argument is that mycotoxins could be the causal agents of effects following exposure to moulds. Reported symptoms include skin and mucous membrane irritation, nausea, headaches, immune-suppression, and systemic effects such as dizziness and cognitive and neuropsychological dysfunction (Goyer *et al*., 2001; Mandal & Brandl, 2011; Pearson *et al*., 2015).

2.3.1.3. Other components

Other bioaerosol components of microbial origin that are considered non-viable but bioactive may be present in the air. For example, ß-(1-3)-D-glucan is a glucose polymer of high molecular weight found in the cell walls of bacteria, moulds and plants (Goyer *et al*., 2001). They consist of glucose polymers with variable molecular weight and degree of branching (Ruzer & Harley, 2012). ß-(1-3)-D-glucan is associated with a dry cough, cough associated with phlegm, hoarseness and atopy and has been reported as prevalent in indoor environments (Adhikari *et al*., 2010; Richter *et al*., 2015). Some of the components of the cell wall of Gram-positive bacteria consist of peptidoglycans. With the inhalation of Gram-positive bacteria, these peptidoglycans may be potential causative agents of lung inflammation (Goyer *et al*., 2001).

During bacterial growth or cell death, proteins are normally secreted. These are bioactive molecules called exotoxins. Exotoxins are usually associated with infectious diseases such as cholera, tetanus and botulism. However, they can also be found on surfaces that can take on an aerosol form and could support bacterial growth (Goyer *et al*., 2001).

2.4. Bioaerosol Detection: Approaches and Challenges

Bioaerosol monitoring is a rapidly emerging area of interest in industrial hygiene (Jensen, 1998). Measurements include especially microbes in both indoor (e.g., industrial, office or residential) and outdoor (e.g., agricultural and general air quality) environments (Yoo *et al*., 2017). It is necessary to evaluate their presence quantitatively (by a count or a determination) and/or qualitatively (by identifying

the genus and species) (Goyer *et al*., 2001). Each bioaerosol sample is unique as its composition varies in time and space (e.g., abundance and diversity of species and the quantity of inflammatory components such as endotoxins and ß-d-glucans). This often leads not only to high variation among samples from the same workplace (which can occur due to external factors), but also to the dynamic evolution of the colonized substrate and fast multiplication rate of microbes (Oppliger, 2014).

2.4.1. Sampling methodologies

A wide variety of bioaerosol sampling equipment is available; however, no standardised protocols have yet been established. There are two primary methods for microbial air sampling, namely passive and active monitoring. Passive monitoring, also referred to as settle plates or petri plates, requires petri dishes containing agar or Petrifilm™ that are opened and exposed to the air for specified periods of time. Microbes that settle on these plates from the ambient air can then be determined qualitatively. This passive approach offers lengthy sampling periods at low cost, however, it does not take into account air movement or airborne populations per volume of air and may miss critical microbes (Moberg & Kornacki, 2015). Active monitoring requires a microbial air sampler to force air onto or into collection media at a specific rate over a specified time period. This approach is less time consuming, better for areas with low microbial loads, and allows for both quantitative and qualitative analyses. However, vigorous air movement may cause injury to vegetative cells (Kornacki, 2014). Three approaches can be used for active monitoring, namely impaction, impingement and filtration.

Impaction involves the use of an air pump to capture air over the surface of a petri dish containing agar. The airflow over the agar is controlled by slits or holes that are arranged to distribute the airflow evenly over the agar surface. Sampling equipment is easy to use and the consumable costs are relatively low. Drawbacks may include loss of microbial cell viability due to impact stress and loss of recovery efficiency due to the failure of microbes to adhere to agar surfaces. Competition for growth and the influence of selective media choices should also be considered when planning a monitoring strategy (Therkorn & Mainelis, 2013). Impaction is often the preferred active monitoring approach for bioaerosol sampling in the food processing environment. Different sampler options are summarized in Table 2.4.

Table 2.4: Available impaction-based bioaerosol sampling devices

Sampler	Information	Difficulty to Use	Flow Rate	References
Single-Stage Viable Andersen Cascade Impactor	• N6 microbial impactor • Meets the specifications of the latest ACGIH Bioaerosol Committee • EPA, OSHA and FDA referenced • Sharp cut-off diametre of 0.65 µm.	Easy to use	28.3 ℓ. min^{-1}	Burge, 1995; Chao *et al*., 2002; Goyer *et al*., 2001; Kim *et al*., 2010; Li & Hou, 2003; Nunes *et al*., 2005; Scott *et al*., 2011
Two-Stage Viable Andersen Cascade Impactor	• Multi orifice cascade impactor • Whenever size distribution is not required • When only respirable segregation or total counts are needed • 95-100% of viable particles above 0.8 µm	Easy to use	28.3 ℓ. min^{-1}	Zhu et al., 2003
Six-Stage Viable Andersen Cascade Impactor	• Multi-orifice cascade impactor • Measures the concentration and particle size distribution of aerobic bacteria and fungi • Viable particles can be collected on a variety of bacteriological agar • Calibrated to collect all particles (regardless of physical size, shape or density) • Can be directly related to human lung deposition	Easy to use	28.3 ℓ. min^{-1}	Benasconi *et al*., 2010; Dillon *et al*., 1996; Gilbert *et al*., 2010; Gorny, 2004; Gorny *et al*., 2002; Goyer *et al*., 2001; Nasir & Colbeck, 2010; Niesler *et al*., 2010; Wang *et al*., 2010; Zhenqian *et al*., 2013
Mattson Garvin Slit-to-Agar	• Accurate and quantitative • Samples even the smallest of viable particles • Collection on 150 mm x 15 mm disposable culture plate	Self-contained	cu ft. min^{-1}	Goyer *et al*., 2001

Sampler	Information	Difficulty to Use	Flow Rate	References
	• No dilution or plating steps are required • Results are expressed as viable particles per unit of air • Time-concentration relationship may be determined			
SAS Super 180	• Considered the international standard for portable air microbiology sampling • Pharmaceutical, food industry, hospital sector and indoor air quality • Used onboard the International Space Station	Easy to use	60-100 ℓ. min^{-1}	Benasconi et al., 2010; Osimani *et al*., 2013; Therkorn & Mainelis, 2013
Biotest RCS	• Evaluates microbiological quality of ambient air, functionality of air treatment equipment and systems, effectiveness of decontamination measures • Collection on agar media strip	Table 1.Push-button operation; remote control	50 ℓ. min^{-1}	Goyer *et al*., 2001
IOM Sampler	• Reusable two-part filter cassette with specified 25 mm filters • Collection of inhalable airborne particles • Available in conductive plastic or stainless steel • Stainless steel model ideal for sampling vapor-phase isocyanates followed by chemical analysis • Sample culturable and non-culturable • Collection on membrane filters	Difficult to use	2 ℓ. min^{-1}	Lecours *et al*., 2013; Wang *et al*., 2015
SKC BioStage®	• Single stage • Viable cascade impactor	Easy to use	28.3 ℓ. min^{-1}	Goyer *et al*., 2001; Therkorn & Mainelis, 2013; Yao & Mainelis, 2007; Zhenqiang *et al*.,

Sampler	Information	Difficulty to Use	Flow Rate	References
	• Meets NIOSH requirements and ACGIH recommendations • Collection on standard-size agar plates • SureLock positive seal ensures sample integrity			2013
SAMPL'AIR™	• 99% microbial collection rate • High efficiency, even with the smallest particles • Ideal for regular, thorough air quality control	Easy to use Flexibility remote control	100 ℓ. min^{-1}	Therkorn & Mainelis, 2013
MAS-100eco	• Sieve impaction systems • Accurately regulates air flow in real time • Collection media: 90-100 mm Petri dish or 55-60 mm contact plate	Easy to use	100 ℓ. min^{-1}	Brandl *et al*., 2008; Haas *et al*., 2007; Kalogeraski *et al*., 2005; Uztan *et al*., 2010; Zorman & Jersek, 2008
RCS	• Rotary Centrifugal Air Sampler • Lightweight and portable • Collection on agar strips	Easy to use	40 ℓ. min^{-1}	Hargreaves *et al*., 2003

Impingement of microbes in a liquid matrix requires particulate-laden air to accelerate as it is drawn through the cassette's tapered inlet slit from where it is directed towards a small slide containing the collection media. Here the particles become impacted and the air flow continues out of the exit orifice. By using this approach, it is possible to measure both the culturable and non-culturable components of bioaerosols and it is ideal for aero-microbiology studies because the liquid matrix can be divided for various analyses. Sampler options are listed in Table 2.5. However, the collection vials are usually constructed of glass and they can be easily damaged or broken. This approach tends to be expensive and may also present low capture rates, loss of collection fluid to evaporation and violent bubbling, low capture rate of virus-sized particles, and loss of cell viability (Urbano *et al*., 2011).

Table 2.5: Avialable impingement-based bioaerosol sampling devices

Sampler	Information	Ease of Use	Flow Rate	Reference
All-Glass (AGI-30) Impinger	• High velocity impinger • Can be used in heavily contaminated environments • Sampling times up to 30 min (dilute impinge solution prior to use)	Easy to use	12-13 ℓ. min^{-1}	Goyer *et al.*, 2001
Burkard May Impinger	• Since 1966 • Fraction collected gently into liquid where clumps separate into viable units • Little danger of sample overload • Sub-samples permit the use of a variety of culture methods • Particle fraction (> 10 µm, 10-4 µm, < 4 µm)	Difficult to use	20 ℓ. min^{-1}	Goyer *et al.*, 2001
BioSampler®	• Collection time up to 8 hours with sonic-flow Vac-U-Go Sampler • Recommended for: infection control investigation in hospitals and veterinary clinics, biological research, infectious disease investigations in public buildings, and safety concerns in the food handling industry	Easy to use	12.5 ℓ. min^{-1}	Goyer *et al.*, 2001
Air-O-cell® cassette	• Use with any standard off-the-shelf area sampling pump (15 LPM open flow) • Unique design for the rapid collection of a wide range of airborne aerosols including mould spores, pollen, insect parts, skin cell fragments, fibres (e.g., asbestos, fiberglass, cellulose, clothing fibers, etc.) and inorganic particulate, e.g., ceramic, fly ash, copy toner, etc.).	Easy to use	15 ℓ. min^{-1}	Godwin & Batterman, 2007

Sampler	Information	Ease of Use	Flow Rate	Reference
	• Collects both viable and non-viable sample specimens • Direct microscopic analysis can be performed immediately • Collection media compatible with a wide range of biological stains and refractive index oils • Direct quantitative analysis of organic and inorganic particulate possible • Suitable for use in confined or restrictive spaces			
Micro-Orifice Uniform Deposition Impactors™ (MOUDI™)	• 18 µm cut-point inlet stage • Additional stages to size-fractionate aerosol particles: 8-stage (0.18 µm) and 10-stage (0.056 µm)	Difficult to use	30 ℓ. min^{-1}	Urbano *et al*., 2011

Filtration involves pumping air through a porous membrane filter to capture bioaerosols. This method can be used to detect both culturable and non-culturable components and has been proven to be highly efficient in trapping microbes larger than the chosen pore size of the filter surface. It does, however, require expensive sampling equipment and sample processing and data analysis may require a high level of expertise (Chen *et al*., 2010). Available cassettes for the filtration sampling of bioaerosols are listed in Table 2.6.

Table 2.6: Available filtration-based bioaerosol sampling devices

Sampler	Information	Ease of Use	Flow Rate	Reference
Burkard Spore Trap (1,7-Day)	• Particle sizes may range (1-10 µm) • Continuous sampling • Spores are impacted on adhesive coated transparent plastic tape (Melinex) • Sensitive to small changes in wind direction	Reliable and simple operation	10 ℓ. min^{-1}	Goyer *et al*., 2001
Button Aerosol Sampler	• Porous curved-surface inlet • Particle sizes 100 µm	Easy to use	4 ℓ. min^{-1}	Goyer *et al*., 2001; Wang *et al*., 2015
Buck BioAire™ Model B520	• Compact, lightweight, controlled flow sampling pump • Uses Allergenco-D™ or Air-O-Cell™ cassettes • Unattended timed programming • 5 hours of continuous operation	Easy to use	15 ℓ. min^{-1}	Rittenouer *et al*., 2014
Zefon 37 mm clear styrene air sampling cassettes	• Meet all applicable NIOSH, OSHA and EPA air sampling standards	Easy to use	4 ℓ. min^{-1}	Wang *et al*., 2015
NIOSH Personal Bioaerosol Cyclone Sampler	• Tube wall impaction • Third-stage filtering	Convenient Easy to use	4 ℓ. min^{-1}	Lecours *et al*., 2013; Wang *et al*., 2015

2.5. Bioaerosol Relevance in Food Manufacturing

Airborne particles and bioaerosols are easily transported, transferred and displaced from one environment to the other. Complex mixtures of bioaerosols such as fungi, allergens and bacteria, along with non-biological particles (e.g., dust, smoke, particles generated by cooking, organic and inorganic gases) are contained in indoor environments (Hargreaves *et al*., 2003). The bioaerosols and their components could pose an environmental hazard when presented in high concentrations in indoor environments, resulting in spoilage/contamination of food products or occupational health risks (Stetzwnbach *et al*., 2004).

2.5.1. Food product related risks: spoilage or contamination

Even before spoilage becomes obvious, microbes have begun the process of breaking down food molecules for their own metabolic needs, and this results in a variety of sensory cues such as off-colours, off-odours, softening of fruits, and a slimy appearance. First, the sugars (carbohydrates) are easily digested, after which plant pectins are degraded, proteins are attacked, and then volatile compounds with characteristic smells such as amines, ammonia and sulphides are produced. Early detection of food spoilage is advantageous in reducing food loss because various interventions could halt or delay deterioration and decay. Several methods to determine the concentrations of spoilage microbes or the volatile compounds produced by spoilage microbes have been devised. However, many of these methods are considered ineffective as they are time-consuming; may not give constant, reliable results; and are labour-intensive (Goyer *et al*., 2001).

Food can also be contaminated by the presence of harmful chemicals and microbes that can cause illness when consumed. For this reason, traceability (i.e., source determination of contamination) remains a relevant topic in food preservation research (Nerin *et al*., 2016). Bioaerosols implicated in respiratory-associated hazards have received much attention; however, the potential that food-associated microbes and foodborne pathogens in bioaerosols may cause food spoilage needs to be clarified. Evidence exists that pathogenic microbes are found in the air and that these microbes can be present in certain products. However, traceable evidence of bioaerosols as the causative agent of spoilage or contamination of food products is not readily available.

2.5.2. Risks associated with food handlers and occupational health threats

Exposure to a high risk of biological hazards is a characteristic of certain industries such as health care, agriculture, fishery, some food industries, construction, and mining. Workers employed in these industries are known to have a high prevalence of respiratory diseases and airway inflammation (Wang

et al., 2015). It is difficult to conduct a comprehensive evaluation of personal bioaerosol exposure in occupational or indoor environments (Hansen et al., 2012) due to: (i) the complex composition of bioaerosols; (ii) the human dose-response (Brooke *et al*., 2013); and (iii) a lack of standardised sampling/analysis methods (Wang *et al*., 2015). Therefore, without appropriate personal exposure assessment and standardised sampling/analysis methods, establishing dose relationships and relevant exposure guidelines is difficult.

Exposure to bioaerosols in the occupational environment is associated with a wide range of health effects such as infectious diseases, acute toxic effects, allergies, and cancer. For example, several cases of pulmonary cancers have been reported in workers exposed to aflatoxins via the respiratory route (Dvorackova, 1976; Hayes *et al*., 1984). These possibilities have been studied for the last 20 years, with data indicating that in Denmark, for example, an increase in the risk of liver cancer has been reported for workers exposed to aflatoxins while processing livestock feed (Olsen *et al*., 1988). A study by Larsson and co-workers (1988) also indicated that asymptomatic dairy farmers who had been exposed to airborne mould dust had symptoms of immune-stimulation and inflammation in their alveolar space. Thus farmers exposed to mould dust may exhibit signs of alveolitis (Larsson *et al*., 1988) and severe toxic irritative reactions can occur after a single inhalation of high levels of spores (Poulsen *et al*., 1995). Studies have suggested that inhalation exposure to mould spores is another cause of organic dust toxic syndrome (Vogelzang *et al*., 1999).

Occupational biohazards of biological origin are grouped as follows: (i) occupational diseases of the respiratory tract and skin caused by allergenic and/or toxic agents forming bioaerosols; and (ii) agents causing zoonoses and other infectious diseases spread through various exposure vectors (Rim & Lim, 2014).

2.5.2.1. Allergenic and/or toxic agents

A wide range of agents may cause different types of allergies. Substances such as microbial enzymes for food processing (e.g., α-amylase in commercial bakeries) and detergents are potent allergens that can cause asthma and rhinitis (Ruzer & Harley, 2012). Many fungal species detected in bioaerosols in the food industry, for example from the genera *Penicillium*, *Aspergillus* and *Cladosporium* (Chang *et al*., 2001; Lee *et al*., 2006; Ma *et al*., 2015), are responsible for respiratory disease and allergies (Flannigan *et al*., 2001). Fungi produce copious amounts of spores that are easily dispersed in polluted air and dust (Zukiewicz-Sobczak, 2013). The genera *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium* and *Fusarium* are highly prone to cause allergy. Fungal allergy often appears as type I (immediate) IgE-

mediated hypersensitivity. In the case of an allergic reaction, it can manifest as rhinitis or conjunctivitis, asthma, urticarial, or atopic dermatitis. This is called a type II hypersensitivity reaction as is the case in response to the mannan–polysaccharide of the cell wall of *Candida* and *Aspergillus*. An example of type III hypersensitivity is allergic alveolitis and bronchopulmonary aspergillosis. Allergy to *Aspergillus fumigatus* is common in atopic asthma. In a large part of the population, allergies occur in the form of rhinitis, also accompanied by ocular signs (Zukiewicz-Sobczak, 2013). It is estimated that approximately 2-6% of the general population in developed countries is allergic to fungi.

2.5.2.2. Infections

Currently, infectious diseases are regarded as the most frequently occurring occupational diseases. Occupational biohazards are infectious agents or hazardous biological materials that exert harmful effects on workers' health, either directly through infection or indirectly through damage to the working environment. Such materials can include medical waste or samples of a microbe, virus or toxin from a biological source (Rim & Lim, 2014). Most agents responsible for respiratory infections are spread through the air, primarily from person to person (anthroponoses), from animal to person (zoonoses), through the abiotic environment (e.g., soil and water), and by means of decaying plant or animal matter (sapronoses) (Ruzer & Harley, 2012). Inhalation is the most important and efficient route by which infectious agents enter the human body and infections contracted by this route are the most difficult to control. Transmission by air allows an infectious agent to reach a larger number of potential hosts than would be possible if infected individuals had to come into direct contact to transfer microbes from person to person (Ruzer & Harley, 2012).

2.6. Relevant International and National Legislation Associated with Bioaerosols

Insufficient occupational exposure limits (OELs) set by regulatory organisations and the diversity of agents in occupational environments often complicate proper risk assessment of exposure to bioaerosols. Regulatory OELs have been adopted for cotton, grain, wood, flour, organic dust and subtilisins (Table 2.7) (TLVs® BELs®, 2010; LIA, 2011). However, these limits are based on dust levels only and do not take specific components present in the dust into consideration. With the exception of subtilisin, even the OEL for "particulates not otherwise regulated" serves as reference where OELs are not specified (TLVs® BELs®, 2010). Furthermore, scientific evidence for certain set exposure limits, such as ≈100 cells.m^{-3} allowed for fungi and actinomycetes, can be difficult to access (Eduard, 2006; MAC, 1993). In some cases, warnings of the risk of infectious agents and guidance on health surveillance and containment levels are provided (Directive, 2000), but no specific guidelines for

bioaerosol concentration level limits are specified for either infectious or non-infectious biological agents.

Table 2.7: Regulatory occupational exposure limits (OEL) for cotton, grain, wood, flour, organic dust and subtilisin

Agent	ACGIH	Norway
Raw cotton dust	0.2 $mg.m^{-3}$	0.2 $mg.m^{-3}$
Grain dust (oat, wheat, barley)	4 $mg.m^{-3}$	none
Flour dust	0.5 $mg.m^{-3}$	3 $mg.m^{-3}$
Wood dust	0.5 $mg.m^{-3}$	1-2 $mg.m^{-3}$
Organic dust	None	5 $mg.m^{-3}$
Particulates not otherwise regulated	10 $mg.m^{-3}$	10 $mg.m^{-3}$
Subtilisin (protease from *Bacillus subtilis)*	60 $ng.m^{-3}$	60 $ng.m^{-3}$

Specific OELs are required to protect workers' health. However, bioaerosol research has thus far only resulted in proposed exposure limits for endotoxins and fungal spores. A criteria document based on inflammatory respiratory effects (Eduard, 2006) proposes a lowest observed effect level (LOEL) of 10^4 m^{-3} for spores, non-pathogenic and non-mycotoxin producing fungal species. Several organisations have also proposed guidelines for fungi in indoor environments, but the criteria were developed for assessing indoor mould problems and are not health-based (Rao *et al*., 1996; WHO, 2009). For other agents, risk assessment may be based on exposure-response associations found in relevant epidemiological studies; e.g., β-(1→3)-glucans and allergens, but lack of standardisation of measurement methods represents a great challenge (Douwes, 2005; Heederick, 2002).

There are no uniform international standards available on levels and acceptable maximum bioaerosol loads (Table 2.8) (Mandal & Brandl, 2011; Kim *et al*., 2018). The American Conference of Governmental Industrial Hygienists (ACGIH) stated in 2009 that "a general threshold limit value [TLV] for culturable or countable bioaerosol concentrations is not scientifically supported" based on the lack of data describing exposure-response relationships (ACGIH, 2009). However, no similar topic or related legislation has been discussed since 2009. Furthermore, no uniform standardised method is available for the collection and analysis of bacterial and fungal bioaerosols, which makes the establishment of exposure limits challenging. Without scientifically proven standards and guidelines, arbitrary criteria

may lead to inappropriate testing and test interpretations (Kim *et al*., 2018). Yet, regardless of these gaps, neither air sampling techniques nor identification and cultivation methods have been internationally standardised; therefore, the prospect of data comparison is still a nebulous area in this field.

Table 2.8: Acceptable maximum bioaerosol limits allowed in various countries/regions

Number of Culturable Organisms as CFU.m^{-3}					
Country	**Bacteria**	**Yeast**	**Mould**	**Total Bioaerosols (Bacteria + Yeast + Mould)**	**Reference**
Brazil		750			de Aquino & de Goes Siqueira, 2004; Nunes *et al*., 2005
			50 (one species of mould) 100 (different mould species)	150	Bratlett *et al*., 2003; EC, 1989; Kim *et al*., 2018; WHO, 1988
Canada		150			
China*	2 500 - 7 000				Gorny, 2004
Finland	4 500				Nevalainen, 1989
Germany	10 000	10 000			IFA, 2001; IFA, 2004
Korea				800	Jo & Seo, 2005; Lee *et al*., 2012
Portugal		500			Pegas *et al*., 2010
Netherlands	10 000			10 000	Heida *et al*., 1995
Russia**		2 000 - 10 000			Eduard, 2009
Switzerland	10 000 (aerobic mesophilic)				Oppliger *et al*., 2005; SUVA, 2007

Number of Culturable Organisms as CFU.m^{-3}					
Country	**Bacteria**	**Yeast**	**Mould**	**Total Bioaerosols (Bacteria + Yeast + Mould)**	**Reference**
USA	1 000 (Gram-negative)	1 000			AGCIH, 2009; OSHA, 2008
European Union	10 000 (private homes) 2 000 (non-industrial indoor locations)	10 000 (private home) 2 000 (non-industrial indoor location)			Wanner & Gravesen 1994

* location dependent

** dependent on fungal species

2.7. Conclusion

Bioaerosol sampling can be a useful tool to study occupational exposure, potential health hazards and the transmission of infectious diseases in occupational and domestic environments. However, bioaerosol sampling has significant limitations and these need to be considered when deciding whether or not to collect bioaerosol samples. It is vital to prepare a sampling plan and to interpret the results meaningfully. The first and most important limitation is the lack of standards and guidelines for acceptable bioaerosol exposure limits. Although numerous studies have suggested a connection between exposure to various bioaerosols and respiratory illnesses, exposure limits do not currently exist. These limits have not been established largely because it is not possible to definitively state that a particular bioaerosol concentration will or will not lead to adverse health outcomes. In addition to the lack of exposure limits for bioaerosols, measuring and interpreting bioaerosol concentrations are more complex than is often appreciated. In addition, bioaerosol concentrations can vary significantly from location to location within a building. It is undeniable, however, that bioaerosol sampling can be beneficial when done in an appropriate context and manner. It is also important to emphasise that bioaerosols are ubiquitous environmental contaminants and, in the majority of cases, they are not an integral part of the industrial process. It would therefore be inappropriate to sample merely 'to-see-what-is-in-the-air' because the presence of microbes in the air can be expected. Moreover, bioaerosol studies are still in their infancy and continued new technological advances are needed to successfully address the questions that persist in this field.

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CHAPTER 3

BIOAEROSOLS AND RELATED ENVIRONMENTAL PARAMETERS IN A PROMINENT FRUIT JUICE MANUFACTURING FACILITY

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3.1. Introduction

Food safety and quality control have become prominent focus areas in recent years, with legislation forcing all food industries to comply with national and, in some cases, international standards. Due to the competitive market faced by each of the different food industries, customer satisfaction has become extremely important. The consumer is focused on personal preference, safety and quality and if one of these elements is under the expected norm, consumer loyalty is easily lost forever. One of the main factors that contributes to obtaining good quality food products is microbiological safety, which is a basic requisite to ensure the integrity of the product and the safety of the product relating to the health of the consumer (Osimani *et al*., 2013).

Fruit juice industries in South Africa play an important economic role because the production and distribution of fruit juices are key contributors to the South African economy, especially as many of the country's fruits and fruit juices are exported to other countries. The consumption of fresh fruit and fruit juices is constantly increasing as consumers strive to maintain healthy diets. Fruit juices contain natural non-pathogenic, epiphytic microflora. However, during the cultivation and harvesting of fruit, as well as during fruit juice processing and handling, contamination by pathogens from humans and other elements is possible. It is thus important for all fruit juice industries to produce a product that the consumer enjoys but that is also safe. The new consumer law encourages microbial and analytic testing of fruit juice products as well as the monitoring of production facilities (South Africa, 2008). Suppliers and distributors of fruit juices spend an enormous amount of money on safety testing with particular focus on equipment and surface swabs, hand swabs, air plates, and water and product testing (South Africa, 2008).

Even though there are many factors that can contribute to a product that is not satisfactory, in recent years the focus has shifted towards the impact of bioaerosol exposure on various environments and the probable health impacts this exposure could have. Although the importance of bioaerosols and their impact on different industries and human health have been recognised, it remains difficult to accurately describe their role in different environments (Kim *et al*., 2018). A problem faced by many fruit juice processing facilities is successful risk assessment and the control of bioaerosols. Bioaerosols are defined as "airborne particles that are living or originate from living organisms, such as microorganisms and fragments of microorganisms, toxins and metabolites from living organisms" (Wang *et al*., 2015). Exposure to naturally occurring bioaerosols has been shown to cause various adverse health effects such as allergies and infections (Ruzer & Harley, 2012; Xu *et al*., 2013; Zukewicz-Sobczak, 2013). Bioaerosols also have the potential to cause major problems in any food industry such as

contamination of the food (spoilage), allergies in individuals (consumers), and infection by means of pathogenic microorganisms.

The fruit juice facility that was selected as the study site is situated in Bloemfontein (Free State, South Africa) and is located in an agricultural and food processing area. Bloemfontein is centrally situated in the country and is therefore an excellent area for the production of various types of food products and the distribution of these products to the rest of the country. At the time of the study, the selected facility had approximately 250 employees and operated between 12 to 24 h per day. The selection criteria for this facility were based on: (i) the product manufactured; (ii) its central location; and (iii) the type of fruit juice (dairy blends, concentrate) that it manufactured. The selected fruit juice industry devotes ample resources towards monitoring and ensuring microbial safety of its products with on-site testing of the product and the processing environment. It is the mission of this specific industry to strive towards a product that is 100% contamination, spoilage and allergen free. It is therefore very important to this facility to find all possible origins of contamination and eliminate them.

However, upon visiting this facility, it was noted that it had no barriers between the clean and unclean areas and no air flow according to the product flow. All three production lines in the facility produced different types of products; however, the lines were located in the same area and this could potentially cause the contamination of one product by another. Furthermore, this plant was not a closed and controlled facility, and the temperature inside the facility was not regulated. The origin of bioaerosols may vary, making the control thereof problematic. At the time of the study, bioaerosols that could have contributed to contamination had not yet been characterised in this facility.

Bioaerosol samplers are increasingly being used to measure airborne microorganisms in occupational- and food processing environments to assess bioaerosol exposure. The purpose of this chapter was therefore three-fold: (i) to collect and analyse the culturable fraction of bioaerosols in this facility during two different seasons in five different sites in the processing section, where the facility monitors the air on a monthly basis; (ii) to determine the effect of basic environmental conditions in these five different sites on the formation of bioaerosols; and (iii) to examine the correlation between different types of bioaerosols.

3.2. Materials and Methods

3.2.1. Sampling protocol

A SAMPL'AIR LITE (AES Chemunex, United States) air sampler, an area heat stress monitor (Questemp, SA) and a Rotating Vane Anemometer were used to determine the environmental conditions (temperature and airflow) in the facility. A purposive sampling methodology was utilised (Etikan *et al*., 2016) to cover both on- and off-peak manufacturing seasons at the same sampling points where the facility monitors the air monthly. The same sampling points where chosen as this represented the areas which bioaerosols might affect the quality of the product. Sampling was performed in duplicate in five distinct areas to ensure a holistic analysis of the facility. These areas were: (i) the entrance to the production area (Area 1); (ii) the area for the preparation and mixing of materials (Area 2); (iii) the area between the production lines (Area 3); (iv) the area where the bottles were dispersed (Area 4); and (v) the area where the bottles were filled with the final product (Area 5) (Figures 3.1a and b).

3.2.2. Measurement of the variation in physical parameters

Temperature and airflow were determined during both sampling occasions (i.e., during the peak and off-peak seasons) and the readings were recorded at 15 min and 30 min respectively after setting up the samplers at breathing height (1.5 m above the ground) in the different areas (Aliakbar *et al*., 2013; Frankel *et al*., 2012). For temperature measurements, an area heat stress monitor (Questemp SA) was used. The heat stress monitor took readings from a dry bulb thermometer that determined the air temperature; a globe thermometer that determined the radiant heat, and a wet bulb thermometer that determined the effect of evaporation in air movement. Based on these measurements, it was possible to determine the WBGT (wet bulb globe temperature) index, which determined the true temperature. A rotating vane anemometer was used for the measurement of air velocity and volume flow. The anemometer uses rotating vane technology where air movement causes rotation of a multi-blade fan mounted in low-friction bearings. An infrared sensor translates fan blade movement into air velocity.

3.2.3. Culture-dependent sampling

The environmental air throughout the facility was evaluated for airborne microbial organisms (bacteria, yeast and mould) by using passive and active sampling.

3.2.3.1. Passive sampling

The facility under study monitored air quality on a monthly basis by using passive monitoring (Haig *et al*., 2016). Passive monitoring is also referred to as settle plates and is done by placing petri dishes containing agar that are exposed to the air for 20 min in the area to be monitored. Microbes that settle out of the ambient air can then be determined quantitatively. Petri dishes containing specific media for total microbial (TPC or total plate count) and yeast and mould (CA - Chloramphenicol Agar) counts were therefore used. Because the facility monitors the air on a monthly basis using this method, the data that had been obtained at the time of the study were also used.

3.2.3.2. Active sampling

Active sampling was performed by using two SAMPL'AIR LITE (AES Chemunex) air samplers simultaneously for each area (Roux *et al*., 2013; Yao *et al*., 2009). Active bioaerosol sampling systems consist of five fundamental elements: (i) inlet into the sampling device; (ii) transport of the air sample through the device; (iii) particle size selection; (iv) collecting medium; and (v) a pump and calibrated flow monitor (Colbeck, 1998). Air samples were collected at a flow rate of 100 ℓ.min^{-1}. The air samplers were disinfected with ethanol between different sampling points in order to prevent cross contamination of the samples. The samplers were turned on for two minutes prior to sampling to allow the ethanol to evaporate, thus ensuring that the number of microorganisms recovered would not be affected. Air samples were taken at a height of 1.5 m from the ground, which was the same level as the working stations in the centre of each area (Shintani *et al*., 2004). Sterile petri dishes containing non-selective and selective media were used. After five minutes, the samplers were turned off and the petri dishes were removed, closed with a lid, and inverted. The petri dishes were stored and transported to a laboratory at the Central University of Technology, Free State, for further analysis.

3.2.4. Selection, enumeration and cryopreserving of the culturable fraction

Active air samples collected on the petri dishes containing the non-selective and selective media were incubated for a specific time at specific temperatures (Table 3.1).

Table 3.1: Media, incubation time and temperature

Enumeration Conditions for Different Microorganisms			
Microorganisms	**Media**	**Incubation Time**	**Temperature**
Total microbial load	PCA (plate count agar)	72 h	30°C
Yeast and mould	RBC (Rose Bengal Chloramphenicol	72 h	25°C

	agar)		
Coliforms and *E. coli*	VRB (Violet Red Bile agar) with MUG (4-Methylumbelliferyl-β-D-Glucuronide)	24 h	37°C
Salmonella spp.	XLD (Xylose Lysine Deoxycholate agar)	24 h	37°C
Staphylococcus spp.	BPA (Baird-Parker agar)	48 h	37°C

The numbers of colony forming units (CFUs) on each plate were counted using a colony counter and converted into airborne concentration (CFU.m^{-3}). Microbial counts were performed using standard guidelines adapted from *The Compendium of Methods for the Microbiological Examination of Foods* (National Advisory Committee, 2018; Swanson *et al*., 1992). Counts above 300 CFUs per plate were marked as too numerous to count (TNTC) and spreader colonies that exceeded 50% of the plate were marked as 'spreaders. TNTC and 'spreader plates were not included in the statistical analyses. The remaining samples were analysed for the presence of total microbial load, yeast and mould, coliforms, presumptive positive *Escherichia coli* (*E. coli*), presumptive positive *Salmonella* spp., and presumptive positive *Staphylococcus* spp. (including presumptive positive *Staphylococcus aureus* [*S. aureus*]). The microbial counts were corrected as per instructions in the manual (SAMPL'AIR LITE, AES Chemunex), using the positive hole conversion method based on Feller's (1950) statistical correction equation (Anderson, 1958; Lee *et al*., 2016). This equation is expressed as follows:

$$\mathrm{Pr} = \mathrm{N}\left[1/\mathrm{N} + 1/\mathrm{N}-1 + 1/\mathrm{N}-2 + \ldots.1/\mathrm{N}-\mathrm{r}+1\right]$$

Where Pr is the statistically corrected count, N is the number of holes in the sampling head, and r resembles each colony count (Anderson, 1958; Lee *et al*., 2016). The microbial concentration of the bioaerosols was calculated using the following formula:

$$\mathrm{C} = \frac{\mathrm{N}}{\mathrm{Q} \times \mathrm{t}}$$

Where C is the bioaerosol concentration in CFU.m^{-3}, N is the total bioaerosol count after the positive hole conversion method, Q is the sampling flowrate or the viable sampler, and t is the sampling duration (Anderson, 1958; Lee *et al*., 2016).

Individual colonies were selected with a sterile inoculation loop and bacteria were stored in 2 mℓ Microbanks (ProLab, United States). These Microbanks were vortexed, labelled and stored at -80°C. Yeast and mould colonies were stored in 1.5 mℓ CryoVials (Fisher Scientific, United States) containing 1 mℓ 15% glycerol. CryoVials were vortexed, labelled and stored at -80°C (De Paoli & Tedeschi, 2011).

3.2.5. Statistical analyses

Pearson's correlation technique was used to measure the relationship between different microbiota across various areas within a selected season, with the rest of intrinsic and extrinsic parameters being kept constant. Furthermore, the relationship between temperature and microbial counts was measured using the same methodology (Table 3.3 and Table 3.4) with P≤0.05 considered to be statistically significant. According to the correlation coefficient (r), the correlations were defined as none (± 0-0.3), weak (± 0.3-0.5), moderate (± 0.5-0.7), and strong (±0.7-1.0) (Cramer, 1987; Li *et al*., 2011). All the analyses were conducted using the Statistical Data Analysis software (2018).

3.3. Results and Discussion

3.3.1. Recording of physical parameters

Like all living organisms, bioaerosols also require a specific environment, nutrition and mode of spreading to survive. This principle also applies to the bioaerosols in this specific fruit juice industry. When the study was conducted, there were no previously recorded data of the physical factors (temperature and airflow) during different seasons for this facility because it was not part of the quality control department's standard operating procedures to monitor these parameters. However, when the study was conceptualised, two basic environmental parameters, namely temperature and airflow, were selected as these are fundamental factors that bioaerosols require to survive and spread. Temperature in particular is an important environmental factor that affects the growth and development of microorganisms because certain microorganisms can only grow and proliferate at specific temperatures. The effect of temperature on microorganisms was highlighted by Farrell and Rose as early as 1967. Historically, the concept of airborne spread was first described in detail by Wells (1934, 1955), but to date little is known about the impact of airflow patterns on the spreading of bioaerosols (Li *et al*., 2007; Seedorf & Schmidt, 2017).

Temperature and airflow sampling points were limited as the temperature and airflow were only measured at the five specific points of interest. These points represented the five areas where the facility monitors air quality as well as the areas where bioaerosols may effect the quality of the product. The results that were obtained indicated no trend in the deviation of temperature and airflow observed during the peak season (onset of summer) and off-peak season (onset of autumn). The average indoor air temperature ranged between 18−22°C (±1.1°C) and airflow between 0 to 4.4. $m.s^{-1}$ as indicated in Figures 3.1a and b. The lowest indoor temperature recorded was in Area 1 (the entrance of the production facility) and varied between 19.79°C in the peak season and 18.28°C in the off-peak

season. Therefore, regardless of the typically high outside temperatures (28°C) for the peak season, these results indicated that the outside temperature had no significant effect on the temperature inside the facility.

The maximum temperature was recorded in Area 5 (filling of bottles with final product) and ranged from 21.95°C during the peak season to 21.90°C during the off-peak season. Area 5 comprised mainly of automated machinery that filled the bottles with the final product. However, the system did not function in a completely automated manner as staff members still needed to assist with packing bottles before filling, filling bottles, closing bottles after filling, labelling bottles, and packing filled bottles for shipment. A further complication that was observed was the fact that more than one product could be filled simultaneously. It was noted that the operating machinery and the presence of the personnel contributed to a warm, compact atmosphere in this area, which was an observation that was also noted by Wyon (2004).

The fact that nearly no airflow was recorded in Area 1 (the highest was an average of 4.4. $m.s^{-1}$) implies that the bioaerosol distribution in this facility was not dependant on air flow (Barberan *et al*., 2015); therefore the lack of an air filtration system might have contributed to the continuous presence of bioaerosols inside the facility.

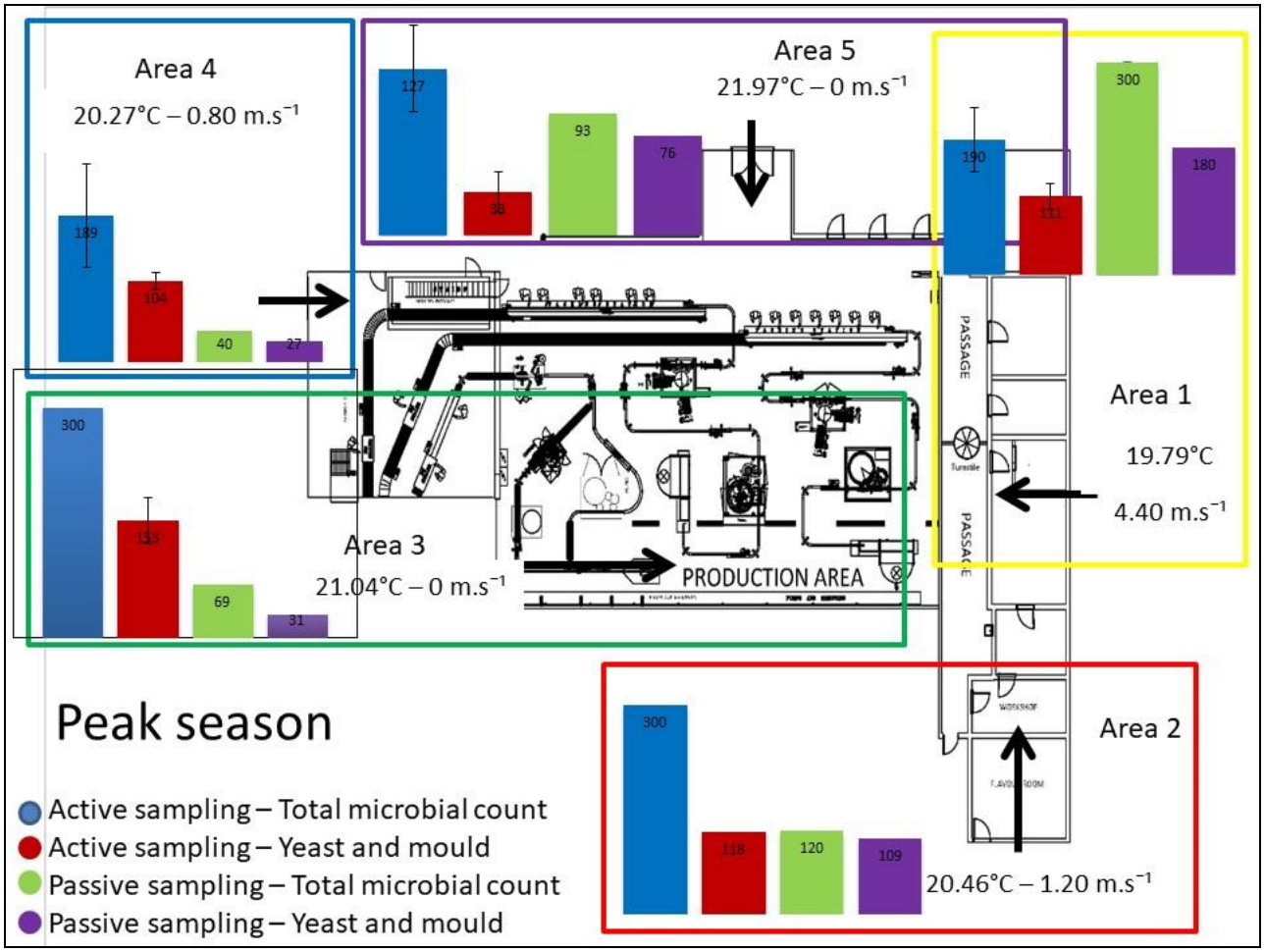

Figure 3.1a:The temperature, airflow and specific bioaerosol composition (total microbial count, yeast and mould) measured by passive and active sampling in different designated areas during peak season: These areas were: the entrance to the production area (Area 1), preparation and mixing of materials (Area 2), between the production line (Area 3), dispersion of bottles (Area 4), and filling of the final product (Area 5). Two test schedules were chosen (peak season (beginning of summer) and off-peak season (onset of autumn) to establish if seasonal variantion impacted the enviromental factors and growth of microorganisms. Data points represent averages of two independent measurements for temperature, airflow, active sampling. Each bar chart represents the counts of the active and passive sampling for total microbial count, yeast and mould (plates only counted to 300) and standard deviation for the active sampling.

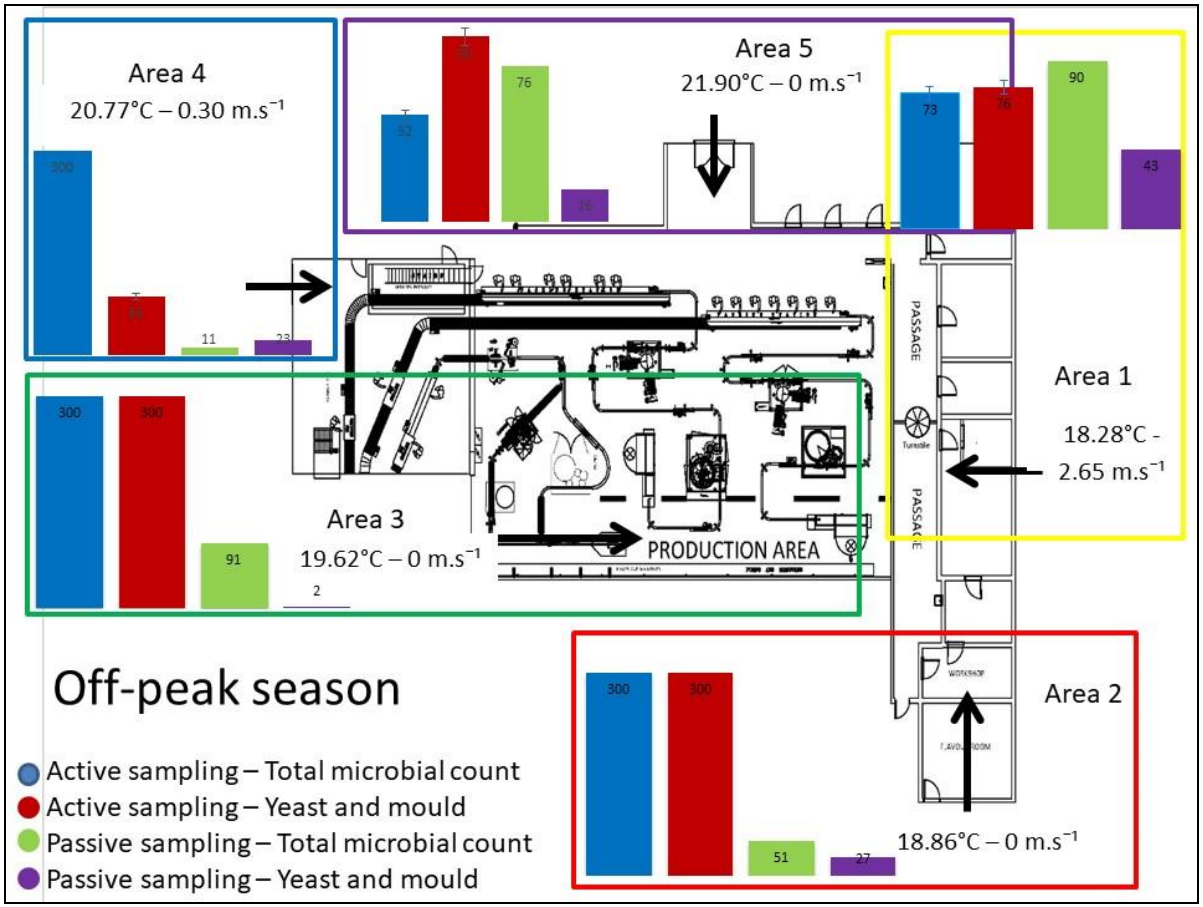

Figure 3.1b:The temperature, airflow and specific bioaerosol composition (total microbial count, yeast and mould) measured by passive and active sampling in different designated areas during off-peak season: These areas were: the entrance to the production area (Area 1), preparation and mixing of materials (Area 2), between the production line (Area 3), dispersion of bottles (Area 4), and filling of the final product (Area 5). Two test schedules were chosen (peak season (beginning of summer) and off-peak season (onset of autumn) to establish if seasonal variantion impacted the enviromental factors and growth of microorganisms. Data points represent averages of two independent measurements for temperature, airflow, active sampling. Each bar chart represents the counts of the active and passive sampling for total microbial count, yeast and mould (plates only counted to 300) and standard deviation for the active sampling.

3.3.2. Comparison of findings for passive and active sampling

Various studies have examined the concentrations of airborne bacteria, yeast and mould in different environments. For this type of investigation, it is important not only to understand what types of microbes are present in the air, but also how many there are (Prussin *et al*., 2015). In some food industries the resources and time to determine the presence of specific types of bioaerosol are not always available, thus determining the number of bioaerosols is a quicker indication of potentially unhygienic effects. Passive and active sampling methods have been used widely in the last decades to assess bioaerosol concentrations in indoor environments (Canha *et al*., 2015; Napoli *et al*., 2012). The passive sampling method appeals to most types of industries due to the fact that they are inexpensive, easy to use, do not require electricity, and are small enough to be transported and deployed almost anywhere. Some studies have revealed that passive sampling provides valid risk assessment as it measures the harmful part of the airborne population that may fall on to a critical surface (Markovic *et al.,* 2015; Napoli *et al.,* 2012).

When the study was conducted at this specific fruit juice industry, it was noted that it had a standard operating procedure (SOP) for environmental monitoring that required passive bioaerosol monitoring once a month in specific designated areas. Specific limits were set for the total microbial load (<100 CFU per plate) and for yeast and mould (<50 CFU per plate) as part of their standard operating procedure. Although the SOP stipulated that, in the case of counts higher than their set limits, the corrective action was to re-test twice in that specific month, this was not efficient as the root cause was not determined and therefore no corrective action could be implemented to ensure that there were no reoccurrences.

Several studies have attempted to compare the values of microbial loads obtained through both active and passive sampling, but inconsistent results were obtained. In certain studies, significant correlations were observed (Orpianesi *et al.,* 1983; Perdelli *et al.,* 2000; Whyte, 1986), while in others no correlations could be reported (Petti *et al.,* 2003; Sayer *et al.,* 1972). Interestingly, international standards offer different techniques (active or passive sampling) and different kinds of samples (living or non-living components), thus leaving the choice of system open (Napoli *et al.,* 2012). In this study, the results from both sampling methods were compared and correlated to determine the reliability and performance of the passive sampling method. Figures 3.1a and b indicate the counts for total microbial load and for yeast and mould respectively in a bar chart. These results were obtained during the onset of summer and autumn in five designated areas in the facility under study.

It was expected that the active sampling method would detect increased microbial counts in every designated area. Active microbiological air sampling physically draws a known volume of air through a particle collection device. Active monitoring requires the microbial air sampler to force air onto or into collection media at a specific rate over a specified time period (Colbeck, 1998; Kornacki, 2014; Roux *et al*., 2013; Yao *et al*., 2009). For the total microbial load, the passive sampling method detected higher total microbial counts during the peak and off-peak season in Area 1 (entrance to the facility) as well as in Area 5 (filling of the final product) during off-peak season for total microbial counts. During the peak season, higher counts of yeast and mould were detected using the passive sampling procedure in Area 1 (entrance to the facility) as well as in Area 5 (filling of the final product). This was contrary to what had been found in previous research where the active sampler had detected higher counts (Markovic *et al*., 2015). These results obtained in this study might have been due to the fact that sampling was not performed on the same day.

The concentrations of yeast and mould observed during the two samplings differed, not only for the reason that different techniques were used for the passive and active sampling of bioaerosols, but also because two different growth mediums were used, namely CA for passive sampling and RBC for active sampling. CA was developed for retrieval of yeast and mould in milk and milk products, while RBC was developed for yeast and mould retrieval in food and environmental samples. The literature suggests that RBC is superior to CA in terms of both number of colonies and number of genera isolated from the air (Mentese *et al*., 2017). In the current study, higher counts of yeast and mould than of total microbial load were observed in the off-peak season using active sampling methods in Area 1 (entrance to the facility) and Area 5 (filling of final product). This was probably due to the fact that the media used for the yeast and mould enumeration were specific for the growth of yeast and mould and that more yeast and mould were identified on the selective media than on the non-selective media (Mentese *et al*., 2017). However, overall, lower yeast and mould counts were observed compared to total microbial load during peak season. This could be attributed to the overgrowth on selective media in the petri dishes that was observed during the enumeration of mould, as this prevented accurate counting and isolation of other moulds or yeast that could have been present (Beuchat & Mann, 2016; Douglas *et al.,* 1979).

3.3.3. Bioaerosol concentrations in different seasons

Bioaerosols and their components could pose an environmental hazard when present in high concentrations in indoor environments, as they result in spoilage/contamination of food products or they may pose various occupational health risks (Stetzwnbach *et al*., 2004). Bioaerosol composition depends on four main factors: (i) different environments (in the current study, these were areas in the

facility); (ii) the season (Frankel *et al*., 2012; Heald & Sprancklen, 2009; Wu *et al*., 2017; Zhen *et al*., 2017); (iii) the weather and environment-related variables (Jones & Harrison, 2004); and (iv) temperature and water availability. At the time of the study, no data could be traced for the bioaerosol composition in this specific fruit juice facility, and this made it difficult to determine which type of bioaerosols needed to be analysed. Therefore, a broad range of bioaerosols was investigated in different designated areas during different seasons to determine where the microbial high-risk areas in the facility were (Table 3.2).

Table 3.2: Culturable bioaerosol fraction counts measured by SAMPL'AIR LITE in different designated areas. Entrance to the production area (Area 1), preparation and mixing of materials (Area 2), between the production lines (Area 3), in the area for the dispersion of bottles (Area 4), and in the area where the final product was filled (Area 5). Two test schedules had been devised: one for the peak season (the onset of summer) and one for the off-peak season (the onset of autumn) to determine whether seasonal varientionimpacted the growth of microorganisms in the facility. Plates with counts higher than 300 were recorded as >300. Data points represent averages of two independent measurements.

Peak Season vs Off-Peak Season										
Area	**Peak Total microbial load**	**Off-Peak Total microbial load**	**Peak Yeast and mould**	**Off-Peak Yeast and mould**	**Peak Coliforms and *E. coli*[a]**	**Off-Peak Coliforms and *E. coli*[a]**	**Peak *Salmonella* spp.**	**Off-Peak *Salmonella* spp.**	**Peak *Staphylococcus* spp.[b]**	**Off-Peak *Staphylococcus* spp.[b]**
Area 1	190	73	111	76	0	0	0	0	41	68
Area 2	>300	>300	118	>300	7	0	2	0	167	43
Area 3	>300	>300	153	>300	10	0	0	2	105	42
Area 4	189	>300	104	86	1	12	0	3	119	138
Area 5	127	52	33	90	6	18	2	12	11	23

[a] Not enough coliforms and *E. coli* to separate – counts combined. [b] No *Staphylococcus aureus* spp. were detected.

Total microbial loads in the air are tested for one main reason, which is that the counts may indicate if the environmental conditions are favourable for microbial growth. The higher the number of microorganisms in a specific area, the greater the probability that the environment in that area is suitable for the prevalence of hazardous microorganisms (Bottari *et al.,* 2015). However, no uniform international standard is available as a guide for acceptable maximum bioaerosol loads (Kim *et al*., 2018; Madal & Brandl, 2011), and this makes it difficult to determine if the total microbial load detected in the air poses a risk or not. What does assist, is that definite evidence has been recorded of the connection between bacteria and spores found in the air and their effect on food-contact surfaces (Bower *et al*., 1996; Di Ciccio *et al*., 2015). The standard operating procedure of the facility for acceptable microbial limits (Table 3.3) on food contact surfaces was therefore used as a guideline to determine which limits were acceptable for microorganisms found in the air (Di Ciccio *et al*., 2015; Hennekinne *et al.,* 2012).

Table 3.3: Acceptable microbial limits for food contact surfaces in the facility under study

Microbial Specifications for Food Contact Surfaces	
Microorganisms	**Specification**
Total microbial load	<100 CFU/area
Yeast and mould	Not detected
Coliforms and *E. coli*	Not detected
Salmonella spp.	Not detected
Staphylococcus spp.	Not detected

When comparing the results obtained with the specifications in the guideline used in this facility for food contact surfaces, the total microbial load observed was beyond the specifications for all five areas during the peak season (>100 CFU.m^{-3}) as well as for Areas 2, 3 and 4 during the off-peak season (>100 CFU.m^{-3}). High counts were reported for all areas in the facility during the peak season as this was the busiest season and the facility was running at full capacity. During the off-peak season, personnel were only present in Area 2 where the preparation and mixing of materials occurred, in the production line area (Area 3), and in the area where the bottles were dispersed (Area 4). The presence of the workers was clearly associated with the high microbial counts observed in these areas. The literature also indicates a correlation between microbial counts and the presence of personnel in specific areas. Airborne microbial levels thus increase when areas are occupied by humans, which is

not the case in unoccupied conditions. This finding was expected as humans have been reported to be a source of bacteria and fungi in settled dust samples (Adams *et al.,* 2015).

Yeast and mould counts were observed during the peak and off-peak seasons in all the designated areas, but higher counts of yeast and mould were observed during the off-peak season in area 2, 3 and 5(an average of 160 CFU.m^{-3}). This may have been due to seasonal variation, which has already been described by several authors (Bonetta *et al.,* 2010; Osimani *et al.,* 2013). Yeast and mould are the microorganisms most frequently responsible for spoilage problems in the fruit juice industry (Groot *et al*., 2018**;** Tournas *et al.,* 2006), which suggests that the counts observed in the facility exposed a threat in these areas (Garnier *et al*., 2017). Conditions in fruit juice facilities and in the fruit juices themselves (the raw materials used for fruit juice production, the low pH of the final product, high sugar concentration, and low water activity) are ideal for the growth of yeast and mould, and this emphasises the importance of a yeast- and mould-free environment in a fruit juice facility, especially because yeast and mould have the ability to cause decay that can be detected in the development of off-flavours, acidification, discolouration, and disintegration (Groot *et al*., 2018).

A detailed summary of the statistical analyses is represented in Table 3.4 and Table 3.5. The statistical analyses revealed only three strong positive Pearson correlations that all occurred during the off-peak season. These correlations were: (i) temperature and coliforms, presumptive *E. coli* (r=0.9451); (ii) temperature and presumptive *Salmonella* spp. (r=0.9034); and (iii) the microbial growth between coliforms, presumptive *E. coli* and presumptive *Salmonella* spp. (r=0.8874). Fruit juice has been acknowledged as a frequent vehicle for transmitting pathogens such as *E. coli* and *Salmonella* spp. since 1922 (Parish, 1998; Park *et al.,* 2017), and with an established food safety management system in place for this facility, low to no counts of coliforms, presumptive *E. coli* and presumptive *Salmonella* spp. were expected. These microorganisms form part of the family Enterobacteriaceae and flourish in the same environmental conditions (such as temperature), which confirms that the same temperature and environment will promote the growth of the same type of microorganism (Park *et al.,* 2017). Pathogenic bioaerosols such as *E. coli* and *Salmonella* spp. have been recorded on food contact surfaces, for example in abattoirs (Joseph *et al*., 2001) and wheat flour facilities (Villa-Rojas *et al*., 2017). Although fruit juice is an acidic food (pH below 4.6), the foodborne pathogens *E. coli* O157:H7 and *Salmonella* spp. have been reported in foodborne disease outbreaks associated with fruit juice (Iqbal *et al*., 2015; Oluwole *et al*., 2016; Tarifa *et al*., 2017; Vantarakis *et al.,* 2011).

Table 3.4: Singular comparisons between temperatures observed during the peak and off-peak seasons and microbial growth using Pearson p scores calculated from R value.

Peak Season (temperature vs microbial growth)				Off-Peak Season (temperature vs microbial growth)			
Microorganisms	**r**	**R^2**	**p**	**Microorganisms**	**r**	**R^2**	**p**
Total microbial load	-0,1546	0,023901	0,8047	Total microbial load	-0,2072	0,042932	0,73833
Yeast and mould	-0,5645	0,31866	0,32203	Yeast and mould	-0,4171	0,173972	0,48488
Coliforms and *E. coli*	0,4801	0,230496	0,41309	Coliforms and *E. coli*	0,9451	0,893214	0,01531*
Salmonella spp.	0,8397	0,705096	0,07516	*Salmonella* spp.	0,9034	0,816132	0,03551*
Staphylococcus spp.	-0,3904	0,152412	0,51633	*Staphylococcus* spp.	0,0146	0,000213	0,98141

r - Pearson's correlation coefficient (measure of the linear correlation between two variables X and Y).

R^2 - R squared (proportion of the variance in the dependent variable that is predictable from the independent variable/s)

P - P-value (probability that would have been found for the current result if the correlation coefficient had in fact been zero [null hypothesis]). If this probability is lower than the conventional 5% ($p<0.05$), the correlation coefficient is deemed statistically significant.

* - $P \leq 0.05$

Table 3.5: Multiple comparisons between different microbial groups during the peak and off-peak seasons using Pearson p scores calculated from R value

Peak Season (microbial growth)				Off-Peak Season (microbial growth)			
Microorganisms	**r**	**R^2**	**P**	**Microorganisms**	**r**	**R^2**	**p**
Total microbial vs yeast and mould	0,8582	0,73650724	0,062717	Total microbial vs yeast and mould	0,6594	0,43480836	0,22603
Total microbial vs coliforms and *E. coli*	0,7551	0,57017601	0,14002	Total microbial vs coliforms and *E. coli*	-0,2564	0,06574096	0,67765
Total microbial vs *Salmonella* spp.	-0,5729	0,32821441	0,31364	Total microbial t vs *Salmonella* spp.	-0,4793	0,22972849	0,41432
Total microbial vs *Staphylococcus* spp.	0,7074	0,50041476	0,18143	Total microbial vs *Staphylococcus* spp.	0,3293	0,10843849	0,58843
Yeast and mould vs coliforms and *E. coli*	0,4462	0,19909444	0,45134	Yeast and mould vs coliforms and *E. coli*	-0,6028	0,36336784	0,28272
Yeast and mould vs *Salmonella* spp.	-0,0246	0,00060516	0,967748	Yeast and mould vs *Salmonella* spp.	-0,4429	0,19616041	0,45613
Yeast and mould vs *Staphylococcus* spp.	0,6458	0,41705764	0,23915	Yeast and mould vs *Staphylococcus* spp.	-0,3981	0,15848361	0,50697
Coliforms and *E. coli* vs *Salmonella* spp.	-0,0247	0,00061009	0,96945	Coliforms and *E. coli* vs *Salmonella* spp.	0,8874	0,78747876	0,04458*
Coliforms and *E. coli* vs *Staphylococcus* spp.	0,2283	0,05212089	0,71187	Coliforms and *E. coli* vs *Staphylococcus* spp.	0,1218	0,01483524	0,8453
Salmonella spp. vs *Staphylococcus* spp.	-0,6024	0,36288576	0,28272	*Salmonella* spp. vs *Staphylococcus* spp.	-0,3277	0,10738729	0,59119

r - Pearson's correlation coefficient (measure of the linear correlation between two variables X and Y).

R^2 - R squared (proportion of the variance in the dependent variable that is predictable from the independent variable(s)).

P - P-value (probability that you would have found the current result if the correlation coefficient were in fact zero (null hypothesis). If this probability is lower than the conventional 5% ($p<0.05$) the correlation coefficient is called statistically significant)

* - $P \leq 0.05$

Staphylococcus aureus and some *Staphylococcus* spp. are extremely important in the fruit juice industry for the following reasons: (i) They are pathogenic bacteria, capable of developing biofilms on surfaces and food processing surfaces in fruit juice industries (Bentanzos-Cabrera *et al*., 2015; Iqbal *et al*., 2015; Madsen *et al*., 2018); (ii) Staphylococcal food poisoning is one of the most common food-borne diseases caused by fruit juice (Di Ciccio *et al*., 2015; Hennekinne *et al.,* 2012; Oluwole *et al*., 2016); (iii) Antibiotic resistance profiles of microbes have been found in fruit juice (Abraha *et al*,. 2018); and (iv) They are frequently part of the microorganisms that cause occupational health risks (Goldstein *et al*., 2014). No *Staphylococcus aureus* were detected during the study. High counts of *Staphylococcus* spp. were observed in Areas 2, 3 and 4 during the peak season (>100 CFU.m^{-3}) and in Area 4 during the off-peak season (>100 CFU.m^{-3}). Area 4 (dispersion of bottles) had poor ventilation (the airflow recorded was 0 m.s^{-1}) and was full of dust, making this a perfect environment for microbial development as *Staphylococcus* spp. is ubiquitous in the environment and can be found in or on the air, dust, sewage, water, environmental surfaces, humans and animals (Henneinne *et al.,* 2012).

3.4. Conclusion

Fruit juice products have been documented as suitable growing environments for certain problematic microorganisms. There is thus a clear need to ensure that the production environment, which includes the air of the facility, is as clean as possible. To determine the risk of bioaerosols and how to control them, different aspects of the facility under investigation needed to be determined during different seasons with specific focus on: (i) environmental parameters (temperature, airflow and seasonal impact); (ii) sampling devices; and (iii) bioaerosol concentration.

Temperature and airflow are basic environmental parameters and thus fundamental requirements for bioaerosols to survive and spread. Just like all living organisms, bioaerosols need a conducive environment, appropriate nutrition and a suitable mode of transportation to survive. Against this background, the lack of temperature control in the facility under study was ideal for bioaerosol growth as the average recorded temperature was 20.30 ±1.1°C. Furthermore, almost no airflow (0 to 4.4. m.s^{-1}) or ventilation systems were observed which may have had either a positive or negative impact on the facility. Because air flow as the main mode of bioaerosol spreading was not available, the bioaerosols needed other means of transportation to spread through the facility, and this was probably facilitated by human workers. Moreover, with no airflow or ventilation system in place, the bioaerosols were not removed from the facility and might either have ended up in the products or could have contributed to occupational diseases.

To ensure a good and hazard-free fruit juice production system, measuring concentrations of bioaerosols and the areas where they occur is important, as bioaerosol counts is a quick and effective indication of potential risk effects. Two main sampling methods that are available for the quantification of bioaerosols were discussed. First, passive sampling requires petri dishes containing agar. These petri dishes are opened and exposed to the air and any bioaerosols that are present will settle in the agar. Secondly, active sampling physically draws a known volume of air through a particle collection device. In the industry under study, the passive sampling technique was habitually used as it was inexpensive, easy to use, did not require electricity, and was small enough to be transported and deployed almost anywhere. As had been expected, the quantities of bioaerosols that were detected by the two methods differed considerably due to the different mechanisms that had been used for sampling.

Although no well-defined association was observed between the two sampling methods used, it was noticed that at least one of the bioaerosol counts observed using the active or passive sampling method was outside the specification of the facility. As was stated before, no specific South African guidelines are currently available for the detection of the concentration of bioaerosols; however, it was deemed important for quality control in the facility and in terms of the products to determine the bioaerosol growth potential and air quality. For this reason, the standard guideline for acceptable microbial limits for food contact surfaces in this facility was used to estimate whether microorganisms found in the air were within acceptable limits.

The data revealed that the total microbial counts were outside most of the specifications during both the testing seasons. This might have been due to increased levels of airborne organisms in areas that were frequented by the personnel. While yeast and mould were observed throughout the facility during the peak and off-peak seasons, higher counts were observed during the off-peak season in specific areas, which was possibly due to seasonal variation or contamination. The yeast and mould counts that were observed could be problematic as they pointed to the fact that the facility had ideal conditions for the growth of yeast and mould. Yeast and mould are the main microorganisms responsible for spoilage problems in the food industry, and their control to limit counts is therefore essential. Puzzling counts of the presumptive coliforms, *E. coli*, *Salmonella* spp. and *Staphylococcus* spp., were observed. These are microorganisms that are capable of developing biofilms on food processing surfaces and they were associated with foodborne disease outbreaks due to contaminated fruit juice before.

Even though bioaerosol exposure assessment is a rapidly evolving field with new sampling techniques and procedures being developed almost daily, there is a clear need to be industry- and product-

specific. Sampling techniques, types of bioaerosols, concentrations of bioaerosols and acceptable limits will differ immensely among industries such as red meat abattoirs, the fruit juice industry, and immunisation processing facilities. Therefore, based on the physical parameters observed in the facility under study, there is a clear need for appropriate temperature control and suitable ventilation systems in the fruit juice industry. Sampling methods could focus on: (i) lowering the acceptable limits for the microorganisms observed during passive sampling and being microorganism specific; and/or (ii) investing in active sampling equipment to gain a true representation of the microorganisms found in the air of a specific facility.

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CHAPTER 4

ENUMERATION, CLASSIFICATION AND CATEGORISATION OF CULTURABLE BIOAEROSOLS IN FRUIT JUICE MANUFACTURING

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4.1. Introduction

Bioaerosols are defined as “aerosols comprising of particles of biological origin or activity which may affect living things through infectivity, allergenicity, toxicity, [and] pharmacological or other processes” (Hirst, 1995; Shale & Lues, 2007). Bioaerosols are emerging as important role players in atmospheric processes, yet they are poorly understood. What is known and is universally accepted is that bioaerosols originate from and may impact various environments. For example, bioaerosols are emitted from terrestrial sources such as soil, forests and desert dust, and from agricultural and composting activities. They are prevalent in urban areas, wetlands, as well as coastal and marine environments. Moreover, they play a key role in the dispersal of reproductive units from plants and microbes where the atmosphere enables their dissemination over geographic barriers and long distances. Bioaerosols are thus highly relevant in the spread of organisms and they allow genetic exchange between habitats and geographic shifts of biomes. These compounds are central elements in the development, evolution and dynamics of ecosystems (Fröhlich-Nowoisky *et al*., 2016). Although bioaerosols may have beneficial characteristics, the opposite is also possible, as the dispersal of plant, animal and human pathogens as well as allergens has major implications for agricultural outcomes and public health. The negative effects that bioaerosols may have on the human respiratory system are particularly well documented (Despres *et al*., 2012).

Modern industrial activities (e.g., waste sorting, organic waste collection, composting, agricultural production, food processing, raising of livestock, and wastewater treatment systems) emit large quantities of bioaerosols, and this results in the release of abundant biological agents into the air. Unfortunately, there is a paucity of evidence on the effects of these bioaerosols on the environment, especially in terms of human health (Yoo *et al.,* 2017), and thus the effects that bioaerosols may have on products and food handlers in the food industry remain controversial. To exacerbate this situation, no legislation is available regarding bioaerosols in the air of food industries in South Africa. Allowable quantities of bioaerosols as proposed by the European Union have been disseminated, but there is no set standard (Bulski *et al*., 2017). What makes the assembly of legislation for bioaerosols so difficult is the fact that, in a specific industry, two or more manufacturing facilities might produce the same product, but the environment, other industries in close proximity, the season, the structure of the facility, and the raw materials used can differ to such an extent that the bioaerosol composition may vary considerably among these facilities.

Monitoring bioaerosols in the food industry environment is one of the many tools that industrial quality control managers can use in the assessment of indoor air quality, agricultural outcomes, and industrial

health. The monitoring process should include: (i) sampling of bioaerosols using either passive or active sampling methods; (ii) measurement of viable (culturable and non-culturable) and non-viable bioaerosols; and (iii) the identification of bioaerosols. Identification of microbial taxa is a critical element in the determination of the bioaerosol load in an industrial environment. Identification of bioaerosols can be performed using a variety of available assessment strategies such as microscopy, immuno-assays as well as various molecular-based assays (Afanou *et al*., 2015; Eduard *et al*., 2012; Rittenour *et al*., 2012). The sensitivity and rapidity of molecular techniques have also led to their use for bioaerosol monitoring in the determination of air quality and the detection of airborne pathogens (Yoo *et al*., 2017). A standard method for the detection of microorganisms in environmental samples is the polymerase chain reaction (PCR) assay. PCR-based approaches are promising because the organism is detected by amplifying the target rather than the signal and it is therefore less susceptible to false positives. PCR is usually followed by Sanger DNA sequencing, which is the most fundamental level of measuring one of the major properties by which terrestrial life forms can be defined and differentiated from one another. Over the years, innovations in sequencing protocols, molecular biology and automation have increased the technological capabilities of sequencing while decreasing the cost and allowing the reading of hundreds of base pairs DNA in length (Heather & Chain, 2016).

The air in food industries can be crowded with various airborne microorganisms that may include bacteria, yeast and mould (Yassin & Almouqatea, 2010; Yoo *et al*., 2017). The compilation of organisms in the air depends on the industry, the facility, the capacity of the facility, as well as the season and the external environment. Airborne microorganisms are a potential source of a wide variety of public and industrial health hazards; however, it is difficult to compile a set standard of acceptable limits for a specific industry as information regarding the types of bioaerosols and their effects is not abundant. Therefore, the aim of this study was to determine if the culturable fraction of bioaerosols sampled during peak and off-peak seasons in a fruit juice manufacturing facility was harmful, innocuous or potentially beneficial to the industry, the personnel and the environment.

4.2. Materials and Methods

4.2.1. Sampling

Two SAMPL'AIR LITE (AES Chemunex, United States) samplers were used to collect culturable bioaerosols in a selected fruit juice manufacturing facility. A purposive sampling methodology was utilised (Etikan *et al*., 2016) that was appropriate for the selected peak and off-peak manufacturing seasons according to which the facility operated. All the sampling was performed in duplicate in five

designated areas to represent a holistic analysis of the facility. These areas were the entrance to the production area (Area 1), the preparation and mixing area of materials (Area 2), the area between the production lines (Area 3), the area for the dispersion of bottles (Area 4), and the area where the bottles were filled with the final product (Area 5) (see Figure 4.1).

The air samplers that were used operated at a flow rate of 100 litres per minute and were disinfected with ethanol between the different sampling points. The samplers were turned on for two minutes prior to sampling to allow the ethanol to evaporate, thereby avoiding interference with the quantities of microorganisms recovered. Air samples were taken at a height of 1.5 meters from the ground (Aliakbar *et al*., 2013; Frankel *et al*., 2012), which was the same level as the working stations in the centre of each area. Sterile petri dishes containing either non-selective or selective media were used appropriately for culture-dependent sampling. After a sampling time of five minutes, the samplers were turned off and the petri dishes were removed and inverted in their covers. At least two independent repeats were conducted to obtain culture-dependent bioaerosols in each environment.

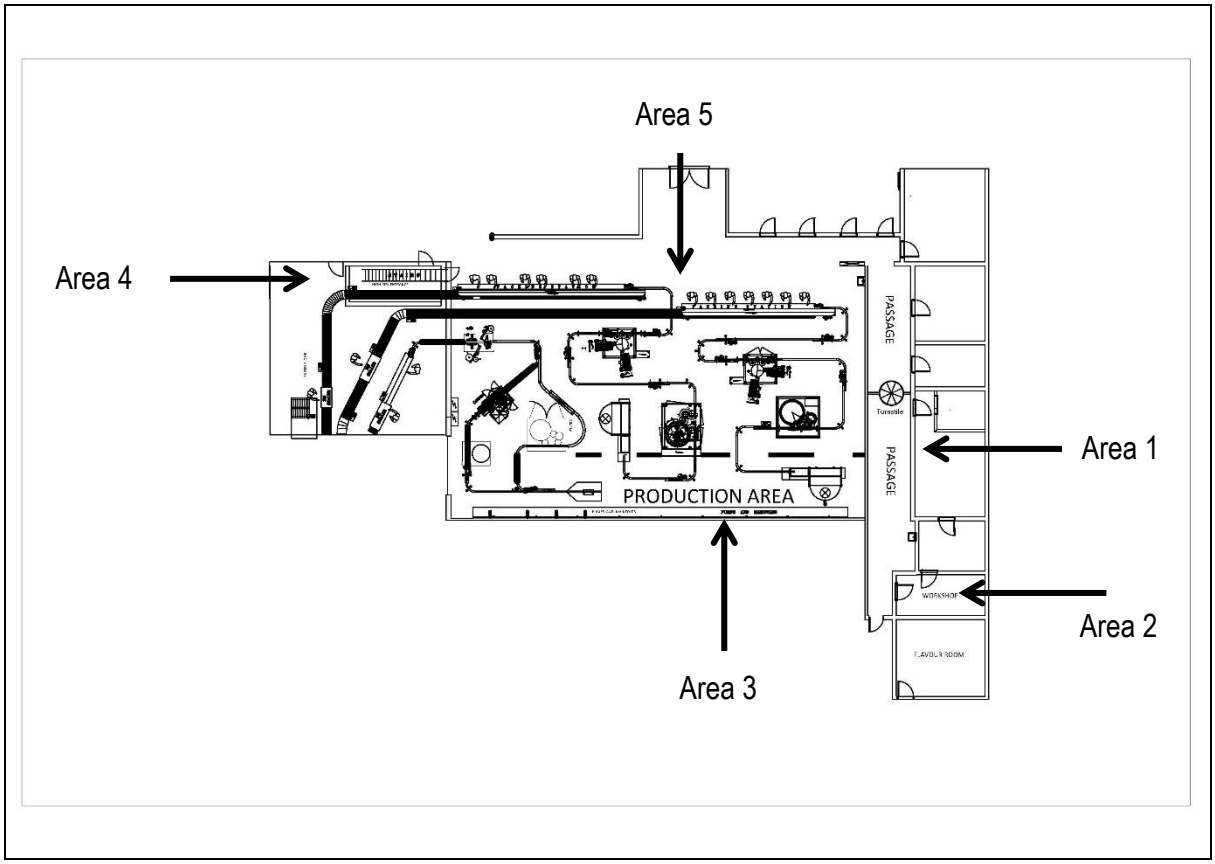

Figure 4.1: Schematic layout of the fruit juice bottling facility: The figure indicates the different sampling areas: Area 1 (entrance to the production area); Area 2 (preparation and mixing of materials); Area 3 (between the production lines); Area 4 (dispersion of bottles); and Area 5 (filling of bottles with the final product).

4.2.2. Culture medium composition used for microbe isolation, cultivation and enumeration

The air samples collected in the petri dishes containing non-selective and selective media were incubated for a specific time at specific temperatures, as was indicated in Table 3.1 (Chapter 3). Selective media are predominantly used for the growth of selected microorganisms. Microbial counts were performed using standard guidelines adapted from *The Compendium of Methods for the Microbiological Examination of Foods* (National Advisory Committee, 2018; Swanson *et al*., 1992). After incubation, the number of colonies on each plate was counted using the Scan® 1200 high-resolution automatic colour colony counter. The colony counts were adjusted using the positive hole correction method (Anderson, 1958; Lee *et al*., 2016) and these colonies are reported as colony forming units per cubic metre (CFU.m^{-3}). Individual presumptive bacterial colonies were selected with a sterile inoculation loop and preserved in 2 ml Microbanks (ProLab). The Microbanks were vortexed, labelled and stored at 80°C. Yeast and mould colonies were stored in 1.5 ml CryoVials (Fisher Scientific) containing 1 ml 15% glycerol. The CryoVials were vortexed and the vials were labelled and stored at -20°C.

4.2.3. Identification of the culturable fraction of bacteria, yeast and mould

Pure cultures of bacteria, yeast and mould were selected from 18 to 72 h agar plates based on colony colour, morphology and cell characteristics using a microscope (Barata *et al*., 2012; Montero *et al*., 2016). The selected colonies were purified onto fresh agar plates. For analysis of bacterial diversity, primer sets were used to target ≈1 300 bp of the 16S rRNA gene. Primers NL1 and NL4 were used for the amplification of the D1/D2 domain of the 26S rRNA gene (≈600 bp) of yeasts. For mould identification, a PCR-mediated reaction was performed targeting the internal transcribed spacer region (ITS1, ITS2) and using primers ITS1 and ITS4 (≈600 bp) (Table 4.1).

Pure culture (20 µl) was used as template DNA. The PCR was carried out in a total volume of 50 µl, containing 1X ThermoPol® reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton®-X-100, pH 8.8 @ 25°C), 0.2 mM dNTPs, 0.52 µM of each primer and 1 unit of Taq DNA polymerase (New England Biolabs). PCR reaction conditions for each primer set are indicated in Table 4.2. PCR products were separated on a 1% agarose gel, stained with 0.05% Ethidium bromide, and visualised using UV light. Digital images were captured with the Molecular Imager® Gel Doc™ XR system (BioRad Laboratories Inc.).

After purification using the Diffinity RapidTip®2 (Sigma), forward and reverse primers were used for sequencing in separate reactions (Frohlich-Nowoisky *et al*., 2016). Sequencing was performed using the ABI Prism 3130 XL genetic analyser and the Big Dye® Terminator V3.1 Cycle Sequencing Kit

(Applied Biosystems). DNA was precipitated with EDTA and ethanol. Contigs of forward and reverse sequence results were assembled using DNA Baser sequence assembly software and compared with sequences accessible in the GenBank database using the BLAST algorithm (megablast) (Altschul, 1997; Frohlich-Nowoisky *et al*., 2016). Sequences with high similarity were then subjected to multiple sequence alignments using Clustal Omega (EMBL-EBI) for identification (Daugelaite *et al*., 2013). Only similarities with a BLAST index of 97% and above were considered for identification (Wei *et al*., 2015). All the analyses were performed at least in duplicate.

Table 4.1: The Nucletide sequence of primers used in this study

Microorganisms	Forward Primer	Reverse Primer	Reference
Bacteria	63F (5'-CAG GCC TAA CAC ATG CAA GTC-3')	1387R (5'-GGG CGG WGT GTA CAA GGC-3')	Marchesi *et al*., 1998
Yeast	NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3')	NL4 (5'-GGT CCG TGT TTC AAG ACG G-3')	Kurtzman & Robnett, 1998; Yang *et al*., 2011
Mould	ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')	ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')	Davolos & Pietrangeli, 2007; Rojo *et al*., 2017 White *et al*., 1990

Table 4.2: PCR workflow for bacteria, yeast and mould

Bacteria			
PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	94°C	180 s	1 cycle
Denaturing	94°C	30 s	30 cycles
Annealing	55°C	30 s	30 cycles
Extension	68°C	90 s	30 cycles (After 30 cycles, final extension was performed once for 6 min.)
Yeast			
PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	95°C	180 s	1 cycle
Denaturing	95°C	30 s	30 cycles
Annealing	55°C	30 s	30 cycles
Extension	68°C	60 s	30 cycles (After 30 cycles, final extension was performed once for 6 min.)
Mould			
PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	95°C	180 s	1 cycle
Denaturing	95°C	30 s	30 cycles
Annealing	55°C	30 s	30 cycles
Extension	68°C	60 s	30 cycles (After 30 cycles, final extension was performed once for 6 min.)

4.3. Results and Discussion

4.3.1. Culturable fraction identified during peak and off-peak sampling

Airborne microorganisms occur ubiquitously in ambient air (Walser *et al*., 2017) and are naturally part of the air in almost any environment. These microbes can originate, not only from humans, but are also spawned by various indoor characteristics (such as ventilation, heating and air conditioning systems) and outdoor environmental sources. Although airborne microorganisms encountered in indoor facilities are still deemed innocuous for healthy individuals, they can cause adverse health effects when high counts are ingested or inhaled (Brandl *et al.,* 2014; Heo *et al*., 2017). Moreover, bioaerosols are easily translocated from one ecosystem to another by wind and air currents, thus making them an important vehicle for the spread of potentially pathogenic organisms (Wijnand *et al.,* 2012). When associated with dust particles or condensation droplets, these organisms can be dispersed among different areas in a food processing unit. International food industries are required by authorities such as the Food and Drug Administration (FDA) to take measures to reduce product contamination by airborne microorganisms (Downes & Ito, 2001; FDA, 2017).

Bacteria, yeast and mould are the main groups of microorganisms categorised as potential pathogenic airborne microorganisms. Bacteria, yeast and mould were identified more than 30 years ago in various food industries as bioaerosols. These industries include dairy processing facilities (Kang & Frank, 1988), poultry-slaughtering facilities (Lutgring *et al*., 1996), automated chicken egg production facilities (Venter *et al*., 2004), and bakeries (Saranraj & Geetha, 2011). In consideration of these earlier findings, it was expected that bacteria, yeast and mould would be recovered during the peak and off-peak seasons (sampling sessions) in the selected facility's air environment.

The study detected a total of 239 bacteria and 41 yeasts and 43 moulds that were isolated from the air in the production environment of the selected facility. An overview of these bioaerosols is presented as a distribution tree where the bacteria, yeast and mould are classified into different phylogenetic orders (Figure 4.2 and Figure 4.3). From the isolates obtained, 92 different species were identified from the culturable fraction. These microorganisms belonged to 15 different taxonomic orders representing five bacteria and ten yeast and mould orders.

.

	Dead organic matter		Actinobacterium
	Found in soil and plant roots		Anti-oxidant, anti-tumor and anti-carcinogenic activities
	Food		Biosynthesis, biodegration, biomedical application, biological control, biocontrol, bioremediation
	Found in the bathroom		Food making
	Found in Spacecraft room		Nitrogen fixation and plant growth promotions
	Found on the skin of mammals and birds		Patent degrade environmental pollution
	Hospital environment		Produce antibiotics
	In humans		Antibiotic resistant, antifungal resistant
	In pillows		Dairy spoilage
	Lives on human skin		Food spoilage
	Lives on wild animal skin		Pathogen
♀	Normal flora of female genital tract		Plant pathogen
	Wetlands and ponds		Bioaerosols
	Wounds and blood		
?	Are not sure where comes from		
	No specific meaning		

Figure 4.2: Symbol key: These symbols are used in Figure 4.3 to link the microorganisms to their origins (from 'Dead organic matter' to 'Wounds and blood), interest (from "Not sure where it comes from' to 'No specific meaning'), and importance (from 'Actinobacterium' to 'Bioaerosols').

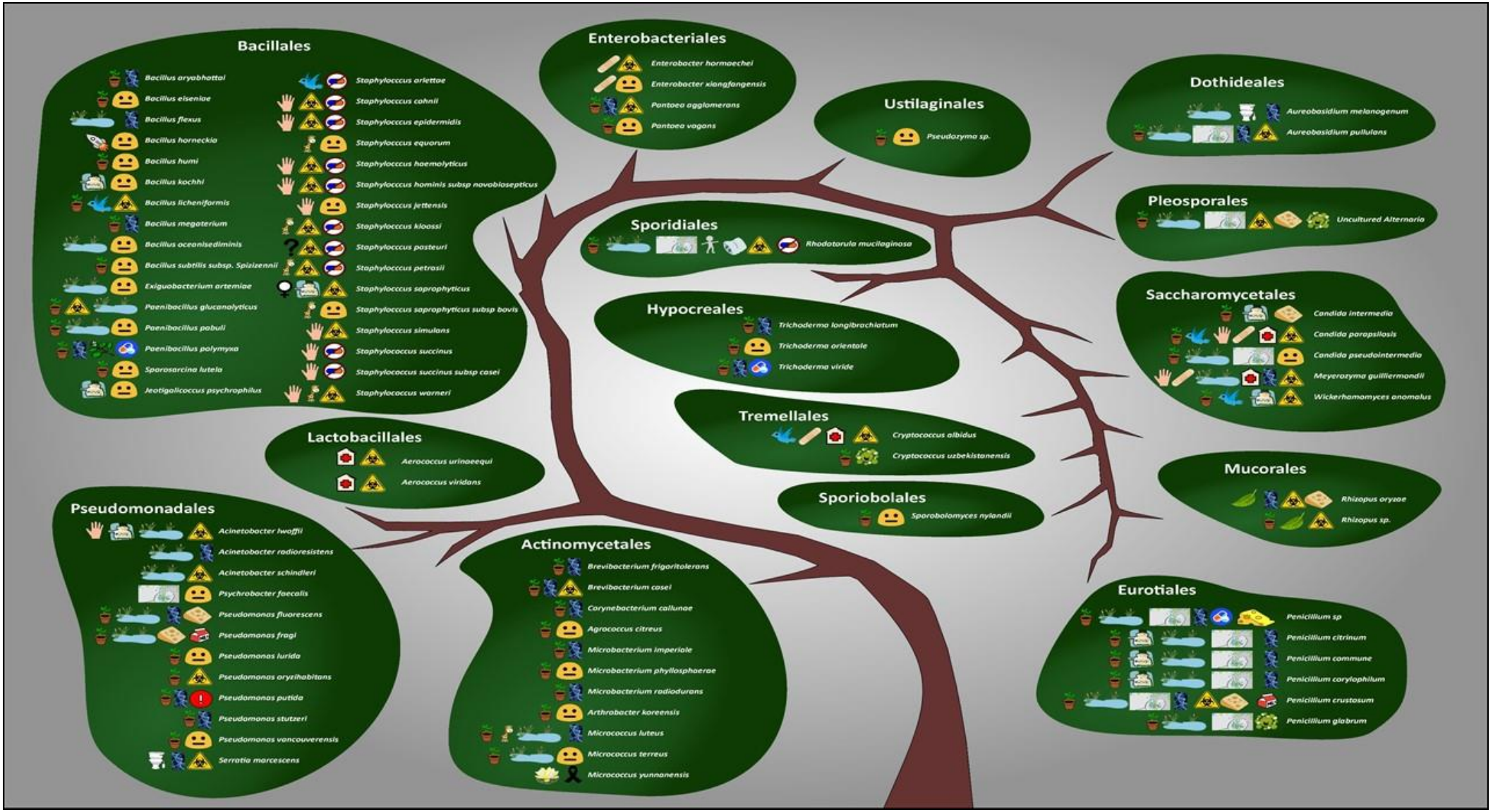

Figure 4.3: Distribution tree: overview of the culturable bioaerosol fraction: As this is a distribution tree, each order is shown as different leaves and the various taxa are indicated in italics. The meaning of each symbol is outlined in Figure 4.2. This is not a phylogenetic tree, nor is there a specific listing order; it merely represents the total diversity detected. Microbial orders are discussed from the bottom left starting with the Actinomycetales in a clockwise direction under the two different kingdoms. The Bacillales, Pseudomonadales and Actinomycetales, and to a lesser extent the Eurotiales and Saccharomycetales orders, were the most prevalent according to the identification of the culturable fraction.

Bacteria are the most abundant and diverse group of organisms (Xia *et al*., 2015) and are ubiquitous in every habitat on Earth. They can be present in soil, water and organic matter as well as in live bodies of plants and animals. Their presence in indoor environments is mostly related to human occupancy and type of indoor environment (Bragoszewska *et al*., 2016). Moreover, bacteria are abundant in the atmosphere where they often represent a major portion of organic aerosols (Bowers *et al*., 2011). Even though bacteria were represented by a smaller group of orders in this study, they represented 62 different species. When the study commenced, it was envisaged that a large number of bacterial species would be detected because four different media types were used for cultivating bacteria.

What is immediately visible in Figure 4.3 is that many bioaerosols that were detected in the facility probably originated from soil and plant roots (), wetlands and ponds (), and human skin (). Many species that were detected had no specific significance (); however, a reasonable quantity could be considered as pathogenic (), specifically in the order Bacillales that is antibiotic resistant (). Mainly fungal isolates and one Pseudomonas have previously been described as bioaerosols (). Only four fungal and two bacterial isolates have previously been associated with food poisoning/spoilage ().

All of the species found in the order Actinomycetales are found in soil and plant roots. Of these species, *Micrococcus* seems to be a predominant specie in indoor air (Bragoszewska *et al*., 2016; Kooken *et al*., 2012). The order Pseudomonadales was represented by the genera *Acinetobacter* spp., *Psychrobacter* spp. and *Pseudomonas* spp., most of which are found in soil, plant roots, wetlands and ponds. Evidence indicates that bioaerosol agents such as *Pseudomonas* spp. cause occupational health threats to immuno-compromised patients (Zemouri *et al*., 2017). *Psychrobacter faecalis* is one species of this order that was discovered in a bioaerosol originating from pigeon faeces (Kampfer *et al*., 2002).

The order Lactobacillales represents a morphologically, metabolically and physiologically diverse group of bacteria (Mekadim *et al*., 2019). In the current study, only one genus, *Aerococcus* spp., with two different species was identified. Both these species are classified as pathogenic. They are prevalent in hospital environments and can form biofilms (Rasmussen, 2016). The order Bacillales represents a wide variety of different species with a wide variety of origins and interests. Genera from the order Bacillales are frequently found to be part of bioaerosols, because genera such as *Bacillus* and *Paenibacillus* can form highly tolerant endospores that can travel long distances (Bragoszewska *et al*., 2016; Hara *et al*., 2015). Two main genera were identified, namely *Bacillus* and *Staphylococcus*, which

are known for the fact that they form part of bioaerosols. Some of the species possess pathogenic abilities and are resistant to antibiotics (Bragoszewska *et al*., 2016; Madsen *et al*., 2018; Wang *et al*., 2015; Zemouri *et al*., 2017). From the Enterobacteriales order, three different genera were identified, namely *Enterobacter* spp. and *Pantoea* spp. (both are found in soil and/or plant roots and in wounds and blood) as well as *Serratia* spp. (which is mostly found in bathrooms). *Pantoea agglomerans* is usually associated with plants and is seen as a bacterium of evil (causing opportunistic human infections) and good (contributing to plant growth) (Dutkiwicz *et al*., 2016).

Eukaryotic cells are considerably more complicated than those of prokaryotic origin and are characterised by a high degree of cellular complexity (lysosomes, peroxisomes, microtubules, mitochondria, cytoskeleton, etc.), which makes the classification of these microorganisms quite difficult (Spang *et al*., 2015). This may explain why yeast and mould could be classified in 10 different orders whereas only 23 different species were identified. Overgrowth of mould in the petri dishes was observed during the enumeration of the culturable fraction (Figures 3.1a and b) and lower yeast and mould counts were observed compared to total microbial load. This may also have contributed to a lower number of identified species (as the overgrowth may have prevented accurate isolation of other moulds and yeasts that could have been present) (Beuchat & Mann, 2016; Douglas *et al.,* 1979). *Pseudozyma,* a yeast found mainly in soil and plant roots (Sajna *et al*., 2015), was the only genus detected from the order Ustilaginales. Of the order Dothideales (microorganisms found mostly in soil, plant roots, wetlands and ponds), only one of the species, *Aureobasidium pullulans*, was previously classified as a bioaerosol (Castoria *et al*., 2001).

Pleosporales is the largest order in the Dothideomycetes species and it comprises a quarter of all the detected Dothideomycetous species. Species in this order occur in various habitats, including bioaerosols (Zhang *et al*., 2012). From the Pleosporales order, one genus was detected, namely an uncultured *Alternaria* spp. that can originate from a large variety of environments such as soil plant roots, wetlands and ponds. The *Alternaria* spp. is seen as a plant pathogen; however, reports have stated that it is also prevalent in the food industry (Fernandez-Rodriquez *et al*., 2015). Of the order Saccharomycetales, three well known genera were detected: (i) *Candida* spp. (one specific *Candida* species, *Candida pseudointermedia,* was previously identified as a bioaerosol), that originates from soil and plant roots and has the ability to cause invasive fungal infection that can have a significant impact on public health (Sowiak *et al*., 2012; Trofa *et al*., 2008); (ii) *Meyerozyma guilliermondii,* which is known for its ability to live on human skin and in wounds and blood and has spoilage abilities (Wrent *et al*., 2015); and (iii) *Wickerhamomyces anomalus,* which is found in food and has pathogenic abilities (Miceli

et al., 2011). The order Mucorales was represented by only one genus, namely a *Rhizopus* spp. This genus is mostly found in dead organic matter and has pathogenic abilities (Spelberg, 2017).

Eurotiales are widespread and abundant fungi that include the well-known genus *Penicillium*. *Penicillium* is recognised as one of the most abundant mould genera in indoor air (Bragoszewska *et al*., 2016; Kobza *et al*., 2018; Sowiak *et al*., 2012). Similarly, in the order Sporobolales, only one species was detected, namely *Sporobolomyces nylandii*, which is normally found in soil and/or plant roots (Limtong & Nasanit, 2017). *Cryptococcus* spp., from the order Tremellales, were also identified. These species are known to have either human or plant pathogenic abilities and have been identified as bioaerosols (Huang *et al*., 2015; Sowiak *et al*., 2012). From the Hypocreales order three different *Trichoderma* spp. were detected, and all originate from soil and/or plant roots (Azin *et al*., 2007; Du Plessis *et al*., 2018; Ghorbani *et al*., 2015). From the Sporidiales order, one microorganism was identified, namely *Rhodotorula mucilaginosa.* This organism is found in soil, plant roots, wetlands, ponds, and in humans and on/in pillows. *Rhodotorula mucilaginosa* has been receiving increased attention because it can be isolated from extreme ecosystems and has the capability to survive and grow in many unfavourable conditions. It is also classified as a bioaerosol and a human pathogen (Deligios *et al*., 2015).

Various microorganisms that were detected support the existing scientific literature that indoor exposure to microorganisms poses a risk for asthma and allergies among occupants of indoor facilities (Lipsa *et al*., 2016). It is undeniable that microbial contamination of a facility has the potential to affect the product and places the occupants at risk of developing airway difficulties. Surprisingly, little research is available with regards to these microorganisms in the food industry, especially in the fruit juice industry, and therefore it was important to clearly classify the microorganisms that were identified. This will aid in better understanding the prevalence and ecology of specific indoor airborne bioaerosols and will be a useful tool in the management and prevention of both long- and short-term problems faced in the fruit juice industrial setting (Bragoszewska *et al*., 2016).

4.3.2. Classification of the bioaerosols that were detected

Bacteria form a large part of airborne particles and comprise bacteria, fungi, viruses, pollen and fragments of these or their metabolic products (endotoxins, mycotoxins). It is reiterated that bioaerosols are of natural origin (such as rotting leaves and mould growth); are ubiquitous in natural environments; play a key role in the dispersal of reproductive units from plants, microbes and organisms; and that they are central elements in the development, evolution and dynamics of ecosystems. The actual identity,

diversity and abundance of different types of bioaerosol particles, as well as their temporal and spatial variability, are not well characterised. Overall, the role of bioaerosols in the atmosphere and their interaction with other ecosystems are not well described and understood. This lack of knowledge is particularly evident with regards to the assessment and prediction of bioaerosols (Frohlich-Nowoisky *et al*., 2016).

Bioaerosols are generated via multiple sources such as different instruments, external environments, and human activity. Bioaerosols have varying microbiological profiles depending on their origin and reason of interest. Bioaerosols can be hazardous to both a product produced in an industry and the workers (Zemouri *et al*., 2017), depending on the kind of bioaerosol that is involved. The literature classifies bioaerosols into three groups, namely: (i) innocuous (Bonadonna *et al*., 2017); (ii) useful (Fröhlich-Nowoisky *et al*., 2016); and (iii) harmful (Majchrzycka *et al*., 2017). After the identification of the culturable fraction of bioaerosols in the current study, it was concluded that 27 innocuous, 26 useful and 39 potentially harmful bioaerosols had been detected.

4.3.2.1. Innocuous bioaerosols

Innocuous microorganisms were classified in 1985 by the European Federation of Biotechnology as "microorganisms that have never been identified as causative agents of disease in man and that offer no threat to the environment" (Kuenzi *et al*., 1985a). For a microorganism to be described as environmentally safe, it should meet the following criteria: (i) be non-pathogenic to humans, animals and plants; (ii) must have a limited ability to compete; (iii) will not indirectly affect other species (by the production of toxic metabolites or biogeochemical changes); (iv) is unable to irreversibly alter equilibria between nutrients, microflora, and higher organisms; (v) is unable, in the open environment, to transfer genetic traits that can be noxious in other species; and (vi) does not contribute to unwanted traits (Frohlich-Nowoisky *et al*., 2016; Lelieveld *et al*., 1996).

During this study, 27 different microorganisms were identified as innocuous bioaerosols (Table 4.3). Of these innocuous bioaerosols, two genera were dominant, namely *Bacillus* and *Staphylococcus*. The genus *Bacillus* includes more than 200 species, is widespread in nature and is found in virtually every environment (Hong *et al*., 2012). Although the *Bacillus* species are ostensibly well-known as pathogens, the overwhelming majority are in actual fact non-pathogenic (Rooney *et al*., 2009). The *Staphylococcus* species are reported as normal microbiota of mammals and birds; however, certain species are important pathogens in humans and animals. It is noteworthy that little is known about the *Staphylococcus* species that are non-pathogenic environmental microorganisms (Gomez *et al*., 2017).

Only four innocuous yeasts and moulds were detected. Although yeast and mould are well-known for their fermentation ability and pharmaceutical properties, it has been found that they are microorganisms that do more harm than good in food and food-related industries (Goyer *et al*., 2001; Kobayaski *et al*., 2009; Mandal & Brandl, 2011; Sorensen *et al*., 1984).

Table 4.3: Innocuous bioaerosols detected and classified alphabetically from order to specie

Innocuous Bioaerosols – Bacteria			
Order	**Family**	**Genus and Specie**	**Reference**
Actinomycetales	Brevibacteriaceae	*Agrococcus citreus*	Wieser *et al*., 1999
Actinomycetales	Microbacteriaceae	*Microbacterium phylloshaerae*	Alcocer *et al*., 2007
Actinomycetales	Micrococcaceae	*Arthrobacter koreensis*	Lee *et al*., 2003
Actinomycetales	Micrococcaceae	*Micrococcus terreus*	Zhang *et al*., 2010
Bacillales	Bacillaceae	*Bacillus eiseniae*	Hong *et al*., 2012
Bacillales	Bacillaceae	*Bacillus horneckiae*	Vaishampayan *et al*., 2010
Bacillales	Bacillaceae	*Bacillus humi*	Heyrman *et al*., 2005
Bacillales	Bacillaceae	*Bacillus kochii*	Seiler *et al*., 2012
Bacillales	Bacillaceae	*Bacillus oceanisediminis*	Zhang *et al*., 2010
Bacillales	Bacillaceae	*Bacillus subtilis subsp. spizizenii*	Rooney *et al*., 2009
Bacillales	Bacillaceae	*Exiguobacterium artemia*	Lopez-Cortes *et al*., 2006
Bacillales	Paenibacillaceae	*Paenibacillus pabuli*	Jemli *et al*., 2007.
Bacillales	Planococcaceae	*Sporosarcina luteola*	Tominaga *et al*., 2009
Bacillales	Staphylococcaceae	*Jeotgalicoccus psychrophilus*	Yoon *et al*., 2003
Bacillales	Staphylococcaceae	*Staphylococcus equorum*	Place *et al*., 2003
Bacillales	Staphylococcaceae	*Staphylococcus jettensis*	De Bel *et al*., 2014
Bacillales	Staphylococcaceae	*Staphylococcus saprophyticus subsp. bovis*	Hajek *et al*., 1996
Enterobacteriales	Enterobacteriaceae	*Enterobacter xiangfangensis*	Chavda *et al*., 2016
Enterobacteriales	Erwiniaceae	*Pantoea vagans*	Palmer *et al*., 2016
Pseudomonadales	Moraxellaceae	*Psychrobacter faecalis*	Kamper *et al*., 2002
Pseudomonadales	Pseudomonadaceae	*Pseudomonas lurida*	Behrendt *et al*., 2007
Pseudomonadales	Pseudomonadaceae	*Pseudomonas vancouverensis*	Gupta & Prakash, 2014
Sphingomonadales	Sphingomonadaceae	*Sphingomonas pseudosanguinis*	Kämpfer *et al*., 2007

Innocuous Bioaerosols – Yeast and Mould			
Order	**Family**	**Genus and Specie**	**Reference**
Saccharomycetales	Saccharomycetaceae	*Candida pseudointermedia*	Nakase *et al*., 1976
Hypocreales	Hypocreaceae	*Trichoderma orientale*	Du Plessis *et al*., 2018
Sporiobolales	Incertae sedis	*Sporobolomyces nylandii*	Limtong & Nasanit, 2017
Ustilaginales	Ustilaginaceae	*Pseudozyma spp.*	Sajna *et al*., 2015

Although these microorganisms would have been innocuous to the products, the workers in the facility and the environment, they were still part of the bioaerosols that were detected during bioaerosol sampling. The high microbial counts that were observed during sampling immediately created the inaccurate assumption that the air was contaminated with hazardous or unsafe bioaerosols (Viegas *et al*., 2018). Therefore, simply analysing bioaerosols for total heterotrophic counts as specified by certain countries (Table 2.8) to determine air quality could be considered a shortcoming.

4.3.2.2. Useful bioaerosols that were detected

Useful microorganisms are generally: (i) environmentally beneficial; (ii) useful in food; (iii) making positive medical contributions; and (iv) advantageous for technology and the future. For example, the use of beneficial/useful microorganisms contributes positively towards environmentally safe agricultural products. The modes of action of these useful microorganisms and their various benefits to plants range from the simple occupation of biological empty spaces to ecological relationships such as antibiosis, competition, predation, and symbiosis, among others (Figueiredo *et al*., 2016). Other beneficial microorganisms represent an important biotechnological approach to decrease the deleterious effects of stress in crops (Egamberdieva *et al*., 2013; Nadeem *et al*., 2014). Studies have also indicated that the growth-promoting ability of some bacteria to synthesise extracellular polysaccharides or exopolysaccharides has commercially significant applications (Nwodo *et al*., 2012).

The use of beneficial microorganisms can potentially revolutionise agriculture and food industries by: (i) integrating crop health with better management practices for specific climatic conditions to improve productivity and quality; (ii) using environmentally friendly approaches to control pests and pathogens, thus reducing the use of chemical pesticides with environmental and health implications; (iii) producing better quality food with less chemical contamination and allergens; and (iv) minimising losses by improving crop fitness in extreme weather conditions (Singh & Trivedi, 2017).

One of the most exciting scientific advances in recent years has been the realisation that commensal microorganisms play key roles in our physiology (including protection against infection) and in drug metabolism, vitamin synthesis, nutrition, as well as in response to disease (Wischmeyer *et al*., 2016). The beneficial influence of microorganisms is still on the border of its' potential and a great deal of future discoveries and technologies are anticipated. In the current study, the useful bioaerosols that were detected during the selected sampling seasons were categorised into three groups, namely: (i) medical contribution; (ii) promoting and protecting plant growth; and (iii) environmental contribution. These benefits are listed in Table 4.4 for each identified microorganism.

Table 4.4: Alphabetical classification of useful bioaerosols detected in the selected facility (peak and off-peak seasons) according to: medical contribution, promoting and protecting plant growth and environmental contribution

Medical Contribution – Bacteria		
Genus and Specie	**Benefit**	**Reference**
Acinetobacter radioresistens	Purification and biochemical properties	Briganti *et al*., 1997
Bacillus flexus	Capable of synthesis of anisotropic silver nanoparticles	Priyadharsshini *et al*., 2012
Bacillus megaterium	Capable of biosynthesis of silver nanoparticles and have antibacterial activity on multi drug resistant clinical pathogens	Saravanan *et al*., 2011
Brevibacterium frigoritolerans	Capable of producing silver nanoparticles	Singh *et al*., 2015
Corynebacterium callunae	Have the function for activity and stability of the enzyme Orthophosphate	Mueller & Nidetzky, 2010
Microbacterium radiodurans	UV radiation-tolerant bacterium	Zhang *et al*., 2010
Micrococcus yunnanensis	Anti-oxidative, anti-tumour-promoting, and anti-carcinogenic activities of adonirubin and adonixanthin	Maoka *et al*., 2013
Medical Contribution – Yeast and Mould		
Genus and Specie	**Benefit**	**Reference**
Meyerozyma guilliermondii	Antifungal activity	Coda *et al*., 2012
Penicillium corylophilum	Antibacterial activity	Silva *et al*., 2004
Penicillium spp.	Capable of biosynthesis of silver nanoparticles	Hemath *et al*., 2010

Promoting and Protecting Plant Growth – Bacteria		
Genus and Specie	**Benefit**	**Reference**
Bacillus aryabhattai	Zinc-solubilizing abilities	Ramesh *et al*., 2013
Brevibacterium casei	Capable of promoting plant growth	Plociniczak *et al*., 2016
Promoting and Protecting Plant Growth – Bacteria		
Genus and Specie	**Benefit**	**Reference**
Microbacterium imperiale	Capable of biodegradation of bromoxynil – to reduce its acute toxicity	Pasquarelli *et al*., 2015
Paenibacillus polymyxa	Capable of nitrogen fixation, plant growth promoting, soil phosphorus solubilisation and production of exopolysaccharides, hydrolytic enzymes, antibiotics and cytokinin. Helps bioflocculation and the enhancement of soil porosity as well as capable of producing optically active 2,3-butanediol (BDL)	Lal & Tabacchioni, 2009
Pantoea agglomerans	Capable of controlling post-harvest diseases on apples	Nunes *et al*., 2002
Pseudomonas fluorescens	Plant protection	Rezzonico *et al*., 2005
Serratia marcescens	Capable of biocontrol against avocado pathogens	Granada *et al*., 2016
Promoting and Protecting Plant Growth – Yeast and Mould		
Genus and Specie	**Benefit**	**Reference**
Aureobasidium pullulans	Biotechnologically important yeast	Chi *et al*., 2009
Penicillium citrinum	Capable of producing plant growth by promoting metabolites	Khan *et al*., 2008
Trichoderma longibrachiatum	Help optimising culture conditions for agricultural purposes	Azin *et al*., 2007

Environmental Contribution – Bacteria		
Genus and Specie	**Benefit**	**Reference**
Micrococcus luteus	Capable of bioremediation of polychlorinated biphenyl (PCB) contaminated environments	Su *et al*., 2014
Pseudomonas putida	Capable of Xenobiotic degrading	Samantha *et al*., 2002
Pseudomonas stutzeri	Capable of denitrification, degradation of aromatic compounds and nitrogen fixation	Lalucat *et al*., 2006
Environmental Contribution – Yeast and Mould		
Genus and Specie	**Benefit**	**Reference**
Aureobasidium melanogenum	Promising biomaterial and can be used for packing food and drugs	Zalar *et al*., 2008
Rhizopus oryzae	Capable of biodiesel production	Rodrigues *et al*., 2016
Trichoderma viride	Capable of enhancement of fungal delignification	Ghorbani *et al*., 2015

Natural products (plants, animals and microorganisms) are essential, reputable resources that originate from Earth's bio-diverse flora and fauna. These natural products are encoded to be bioactive and have been used as medicines for ages. Today, they continue to be a reservoir of potential resources (David *et al*., 2014). Recently, the global threat of anti-microbial resistance has increased the need for urgent therapeutic discoveries and the improvement of existing antimicrobial practices (Adukwe *et al*., 2016). Numerous medical conditions are the focus of these efforts; however, one of the medical areas in which microorganisms have contributed tremendously in the last few years is cancer research. Cancer is a collective term used for diseases that are characterised by the loss of control of growth and the division and the spread of cells that lead to primary tumours that invade and destroy adjacent tissues. Cancer is undeniably one of the most serious health threats worldwide (Chen *et al*., 2014). By loading anti-cancer drugs into nanoparticles, more favourable pharmacokinetics and adjustable biodistribution of nanoparticles can increase the efficacy of the drug (Quinto *et al*., 2015). It is noteworthy that the current study detected four microorganisms that have the capability of producing silver nanoparticles. Silver nanoparticles are an arch product from the field of nanotechnology and have gained boundless interest because of their unique properties such as chemical stability, good conductivity, catalytic properties and, most importantly, antibacterial, anti-viral and antifungal activities (Ahmed *et al*., 2016).

The urgency of feeding the world's growing population while at the same time combating soil pollution, salinization and desertification has given plant and soil productivity research vital importance. It requires suitable biotechnology not only to improve crop productivity, but also to improve soil health through interactions of plant, root and soil microorganisms (Shrivastava & Kumar, 2014). Some plant growth-promoting rhizobacteria may exert a direct stimulation on plant growth and development by providing plants with fixed nitrogen, phytohormones and iron sequestered by bacterial siderophores and soluble phosphate (Hayat *et al*., 2010). Others do this indirectly by protecting the plant against soil-borne diseases (Lutgtenberg & Kamilova, 2009).

In order to make the environment healthier for human beings, contaminated water bodies and land need to be rehabilitated to make them free from toxic waste, heavy metals and trace elements. With the escalated growth of various industries, there has been a considerable increase in the discharge of industrial waste into the air, soil and water, and this has led to the accumulation of heavy metals and toxic waste in these environments, especially in urban areas. The use of microorganisms (*Micrococcus luteus* for example) for remediation technologies and bioremediation to rehabilitate and re-establish the natural condition of the environment is an emerging science (Dixit *et al*., 2015; Singh *et al*., 2013). Other ways of environmental rehabilitation using microorganisms, such as fungal delignification

(*Trichoderma viride*) (Thomsen *et al*., 2016) and biodiesel production (*Rhizopus oryzae*) (Aransiola *et al*., 2014) have also been investigated during the last few years.

The 26 different innocuous species that were identified in the selected facility could all be extremely beneficial in various fields of technology; however, not one of these microorganisms was likely to have a direct impact on the product or the food handlers in the facility. Therefore, because there are still no standards nor an implementation plan available (Crook et al., 2016), it is important to create awareness of what needs to be monitored in each industrial environment. Moreover, these criteria should be standardised.

Although innocuous and useful bioaerosols do not negatively influence human health, it is critical to mention that the presence of innocuous and useful bioaerosols still serves as an indicator that an ideal environment exists for possible harmful bioaerosols to emerge. In addition, any type of bioaerosol that occurs in excess may have a negative influence on the food product and this should also be considered (Adams *et al.,* 2015; Crook et al., 2016; Frankel *et al*., 2012; Viegas *et al*., 2018.; Wu *et al*., 2017; Zhen *et al*., 2017).

4.3.2.3. Potentially harmful bioaerosols

Various bioaerosols can have infectious, allergenic or toxic effects on living organisms and may impact human and animal health and agricultural outcomes on a local, regional or global scale. Many plant, animal and human pathogens are dispersed through the air (Fisher *et al*., 2012), and thus the occupational health of workers is easily affected. Various major infectious diseases in humans such as foot-and-mouth disease, tuberculosis, Legionnaire's disease, influenza and measles can be spread by airborne bacteria or viruses (Frohlich-Nowoisky *et al*., 2016; Lipsa *et al*., 2016). Moreover, the inhalation of pathogenic, viable airborne fungi such as *Aspergillus*, *Cryptococcus* and *Pneumocystis* spp. into the lungs can cause invasive infections associated with mortality rates of up to 95% in infected populations (Brown *et al*., 2012; Lin *et al*., 2001; Lipsa *et al*., 2016; Yu *et al*., 2010).

Food safety is a complex issue that has an impact on multiple segments of society. Usually a food is considered too adulterated if it contains a poisonous or otherwise harmful substance that is not an inherent natural constituent of the food itself; if it poses a reasonable possibility of injury to health or is presented in a substance that is an inherent natural constituent of the food itself; if it is not the result of environmental, agricultural, industrial, or other contamination; and if is present in a quantity that ordinarily renders the food injurious to health (Sowiak *et al*., 2012).

Harmful microorganisms can: (i) be pathogenic/infectious; (ii) multidrug resistant; (iii) cause food poisoning; (iv) cause food spoilage; (v) be used in biological warfare; and (vi) cause negative occupational health effects. As was expected, no biological warfare microorganisms were detected in the facility under study, but it was expected that a large number of allergenic and/or toxic agents forming bioaerosols and causing occupational diseases of the respiratory tract and skin would be present due to the layout (no airflow, production lines in close proximity to one another) and the type of product the facility produced (Wang *et al*., 2015). Table 4.5 depicts the four types of 39 harmful bioaerosols that were detected during the two sampling seasons.

Table 4.5: Harmful bioaerosols detected and classified alphabetically according to their pathogenicity and infection potential, multidrug resistance, food poisoning and food spoilage potential

Pathogenicity/Infection Potential – Bacteria					
Genus and Specie	**Reference**	**Genus and Specie**	**Reference**	**Genus and Specie**	**Reference**
Acinetobacter woffii	Rosa *et al*., 2015	*Pantoea agglomerans*	Dutkiewicz *et al*., 2016	*Staphylococcus kloosii*	Mascarenhas dos Santos *et al*., 2018
Acinetobacter schindleri	Wong *et al*., 2017	*Pseudomonas oryzihabitans*	Choi *et al*., 2018	*Staphylococcus pasteuri*	Savini *et al*., 2009
Aerococcus urinaeequi	Rassmussen, 2015	*Pseudomonas stutzeri*	Lalucat *et al*., 2006	*Staphylococcus petrasii*	Pantucek *et al*., 2013
Aerococcus viridans	Mohan *et al*., 2017	*Serratia marcescens*	Quinn *et al*., 2018	*Staphylococcus saprophyticus*	Trivedl *et al*.., 2015
Bacillus licheniformis	Ronning *et al*., 2015	*Staphylococcus cohnii*	Garg, 2017	*Staphylococcus simulans*	Shields *et al*., 2016
Brevibacterium casei	Bal *et al*., 2015	*Staphylococcus epidermidis*	Otto, 2013	*Staphylococcus succinus*	Novakova *et al*., 2006
Enterobacter hormaechei	Rafferty *et al*., 2011	*Staphylococcus haemolyticus*	Czekaj *et al*., 2015	*Staphylococcus succinus subsp. casei*	Novakova *et al*., 2006
Paenibacillus glucanolyticus	Athan, 2014	*Staphylococcus hominis* subsp *novobiosepticus*	Ahmed *et al*., 2017	*Staphylococcus warneri*	Dimitriadi *et al*., 2014

Pathogenicity/Infection Potential – Yeast and Mould					
Genus and Specie	**Reference**	**Genus and Specie**	**Reference**	**Genus and Specie**	**Reference**
Alternaria spp.	Fernandez-Rodriquez *et al*., 2015	*Cryptococcus albidus*	Huang *et al*., 2015	*Rhodotorula mucilaginosa*	Dellgios *et al*., 2015
Aureobasidium pullulans	Castoria *et al*., 2001	*Cryptococcus uzbekistanensis*	Dehghan-Niri, *et al*., 2015	*Wickerhamomyces anomalus*	Miceli *et al*., 2011
Candida intermedia	Sheng-Yuan *et al*., 2010	*Rhizopus oryzae*	Han *et al*., 2018		
Candida parapsilosis	Trofa *et al*., 2008	*Rhizopus spp.*	Spellberg, 2017		
Multidrug Resistance – Bacteria					
Genus and Specie	**Reference**	**Genus and Specie**	**Reference**	**Genus and Specie**	**Reference**
Staphylococcus arlettae	Liu *et al*., 2017	*Staphylococcus epidermidis*	Otto, 2013	*Staphylococcus hominis* subsp *novobiosepticus*	Ahmed *et al*., 2017
Staphylococcus cohnii	Garg, 2017	*Staphylococcus haemolyticus*	Czekaj *et al*., 2015	*Staphylococcus succinus*	Novakova *et al*., 2006
Food Poisoning – Bacteria					
Genus and specie	**Reference**	**Genus and specie**	**Reference**	**Genus and specie**	**Reference**
Bacillus licheniformis	Ronning *et al*., 2015				

Food Poisoning – Yeast and Mould					
Genus and Specie	**Reference**	**Genus and Specie**	**Reference**	**Genus and Specie**	**Reference**
Penicillium commune	Sosa *et al*., 2002	*Penicillium crustosum*	Sonjak *et al*., 2005		
Food Spoilage - Bacteria					
Genus and Specie	**Reference**	**Genus and specie**	**Reference**	**Genus and specie**	**Reference**
Pseudomonas fluorescens	Andreani *et al*., 2014	*Pseudomonas fragi*	Decimo *et al*., 2018		
Food Spoilage – Yeast and Mould					
Genus and Specie	**Reference**	**Genus and Specie**	**Reference**	**Genus and Specie**	**Reference**
Meyerozyma guilliermondii	Wrent *et al*., 2015	*Penicillium commune*	Sosa *et al*., 2002	*Penicillium crustosum*	Sonjak *et al*., 2005

Staphylococcus spp. are indicators of the severity of air pollution and their presence may indicate the further presence of pathogenic bacteria (Bragoszewska *et al*., 2016; Kim *et al*., 2007; Kubera *et al*., 2015). In the current study, five *Staphylococcus* spp. (*cohnii*, *epidermidis*, *haemolyticus*, *hominis* subsp *novobiosepticus* and *succinus*) were detected on more than ten occasions in different areas in peak and off-peak air samples (Figure 4.4). *Staphylococcus cohnii, epidermidis, haemolyticus, hominis subsp novobiosepticus* and *succinus* are coagulase-negative staphylococci that may be responsible for bloodstream infections in immuno-suppressed patients (Ahmed *et al*., 2017; Czekaj *et al*., 2015; Garg, 2017; Novakova *et al*., 2006; Otto, 2013). Even though these species can only affect immuno-suppressed individuals, their multidrug resistance capacity against available antimicrobial agents is considered a problem and is the reason why these species are of clinical significance (Carvalhais *et al*., 2015).

Although *Staphylococcus* spp. are opportunistic pathogens and rarely cause human infections, their ability to form biofilms on different equipment surfaces had the potential to negatively influence the hygiene of workers in this specific production facility. Despite the low pH as well as the water activity and high sugar content that are characteristics of fruit juice, various *Staphylococcus* spp. have been detected in fruit juice in earlier studies (Abraha *et al*., 2018; Bentanzos-Cabrera *et al*., 2015; Carvalhais *et al*., 2015). Even with regards to infectious diseases only, no clear correlation was found between concentrations of culturable microorganisms in the air and infection. One reason for this could be that infection should be correlated with the dose-response rather than the concentration. Unfortunately, dose-response relationships still have not been established for most biological agents (Bragoszewska *et al*., 2015).

Yeast and mould have been used for centuries in the production of diverse foods and beverages. They have also been shown to be involved in the spoilage of an extensive range of foods. Yeasts, predominantly *Saccharomyces* and *Zygosaccharomyces* spp., are able to grow at low pH values in foods with a high sugar content and at refrigeration temperature, making them potential spoilers of refrigerated or concentrated fruit juices (Ferrario *et al*., 2015; Goyer *et al*., 2001; Rojo *et al*., 2017). However, none of these yeasts were isolated during the two sampling seasons using this specific sampling methodology. This suggests that they were possibly present but were not isolated; were not present in the environmental air of the facility; or were not present in the environment of this facility at all.

Fungal spoilage encompasses the decay of foods, including the development of off-flavours, acidification, discolouration, and disintegration. Moulds that are typically isolated from fruit juice belong

mainly to the *Penicillium* genus and have been identified in several earlier studies (Groot *et al*., 2018; Lipsa *et al*., 2016). In the current study, *Penicillium commune* was detected on more than ten occasions in peak and off-peak samples that had been collected in different areas (Figure 4.4). Fungal spoilage endangers the health of humans by exposing consumers to toxic secondary metabolites such as mycotoxins. The mycotoxin (Cyclopiazonic acid) producing ability displayed by this isolate is a disturbing fact as it causes poisoning in humans when ingested. It is widely known that there is an active metabolism and dissemination of hyaline fungal hyphae inside substrates before the formation of visible colonies on the surface of food. In the interstitial period, there is a risk of consumer exposure to mycotoxins (Wigmann, *et al*., 2015).

Microorganisms that have pathogenicity/infectious capacity, multidrug resistance and food poisoning/spoilage abilities can be found in the air and they also form part of certain environments as bioaerosols. Although the importance of bioaerosols and their impact on human health have been recognised, it is still difficult to accurately describe their role in the initiation or worsening of diverse symptoms and diseases. Diseases and food spoilage arise from exposure to biological agents through the transmission of infectious agents by direct and/or indirect contact, airborne transmission, and vector-borne transmission (Kim *et al*., 2018).

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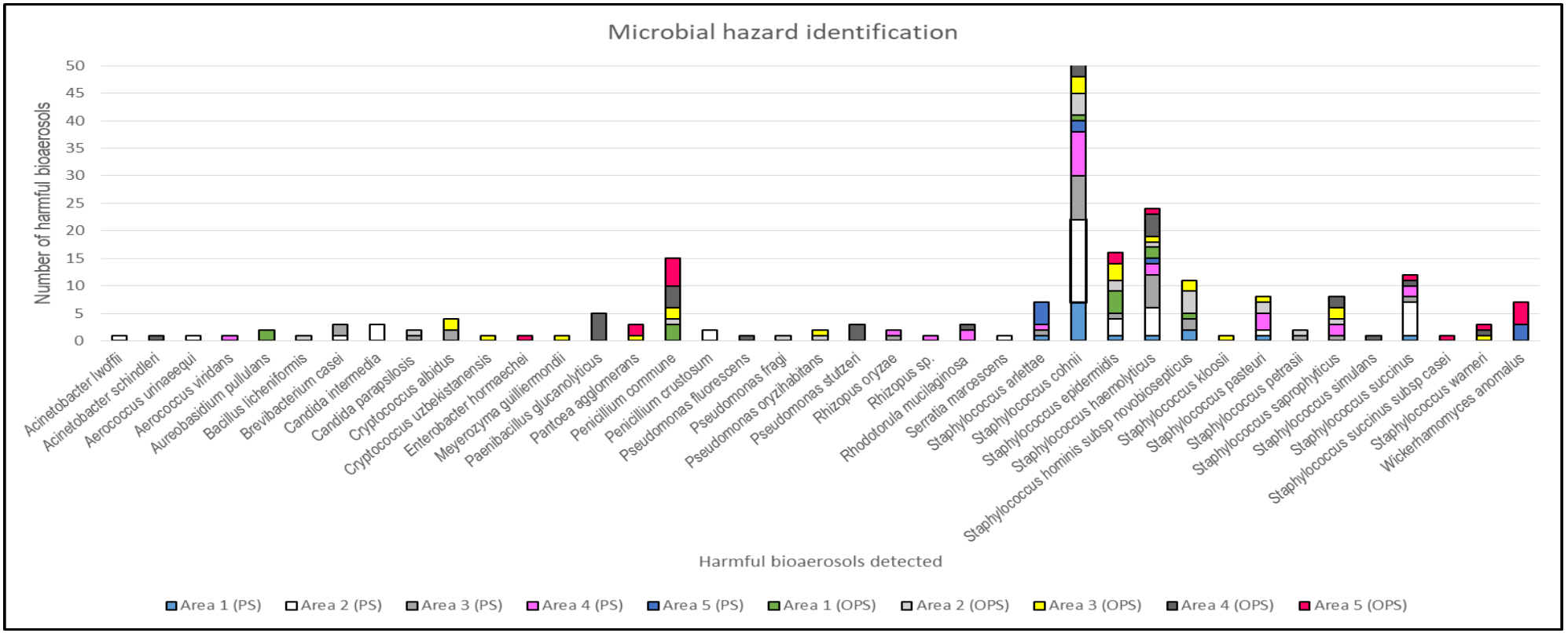

Figure 4.4: Number of identified harmful bioaerosols detected during the two sampling seasons in the designated areas: entrance to the production area (area 1), preparation and mixing of materials (area 2), between the production lines (area 3), dispersion of bottles (area 4) and filling of the final product (area 5). The two sampling phases are indicated as peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn).

The transmission of pathogens and other bioaerosols among humans has been a topic of research for centuries as humans harbour diverse microbes (including pathogens) in and on their bodies. The presence and activities of humans, particularly in indoor environments, can influence bioaerosol concentrations negatively. This is depicted in Figure 4.4 where, in Area 5 (filling of final product), more personnel were involved and thus higher and more diverse harmful bioaerosols were observed. The emission of particles by breathing, sneezing, coughing, talking and movement, as well as from resuspension of dust due to human activity, has been the focus of numerous indoor bioaerosol studies (Adams *et al*., 2015; Bhangar *et al*., 2014, 2015; Castillo *et al*., 2012; Hospodsky *et al*., 2012; Meadow *et al*., 2014, 2015; Morawska, 2006; Nazaroff, 2015; Noble, 1975; Qian *et al*., 2012, 2014; You, 2013).

The conditions in the fruit juice facility and of the fruit juices themselves (raw materials used in fruit juice production, low pH of the final product, high sugar concentration, and low water activity) were ideal for the growth of yeast, mould and fungal spore concentrations and also increased the risk of fungal contamination. For example, mould spores are associated with 'sick building syndrome' and can cause allergic reactions (Kobayaski *et al*., 2009). Toxic fungal metabolites such as mycotoxins (Sorensen *et al*., 1984) can cause severe adverse health problems. Apart from the fact that *Candida* spp. and *Staphylococcus* spp. are responsible for a substantial number of infections independently, there is increasing evidence that they can be co-isolates in cases of biofilm associated infections (Zago *et al*., 2015). Interestingly, in Area 2 and Area 3 where *Candida* spp. were detected, *Staphylococcus* spp. were also observed (Figure 4.4). The clinical outcome of these mixed bacterial-fungal interactions is that the resultant infections can correlate with an increased frequency or severity of diseases (Zago *et al*., 2015).

Staphylococci constitute the main part of the human skin microbiome, and for this reason their role as pathogens has been underestimated (Czekaj *et al*., 2015). The genus *Staphylococcus* is a major cause of both hospital-acquired and community-onset infections and there is a clear need to control antimicrobial-resistant staphylococci (Diekema *et al*., 2001; Madsen *et al*., 2018). The *Pseudomonas* genus also observed in Figure 4.4 was classified as a human pathogen in 1972 (Gilardi, 1972). *Pseudomonas* is one of the more diverse genera, and its taxonomy has undergone many changes since earlier descriptions (Mulet *et al*., 2010). Today *Pseudomonas* spp. has established itself as one of the most troublesome agents causing nosocomial infections (Shyamala & Rao, 2015).

Climatic conditions have a significant impact on the concentrations and diversity of airborne microorganisms (Wu *et al*., 2017; Zhen *et al*., 2017). This study thus considered climatic conditions to determine if seasonal variation influenced the diversity, distribution and occurrence of harmful

bioaerosols, and they were in fact detected in the various designated areas in the facility during both seasons (Figure 4.5). A clear trend was noted between Area 2 (preparation and mixing of materials), Area 3 (between the production lines) and Area 4 (dispersion of bottles) with distinguished higher diversity and representability of the same species in both seasons. In Area 5 (filling of final product), where more personnel were involved, diverse harmful bioaerosols were detected, but the same species were not present during both seasons as the lowest diversity and representability of the same species were observed in Area 1 (entrance to the facility) during both seasons. During both seasons 39 different species were detected; *Staphylococcus* spp. (13) and *Pseudomonas* spp. (4), and to a lesser extent (with two species each) *Aerococcus* spp., *Acinetobacter* spp., *Penicillium* spp., *Candida* spp., *Cryptococcus* spp. and *Rhizopus* spp. were the most prevalently harmful bioaerosols that were identified. Two of these prominent species, namely *Aerococcus* spp. and *Rhizopus* spp., were only detected during the peak season whereas *Acinetobacter* spp., *Penicillium* spp., *Candida* spp. and *Cryptococcus* spp. were detected during both the peak and off-peak seasons. The second most prominent genera, *Pseudomonas* spp., with a prevalence of the species detected during the off-peak season in Areas 2, 3 and 4, is the most frequently reported genus of the bacteria found after sanitation of food processing surfaces across all types of food production. *Pseudomonas* spp. occur ubiquitously as they are associated with a wide range of niches in food production environments with respect to nutrients, temperature, surface materials, and stress factors. This genus has established itself on stainless steel coupons placed in the processing environments of fruit juice related industries (Moretro & Langsrud, 2017). *Staphylococcus* spp. is one of the most common Gram-positive genera found in food production environments, and it came as no surprise that *Staphylococcus* spp. were the most prominent genera detected in the current study. These genera were detected during both seasons and were prevalent consistently throughout the facility in all high-risk areas. The biofilm-producing ability of staphylococci may contribute to their persistence in food processing environments, which also occurs in clinical environments (Moretro & Langsrud, 2017). Three *Staphylococcus* spp. (*Staphylococcus cohnii*, *haemolyticus* and *succinus*) were found in all five designated areas.

In most studies, bacteria have been reported as the dominant microorganisms and they seem to have dominated in most production environments. However, research has shown that in production environments that are more ideal for eukaryotic microorganisms (dry environments and low water activity), yeasts and moulds may be present in significant numbers (Calasso *et al*., 2016; Minervini *et al*., 2015; Moretro & Langsrud, 2017). Four significant eukaryotic microorganisms were detected in this study during both seasons, namely *Cryptococcus albicans*, *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus,* and *Penicillium commune*.

Even though research has indicated that seasons have an influence on the concentration and diversity of microorganisms, any increase in temperature and air exchange rate will cause an increase in airborne bacteria, yeast and mould (Frankel *et al*., 2012; Wu *et al*., 2017; Zhen *et al*., 2017). The temperature in the production facility under study did not fluctuate significantly during the two study seasons, and thus external seasonal variation did not influence the microbial concentration or diversity in the different sampling areas. The only variation that was observed was that more personnel were present during the off-peak season in all the areas, which might explain the additional species observed during this season. Moreover, the airborne microbial levels increased significantly in the occupied areas compared to the unoccupied areas. This finding supports the argument that humans are a source of bacteria and fungi in settled dust samples (Adams *et al.,* 2015).

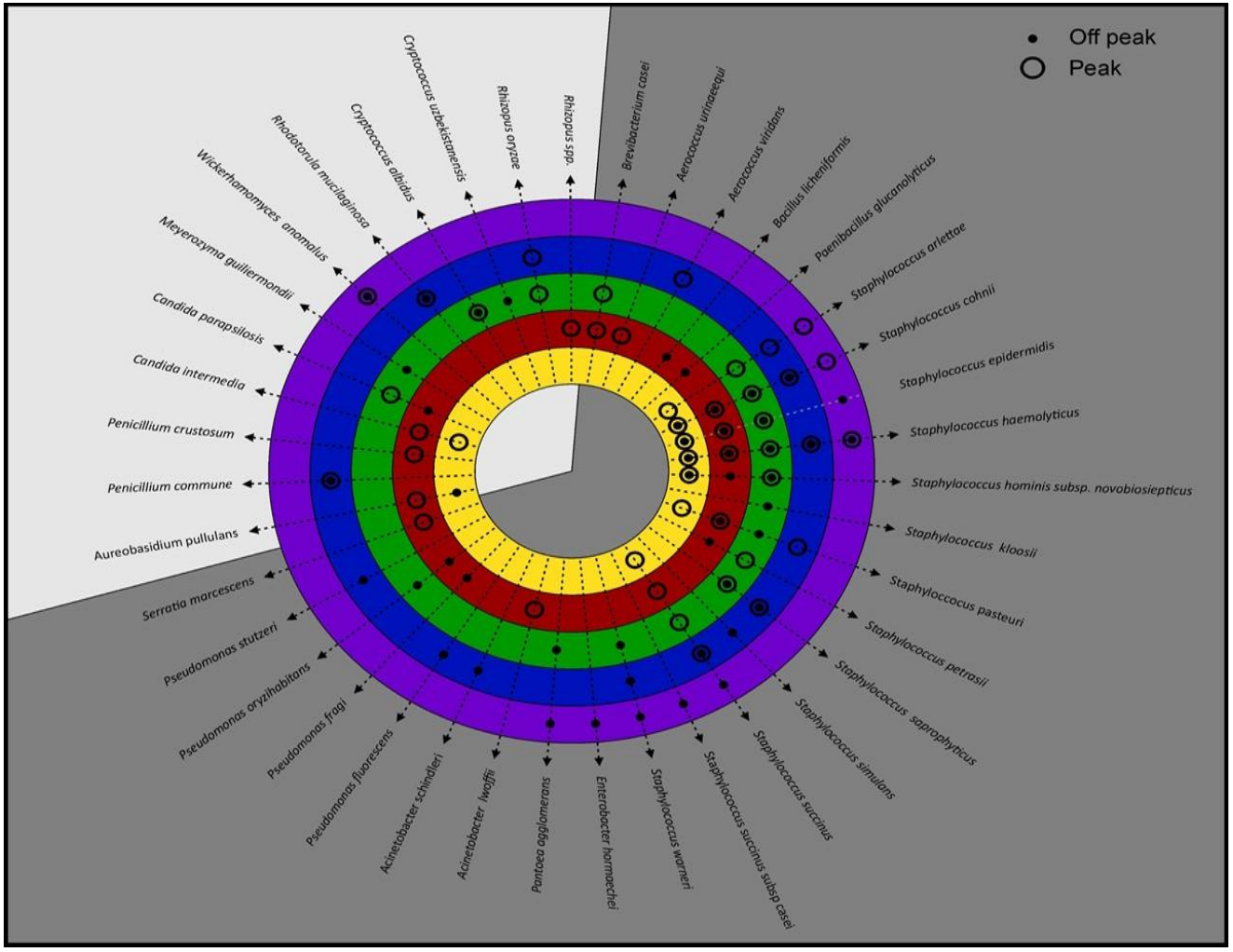

Figure 4.5: Harmful bioaerosols detected in samples from the different designated areas: entrance to the production area (Area 1: yellow), preparation and mixing of materials (Area 2: red), between the production lines (Area 3: green), dispersion of bottles (Area 4: blue) and filling of the final product (Area 5: purple). Sampling occurred during peak season (onset of summer) and off-peak season (onset of autumn) to establish if seasonal variation would impact the accumulation and spread of the harmful bioaerosols. Bacteria are represented by the dark grey region and the yeast and mould are represented by the light grey region.

When comparing the densities of the harmful bioaerosols that were detected, only a small group of the species (Table 4.5) had the potential ability to affect the products manufactured at the facility. Kim *et al*. (2018) argue that although food poisoning and/or spoilage microorganisms are present in the air, it is not guaranteed that they will cause harm as there are still factors that affect their capability to cause harm (e.g., dose relationship, microbial competition and contact with host). With this in mind, it may explain the fact that even though these food poisoning and/or spoilage microorganisms were present in the air, there were no reports of these specific microorganisms influencing the products produced in this fruit juice industry. A great number of pathogenic bioaerosols was detected, and these all had the potential to impact the occupational health of the personnel in the facility negatively. This confirms the argument that the measurement of bioaerosols should be performed according to a protocol that is representative of exposure patterns and duration and that relates to the dose (Bragoszewska *et al*., 2016). Therefore, estimating the dose of culturable bacteria that affect people who inhale it in a factory seems to be important for future exposure analyses.

As studies will continue to examine the microbiology of indoor environments, we should maintain a central focus on people, as human occupants are a major source of indoor bacteria. However, the type of measurement tool we use should be carefully considered as measurement limitations continue to be daunting (Nazaroff, 2014). For example, for easier interpretation of the results, the reference limit values for bacteria, yeast and mould concentrations in the indoor air of the facility under study should have been facility and product specific. The categorisation that is used in indoor bioaerosol studies should also describe the parameters for interpretation of the investigated events. Moreover, research that focuses on processes and that is framed in the context of well-established fact and research-based knowledge can be a valuable way to proceed in this field.

Despite tremendous scientific progress globally, the body of knowledge about biologically originated indoor air pollution seems to remain relatively narrow and insufficient. The reasons for this limited scope could be attributed to: (i) a lack of modern sampling instrumentation (that is industry-bioaerosol specific); (ii) common use of old methods to evaluate the microbiological quality of air; (iii) relatively high costs of instrumental analyses for bacterial and fungal toxins and their markers; (iv) lack of common approved criteria for assessing exposure to biological factors; and (v) a very low number of institutions/organisations interested in (or obligated to perform) comprehensive environmental monitoring of bioaerosols.

It has been argued that, although the complexity and importance of the subject of indoor bioaerosol dynamics have been underscored by various studies, our understanding of this phenomenon is not yet

mature. One might therefore anticipate fundamental paradigm shifts as knowledge grows and the ability to ask and answer incisive questions improves. Therefore, because the gap between what we know and what we would like to know is quite large, our current knowledge is insubstantial, and we need to realise that we will probably never measure everything. Nevertheless, we need to accurately measure what can reasonably be expected within scientifically determined parameters.

In light of the above arguments, the diversity and complexity of fruit juice facilities will continue to pose great challenges for studies on indoor bioaerosol dynamics. This is because mere basic identification and simply analysing bioaerosol concentrations in the air can lead to large misclassification errors of aerosol sources, and misidentification can also lead to misattribution. In this context, the findings of the current study may serve as a reference for future assessments and they may contribute to: (i) policy reviews for product and occupational health; (ii) research efforts in the field to be more outcomes specific; (iii) the implementation of preventative occupational health programs; (iv) the formulation of recommendations aimed at providing healthier production and working environments; and (v) the setting of a clear standard with scientifically established limits in order for facilities to operate within a safe range concerning bioaerosols, the safety of employees, and product quality and safety.

4.4. Conclusion

Bacteria, yeast and mould are the main groups of microorganisms found in bioaerosols. The literature has revealed that the actual identity, diversity and abundance of different types of bioaerosol particles, as well as their temporal and spatial variability, have not been well characterised. Overall, the role of bioaerosols in the atmosphere and their interaction with other ecosystems are not well described and understood. This study thus attempted to fill this gap.

The analyses that were conducted isolated a total of 239 bacteria, 41 yeasts and 43 moulds from the air in the selected fruit juice production environment. From the isolates that were obtained, 92 different species were identified from the culturable fraction. These microorganisms belonged to 15 different taxonomic orders that were divided into five orders representing bacteria and ten orders representing yeast and mould. Based on the data elicited by the study, the culturable fraction of the bioaerosols identified were categorised into three main groups, namely 27 innocuous, 26 useful and 39 harmful bioaerosols.

In the innocuous bioaerosol group, two genera were dominant, namely the *Bacillus* and *Staphylococcus* species, and only four innocuous yeasts and moulds were detected. Useful bioaerosols detected during the sampling seasons were categorised into three different groups according to their known

capabilities, namely: (i) medical contribution; (ii) promoting and protecting plant growth; and (iii) environmental contribution. Although innocuous and useful bioaerosols do not negatively influence human health, it is critical to mention that the presence of innocuous and useful bioaerosols serves as an indicator that an ideal environment is present for the possible emergence of harmful bioaerosols. In addition, any type of bioaerosol that is in excess will have a negative influence on the food product and must also be considered a threat.

The fact that harmful bioaerosols were detected is reason for concern, especially as species such as *Staphylococcus* spp., *Pseudomonas* spp., *Penicillium* spp. and *Candida* spp. were detected. These species in particular have been reported extensively as problematic in the fruit juice industry as they are harmful and have pathogenic/infectious, multidrug resistance, and food poisoning/spoilage abilities. However, even though the air in this facility contained pathogenic/spoilage microorganisms, various factors that affect their harmful capabilities (such as dose relationship, microbial competition and contact with host) should be considered.

This study demonstrated that all types of culturable airborne microorganisms occur ubiquitously and are naturally part of the air environment. It is therefore important that food processing facilities ensure that measures are taken to reduce bioaerosols that may cause product contamination or even occupational health issues. However, there is clearly a need to be more industry- and outcome-specific before monitoring the prevalence of bioaerosols in a specific industry. Culture-dependent methods remain important if information regarding the viability and metabolic activity of these organisms is to be obtained. It is also important that the role that different microbes play in distinctive processes is ascertained and that a clear standard with scientifically established limits be disseminated so that facilities may operate within a safe range concerning bioaerosols. This is especially important in light of the safety of employees and the quality and safety of reliable products.

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CHAPTER 5

CULTURE-INDEPENDENT ANALYSES OF THE BIOAEROSOL MICROBIOME IN FRUIT JUICE

5.1. A Critical Assessment

The term 'bioaerosols' is used to refer to all the particles originating from a biological source that are in suspension in the air. This includes microorganisms such as bacteria, fungi, viruses, protozoa and algae as well as biomolecules such as aflatoxins, mycotoxins, and debris from membranes (Wery, 2014). One way of studying the identity, behaviour, movement and survival of such airborne organic particles that are passively transported in the atmosphere is through the field of aerobiology. Aerobiology seeks to understand interactions between biological aerosols and the atmosphere, including the role of weather and climate in what has been described as the aerobiology pathway (Beggs *et al*., 2017).The impact of aerobiology is especially notable in such diverse basic applied sciences as allergology, bioclimatology, palynology, biological pollution, biological warfare and terrorism, mycology, biodiversity studies, ecology, plant pathology, microbiology, indoor air quality, biological weathering, industrial aerobiology, and cultural heritage (Despres *et al*., 2012). Epidemiological and toxicological studies have indicated a close association between exposure to bioaerosols and many adverse health effects such as infectious diseases, acute toxic effects, allergies and cancer. Therefore, exposure to bioaerosols is a crucial occupational and environmental health issue that warrants close attention (Li *et al*., 2017; Wang *et al*., 2015) not only through of aerobiological studies, but also by finding novel ways of monitoring air quality and determining the specific risks associated with bioaerosols detected in various industries.

Bioaerosol monitoring is useful for controlling air quality, assessing possible product exposure, identifying emission sources, and estimating the performance of air cleaning devices (Park *et al*., 2015). Bioaerosols can be isolated from the environment using various methods that either enumerate viable bioaerosols (i.e., culture-dependent methods), or that involve the collection of viable but non-culturable bioaerosols (i.e., culture-independent methods). Microorganisms may lose the ability to grow (or to be cultured) during the sampling proses due to damaging of the cells during sampling, microbial competition, and unfavourable growth conditions. There is a risk that the inability of microorganisms to grow (or to be cultured) may be wrongly attributed to underperforming bioaerosol samplers, which may result in their efficiency being underestimated. Therefore, culturability losses need to be determined to give an improved overall picture (Zhao *et al*., 2011). A culture-based, colony-counting method is the most widely used analytical technique for monitoring bioaerosols. However, this method requires several days for colony formation, which is one of its most debilitating limitations. In addition, the culture-based method is only applicable to: (i) microbes that are culturable using specific growth conditions; (ii) culturable microbes that can divide at a sufficient rate to form colonies; and (iii) can

survive the stress of aerosolization and sampling. The latter method could underestimate the number of cells due to the presence of viable but non-culturable cells that have the ability to proliferate under more favourable conditions (Alvarez *et al*., 1995; Tringe & Hugenholtz, 2008).

While culture-dependent methods must be used to isolate new strains of potential interest and give quantitative counts of viable microorganisms, research has indicated that culture-dependent methods may underestimate the overall diversity of the microbial community present in different ecosystems (Motato *et al*., 2017). Culture-independent analysis enables the examination of culturable as well as non-culturable bioaerosols, viable and dead cells, and plant and animal fragments. To analyse biological aerosols with molecular genetic tools, bioaerosols need to be collected on appropriate air filters and the deoxyribonucleic acid (DNA) needs to be extracted. The basis for most molecular analysis techniques is the successful extraction of DNA. DNA extraction protocols vary according to the type of tissue undergoing extraction and extractions are therefore performed using a method specific to a particular organism or tissue type. Therefore, ambient samples, which include a mixture of many types of biological material, may lead to the underestimation of some bioaerosols (Park *et al*., 2015).

Filtration is one of the most widely used atmospheric bioaerosol sampling methods; however, it has various limitations for the collection of bioaerosols (Xu & Yao, 2013). On the other hand, the SAMPL'AIR LITE air sampler has been extensively used to monitor bioaerosol concentrations (Gorny *et al*., 1999; Kim & Kim, 2007; Meklin *et al*., 2002; Nasir & Colbeck, 2010; Sanchez-Monedero *et al*., 2005; Xu & Yao, 2013). Although airborne microbes in certain environments have been reported, comparisons among them are rather limited. Moreover, most of these studies were limited to the total or culturable aerosol concentration while information about bioaerosol diversity in different environments is lacking. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) has been widely used for profiling environmental or food-associated microbial ecosystems (Laforgue *et al*., 2009; Osimani *et al*., 2016; Xie *et al*., 2018). These molecular methods have been successfully used to describe the bacterial, yeast and mould communities found in meat, dairy products, fruit juice and various other ecosystems such as aerosols (Cocolin *et al*., 2001; Ercolini *et al*., 2001; Ndiaye *et al*., 2016; Nieguitsila *et al*., 2007; Ogier *et al*., 2002). PCR-DGGE is also a powerful molecular method for rapid detection of microbial community changes or for comparative analyses of environmental samples, and it offers more accurate information about the distribution and composition of microbial species present in bioaerosols (Aydin *et al*., 2015).

However, the various sampling and identification methods mentioned above are of no use without considering the layout of the facility, the product being produced, and the assessment of personnel

working in the facility. Moreover, a lack of standardised sampling/analysis methods for each product, or at least each industry, also makes the determination of a relevant exposure guideline difficult (Wang *et al*., 2015). Current bioaerosol research thus primarily focused on the monitoring and control of ambient or target bioaerosols. A better understanding of the composition and concentration of bioaerosols in various environments is therefore needed. For example, there are different sets of factors that affect bioaerosol composition and concentration in indoor versus outdoor environments (Soleimani *et al*., 2016). Also, the effective monitoring of bioaerosols requires efficient collection of microorganisms from the air, and thus an appropriate air sampling technique for a specific industry must be selected (Yoo *et al*., 2017).

To investigate the true reflection of bioaerosols in the air, a detailed assessment study is necessary using the following steps: (i) pre-sampling assessment; (ii) a sampling process (i.e., the collection of bioaerosols with samplers); and (iii) a post-sampling process (i.e., the air sample handling procedure) (Yoo *et al*., 2017; Zhao *et al*., 2011). The aim of this phase of the study was therefore to determine the non-culturable fraction of bacterial, yeast and mould diversity during the peak and off-peak seasons in the selected fruit juice manufacturing plant and to compare the data to results obtained when only the culturable fraction was determined. An attempt was therefore made to create the same baseline as the culture-dependent sampling by standardising the sampling conditions as far as possible for culture-independent sampling.

5.2. Materials and Methods

5.2.1. Sampling

Two SAMPL'AIR LITE (AES Chemunex, United States) air samplers were used to collect culture-independent bioaerosols in the fruit juice production plant referred to in earlier chapters. All sampling was performed in duplicate before production and during the peak and off-peak seasons at the facility. Five distinct areas were identified to ensure a holistic representation of the facility. These areas were: the entrance to the production area (Area 1), the area for the preparation and mixing of materials (Area 2), the area between the production lines (Area 3), the area for the dispersion of bottles (Area 4), and the area where the bottles were filled with the final product (Area 5). The air samplers operated at a flow rate of 100 litres per minute. The air samplers were disinfected with ethanol when changing from sampling point to sampling point. The samplers were turned on for two minutes prior to sampling to allow the ethanol to evaporate. Air samples were taken at a height of 1.5 m from the ground, which is the same level as the working stations in the centre of each area. For culture-independent sampling, Hydrophilic Polypropylene Membrane Filters (25 mm in diametre and 0.2 µm pore size) (Pall

Corporation) were placed directly on the media in the agar plates to ensure that precise conditions were met that would be similar for the culture-dependent sampling method. After 20 min, the samplers were turned off, the filters were removed from the centre of the sterile petri dishes using sterile forceps, and placed in sterile falcon tubes. Immediately after collection, the samples were transported to the laboratory where the filters were stored at -20°C until analysis.

5.2.2. Strategies for the total community DNA extraction and PCR amplification

A comprehensive literature review was conducted to determine which filters and DNA extraction kits had been used successfully in culture-independent bioaerosol investigations. The following criteria were subsequently used to organise the data: (i) filter type; (ii) pore size; (iii) flow rate of sampler; (iv) sampling period; (v) extraction method; (vi) identification method; and (vii) bioaerosols targeted. Several successful extractions of bioaerosol DNA were conducted (Table 5.4) using various techniques. With reference to the successful extractions of bioaerosol DNA, four commercial DNA extraction kits and one manual harsh lysis extraction method were compared to extract total genomic DNA from the Hydrophilic Polypropylene Membrane Filters used for sampling (5.2.1) (Table 5.5). The commercial extraction kits were used following the manufacturers' instructions and the protocol for harsh lysis extraction as described by Labuschagne and Albertyn (2007). Based on the results presented later in Table 5.5, the most appropriate extraction/analysis methods were selected for this study.

Total genomic DNA extracted from each filter sample was used as a template for PCR to amplify the 16S, 18S and D1/D2 domains of the 26S rRNA genes. As a quality measure, unused filters were stored and analysed as controls. No contamination was observed. For the analyses of bacterial, yeast and mould diversity, different primer sets were used to target the 16S (≈1 300 bp), 18S (≈1792 bp) and D1/D2 domains of the 26S (≈600 bp) rRNA genes (Table 5.1). The PCR was carried out in a total volume of 50 µl, containing 5 µl template DNA, 1X ThermoPol® reaction buffer, 0.2 mM dNTPs, 0.52 µM of each primer, and 1 unit of Taq DNA polymerase (New England Biolabs). Reaction conditions are presented in Table 5.2 and Table 5.3. Successful amplification was verified by separating PCR products on a 1% agarose gel stained with 0.05% Ethidium bromide and visualised with exposure to UV light. Digital images were captured with the Molecular Imager® Gel Doc™ XR system (BioRad Laboratories Inc.).

Where low product yield was observed for the direct amplification, a pre-amplification approach was attempted to increase yield. The extracted DNA was enhanced by pre-amplifying 1 µl of gDNA using the SSoAdvanced™ PreAmp Supermix (BioRad Laboratories Inc.) and a 50 nM mixture of all specified

forward and reverse primers according to the manufacturer's instructions (Hartung *et al*., 2019; Zhou *et al*., 2018). The PreAmp DNA was then used as a template for downstream application.

Table 5.1: Primers used in this study

Bacteria			
Application	**Forward Primer**	**Back Primer**	**Reference**
PCR	63F (5'-CAG GCC TAA CAC ATG CAA GTC-3')	1387R (5'-GGG CGG WGT GTA CAA GGC-3')	Marchesi *et al*., 1998
DGGE	341-F^{GC} (5'-CCT ACG GGA GGC AGC AG-3') with incorporated 40 bp GC at the 5'-end	907R (5'-CCG TCA ATT CMT TTR AGT T-3")	Muyzer *et al*., 1993
Yeast and Mould			
Application	**Forward Primer**	**Back Primer**	**Reference**
PCR	NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3')	NL4 (5'-GGT CCG TGT TTC AAG ACG G-3')	Kurtzman & Robnett, 1998; Yang *et al*., 2011
PCR	EukA (5'-AACCTGGTTGATCCTGCCAGT-3')	EukB (5'-TGATCCTTCTGCAGGTTCACCTAC-3')	Medlin *et al*., 1988; Gonzalez & Saiz-Jimenez, 2005
DGGE	Euk1A^{GC} (5'-CTG GTT GAT CCT GCC AG-3')	Euk516-R (5'-ACC AGA CTT GCC CTC C-3")	Amann *et al*., 1990; Sogin & Gunderson, 1987

Table 5.2: Reaction conditions for bacteria amplification

Bacteria			
PCR steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	94°C	180 s	1 cycle
Denaturing	94°C	30 s	30 cycles
Annealing	55°C	30 s	30 cycles
Extension	68°C	90 s	30 cycles (After 30 cycles, final extension was performed once for 6 min.)
DGGE-PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**

Initial denaturing	95°C	300 s	1 cycle
Denaturing	95°C	45 s	30 cycles
Annealing	55°C	45 s	30 cycles
Extension	68°C	60 s	30 cycles (After 30 cycles, final extension was performed once for 7 min.)

Table 5.3: Reaction conditions for yeast and mould amplification

Yeast and Mould			
PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	95°C	180 s	1 cycle
Denaturing	95°C	30 s	30 cycles
Annealing	55°C	30 s	30 cycles
Extension	68°C	60 s	30 cycles (After 30 cycles, final extension was performed once for 6 min.)
DGGE-PCR Steps	**Thermal Conditions**	**Time Interval**	**Notes**
Initial denaturing	94°C	130 s	1 cycle
Denaturing	94°C	30 s	35 cycles
Annealing	56°C	45 s	35 cycles
Extension	68°C	130 s	35 cycles (After 35 cycles, final extension was performed once for 5 min.)

A nested approach was then used to amplify shorter fragments for DGGE analysis. A ≈606 bp section of 16S and a ≈600 bp of 18S rRNA gene were amplified using primer pairs 341-F^{GC}/907RM and Euk1A^{GC}/Euk516-R respectively (Table 5.1). Amplification reactions were performed in a total volume of 50 µl with reaction constituents and conditions as described previously (Table 5.2 and Table 5.3). In order to reduce possible inter-sample PCR variation, two sets of PCRs were performed as independent duplicates and pooled before loading on the DGGE gel. DNA fragments were separated on a 2% agarose gel, stained, and visualised under UV light.

5.2.3. Denaturing gradient gel electrophoresis

DGGE analyses were performed on 30 µl of the ≈606 bp GC-clamped PCR fragments for the 16S rRNA gene and ≈600 bp GC-clamped PCR fragments for the 18S rRNA gene using the D-Code

Universal Mutation Detection System (BioRad Laboratories) essentially as described by Muyzer and co-workers (1993) and Sogin and Gunderson (1987). Several attempts were made to optimise the DGGE resolution by varying the polyacrylamide concentration (7%, 8% and 10%), urea gradient (40-60% and 40-50%), and the electrophoresis parameters (for 4.5 h at 130 V at 60°C, for 5 h at 130 V at 60°C, for 5 h at 200 V at 60°C, and for 12 h at 130 V at 60°C). Samplers were finally applied to 8% (w/v) polyacrylamide gels (acrylamide/bis 37.5:1) in 1X TAE buffer. Optimal separation was achieved with a 40-60% urea-formamide denaturing gradient (100% denaturant corresponds to 7 M urea and 40% [v/v] formamide). Electrophoresis was carried out for 5 h at 130 V at 60°C. Gels were stained with 0.05% GelStar® (Lonza) for 15 min, rinsed with ultra-pure water, and photographed while exposed to UV light. DGGE digital images were captured on the Molecular Imager Gel Doc™ XR and analysed with the Quantity One® 1-D analysis imaging software (BioRad Laboratories). Densitometric profiles were generated with the band selection threshold set at 5% intensity. Individual bands were matched according to their positions in the gel with a 1.5% position tolerance and peak areas were used to determine intensity (Julien *et al*., 2008). Cluster analysis describing pattern similarities among different samples was performed using an unweighted pair-group method with an arithmetic mean algorithm (UPGMA) (Martinez-Alonso *et al*., 2010). Dominant bands for further investigation were selected based on band intensity (≥3.715).

5.2.4. DGGE profile analyses

Diversity and dissimilarity indices were analysed according to the DGGE banding profiles.

5.2.4.1. Operational taxonomic units (OTU's)

The number of bands were taken as a measure of different operational taxonomic units and the respective intensity as their proportion in the population (Ahmed *et al*., 2019).

5.2.4.2. Range weighted richness (Rr)

Species richness was calculated by range of weighted richness:

$$Rr = (N2 \text{ x } \mathrm{Dg})$$

where N is the number of bands and Dg is the range of denaturant gel in which the top and bottom bands were separated (Marzorati *et al*., 2008).

5.2.4.3. Shannon-Weaver diversity index (H)

The Shannon-Weaver diversity index (H) of bacterial diversity was calculated by using the following equation:

$$H = -\sum Pi \, \mathrm{Ln} Pi$$

where P_i is the proportional intensity of each band or OTU and Ln (Pi) is the natural logarithm of proportional intensity of each band (OTU) (Shannon & Weaver, 1999).

5.3. Results and Discussion

5.3.1. Experimental design, sampling and gDNA extraction

Bioaerosols originate from all types of environments, including the atmosphere, soil, freshwater and oceans, and their dispersal into air is temporally and spatially variable. The variability of bioaerosol composition is subjected to: (i) the fact that biological material does not necessarily occur in the air as independent particles; (ii) bacteria may occur as agglomerations of cells or may be dispersed into the air on plant or animal fragments, on soil particles, on pollen, or on spores that have become airborne (Yoo *et al*., 2017); (iii) the correlation between the variations in atmospheric bacterial community structures over time and their physical and chemical characteristics (Fierer *et al*., 2008); (iv) variations in the robustness of different species of microorganisms; and (v) the difficulty of differentiating strains of the same species (Griffin *et al*., 2001). Therefore, examining only the culturable fraction leads to an underestimation of the total bioaerosol diversity. With this in mind, the culture-independent analyses revealed a greater diversity of airborne microorganisms compared to the traditional culture-dependent method (Lee *et al*., 2009; Yoo *et al*., 2017). The sensitivity, specificity and high speed of molecular techniques have also led to their use for bioaerosol monitoring in the determination of air quality and the detection of airborne pathogens (Han *et al*., 2012).

Although culture-independent analysis is ideal for diverse bioaerosol composition, diversity analysis is still complicated due to: (i) bioaerosol composition consisting of various molecular components; (ii) different molecular components that can interfere in the detection of target bioaerosols; and (iii) the fact that a large number of the same genus with different species of bioaerosols may occur, making it difficult for some culture-independent methods to distinguish between them (Yoo *et al*., 2017; Zhao *et al*., 2011).

For accurate identification of culture-independent bioaerosols in a specific environment, four essential steps need to be established for optimal results, namely: (i) the sampling process; (ii) an appropriate DNA extraction method; (iii) amplification/identification; and (iv) interpretation of data (Yoo *et al*., 2017; Zhao *et al*., 2011). With these factors in mind, this study attempted to obtain a true reflection of the culture-independent bioaerosols. Membrane filters placed directly on the media in the agar plates were

used to attempt to create the same conditions as those that had been used for the culture-dependent approach (Chapter 4). An attempt was thus made to compare the culture-dependent and culture-independent data and to determine commonalities and potential relevance. Currently, there is no published information available on the composition of non-culturable bioaerosols in fruit juice bottling facilities. In fact, only broad research on sampling procedures to determine the bioaerosol composition of indoor and outdoor environments, specifically using culture-independent approaches, has conducted, and thus there is a lack of literature on the culture-independent analysis of bioaerosols in indoor environments. Indoor air is a very dynamic system in which particles of biological and non-biological origin are distributed and displaced. Culture-independent analyses that focused on indoor bioaerosols were conducted by researchers such as Angenent *et al*. (2005), Norris *et al*. (2011), O'Brien *et al*. (2016), Robertson *et al.* (2013) and Tanaka *et al*. (2015), as it is an increasingly important issue for occupational and public health. Not only sampling procedures, but also DNA extraction methods were considered because a combination of both is crucial for DNA recovery (Ferguson *et al*., 2019). The data that are presented in Table 5.4 show that membrane and fibrous filters were mostly used with pore sizes ranging from 0.05-18 µm. Although fibrous filters seem popular and have demonstrated good loading capacity for bioaerosol detection, particles are not easily released and may remain trapped between the filaments (Cao *et al*., 2014; Pankhurst *et al*., 2012). The remaining filters used were membrane filters, but no specific type seemed popular. After careful consideration, the decision was taken to use Hydrophilic Polypropylene Membrane Filters (25 mm in diameter and 0.2 µm pore size) (Pall Corporation) in the current study. Membrane filters typically have high collection efficiencies (>95%) for particles >0.5 µm (bioaerosol size range: ~5-100 µm) in diameter and are simple to use. However, membrane filters have a complex internal structure of pores within which particles are deposited. With these filters, extraction occurs directly from the filter for downstream analysis (Ferguson *et al*., 2019). Airflow that was involved in earlier studies varied from 0.6-70.86 $L.h^{-1}$ and sampling periods from 10 min to 10 days. The most successful extraction methods seemed to be commercially available DNA extraction kits for soil and mechanical lysis using beads and chemicals. The DNA extraction kits for soil demonstrated that the extraction method is suitable for different aerosol filter types as samples had been successfully sequenced (Despres *et al*., 2007; Frohlich-Nowoisky *et al*., 2012).

Table 5.4: Different ambient air filters and extraction methods used since 2002 for DNA analysis of bioaerosols, arranged by type of extraction method

Filter Type, Pore Size, Flow Rate and Time	Extraction Method	Bioaerosols Targeted	Identification Method	Reference
Poretics polyester membrane filters (1 µm pore size, 1000 liters.min^{-1}, 24 h)	MoBio UltraClean Soil DNA kit	Bacteria	PCR amplification and sequencing	Radosevich *et al*., 2002
Glass fibre filters (15 mm diameter, 500 liters.min^{-1}, 4 to 5 d)	Fast DNA spin kit for soil	Bacteria, Archaea, Mould, Plants and Animals	PCR amplification, cloning and sequencing	Despres *et al*., 2007
Cellulose nitrate filters (15 mm diameter, 19.68 liters.min^{-1}, 24 h)	Fast DNA spin kit for soil	Bacteria, Archaea, Mould, Plants and Animals	PCR amplification, cloning and sequencing	Despres *et al*., 2007
Polypropylene filters (44 mm diameter, 11.67 liters.min^{-1}, 10 d)	Fast DNA spin kit for soil	Bacteria, Archaea, Mould, Plants and Animals	PCR amplification, cloning and sequencing	Despres *et al*., 2007
Glass fibre filters (3 µm pore size, 300 liters.min^{-1}, 1 to 7 d)	Fast DNA spin kit for soil	Yeast and Mould	PCR amplification, cloning and sequencing	Fröhllich-Nowoisky *et al*., 2009
Glass fibre filters (50 mm diameter, .225 liters.min^{-1}, 24 h)	Soil DNA isolation kit	Bacteria and Mould	PCR amplification, cloning and sequencing	Lee *et al*., 2010
Quartz fibre filters (NA, 1130 liters.min^{-1}, 3 d)	PowerSoil DNA isolation kit	Bacteria	PCR amplification, cloning and sequencing	Bowers *et al*., 2011
HVAC filters (NA, 12.5 liters.min^{-1}, 1 h)	PowerSoil DNA isolation kit	Bacteria and Mould	PCR amplification, cloning and sequencing	Norris *et al*., 2011
Glass fibre filters (50 mm diameter, 225 liters.min^{-1}, 24 h)	Soil DNA isolation kit	Bacteria and Mould	PCR amplification, cloning and sequencing	Lee *et al*., 2010

Filter Type, Pore Size, Flow Rate and Time	Extraction Method	Bioaerosols Targeted	Identification Method	Reference
Quartz fibre filters (NA, 1130 liters.min^{-1}, 3 d)	PowerSoil DNA isolation kit	Bacteria	PCR amplification, cloning and sequencing	Bowers *et al*., 2011
HVAC filters (NA, 12.5 liters.min^{-1}, 1 h)	PowerSoil DNA isolation kit	Bacteria and Mould	PCR amplification, cloning and sequencing	Norris *et al*., 2011
Polyvinyl chloride filter (25 mm diameter, 5.0 µm pore size, 2-4 liters.min^{-1}, 30-990 min)	PowerSoil DNA isolation kit	Bacteria	Whole genome sequencing	O'Brien *et al*., 2016
Quartz fibre filters (150 mm diameter, 500 liters.min^{-1}, 24 h)	Fast DNA spin kit for soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Palliflex quartz filters (3 µm pore size, 272 liters.min^{-1}, 10-50 h)	Fast DNA spin kit for soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Quartz fibre filters (NA, 1000 liters.min^{-1}, 2-26 h)	Fast DNA spin kit for soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Dichotomous sampler (self-built) (102 mm diameter, 30 liters.min^{-1}, 7 d)	Fast DNA spin kit for soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Quartz fibre filters (8.0 µm pore size, 50 liters.min^{-1}, 48-72 h)	Fast DNA spin kit for soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Quartz fibre filters (NA, 1130 liters.min^{-1},		Mould	PCR amplification, cloning and	Fröhlich-Nowoisky *et al*.,

Filter Type, Pore Size, Flow Rate and Time	Extraction Method	Bioaerosols Targeted	Identification Method	Reference
12-24 h)	Fast DNA spin kit for soil		sequencing	2012
Glass fibre filters (NA, 1120 liters.min^{-1}, 21-35 h)	Fast DNA spin kit for Soil	Mould	PCR amplification, cloning and sequencing	Fröhlich-Nowoisky *et al*., 2012
Polycarbonate filters (47 mm diameter, 0.2 µm pore size, 10 liters.min^{-1}, 3 h)	Ultra Clean Soil DNA isolation kit	Bacteria	PCR-DGGE analysis, cloning and sequencing	Tanaka *et al*., 2015
Automobile air conditioning filters (NA, 1 liters.min^{-1}, 10 min)	E.Z.N.A soil DNA Kit	Bacteria	PCR amplification, cloning and sequencing	Wei *et al*., 2015
Filter pack – not specified (NA, 16.7 liters.min^{-1}, 24 h)	PowerSoil DNA isolation kit	Bacteria	PCR amplification, cloning and sequencing	Lee *et al*., 2017
Quartz aerosol collection filters (47 mm diameter, 5 liters.min^{-1}, 24 h)	PowerSoil DNA isolation kit	Mould	PCR amplification, cloning and sequencing	Yan *et al*., 2016
Cellulose ester filters (1.4 µm pore size, 4 liters.min^{-1}, 8 h)	MO BIO PowerWater DNA isolation kit	Bacteria	PCR amplification, cloning and sequencing	Meadow *et al*., 2014
Teflon filters (2.0 µm pore size, 4 liters.min^{-1}, 4 h)	NucliSense Magnetic Extraction kit	Bacteria	PCR amplification and microarray analysis	Hogerwerf *et al*., 2012
γ-radiated filter cassette (0.45 µm pore size, 12.5 liters.min^{-1}, 1 h)	Bead beating method	Bacteria	PCR amplification, cloning and sequencing	Angenent *et al*., 2005
Glass fibre filters (0.6 µm pore size, 500 liters.min^{-1}, 200 min)	Bead beating method	Bacteria	PCR amplification, cloning and sequencing	Park *et al*., 2016
Celanex polyethylene terephthalate (1 µm pore size, 10 liters.min^{-1}, 24 h)	Single bead beating	Bacteria	PCR amplification and microarray analysis	Brodle *et al*., 2007

Filter Type, Pore Size, Flow Rate and Time	Extraction Method	Bioaerosols Targeted	Identification Method	Reference
Polycarbonate filters (25 mm diameter, 0.8 µm pore size, NA,NA)	Mini-bead Beater, DNA-EZ kit	Mould	PCR amplification and microarray analysis	Vesper *et al*., 2007
Mixed cellulose ester membrane filters (0.8 µm pore size, 2 liters.min^{-1}, 10-90 min)	Bead beater kit	Mould	PCR amplification, cloning and sequencing	Rittenour *et al*., 2013
Borosilicate filters (18 mm diameter, NA, 6 h)	Fast Prep 120	Bacteria	PCR amplification and microarray analysis	DeSantis *et al*., 2005
Fluoropore membrane PTFE filters (0.056 to 18 µm, 30 to 48 liters.min^{-1},40 min to 6 h)	Chloroform method	Bacteria, Yeast and Mould	PCR amplification, cloning and sequencing	Urbano *et al*., 2011
Polycarbonate filters (0.2 µm, 300 liters.min^{-1}, NA)	Chloroform method	Bacteria	PCR amplification and microarray analysis	Robertson *et al*, 2013
HEPA filters (8cm x 4cm, NA, NA)	Modified Miller Method	Bacteria	PCR amplification and microarray analysis	Korves *et al*., 2013
PTFE filters (47 mm diameter, 0.45 µm pore size, NA, 20 min)	Hexadecyltrimethylammonium bromide (CTAB) protocol	Bacteria	PCR amplification and microarray analysis	Fahlgren *et al*., 2015

NA – Not applicable.

Table 5.5: Comparison of recommended DNA extraction protocols for DNA analysis of bioaerosols

Extraction Kit/Steps	Supplier	Outcome	Observations	Reference
ZR Fungal/Bacterial DNA MiniPrep™	Zymo Research	No bacterial, yeast or mould product observed	Mould detected in air and surface samples using RT-PCR analysis. Bacterial spores detected from powder samples using RT-PCR analysis	Molsa *et al*., 2016; Viegas *et al*., 2016
ZR Soil Microbe DNA MiniPrep™	Zymo Research	No bacterial, yeast or mould product observed	Bacteria detected in faecal specimens using PCR-DGGE analysis	Huges *et al*., 2017; Shepherd *et al*., 2015
Harch lysis, extraction method	Manual	No bacterial, yeast or mould product observed	Modified extraction method for yeast identification using PCR analysis	Labuschagne & Albertyn, 2007
QIAamp® DNA Stool Mini Kit	QIAGEN	No bacterial, yeast or mould product observed	Bacteria detected in human gut samples using PCR-DGGE analysis. Bacteria detected in faecal specimens using PCR-DGGE analysis	Ariefdjohan *et al*., 2010; Collado *et al*., 2016
Xpedition™ Soil/Fecal DNA MiniPrep Kit	Zymo Research		Bacteria detected in human gut samples using PCR-DGGE analysis. Bacteria detected in environmental samples using PCR-DGGE analysis	Barros *et al*., 2015: Wilmeth *et al*., 2018

Culture-independent methods have revolutionised our understanding of the microbiology of different communities. More especially, DNA-based methods for phylogenetic analysis are increasingly applied. The analytical success of molecular techniques, including PCR-DGGE, is greatly affected by the reliance on cell lysis efficiency and the quality of DNA recovered from environmental samples. However, DNA isolation methods that contribute to insufficient cell lysis or shearing of DNA may cause bias in PCR amplification. It is therefore important that upstream protocols (e.g., DNA extraction) are optimised in order to obtain accurate results (Ariefdjohan *et al*., 2010). However, 'you only see what you sequence and only sequence what you can extract and amplify', and this understanding highlights that the extraction step plays a big role in the effectiveness of DNA-based analysis of environmental samples. With a lack of information regarding aerosol bacterial diversity in the food industry that existed at the commencement of the study, five different extraction methods were chosen after a thorough review of the literature to explore extraction methods used for successful identification of environmental bioaerosols (Table 5.5). DNA extraction consists of three main steps: (i) cell lysis to expose the intracellular material; (ii) isolation of DNA from contaminants; and (iii) final elution. The Xpedition™ Soil/Fecal DNA MiniPrep Kit was revealed as the only method that is able to extract sufficient DNA from filters usable for PCR.

Higher extraction efficiency allows for better recovery of DNA from environmental samples and this results in a more comprehensive and complete profile of the bacterial community within a sample. As soil is considered to be a highly diverse microbial habitat with an estimate of up to 1 million distinct genomes per gram (Brodle *et al*., 2007; Santamaria *et al*., 2018), it is not surprising that this method had a relatively high success rate. Various commercial DNA extraction kits have been developed to simplify and speed up the extraction process. However, none of these different techniques have been quantitatively compared and the relative efficacy of these kits and the optimum range of sample weight for extraction need further evaluation. Clearly, the choice of DNA extraction and amplification protocols is pivotal to the outcome of any amplicon sequencing study (Albertsen *et al*., 2015; Luhung *et al*., 2015).

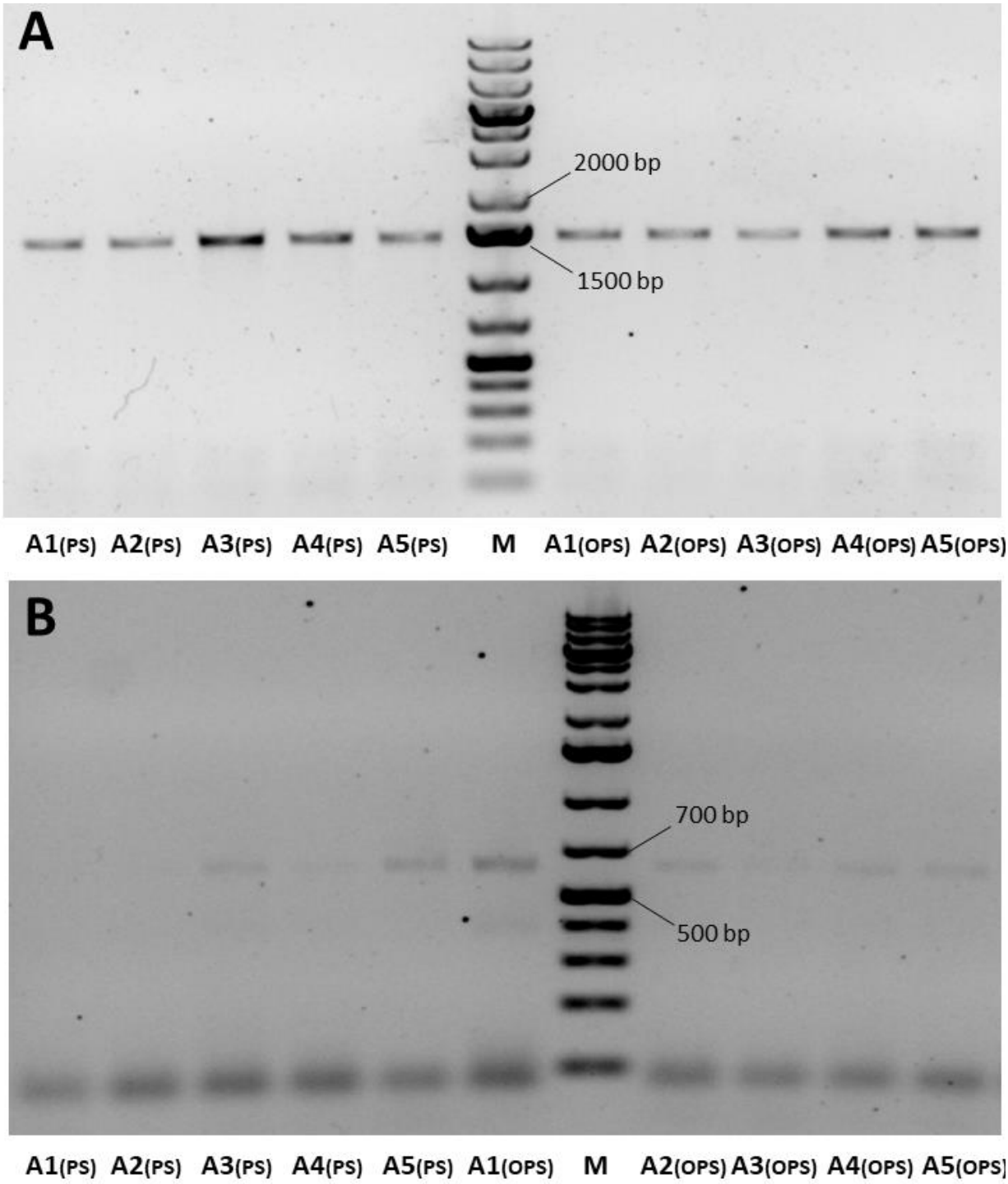

Figure 5.1: PCR products of the 16S rRNA gene amplified from bacterial communities (A) and 26S rRNA gene amplified from yeast and mould communities (B) present on the sample filters: Areas sampled are represented by A1–A5: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Samples were collected during peak season (PS) and off-peak season (OPS). Lane M contains GeneRuler™ 1 kb plus DNA ladder (ThermoFisher Scientific).

5.3.2. PCR-DGGE analysis and bacterial diversity

The microbiological contents of nominally similar environmental samples tend to vary from site to site over time. Variation is particularly expected in the diversity of bacteria, yeast and mould in different

areas. In order to explore a scattered collection of the phylogenetic distribution, the usefulness of extracted DNA for downstream application was analysed in a range of different steps to identify the bioaerosol diversity in the industry under study. Figure 5.1 presents the PCR products amplified from total DNA extracted from filters using the Xpedition™ Soil/Fecal DNA MiniPrep Kit and targeting the 16S rRNA gene. Amplification of the 18S rRNA gene and D1/D2 region of the 26S rRNA was also attempted to assess yeast and mould diversity (Figure 5.1). Low PCR products were obtained where primer pair NL1 and NL4 was used to amplify the D1/D2 region. Subsequently, the 18S rRNA gene region was targeted for further analysis of yeast and mould diversity, although no 18S rRNA gene amplified products using primer pair EukA and EukB could be obtained.

PCR-DGGE is a useful tool for detecting microbial community structure, dominant populations and changes of predominant microbiota in specific microhabitats, and it has been widely applied for comparative analyses of parallel samples (Lv *et al*., 2012; Tanaka *et al*., 2015). Figure 5.2 represent the PCR-DGGE products generated from extracted DNA using primer sets modified with GC clamps that represent bacterial and yeast/mould communities as the V3 region of the 16S rRNA (Figure 5.2) and partial 26S rRNA gene products (Figure 5.2). These products were resolved in a polyacrylamide gel with a urea gradient (40-60%) to assess microbial community structure. Figure 5.3 and Figure 5.4 illustrate the bacterial and yeast and mould diversity respectively. PCR products representative of bacterial diversity were successfully separated, but the separation of yeast and mould PSR products was unsuccessful. Despite numerous attempts to optimise different conditions, DGGE resolution could not be improved.

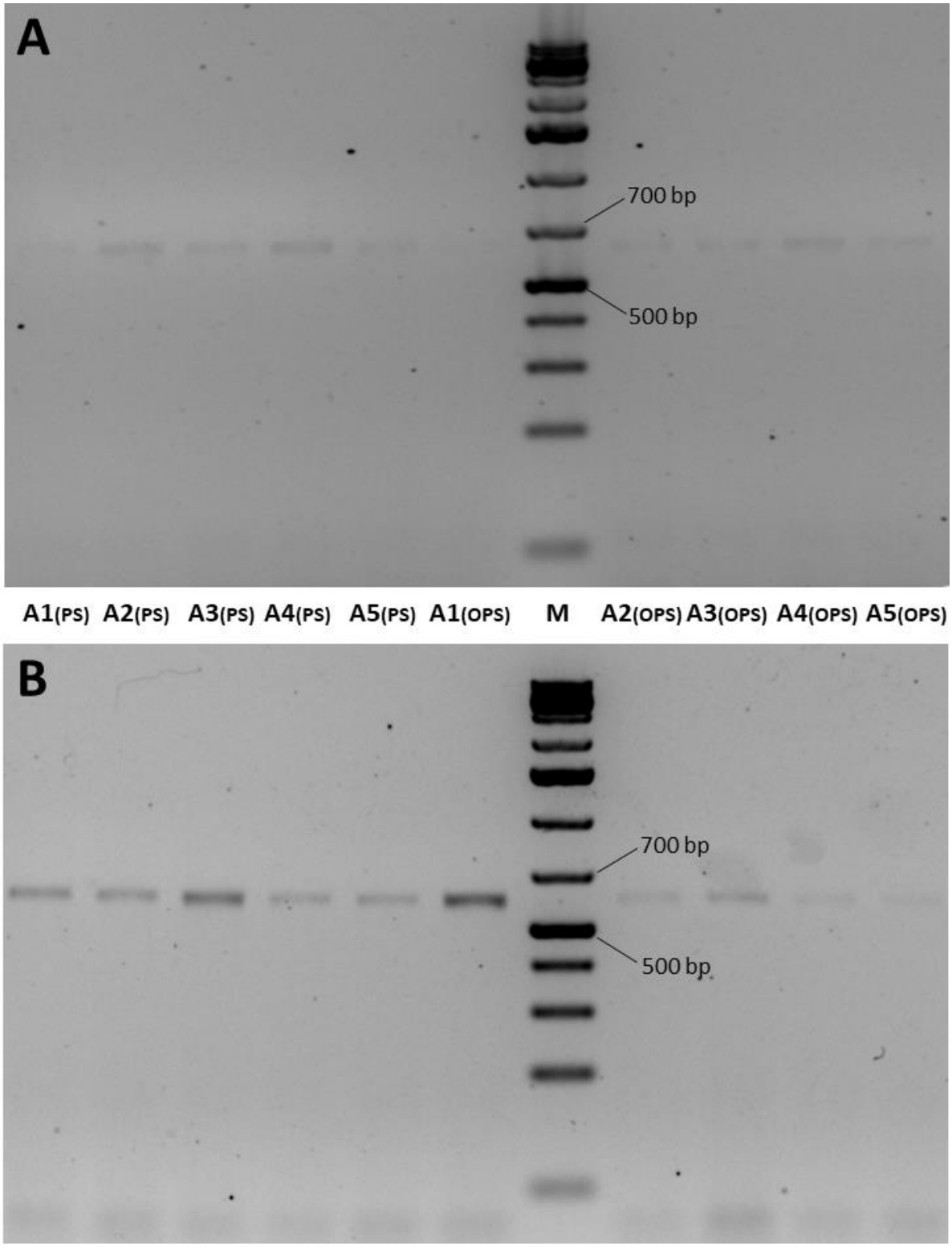

Figure 5.2: PCR-DGGE amplification products of the 16S (A) and 26S (B) rRNA genes: Areas sampled are represented by A1–A5: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Samples were collected during peak season (PS) and off-peak season (OPS). Lane M contains GeneRuler™ 1 kb plus DNA ladder (ThermoFisher Scientific).

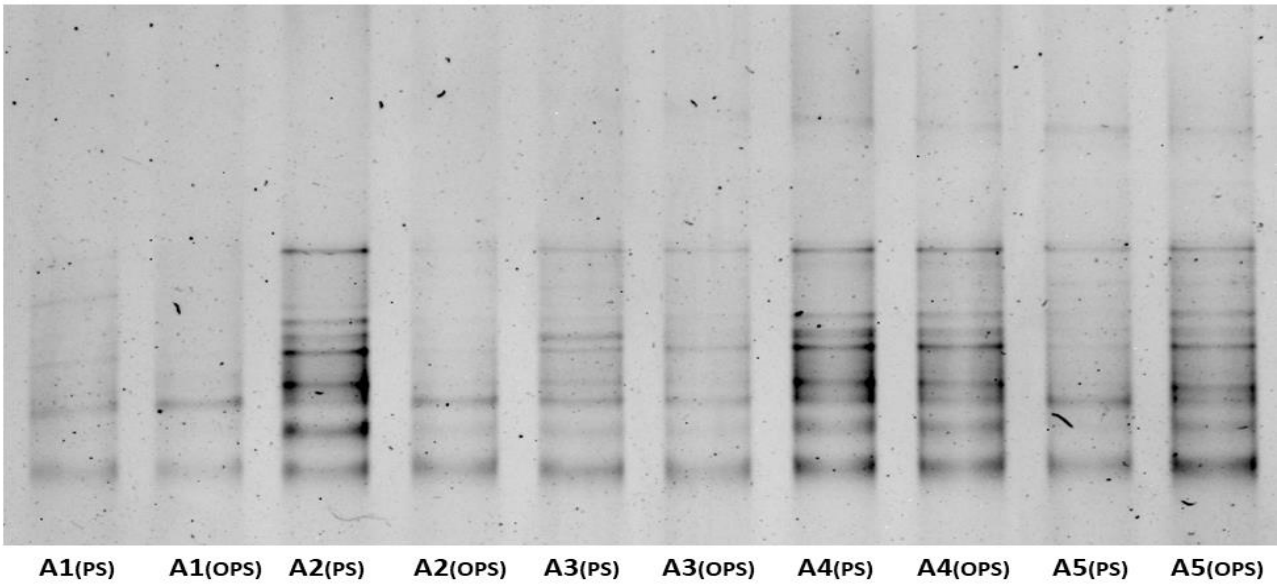

Figure 5.3: Denaturing gradient gel electrophoresis (DGGE) profiles of amplified 16S rDNA from genomic DNA extracted from air samples: The samples were collected in a fruit juice production facility during different sampling seasons in various designated areas: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Two test schedules were chosen: peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn).

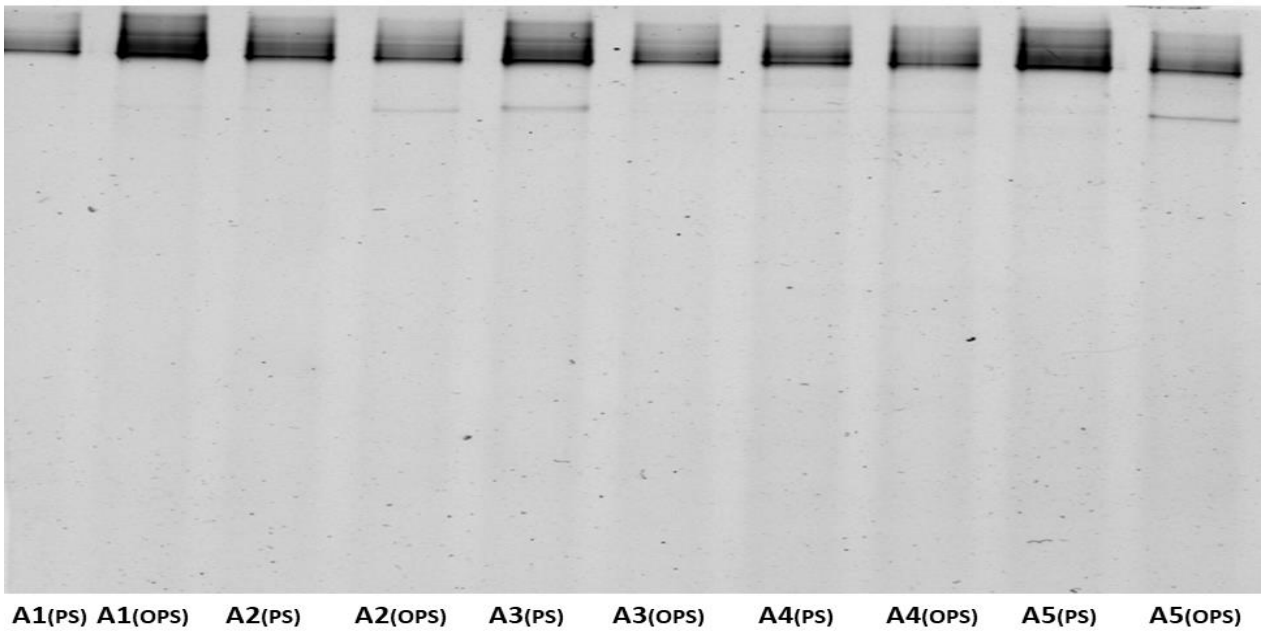

Figure 5.4: The denaturing gradient gel electrophoresis (DGGE) profiles of amplified 26S rDNA from genomic DNA extracted from air samples: The samples were collected in a fruit juice production facility during different sampling seasons in various designated areas: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Two test schedules were chosen: peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn).

The wide-ranging diversity of DNA molecules in bioaerosol samples could also be considered a limiting factor in culture-independent studies. To overcome this limitation, different enhancing approaches are proposed by O'Brien *et al*. (2016), of which pre-amplification of genomic DNA and the use of a nested PCR approach have been successful. SsoAdvanced™ PreAmp Supermix was used for unbiased target-specific pre-amplification in an attempt to improve PCR-DGGE product yield. However, no PCR products were obtained for either of the rRNA gene targets. A further attempt to increase target DNA yield was to use the amplified PCR products of longer 16S fragments as templated for PCR-DGGE (Figure 5.1A). For the yeast and mould amplified products, longer 18S rRNA fragments as templated for PCR-DGGE were used using primer pair EukA and EukB. The nested PCR approach resulted in better PCR product yield for 16S rRNA targets (Figure 5.5), but no successful amplification of the 18S rRNA gene occurred. Furthermore, PCR-DGGE products represented in Figure 5.5 did not yield usable results when resolved in polyacrylamide gels with urea gradient (Figure 5.6).

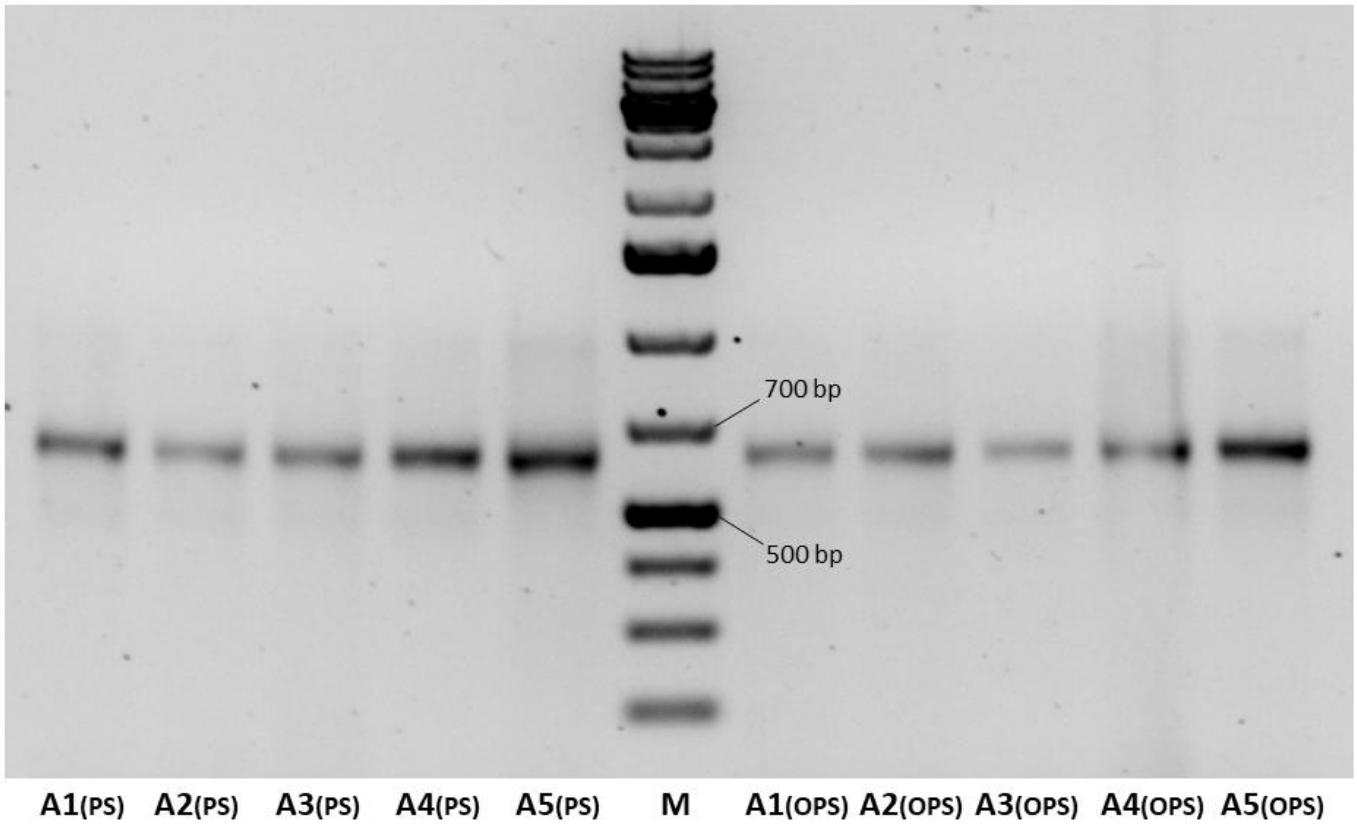

Figure 5.5: Bacteria representative electrophoresis photo of Nested PCR-DGGE amplification products during peak season (A) and off-peak season (B): Samples were collected in five distinct areas: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Lane M contains GeneRuler™ 1 kb plus DNA ladder (ThermoFisher Scientific).

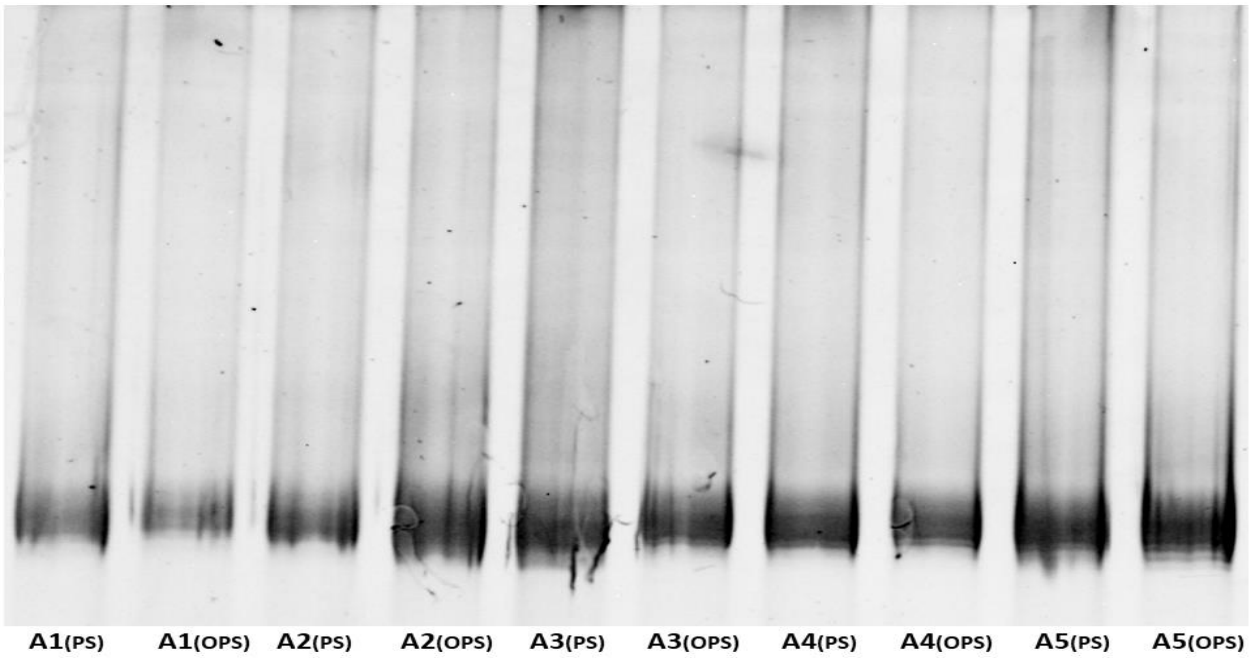

Figure 5.6: The denaturing gradient gel electrophoresis (DGGE) profiles of amplified 16S rDNA from genomic DNA directly extracted from air samples collected after Nested DGGE-PCR analysis: The samples were collected in a fruit juice production facility during different sampling seasons in different designated areas: Area 1: the entrance to the production area; Area 2: preparation and mixing of materials; Area 3: between the production lines; Area 4: dispension of bottles; and Area 5: filling of bottles with the final product. Two test schedules were chosen: peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn).

For eukaryotic diversity, it is well known that the quantification of organisms by PCR-based methods is fraught with many uncertainties. Some biases may be due to differences in rRNA gene copy numbers, and this could be especially important for eukaryotic organisms that may contain up to several thousand copies of the rRNA gene. During PCR, some phylotypes can be amplified preferentially due to preferential priming or differences in elongation rates between amplicons. Another bias can occur when the PCR includes many cycles; according to the kinetic model, when the number of cycles is increased, there is a tendency for the different amplicons to reach equimolarity. All of these potential biases can change the relative concentrations of PCR products so that the resulting profile of phylotypes no longer reflects the composition of the native community (Diez *et al*., 2001; Laforgue *et al*., 2009; Lv *et al*., 2012). Moreover, molecular investigation of the fungal diversity from environmental samples is highly dependent on the primers used (Laforgue *et al*., 2009). As observed during the testing of the culturable fraction, the quantity of the bacteria was considerably more than the yeast and mould quantities, as this could be the situation during the non-culturable analysis as well. Recent research indicated that, for culture-independent identification of yeast and mould in the air, a species-specific approach seemed to be successful (Libert *et al*., 2017). For this approach to work, there is a clear need to create/improve the database of culturable yeast and mould populations in certain indoor environments.

5.3.3. DGGE profile analyses

DGGE fingerprinting can be coupled with the calculation of biodiversity indices, similarity indices, cluster analysis, and banding patterns, and it can compare bacterial communities occurring in different environmental samples to evaluate the diversity and the dynamics of bacterial communities (Aydin *et al*., 2015; Marzorati *et al*., 2008).

The gel that is presented in Figure 5.3 was used to analyse the non-culturable bacterial diversity for each sample area. By using the same quantity of template DNA for PCR-DGGE, the band position and intensity correspond to the abundance of specific species (Wang *et al*., 2016). Regrettably, the resolution (intensity) of the bands represented in Figure 5.3 was not sufficient to allow excision under blue or UV light, and no sequence data to identify individual band positions could be obtained. However, comparative analyses of bacterial diversity richness in the different sampling areas were still possible.

Each vertical lane in Figure 5.6 represents a sample corresponding to a designated area during the two sampling seasons, and each band position ideally represents a bacterial species. The diversity observed with this technique was relatively uncomplicated as a few dominant bands and a larger number of faint bands were revealed. The number of DGGE bands varied from 3 to more than 20 depending on the sample. Bacterial community profiles were different between the two seasons for Area 1 and Area 2 (A1 - PS; A1 - OPS; A2 - PS and A2 - OPS), but with minimal sample-to-sample variation for Areas 3 to 5 (A3 – PS; A3 - OPS; A4 – PS; A4 – OPS; A5 - PS and A5 - OPS) for both seasons.

UPGMA Cluster analysis estimated the order of relatedness among the different samples (Figure 5.7 and Figure 5.8). The cluster analyses that were conducted based on the genetic polymorphism detected by PCR-DGGE segregated the diversity profiles into two main cluster groups, namely Cluster 1 and Cluster 2, which allowed differentiation between the two groups. Cluster 2 contained the samples collected during the peak and off-peak seasons in Area 1 and peak season in Area 5. The entrance to the facility is denoted by Area 1 while the exit is denoted by Area 5. Research has demonstrated that outdoor bioaerosols can penetrate indoors (Chen *et al*., 2015; Meadow *et al*., 2014; Soleimani *et al*., 2016), and this was confirmed by the results that indicated that different groups of bioaerosols were detected in these two areas from those that were detected further inside the plant. This was due to outdoor bioaerosols being present near the outside doorway. The other samples were combined in Cluster 1. Eleven samples had a high similarity of more than 70%. Conversely, earlier studies reported

a low degree of similarity (about 10-40%) with outside airborne bacterial communities (Jeon *et al*., 2011; Tanaka *et al*., 2015).

High similarities (>80%) were detected amongst samples obtained from Area 4 during the peak and off-peak seasons (82.5%). Area 4 (dispersion of bottles) had poor ventilation (the airflow recorded was 0 $m.s^{-1}$), was full of dust and was occupied by personnel who dispensed the bottles. Literature refers to a correlation between microbial counts and the personnel observed in specific areas, and this was confirmed by the current study as airborne microbials were more prevalent when the areas were occupied compared to counts in unoccupied conditions. In addition, humans have been reported to be a source of bacteria and fungi in settled dust samples (Adams *et al.,* 2015). During the culture-dependent identification, the only bacteria (innocuous, useful and harmful) that were detected during both seasons were *Micrococcus terreus, Staphylococcus cohnii*, *S. haemolyticus*, S. *saprophyticus* and *S. succinus*. The same was observed for Area 4 and Area 5 for samples taken during the off-peak season based on culture-independent analyses (85% similarity). The only culturable bacteria (either innocuous, useful or harmful) that were detected during both seasons in this area were *Brevibacterium frigoritolerans, Staphylococcus haemolyticus, S. jettensis,* and *S. succinus*.

Earlier research suggests that PCR-DGGE analysis utilising 16S rRNA genes usually yields patterns that reflect the composition of dominant microorganisms, including non-culturable members. (El-Sayed *et al*., 2015; Head *et al*., 1998). This explains why the similarities displayed in the bacterial diversity (Figure 5.7 and Figure 5.8) obtained during the culture-independent analyses differed from what had been observed during the culture-dependent analyses. Moreover, Shade et al. (2012) demonstrated that culture-dependent methods from the murine gut and soil could reveal the presence of rare bacterial species in a community. Their results indicated that bacteria detected by culture-dependent methods were either less abundant, absent, or it was not possible to distinguish any among the different species. Various researchers also highlight the importance of culture-dependent methods for analysing outdoor and indoor bioaerosols (Douwes *et al*., 2003; Griffin, 2007; Tringe & Hugenholtz, 2008), although they also point out various limitations such as loss of important microorganisms, a long detection period, cells that can be non-viable, and dead microorganisms. With this in mind, it may be untimely to conclude that the results of the culture-dependent and the culture-independent analyses corresponded in this specific study. Rather, it confirms the need to select a bioaerosol approach that depends on the information that is needed, such as: (i) is it (the data) qualitative or quantitative; (ii) specific or general; and (iii) highly localised or over a broader landscape? (Yoo *et al*., 2017).

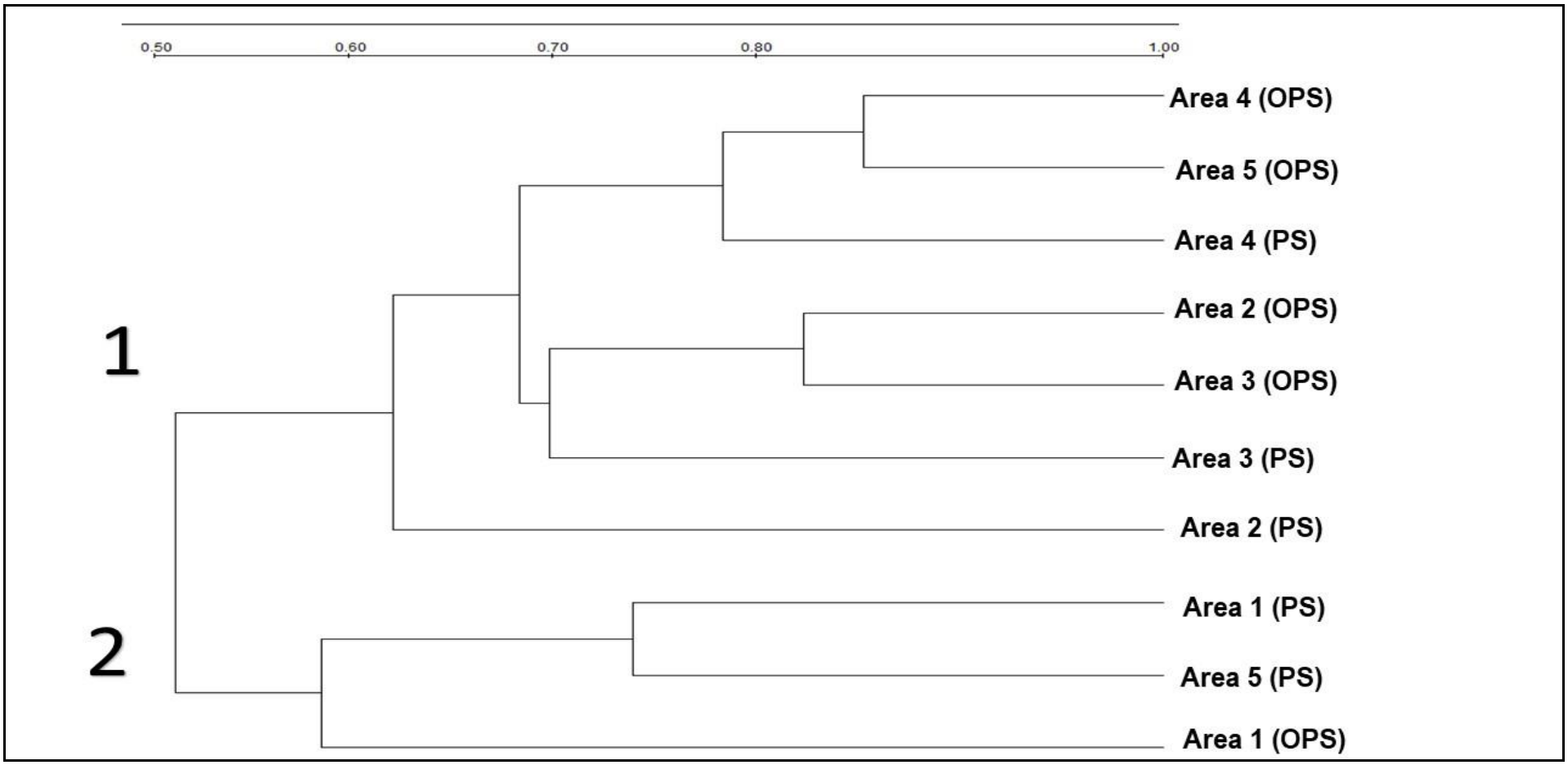

Figure 5.7: Cluster analysis demonstrated graphically as an UPGMA dendogram: The results depict all the DGGE profiles of bacterial 16S rRNA genes amplified using genomic DNA extracted from air samples collected in a fruit juice production facility from five different designated areas: entrance to the production area (A1); preparation and mixing of materials (A2); between the production lines (A3); dispersion of bottles (A4); and filling of the final product (A5). Two test schedules were chosen: peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn).

Sample name	Area 1 (PS)	Area 1 (OPS)	Area 2 (PS)	Area 2 (OPS)	Area 3 (PS)	Area 3 (OPS)	Area 4 (PS)	Area 4 (OPS)	Area 5 (PS)	Area 5 (OPS)
Area 1 (PS)	100									
Area 1 (OPS)	66,4	100								
Area 2 (PS)	25,5	20,6	100							
Area 2 (OPS)	61,7	53,1	63,3	100						
Area 3 (PS)	59,1	35,2	64	74	100					
Area 3 (OPS)	61,8	49,8	61,2	81,9	64,4	100				
Area 4 (PS)	41,4	36,4	70,3	72,7	69,1	70,7	100			
Area 4 (OPS)	45	34,4	53,2	61,9	61,4	74,6	82,5	100		
Area 5 (PS)	73,4	49,2	44	63,5	59,2	76	66	67,8	100	
Area 5 (OPS)	54	30,5	56,2	60,9	66,2	71,9	73,4	85	74,8	100

Figure 5.8: Dice coefficient matrix derived from DGGE profiles of 16S rDNA analysis. Areas: entrance to the production area (A1); preparation and mixing of materials (A2); between the production lines (A3); dispersion of bottles (A4); and filling of the final product (A5). Two test schedules were chosen: peak season (PS) (onset of summer) and off-peak season (OPS) (onset of autumn). Samples highlighted in blue had a high similarity rate of more than 70%.

Sequence technologies have become increasingly popular to describe microbial communities (Poulsen *et al*., 2019), but technical bias problems related to the molecular analysis of microbial communities in environmental samples are well documented (Al-Mailem *et al*., 2017). Such problems may be due to: (i) template annealing in the amplification of 16S rRNA genes (Suzuki & Giovannon, 1996); (ii) template-to-product ratios in multi-template PCR (Polz & Cavanaugh, 1998); (iii) limitations inherent in 16S rRNA genes interspecies heterogeneity (Dahlof *et al*., 2000); (iv) single DGGE bands not always representing single bacterial strains (Sekiguchi *et al*., 2001); (v) primer mismatch, annealing temperature and PCR cycle number affecting the 16S rRNA targeted gene (Sipos *et al*., 2007); (vi) intraspecific polymorphism of 16S rRNA genes (Cui *et al*., 2009); and (vii) differential 16S rRNA gene amplification by primers (Al-Awadhi *et al*., 2013).

To facilitate taxonomy-independent analyses and to reduce the computational resources necessary, marker gene sequence similarity analyses can be carried out under the assumption that sequences with greater similarity represent more phylogenetically similar organisms. These clusters, or operational taxonomic units (OTUs), are widely used as analytical units in microbial ecology studies (He *et al*., 2015). The DGGE profiles (Figure 5.3) reveal several bands, with each band representing an OTU. The highest numbers of OTUs occurred for Area 3 (peak season), Area 4 (peak and off-peak season) and Area 5 (off-peak season), ranging from an OUT of 10.00-12.00 indicating the largest bacterial community diversity (Table 5.6).

Table 5.6: Number of OTUs, weighted richness, and Shannon-Weaver diversity index observed in PCR-DGGE. Data were collected from five designated areas (A1-A5) during two different sampling seasons (peak and off-peak season)

	Sample	Peak Season	Off-Peak Season
Number of OTUs	Area 1	4.00	2.00
	Area 2	8.00	6.00
	Area 3	10.00	6.00
	Area 4	11.00	10.00
	Area 5	7.00	12.00

	Sample	Peak Season	Off-Peak Season
Range weighted richness	Area 1	0.48	0.12
	Area 2	1.92	1.08
	Area 3	8.00	2.88
	Area 4	9.68	8.00
	Area 5	3.92	11.52
	Sample	**Peak Season**	**Off-Peak Season**
Shannon-Weaver diversity index	Area 1	1.38	0.69
	Area 2	2.70	2.10
	Area 3	3.41	2.00
	Area 4	3.58	3.57
	Area 5	2.42	3.55

If an environment is highly habitable, it can host a vast number of different microorganisms with genetic variability; hence a wide gradient is needed to describe the total microbial diversity. Conversely, if the environment is adverse or exclusive, a smaller number of microorganisms will be part of the microbial community and hence a narrow, denaturing gradient will be used to describe the total diversity. In this context, the Range weighted richness (Rr) is the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analysed (Marzorati *et al*., 2008). In the current study, the DGGE bands were used to calculate species richness which is expressed as Range weighted richness (Rr). The Range weighted richness (Rr) values for both peak and off-peak seasons were less than 12 (Table 5.6). However, for both seasons, the rates for Area 1 and Area 2 were considerably lower than for the rest with an Rr <2.00. Based on the DGGE, Rr <10 can be attributed to environments that are particularly adverse or restricted to colonisation (which suggests contaminated soil), or it may be due to poor DNA extraction that resulted in DGGE fingerprinting profiles that are not representative of the bacterial community and are characterised by a low Range weighted richness (Ariefdjohan *et al*., 2010; Marzorati *et al*., 2008). For Area 5 (filling of bottles with final product) in the off-peak season, the highest Rr value of 11.52 was obtained which, according to the literature, is classified as a medium Range weighted richness that is found in food (Biradar *et al*., 2017; Lara *et al*., 2012; Marzorati *et al*., 2008). According to the literature, outdoor and

indoor bioaerosol samples collected a few days apart can harbour very different types of microorganisms, confirming that the air in the same area may differ considerably during two different sampling seasons (Tanaka *et al.*, 2015; Xu & Yao, 2013). The same was observed for Area 5, for which an Rr value of 3.92 was obtained during the peak season. Processes in this area are mostly performed by automated machinery, although personnel are involved in this area by assisting, especially in the peak season. This indicates that this area might have contained the highest bacterial richness, because studies have indicated that humans are a source of bacteria and increase the composition of bacteria in an area (Adams *et al.,* 2015).

The Shannon-Weaver diversity index provides important information about the rarity and commonness of species in a community (Biradar *et al*., 2017). For this reason, Shannon-Weaver diversity index (H) values were obtained from the DGGE profile of each lane. This diversity index showed that the index of species diversity from low to medium was 0.69-3.58 (Table 5.6). The lowest OTUs, Ranged-weighted-richness and Shannon-Weaver diversity rates were detected in Area 1 for the off-peak season. Earlier studies showed changing values for the Shannon-Weaver diversity index during different seasons in different designated areas, which clearly suggests that both the number of species and the number of individuals within species may change during different seasons as well as within different regions (Bonetta *et al.,* 2010; Osimani *et al.,* 2013). This might have been the case in this study for Area 1 during the off-peak season.

When both diversity and richness increase, the Shannon-Weaver diversity index value also increases (Biradar *et al*., 2017). The highest bacterial diversity was observed for Area 4 (peak and off-peak seasons) and Area 5 (off-peak season), and this corresponded with the number of OTUs, range weighted richness and the Shannon-Weaver diversity index. These three samples were also clustered together (Figure 5.9) with a similarity of >80%. These results might indicate that these three areas had bioaerosols of the same composition corresponding with the DGGE image (Figure 5.8). Area 4 (dispersion of bottles) had poor ventilation (airflow recorded was 0 $m.s^{-1}$), was full of dust and was occupied by personnel who dispensed the bottles. These results may indicate that the high bacteria diversity in Area 4 might have affected Area 5, where the final product was filled.

The journey to develop much-needed standardised methods for bioaerosol research has been challenging due to major technical limitations. One challenge is that bioaerosol concentrations are naturally diluted in the environment (Luhung *et al*., 2015). Moreover, low concentrations of interest led to detection limits and sensitivity problems in subsequent analyses. In consideration of the possible limitations in the detection of the non-culturable fraction of bioaerosols, the following can be done to

optimise the process: (i) the source of the bioaerosols must be understood; (ii) a clear result-focused sampling design must be followed (e.g., a higher flowrate or longer duration); (iii) a focused and improved sampling extraction process must be selected; (iv) if needed, extract concentrated DNA; (v) choose the primer carefully as it is one of the most important factors in achieving accuracy in culture-independent analyses (with low DNA content, this needs to be species-specific); (vi) utilise appropriate sequencing technology; and (vii) workflow should be carefully chosen and specifically adapted to meet the requirements of the project.

5.4. Conclusion

The bacterial community structure was analysed using the PCR-DGGE method. The PCR-DGGE method could rapidly analyse a large number of samples without having to know any specific sequences in the sample. This not only makes it possible to resolve complex ecosystems, but also enables the analysis of dynamic changes of the microbial community in different environments or time periods. This method is a useful way to study the microbial community in bioaerosols. Cluster, OTU, Range weighted richness and the Shannon-Weaver diversity index were used to determine the richness and diversity of the bioaerosols.

The PCR-DGGE results indicated that the diversity of bacteria was moderately distributed. Three samples were significant: (i) Area 4 during peak season; (ii) Area 4 during off-peak season; and (iii) Area 5 during off-peak season. These samples had the highest similarity (>80%), the highest OTUs (10.00-12.00), the highest specie richness (8.00-11.52), and the highest diversity (3.55-3.58). Area 4 (dispersion of bottles) had poor ventilation and was full of dust, which made this the perfect environment for bacteria to grow. Area 5 comprised mainly of automated machinery for filling the bottles with the final product. The system did not function in a completely automated manner and staff members still needed to assist with packing bottles before filling, closing bottles after filling, labelling bottles, and packing filled bottles for shipment. A further complication was the fact that more than one product was filled simultaneously. The combination of machinery and personnel contributed to a compact atmosphere in this area, making it ideal for bacteria to grow. Moreover, in this designated area the similarities that were detected indicated an evolutionary relationship, spread and interaction among the bacterial communities.

Because the PCR-DGGE technique was used to determine the diversity of bacteria in the air, it was possible to analyse the differences in the number of bacteria and their diversity using samples from different areas and seasons. Nevertheless, one of the disadvantages in molecular methods is that protocols tend to be specific to each project and thus differ from one study to another. There is thus clearly a need to establish the relationship between culture-dependent and culture-independent approaches when studying bacterial diversity in bioaerosols.

Evidence has increasingly indicated that there is a need for combining molecular tools and environment-specific culture-dependent approaches when studying bioaerosols. Optimisation can sometimes be a long and tedious process; however, omitting this critical step will greatly decrease the accuracy of the results. In light of the potential limitations in detecting the non-culturable fraction of

bioaerosols, the following can be done to optimise the process: (i) the source of the bioaerosols must be understood; (ii) a clear result-focused sampling design must be followed (e.g., a higher flowrate or longer duration); (iii) a focused and improved sampling extraction process must be selected; (iv) if needed, extract concentrated DNA; (v) choose the primer carefully as it is one of the most important factors in achieving accuracy in culture-independent analyses (e.g., with low DNA content, this needs to be specie-specific); (vi) utilise appropriate sequencing technology; and (vii) workflow should be carefully chosen and specifically adapted to meet the requirements of the project. If these considerations are not attended to, this field may never be comprehensively understood.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1. Introduction

Limited data, the enormous variability in the potential effects of different types of bioaerosols, and non-existent standards in South Africa for bioaerosol prevalence render bioaerosol risk assessments challenging and often impractical. Moreover, researchers' methodologies to collect and analyse bioaerosols have differed significantly, and it is thus important to find the ideal middle ground between science and industrial practices in the interest of healthy industrial environments for both workers and products. The targeted facility monitored air quality on a monthly basis by using passive (air plate) monitoring.

6.2. Concluding Remarks

Exposure to bioaerosols has become a hotly debated topic in the past decade, but there are still many unanswered questions such as the following: (i) What complications do bioaerosols cause? (ii) How does the environment influence bioaerosol composition? (iii) Why is there controversy regarding sampling procedures and methods? (iv) What impact does sampling procedures have on bioaerosol recovery? (v) Which dose-response relationships of bioaerosols have an effect on the product and/or occupational health? and (vi) Why are there still only limited guidelines and standards available?

Bearing these questions in mind, the main objective of this study was to collect both the culturable and non-culturable fraction of bioaerosols by active sampling in different areas of a fruit juice facility during peak and off-peak seasons. In addition, this study also aimed to: (i) determine, by using statistical data, if the temperature and airflow in the manufacturing plant affected the growth of organisms in the bioaerosols; (ii) compare the outcomes of passive and active sampling methods; (iii) conduct a survey of the bioaerosols that were detected by cultivating and enumerating the culturable fraction of the bioaerosols; (iv) identify the culturable and non-culturable fraction of the bioaerosols using PCR and PCR-DGGE analyses; (v) categorise the detected bioaerosols as harmful, innocuous or even useful; and (vi) compare the data obtained for the culturable and non-culturable bioaerosol fraction.

All living organisms, including bioaerosols, require a specific environment, nutrition and modes of distribution in order to survive. Two basic environmental parameters, namely temperature and airflow, are fundamental requirements for bioaerosols to survive and spread. With no temperature control in the

facility under study, the average recorded temperature of 20.30 ± 1.1°C was ideal for bioaerosol growth. Furthermore, no airflow (0 $m.s^{-1}$) or ventilation systems were observed, which may have had a positive or negative impact on the quality of the air in the facility. For example, as the main mode of bioaerosol distribution (airflow) was not available, the bioaerosols would have relied on other means to spread through the facility. Moreover, as no airflow or ventilation system was in place, the bioaerosols were not removed from the facility and might either have ended up in the product or contributed to potential occupational diseases.

To ensure good air quality in any facility, it is important to measure the concentrations of bioaerosols that are present, because the number and density of bioaerosols are quick indications of the potential risks that air poses. Two main sampling methods are available for the quantification of bioaerosols: (i) passive sampling (this requires petri dishes containing agar that are opened and exposed to the air); and (ii) active sampling, which physically draws a known volume of air through a particle collection device. As had been expected, the quantities of bioaerosols observed between the two methods differed considerably due to the different sampling approaches. Although a well-defined association was not observed between the two sampling methods, it was notable that at least one of the bioaerosol counts was outside the specification of the facility, irrespective of whether active or passive sampling had been used.

The total microbial counts observed were outside the specifications for the majority of the facility during both seasons. This may have been due to increased levels of airborne organisms in areas that were frequented by personnel. While yeast and mould were observed throughout the facility during the peak and off-peak seasons, higher counts were observed during the off-peak season. This was possibly due to seasonal variation that had already been described by several authors in the past. The microbial, yeast and mould counts that were observed were elevated with high counts obtained during both sampling periods. This indicates that the air in this facility created ideal conditions for all the bioaerosols that were detected. This finding exposed a serious problem as yeast and mould are the main role-players responsible for spoilage in fruit and more specifically fruit juice, which was what this specific industry was producing. It is also noteworthy that traces of presumptive positive pathogens were observed in each of the five areas where samples had been collected. Presumptive positive pathogens are microorganisms that are capable of developing biofilms on food processing surfaces and have been associated with foodborne disease outbreaks caused by fruit juice consumption in the past.

Bacteria, yeast and mould are the main groups of microorganisms found in bioaerosols. This study isolated a total of 239 bacteria, 41 yeasts and 43 moulds from the air in the production environment. Of

the isolates, 92 different species were identified from culturable fraction. These microorganisms belonged to 15 different taxonomic orders that were in turn divided into five orders representing bacteria and ten orders representing yeast and mould. Based on the data, the culturable fraction of the bioaerosols identified was categorised into three main groups, namely 27 innocuous, 26 useful and 39 harmful bioaerosols.

Innocuous bioaerosols included two dominant genera, namely *Bacillus* and *Staphylococcus*. Only four innocuous yeast and mould genera were detected. Useful bioaerosols detected during the sampling seasons were again categorised into three groups, namely: (i) medical contribution; (ii) promoting and protecting plant growth; and (iii) environmental contribution. The fact that harmful bioaerosols were detected was certainly an aspect that raised concern, especially as *Staphylococcus* spp., *Pseudomonas* spp., *Penicillium* spp. and *Candida* spp. were isolated. All these have been reported extensively as problematic in the fruit juice industry. These harmful microorganisms have the ability to be pathogenic/infectious, have multidrug resistance, and have food poisoning/spoilage abilities. Their detection was quite significant and was considered an aspect that the facility should investigate as a matter of urgency.

The bacterial community structure was also analysed using PCR-DGGE. The PCR-DGGE method can be used to rapidly analyse a large number of samples without having to know any specific sequences in the sample. This not only makes it possible to resolve complex ecosystems, but also to analyse the dynamic changes of the microbial community in different environments during various time periods.

Diversity, similarity indices and range weighted richness indicated that the diversity of bacteria was moderately distributed. Three samples were significant: (i) Area 4 (dispersion of bottles) during the peak season; (ii) Area 4 during off-peak season; and (ii) Area 5 (filling of bottles with the final product) during off-peak season. These samples had the highest similarity (>80%), the highest OTUs (10.00-12.00), the highest specie richness (8.00-11.52), and the highest diversity (3.55-3.58). Area 4 had poor ventilation and was full of dust, making this the perfect environment for bacteria to grow. Area 5 comprised mainly of automated machinery that filled the bottles with the final product. However, the system was not completely automated as personnel still needed to pack bottles before filling, fill bottles, close bottles after filling, label bottles, and pack filled bottles for shipment. A complication was that more than one product had to be filled simultaneously. The machinery and personnel contributed to a compact atmosphere in this area which made it ideal for bacterial growth. Although these were designated areas, the similarities indicated an evolutionary relationship, spread and interaction among the bacterial communities.

It is important to emphasise that bioaerosols are ubiquitous environmental contaminants and, in most cases, they are not an integral part of the standard production process. It would therefore be inappropriate to simply 'sample-to-see-what-is-in-the-air' because the presence of microbes in the air can be expected. The bioaerosol field is dominated by a lack of consistent data and an abundance of speculation. The lack of standard methods, environmental guidelines and databases further complicates the interpretation and comparison of results. In addition, because no single method can fully characterise all bioaerosol components, it is imperative to do a proper evaluation/investigation before choosing a sampling method or initiating a sampling protocol. The following comments summarise important aspects to address when planning a bioaerosol monitoring approach, and these can also be used as guidelines when future studies are conceptualised.

6.3. Bioaerosol Monitoring Considerations

6.3.1. Sampling motivation

Formulate the objectives for sampling clearly and unambiguously. There is a clear need to be industry-specific when bioaerosols are sampled. It is also important to establish whether the sampling of bioaerosols is necessitated by baseline monitoring for compliance or to confront an existing quality (product) and/or safety (food handler health) problem for which bioaerosols, as causative agents, need to be ruled out.

6.3.2. Sampling locality

The notion of sampling before doing a critical assessment of the facility is a current shortcoming. This approach can even be misleading because it produces information that is difficult to interpret, might create unnecessary concern, and may lead almost inevitably to the sampling having to be repeated professionally/by external consultants. There is therefore a clear need to be industry- and outcome-specific before monitoring the presence of bioaerosols in a facility. The focus of the assessment should include environmental factors, factory design/layout, nature of the equipment, product type, and conditions impacting food handlers (e.g., their health, shifts/placement, skills level, training, behaviour). Certain environmental factors such as temperature, airflow and relative humidity can be associated with bioaerosol levels and factors such as heating, air-conditioning, or ventilating systems may provoke fluctuations in temperature and relative humidity. Detectable bacterial and fungal levels can also be affected by these factors because they require specific environmental conditions to grow and propagate. Sampling sites to consider include areas with negative air pressure, raw material storage

areas, areas where a lot of dust is generated, under air vents, areas where water spraying or misting can occur, active floor drains, and areas with high worker activity or other movement.

6.3.3. Selection of bioaerosol components for measurement

The evaluation/investigation should provide information that can be used to establish which bioaerosol component is of interest: viable microbial components (culture-dependent); or non-viable, but still bioactive (culture-independent), components. Culture-dependent methods remain important to obtain information regarding the viability and metabolic activity of these organisms and it is also desirable for ascertaining the role different microbes play in distinctive processes. Although culture-dependent methods are by far the most widely used procedures for assessing the microbiological content of bioaerosols, it is now widely accepted that such methods significantly underestimate the total quantity of prevalent microbes. General plate count media is a well-known problem as only a small fraction (10%) of airborne microbes form colonies on a typical culture media, thus leading to a significant underestimation of the actual viable airborne bioaerosol concentrations. Moreover, a vast number of remaining airborne microbes can be described as viable but non-culturable, indicating very low metabolic activity or a resting, dormant state. Dead airborne bacteria or fungi, debris or toxins retain their allergenic or toxic properties and are therefore also relevant to any occupational health assessment process. Evidence has increasingly indicated that a clear need exists for combining molecular tools and environment-specific, culture-dependent approaches when studying bioaerosols.

6.3.4. Choice of equipment

Impingement sampling devices can be used to detect both viable and non-viable bioaerosol components. Moreover, either viable or non-viable components can be assessed using impaction or filtration. Choosing a sampling device will also depend on its availability, the level of expertise of the investigator, and funding.

6.3.5. Sample design and intervals between sampling

When embarking on a new program for compliance monitoring, it is advisable to start with more frequent data collection sessions as this will allow for baseline establishment. When data are available to show that the bioaerosols in a system/area are stable enough, the number of data collection points can be reduced. It is noteworthy that microbial results can differ depending on the activity in a specific area. Sampling times should also occur during both ‘dynamic’ and ‘static’ conditions for optimal monitoring.

6.4 Closing Statements: Future Research, Recommendations to Industry

Until new and reliable techniques for bioaerosol monitoring have been introduced, a common protocol for their quantification based on currently available methods should be employed to offer a near-uniform basis to allow cross-comparisons between different experimental data sets. Therefore, further research is required to establish more appropriate and industry-specific assessment tools for the exposure of bioaerosols. In this context, the following are recommended: (i) an open network approach; (ii) shared infrastructure, technical protocols and training programs; (iii) identifying and collaborating with knowledge-users; and (iv) enhanced capacity-building for response measures.

Information that was elicited by this study may be useful in addressing the gaps in knowledge mentioned above and to aid the fruit juice industry in better understanding and controlling bioaerosols in their facilities. This may also relate to other industries where it may be necessary to use more specific and valid risk assessment protocols for the control of bioaerosols in order to ensure product and occupational health safety. Future research should focus on designing an application that is industry- and outcome-specific in order to aid industries in monitoring bioaerosols and thereby limiting detrimental spoilage and the health risks associated with bioaerosol exposure.