Article

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Culture-dependent diversity profiling of spoilage yeasts species by PCR-RFLP comparative analysis

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Abstract

Spoilage caused by yeasts is a constant, widespread problem in the beverage industry that can result in major economic losses. Fruit juices provide an environment that allows the proliferation of yeast. Some factories in South Africa are not equipped with laboratory facilities to identify spoilage yeasts and outsourcing becomes a prolonged process which obstructs corrective action planning. This study aimed to establish yeast diversity and apply a rapid method for preliminary identification of spoilage yeasts associated with a small-scale fruit juice bottling factory. Yeast population in the factory was determined by isolation from the production environment, process equipment and spoiled products. PCR-RFLP analysis targeting the 5.8S-ITS region and D1/D2 sequencing was used for identification. A total of 207 yeasts belonging to 10 different genera (*Candida, Lodderomyces, Wickerhamomyces, Yarrowia, Zygosaccharomyces, Zygoascus, Cryptococcus, Filobasidium, Rhodotorula*/*Cystobasidium* and *Trichosporon*) were isolated and identified from the production environment and processing equipment. *Candida intermedia, C. parapsilosis* and *Lodderomyces elongisporus* were widely distributed in the factory. *Zygosaccharomyces bailii, Z. bisporus, Zygoascus hellenicus* and *Saccharomyces cerevisiae* were isolated from the spoiled products. The data provided a yeast control panel that was used successfully to identify unknown yeasts in spoiled products from this factory using polymerase chain reaction-restriction length polymorphism (PCR-RFLP) comparative analysis.

Keywords

Fruit juice, spoilage yeast, 5.8S-ITS region, yeast diversity, RFLP analysis

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INTRODUCTION

The food and beverage industry is one of the most important components of South Africa's manufacturing sector. Beverages account for just over 4% of all manufacturing sales, while in the food and beverage sector, beverages represent 24% of sales (Food and Beverages Manufacturing Sector Education and Training Authority. SSP 2011–2016). Increased consumption of fruit juices has a direct influence on the economy in a positive way, but becomes negatively affected when foodborne disease outbreaks and spoilage problems occur (Tribst et al., 2009).

Yeast spoilage is a constant and widespread problem in the beverage industry (Aneja et al., 2014). This type of spoilage is predictable and mainly occurs in those products where bacterial growth is either impeded or prevented by predominating intrinsic, extrinsic and processing factors. Typically, acidic low pH foods and products with a high sugar content, such as fruit juice concentrates, are affected (Tribst et al., 2009). The most visible sign of yeast spoilage is recognised by swelling of the product container due to gas production that

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results from fermentation, referred to as 'blowing' (Kurtzman and James, 2006; Wang et al., 2016). This form of spoilage causes major financial losses to the bottling factories involved (Arias et al., 2002).

Industrial facilities harbour yeast strains that are adapted to the processing environment and circumvent the barriers set up to prevent them (Doyle, 2007; Martorell et al., 2005). Precautionary measures to avoid or reduce microbial spoilage require high expenditure. Despite quality control practices in bottling factories that entail rigorous microbial monitoring of pulps, water, air and equipment, yeast spoilage continues to occur. The origin of spoilage yeast seldom follows a set pattern and the modes of contamination are often specific to each processing facility. It therefore seems mandatory to study each particular manufacturing plant to determine the origin of spoilers (Hernández et al., 2018).

To design adequate strategies to prevent spoilage, it is advantageous to know the identity of the spoilage organisms present in the product and gain insight into the source of contamination (Loureiro, 2000). Improved techniques with increased specificity, discriminatory power and shorter detection times for the identification of spoilage yeasts in foods and drinks are becoming increasingly important in the food sector (Casey and Dobson, 2004; Hernández et al., 2018). Many recent, complex and sensitive identification tools for rapid identification of pathogens and spoilers have become available, but for small-scale fruit juice bottling factories, more basic methods are still required, at least for preliminary identification (Kesmen et al., 2018).

One such fruit juice bottling factory in Bloemfontein, South Africa, experiences problems with 'blowing' of certain juice concentrates on an annual basis. The problem is aggravated by long waiting periods for identification of the spoilage contaminants, which have delayed corrective actions in the past. Therefore, the aim of this research was to compile a culture-dependent yeast diversity profile of the factory environment/equipment and apply comparative PCR-RFLP analysis to identify spoilage yeasts associated with its products.

MATERIALS AND METHODS

Sampling protocol

The factory has been operating for 24 years producing fruit juice concentrates of different flavours. It consists of nine blending tanks and three filling lines. Approximately 24,000 l of juice is produced per day. Surface swabs were obtained from the production environment and processing equipment. Areas included the refrigerator, powder blenders, pipes, blending tanks, holding tanks, nozzles, ramp, bottle and caps. Yeast isolates originating from weekly routine analysis of surface swabs and air samples taken after Cleaning in Place (CIP) protocols were carried out and were isolated on chloramphenicol agar plates. All isolates were collected over a period of one year and cryopreserved in 15% glycerol at −80 °C. Eight fruit juice samples, each representing a different batch affected by spoilage (blowing) during the sampling period, were collected, retained on ice during transportation and analysed without delay. Spoiled sample flavours included ice tea, fruit flavour, cordial and fruit/dairy blend.

Enumeration and isolation

Surface swabs were suspended in 10 ml peptone water and serially diluted. Dilutions were plated onto Rose Bengal Chloramphenicol (RBC) agar and incubated at 30 °C for 48–120 h. Yeast colonies from chloramphenicol agar plates that were provided by the factory technician were also transferred to RBC agar and incubated at the same conditions. The resulting colonies were selected based on differences in colony morphology and purified by repeated sub-culturing (Barata et al., 2008).

Spoiled fruit juice samples were also serially diluted in sterile peptone water. The series of dilutions were plated onto RBC agar and Malt Extract agar, and incubated for up to three days at 30 °C. Of the resulting colonies, approximately 10% were randomly selected based on colony morphology and used as template in whole cell PCR (Barata et al., 2008). Isolates were purified by repeated sub-culturing and cryopreserved in 15% glycerol at −20 °C.

PCR amplification and RFLP analysis of 5.8S-ITS region

Whole cell PCR amplification and RFLP analysis of the 5.8S-ITS region were performed on all isolates and reference strains. For comparison and preliminary identification, reference strains frequently isolated from fruit juice were obtained from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free State. The 17 reference strains, also cryopreserved as described before, included *Candida intermedia* (UOFS Y-0649), *Candida parapsilosis* (UOFS Y-0206), *Candida tropicalis* (UOFS Y-0534), *Dekkera anomala* (UOFS Y-1062), *Hanseniaspora occidentalis* (UOFS Y-0153), *Kluyveromyces marxianus* (UOFS Y-0797), *Lodderomyces*

elongisporus (UOFS Y-2394), *Millerozyma farinosa* (UOFS Y-0203), *Pichia kudriavzevii* (UOFS Y-0814), *Rhodotorula/Cystobasidium slooffiae* (UOFS Y-0972), *Saccharomyces bayanus* (UOFS Y-0912), *Saccharomyces cerevisiae* (UOFS Y-0792), *Saccharomycodes ludwigii* (UOFS Y-0540), *Torulaspora delbrueckii* (UOFS Y-1016), *Wickerhamomyces anomalus* (UOFS Y-0810), *Zygosaccharomyces bailii* (UOFS Y-1535) and *Zygosaccharomyces rouxii* (UOFS Y-0763).

Yeast cells from 48 h single colonies were suspended in 50 µl PCR reaction mix containing 0.52 µM primer ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (Rojo et al., 2017), 0.2 µM dNTPs, 1X reaction buffer ThermoPol®, and 1 U of NEB Taq ThermoPol® (New England Biolabs). Amplification conditions included one cycle at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min. A final elongation step was performed at 68 °C for 7 min. Successful amplification was confirmed by agarose gel (1%) electrophoresis. All amplicons (10 µl) were digested with 1 unit of *Cfo*I, *Hae*III and *Hinf*I restriction enzymes using FastDigest™ and Tango™ buffers (Thermo Scientific) in separate reactions (Rojo et al., 2017). Fragments were separated in a 3% agarose gel and digital images were captured with the Molecular Imager® Gel Doc™ XR system (BioRad Laboratories Inc.). Band sizes were calculated with reference to a GeneRuler™ 1 kb DNA ladder Plus and GeneRuler™ 50 bp DNA ladder (Thermo Scientific), using Quantity One® 1-D Analysis software (BioRad Laboratories Inc.). Resulting PCR-RFLPs were grouped according to profiles (Table 1), compared to the reference strain profiles and yeast-ID database (rank ± 20 bp) for preliminary identification and Sanger sequenced for confirmation.

Amplification and sequencing of D1/D2 domain

The D1/D2 domain of the 26S rRNA gene was amplified from at least three isolates representative of a specific PCR-RFLP profile and sequenced for identity confirmation. Amplification of the D1/D2 domain was performed using primers NL-1 (5′-GCATA TCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (White et al., 1990). Sequencing reactions were carried out using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Data were analysed using Chromas LITE version 2.1.1 and compared with previously published sequences using the BLAST algorithm (Altschul, 1997) for species identification (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were deposited into the NCBI database with accession numbers KU708234.1–KU708252.1.

RESULTS AND DISCUSSION

Identification of spoilage yeast using PCR-RFLP comparative analysis

A total of 207 yeasts were isolated and identified according to 5.8S-ITS polymorphisms (Ting et al., 2018). The isolates showed different PCR product sizes, ranging from 300 to 900 bp. The PCR products digested with *Cfo*I, *Hae*III and *Hinf*I enzymes were analysed for all isolated strains and 18 distinct profiles were obtained (Table 1), designated by a letter of the alphabet. For comparison, PCR-RFLP of the 5.8S-ITS region was applied simultaneously to reference strains from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection as controls. Representatives of each of the 18 profiles were confirmed by sequencing the D1/D2 domain of the 26S rRNA gene.

It could be argued that an indigenous yeast control panel might appear redundant, since yeast identification databases constructed on RFLP data are available. However, discrepancies can be observed in amplicon sizes and restriction profiles among species in different studies. It may be expected that differences in recorded fragment sizes as great as 20 bp are possible simply due to the manner in which the bands sizes were determined. This would account for many of the small size variations reported by different researchers for the same strain of a species (Coton et al., 2006; Jeyaram et al., 2008; Pham et al., 2011; Satora et al., 2013; Sun and Liu, 2014). Although innovative and user friendly, the yeast-ID database (CECT-IATA, Spanish Type Culture Collection, Universitat de Valencia, Valencia, Spain; www.yeast-id.org) could identify only 22% of the isolates (Table 1). It is also possible that the database is limited to the specific culture collection and may not contain the yeast species of interest, which was the case with *Candida quercitrusa, Candida sojae, C. slooffiae, Filobasidium uniguttulatum* and *Trichosporon ovoides* isolated from the factory environment in this study.

Yeasts that were isolated from eight spoiled fruit juices each representing a different batch affected by spoilage yielded populations ranging from 2.80×10^3 to 2.23×10^7 CFU/ml. PCR product lengths were between 600 and 900 bp and was already an indication that spoilage of the different juices was likely caused by the presence of different yeast species. PCR products were digested with *Cfo*I, *Hae*III and *Hinf*I. Table 2 presents the digestion profiles of unknown yeasts. Restriction profiles were compared to that of the control panel and subsequently identified. The restriction profile of unknown yeasts 1 and 3 was not identical to any of the isolates from the control panel and preliminary identification was not possible. The isolates were

Table 1. Characterisation of yeast isolates based on 5.8S-ITS region PCR and RFLP data, and D1/D2 domain sequence identifications[1]

		RFLP based identification		Fragment lengths (bp)				
Profile	Isolates	UNESCO-MIRCEN Biotech Yeast Culture Collection	Yeast-ID.org database (% identity)	PCR	*Cfo*I	*Hae*III	*Hin*fI	Identification (D1/D2 domain sequencing)
G	6	No match	*Y. lipolytica* (100%)	377	214, 174	377	182	*Y. lipolytica*
C1	27	*C. intermedia*	*C. intermedia/pseudointermedia/catenulate/haemulonis* (100%)	400	212, 174	400	215, 193	*C. intermedia*
V	8	No match	*Cryptococcus hungaricus/luteolus* (88%)	480	272	476	234	*T. ovoides*
A1	38	*C. tropicalis*	*C. montana* (62%)	515	290, 220	410, 112	281, 257	*C. parapsilosis*
P	5	*C. parapsilosis*	*C. parapsilosis* (100%)	515	252, 202, 64	399	236	*C. laurentii*
R1	7	No match	*Cryptococcus skinneri* (62%)	527	293, 238	468	274	*C. sojae*
O	12	*L. elongisporus*	*C. maltose/ L. elongisporus* (100%)	576	323, 240	527	298, 261	*L. elongisporus*
X	8	No match	*C. multigemmis/C. valdiviana* (75%)	596	317	423, 141	318	*C. oleophila*
U	11	No match	*C. laurentii/Wickerhamomyces bovis/ Pichia dryadoides* (75%)	611	612	586	339, 265	*C. spandovensis*
A	14	No match	*Cyberlindnera bimundalis/ Wickerhamomyces strasburgensis* (88%)	612	281, 310	508	357, 280	*F. capsuligenum*
H	8	No match	*Kuraishia capsulate/ C. ergatensis* (79%)	624	392, 308	487	261, 242, 146	*F. uniguttulatum*
I1	5	No match	*C. santamariae/atlantica Schwanniomyces pseudopolymorphus Yamadazyma Mexicana* (92%)	634	320, 226, 66	420, 137	312	*C. quercitrusa*
E	4	No match	*Cyberlindnera meyerae/ F. capsuligenum* 62%)	635	385	536	350, 292	*C. saitoi*
F	25	*W. anomalus*	*W. anomalus* (88%)	640	569	640	308	*W. anomalus*
K1	7	No match	*Z. hellenicus/Wickerhamomyces bisporus* (92%)	643	331	643	343, 171, 122	*Z. hellenicus*
W	10	*R. slooffiae*	*C. glaebosa* (75%)	647	647	647	325, 253	*R. slooffiae*
R	6	No match	*Saccharomyces kluyveri* (73%)	687	341, 321	387, 209	221, 215, 103	*R. dairenensis*
D	6	*Z. bailii*	*Z. bailii* (69%)	782	336, 284, 89	712	331, 230, 160	*Z. bailii*

[1]Data arranged according to PCR product size; fragments smaller than 50 bp were not included.

Table 2. Characterisation of yeast isolated from spoiled fruit juices based on 5.8S-ITS region PCR and RFLP data, and D1/D2 domain sequence identifications

Isolate	Juices	RFLP based identification	Fragment lengths (bp)				Identification (D1/D2 domain sequencing)
		Factory control panel yeast	PCR	*Cfo*I	*Hae*III	*Hin*fI	
Unknown 1	Cordial	*No match*	765	286, 252	692	393, 228, 148	*Z. bisporus*
Unknown 2	Ice tea Fruit Cordial Fruit/dairy blend	*Z. bailii*	781	322, 270, 85	697	349, 220, 160	*Z. bailii*
Unknown 3	Fruit/dairy blend	No match	844	357, 335, 127	306, 224, 169	356, 114	*S. cerevisiae*
Unknown 4	Cordial	*Z. hellenicus*	630	317	623	327, 161, 110	*Z. hellenicus*

sequenced (D1/D2 domain) and identified as *Zygosaccharomyces bisporus* and *S. cerevisiae*, respectively. The restriction profiles of unknown yeasts 2 and 4 were identical to that of *Z. bailii* and *Zygoascus hellenicus* and were verified by D1/D2 domain sequencing. Spoiled ice tea and fruit flavours contained only *Z. bailii*. Cordial contained *Z. bailii, Z. bisporus* and *Z. hellenicus*. Both *Z. bailii* and *S. cerevisiae* were isolated from the fruit/dairy blend.

Yeast diversity in the production environment and processing equipment

Many types of yeasts are potential spoilage agents of fresh and concentrated fruit juices due to favourable pH conditions and high sugar levels of these beverages (Tribst et al., 2009). Contamination may originate from any step along the manufacturing process. Raw materials, factory environment, packaging and processing equipment are all potential sources of contamination (Hernández et al., 2018). In a 'forensic approach' to spoilage of soft drinks, Davenport (1996) noted that most yeast contaminants encountered could be divided into four categories, which he referred to as Groups 1–4. Group 1 constitutes spoilage yeasts that are fermentative and preservative resistant. Group 2 comprises spoilage or hygiene types and Group 3 are indicators of poor factory hygiene. Group 4 yeasts are 'aliens' which are out of their normal environment (Davenport, 1996).

The yeast population from the production environment and processing equipment comprised 10 different genera. Ascomycetous yeast species included *C. intermedia* (13%), *C. parapsilosis* (18%), *C. sojae* (3%), *C. quercitrusa* (2%), *C. spandovensis* (5%), *C. oleophila* (4%), *L. elongisporus* (6%), *W. anomalus* (12%), *Yarrowia lipolytica* (3%), *Z. bailii* (3%) and *Z. hellenicus* (3%). Yeast diversity in this factory was dominated by Ascomycetes, which was not surprising since most ascomycetous yeasts have been found in environments with high concentrations of sugar (Molnárová et al., 2014). Basidiomycetous yeasts were represented by *C. slooffiae* (5%), *Rhodotorula dairenensis* (3%), *Cryptococcus laurentii* (2%), *Cryptococcus saitoi* (2%), *F. uniguttulatum* (4%), *Filobasidium capsuligenum* (7%) and *T. ovoides* (4%). Basidiomycetous yeasts are generally found in soil, plant materials and bird droppings, and are not usually associated with spoilage and industrial processes, but are regarded as hygiene-indicator species (Tekolo et al., 2010). As such, these yeasts were also less abundant in the factory equipment compared to ascomycetous yeasts (Figure 1).

Diversity distribution varied among the different equipment. *C. slooffiae, Z. hellenicus, W. anomalus* and *Z. bailii* were isolated from the air samples taken from the refrigerator where the concentrated pulps were stored. *C. slooffiae*, previously known as *Rhodotorula slooffiae* (Yurkov et al., 2015), was the dominant yeast isolated from this area.

In the powder blenders that are used for mixing powder ingredients, eight different yeast species were detected, of which *C. intermedia* and *C. parapsilosis* dominated. Both yeasts have been isolated previously from reconstituted fruit juice (Maciel et al., 2013) and *C. parapsilosis* has been reported as an opportunistic pathogen responsible for various mycoses (Jacques and Casaregola, 2008). It is reasonable to assume that contamination of equipment by *C. parapsilosis* is a result of food handlers, since this organism is frequently found in blood, skin and nails (including hands of healthcare workers) (Nosek et al., 2009). Furthermore, Welthagen and Viljoen (1998) reported that workers' hands and aprons were also responsible

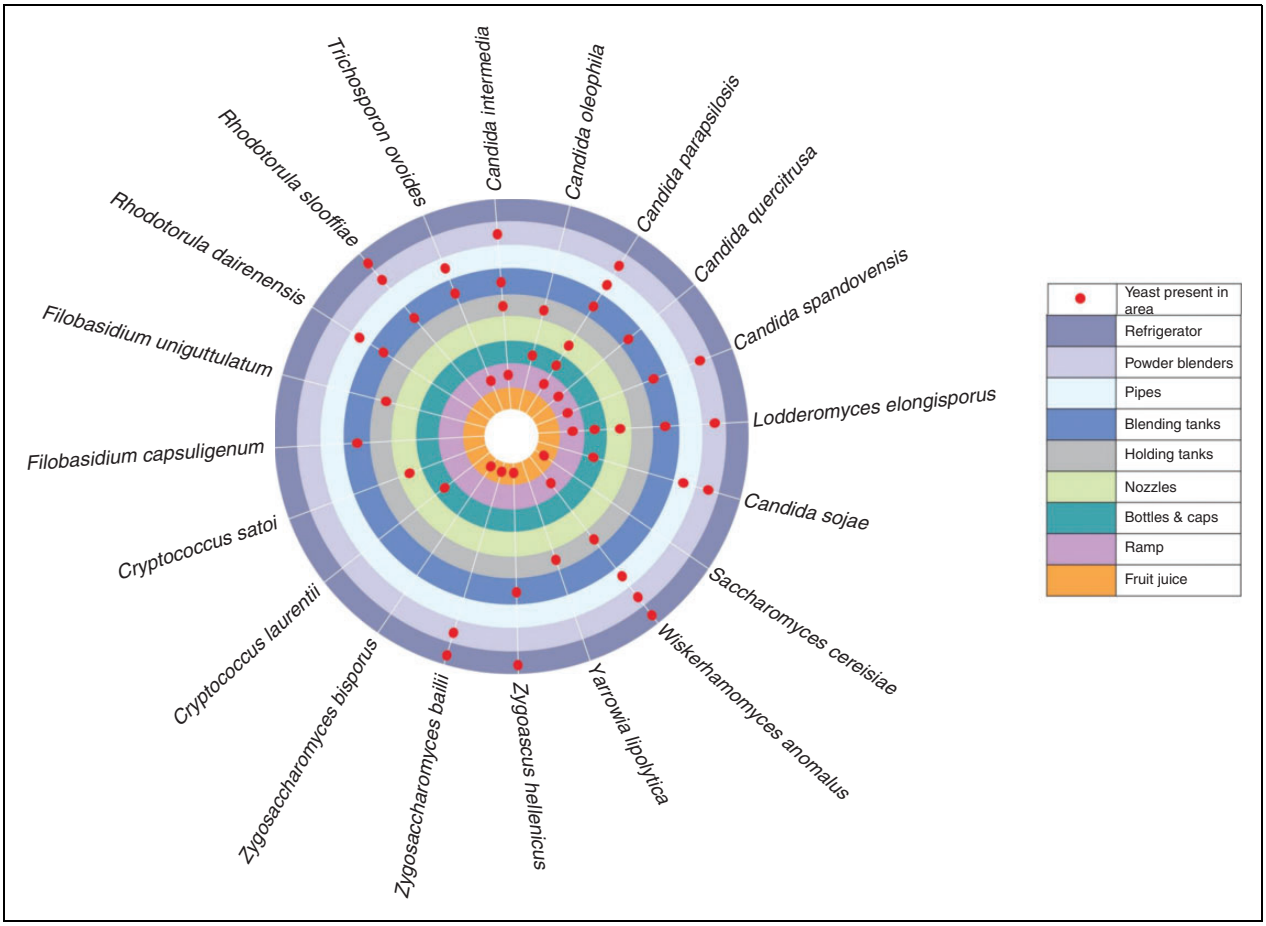

Figure 1. Data wheel showing the yeast distribution in the fruit juice bottling factory and spoiled products. View clockwise from *C. intermedia* to *Z. bisporus* shows the distribution of Ascomycetes, and from *C. laurentii* to *T. ovoides* that of Basidiomycetes. The coloured legend indicates the area/origin sampled and a red dot when the specific yeast species was detected.

for a high rate of yeast contamination in other food processing environments. The isolates from the powder blenders comprised Group 2 yeasts that are spoilage and hygiene types (Davenport, 1996). This group is able to cause spoilage of fruit juices, but only if complications arise during manufacturing, such as low level or absence of preservative, ingress of oxygen, pasteurisation failure or poor standards of hygiene (Davenport, 1997).

The pipes that connect the powder blenders to the blending tanks were largely colonised by *C. parapsilosis*. *T. ovoides* is classified by Davenport (1996, 1997, 1998) as belonging to Group 3 organisms that are hygiene indicators, not causing spoilage. These species are able to utilise different carbohydrates and carbon sources and degrade urea, but members of this genus are non-fermentative. *W. anomalus, C. sojae* and *R. dairenensis* were also isolated from the pipes. Not unexpectedly, the blending tanks shared similar diversity with the pipes and also contained *F. capsuligenum, C. intermedia, L. elongisporus, C. slooffiae, Z. hellenicus, C. quercitrusa* and *C. spandovensis*. The high sugar content and low water activity of the ingredients in the blending tanks favour the growth of yeasts, which contributed to the considerable diversity isolated from this equipment.

C. parapsilosis, L. elongisporus and *C. saitoi* were present in the nozzles that release the fruit juice into the bottles during filling. These yeast are common contaminants in bottling factories, but can be effectively controlled if Good Manufacturing Practices (GMPs) are strictly adhered to (Davenport, 1996). *C. parapsilosis, L. elongisporus, C. sojae, C. oleophila* and *C. laurentii* were isolated from the bottles and caps used for packaging during filling. Caps and bottles are not washed prior to filling and are stored in the roof area, which is not properly insulated. The latter is likely to introduce soil and dust-related yeasts, such as *L. elongisporus* and *C. laurentii*, into the packaging material (Cloete et al., 2010; Sláviková et al., 2007; Stratford and James, 2003).

Relevance of detected spoilage yeasts

Z. bailii, Z. bisporus, Z. hellenicus and *S. cerevisiae* isolated from the spoiled fruit juices are categorised as Group 1 yeasts (Davenport, 1996) that have been described as spoilage organisms adapted to growth in fruit juices and being able to cause spoilage from very low cell numbers. The characteristics of Group 1 yeasts are osmotolerance, aggressive fermentation, resistance to preservatives (particularly weak organic acids) and a requirement for vitamins. The high sugar concentrations in fruit juice concentrates favour the growth of yeasts with a higher fermentative activity (Brugnoni et al., 2012).

Osmotolerant *Z. bailii* is commonly encountered in high sugar (40–70%) environments responsible for considerable economic losses in the beverage industry (Loureiro, 1994; Rojo et al., 2014; Stratford et al., 2013; Wang et al., 2016). *Z. bailii* was detected in all the spoiled fruit juice flavours, which was not unexpected given its extreme resistance to preservatives and ability to grow in concentrations of preservatives in excess of the legally permitted levels (Harrison et al., 2011). The low permeability of *Z. bailii* to weak acid preservatives at low pH values, and its ability to metabolise acid compounds, even in the presence of glucose, are some of the physiological traits associated with its high tolerance to acidic environments (Sousa et al., 1996). The factory from which the spoiled fruit juices were obtained uses sodium benzoate and sodium metabisulphite preservatives, which are classified as weak acid preservatives, and are thus easily resisted by members of the genus *Zygosaccharomyces*.

Z. bailii was not widely distributed in the processing equipment analysed. This observation could be linked to the fact that it exhibits the lowest capacity of adhesion to stainless steel and also the lowest percentage of hydrophobicity (Brugnoni et al., 2007). Stainless steel is the most frequently used food contact material in the fruit juice processing industry and the factory investigated was no exception. The combination of the two parameters pertaining to adhesion and hydrophobicity leads to a significant decrease of the adhesion capacity of this species (Brugnoni et al., 2007). Although *Z. bailii* may be present in low numbers on stainless steel, it can show gradual proliferation in concentrates and only one cell per container of diluted stock can cause spoilage (Wareing and Davenport, 2005). Although *Z. bisporus* is isolated from foods at a substantially lower frequency than *Z. bailii*, it has a similar ability to cause food spoilage and is also preservative resistant (Barata et al., 2012).

Zygoascus hellenicus is highly fermentative and has been described extensively as being associated with grape berries or must in the winery environment (Barata et al., 2008; Simões and Gomes, 2015). The cordial, which was the only fruit juice type contaminated by *Z. hellenicus*, consisted of strawberry, cranberry and raspberry pulp. It could be possible that this species originated from the pulp since it has been associated with contamination of berries. It has also been described as a contaminant often associated with damaged grapes (Barata et al., 2008) and some studies indicated that it has been isolated from fruit juices (Maciel et al., 2013; Nyanga et al., 2013). It was not unusual to isolate *S. cerevisiae*, also a fermentative yeast associated with microbial decomposition of fruit juices (Turtoi, 2014). This yeast can grow in a range of conditions and is characterised for its optimal growth on high sugar media. *S. cerevisiae* has also been isolated from a variety of dairy products, especially those containing sugar and fruit (Mayoral et al., 2005). Not surprisingly in this study, the fruit/dairy blend containing milk powder was populated by *S. cerevisiae*.

CONCLUSION

Culture-dependent PCR-based techniques are primarily chosen to identify yeasts due to their technical simplicity and relatively low cost. Restriction enzyme digestion of the ITS regions is still commonly used for yeast species identification. A culture-dependent approach was also chosen for this study to capitalise on the sampling protocol and yeast enumeration methods that already formed part of the factory's microbial quality control. Consequently, this approach not only ensured consistent standard operating procedures and sufficient coverage of the area for diversity analysis, but also a control panel of yeasts that could be used as comparative references to identify isolates from spoiled products. Yeast diversity data revealed that the processing environment and equipment of the fruit juice bottling factory investigated harboured a variety of yeast species, despite rigorous cleaning and disinfection. The dominant presence of hygiene indicator species *C. intermedia, C. parapsilosis* and *W. anomalus* in the factory environment after CIP suggests that the current GMP requires attention. *Z. bailii* and *Z. hellenicus* were both mainly isolated from the air in the refrigerator where the fruit pulp was stored, which might imply the refrigerator as the likely source of contamination. *S. cerevisiae* and *Z. bisporus* were not detected in the factory environment, which showed that spoilage yeasts did not necessarily originate from the factory environment. Although it is unlikely that these micro-organisms were missed during sampling, it cannot be excluded as a possibility. It is, however, more reasonable to assume that they originated from an external source, presumably the concentrated pulps. Pre-screening fruit pulp for spoilage yeast could be a useful approach towards spoilage prevention for this particular factory.

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