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Full Length Research Paper

Diversity and bioprospecting potential of rhizo and endophytic bacteria from two mangrove plants in Saudi Arabia

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Mangrove plants are located on coastal area of sea and harbor diverse communities of microorganisms. The aim of our present study was to isolate bacteria from two different mangroves collected from the coastal area of Thuwal, Saudi Arabia and to further screen them for their antimicrobial activities. We have isolated 317 different rhizo and endophytic bacteria from mangroves using soil, roots and leave tissues. Bacteria were screened for their antifungal activities against oomycetes pathogens, *Phytophthora capsici, Pythium ultimum*. Only 25 bacterial strains found to be active against oomycetes fungal pathogens. These bacteria were tested further against other fungal pathogens like *Magnaporthe grisea, Altenaria malli,* and *Fusarium oxysporum*. Antagonistic bacteria were further screened for antibacterial activities against human pathogenic bacteria. Only few isolates exhibited antagonistic activity against these pathogenic bacteria. Hydrolytic enzymes production (cellulase, protease, lipase, and amylase) was also assessed. Most of the active isolates exhibited amylase and protease activities. Identification based on 16S rRNA gene revealed 92.8 to 99.9% sequence, similar to type strains of related species. Antagonistic bacteria belong to 5 different classes that is Gammaproteobacteria (*γ-Proteobacteria*), Alphaproteobacteria (*α-Proteobacteria*), *Firmicutes*, *Bacteroidetes* and *Actinobacteria.* Our results provide evidence that, mangroves plants harbor potentially useful bacteria producing active metabolites and enzymes.

Key words: Mangrove plants, antagonistic bacteria, enzymatic activities, 16S rRNA gene sequence, phylogenetic analysis.

INTRODUCTION

Mangroves, the coastal ecosystems, are found in transitional zones between rivers, sea and land (Schaeffer-Novelli et al., 2000; Kathiresan and Bingham, 2001; Walters et al., 2008) in tropical and subtropical regions all around the world. Their distribution lies in 123 countries covering about 152,000 km^2 (Spalding et al., 2010). Mangrove forests also have diverse microbial communities, which play critical role in maintenance and functioning of these complex and sensitive ecosystems (Sahoo and Dhal, 2009).

Mangroves sedimentation is the actual base for mangrove forests and organisms inhibiting them. In turn, these microorganisms release nutrients from the sediments and provides base for an enormous food web (Holguin et al., 2001; Spalding et al., 2010).

Mangrove plants can tolerate a wide range of environmental factors. This tolerance is facilitated by rhizospheric and endophytic microorganisms, which play vital role in biogeochemical cycles and nutrient transformations (Kathiresan and Bingham, 2001). Bacteria are primary decomposers of organic matter (Saxena et al., 1988) and key players in nitrogen fixation (Abraham et al., 2004; Miransari, 2011). Mangrove sediments and organisms controlling these ecosystems are good targets to study (Kathiresan and Bingham, 2001). High salinity, organic matter, low aeration in mangroves provides conditions, favorable for the growth of diverse microbes of biotechnological importance (Sivaramakrishnan et al., 2006; Dias et al., 2009). Organisms present in mangrove ecosystems remains largely unexplored therefore, there is an excellent source for finding new and novel bioactive secondary metabolites with distinct functions such as enzymes, antibiotics and antitumor compounds (Das et al., 2014). Both rhizo and endophytic bacteria performing diverse functions have been isolated previously from mangrove ecosystem (Hong et al., 2015).

Endophytes are microorganisms harboring internal tissues of plants and are important source of secondary metabolites for development of novel drugs against different diseases of human (Strobel et al., 2004). From biotechnological perspective, bacteria from mangrove plants are an important source of functional metabolites including enzymes and antibiotics (Dias et al., 2009; Thatoi et al., 2013). Several previous studies highlighted importance of beneficial bacteria isolated from mangrove habitats (Vazquez et al., 2000; Soares Junior et al., 2013; Zainal Abidin et al., 2016).

Many rhizo and endophytic bacteria from mangrove plants is a potential producers of important enzymes like amylase, esterase, cellulose and proteases (Dias et al., 2009; Castro et al., 2014). By considering mangroves as an important source of bacteria, these bacteria can produce both enzymes and antimicrobial compounds which are of great interest. Therefore, present study was designed to isolate and characterize rhizo and endophytic bacteria from two mangroves, *Haplopeplis perfoliata* and *Cyperus conglomerates*. Their antifungal and enzymatic characteristics have been examined and 16 SrRNA analysis placed them in different groups of bacteria.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

Plant samples were collected from the carbonated shore of Red sea in Thuwal, (22°15' 54" North, 39°6' 44" East) located in Jeddah, Saudi Arabia. Both plant samples that are *H. perfoliata and C. conglomerates* were put in sterile bag after collection and transferred to laboratory for bacterial isolation.

Soil, roots and leaves samples of plants were used for isolation of bacteria. For bacterial isolation from adhering soil, dipped roots in filtered autoclaved sea water (FAS) to remove adhering soil and serial dilutions were made (10^{-3}, 10^{-4} and 10^{-5}) in a filtered autoclaved sea water (FAS), to spread on different media used for culturing of bacteria for maximum isolation.

Half strength R2A (½ R2A) [0.25 g yeast extract, 0.25 g proteose peptone No. 3 (Difco), 0.25 g casamino acid, 0.25 g dextrose, 0.25 g soluble starch, 0.15 g sodium pyruvate, 0.15 g K_2HPO_4, 0.03 g $MgSO_4$], half Tryptic soy agar (½ TSA) [Pancreatic digest of casein, 7.5 g Papaic Digest of soybean, 2.5 g sodium chloride, 2.5 g agar, 15.0 g], marine agar (MA) [peptone, 5.0 g yeast extract, 1.0 g ferric citrate, 0.1 g sodium chloride, 19.45 g magnesium chloride, 8.8 g sodium sulfate, 3.24 g calcium chloride, 1.8 g potassium chloride, 0.55 g sodium bicarbonate, 0.16 g potassium bromide, 0.08 g strontium chloride, 34.0 mg boric acid, 22.0 mg sodium silicate, 4.0 mg sodium fluoride, 2.4 mg ammonium nitrate, 1.6 mg disodium phosphate, 8.0 mg agar,15.0 g] and half nutrient agar (½ NA) [beef extract, 1.5 g peptone, 2.5 g agar, 15.0 g] (Difco Laboratories, Detroit, MI) for bacterial culturing.

Surface sterilization and isolation of endophytic bacteria

Roots and leaves tissues were also used for isolation of bacteria. To isolate endophytic bacteria from plant, roots and leaves samples were washed several times with tap water and was further sterilized by washing with disinfectants as described previously (Bibi et al., 2012).

To check sterilization, washed roots and leaf segments were placed on ½R2A agar to check the growth of bacteria, from these plant parts after incubation at 28°C for 5 days. After confirming sterilization of root and leaf segments, small pieces of sterilized root and leaf segments were ground in FAS using sterile mortar and pestle. Aliquots were further serially diluted (10^{-3}, 10^{-4} and 10^{-5}) and plated in duplicate on different media mentioned above.

To inhibit fungal contamination, 50 µg/ml cycloheximide was mixed to the medium before pouring. The plates were incubated at 25°C for 2 weeks for bacterial growth. Individual colonies were streak to check purity of the strains and all bacterial isolates were further subculture and stored in, 15% (v/v) glycerol stock of strains at -70°C.

Screening for antifungal activity

Bacteria isolated from soil, roots and leaves of the mangroves were used to check their antifungal potential. Five different test fungal pathogens *Phytophthora capsici, Pythium ultimum* and *Magnaporthe grisea* were obtained in our laboratory while *Altenaria malli* (KCTC

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6972), and *Fusarium oxysporum* (KCTC 6149) were obtained from Korean type culture collection centre (KCTC).

Antagonistic activity against fungal pathogens was determined by using cross streak method. All isolates were striated on PDA media supplemented with ½ R2A in sea water. Each 6 mm mycelial disc of 4-day-old test fungal pathogens was placed in center of plate perpendicular to streak of isolates at 4 cm distance from edges of plate and incubated at 28°C for 4 to 6 days. All strains were checked twice for antagonistic activity. The antagonistic activity was then evaluated by measuring the inhibition zone of fungal mycelia around bacterial colony.

Screening of bacteria for antibacterial activity

Using overlay assay, all antagonistic bacteria have been checked for their antibacterial activity. Firstly, bacterial isolates were grown on ½ R2A in sea water at 28°C for 48 h. Pathogenic bacteria were grown in culture media for 24 h, mixed with 0.1% soft agar and overlaid on strains to be screened.

All test pathogenic bacterial strains were diluted to final concentration A_{600}= 0.1. After applying overlay of soft agar, plates were incubated at 28°C for 36 h and the zone of inhibition was determined. The test strains of bacteria (*Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Pseudomonas aeurigenosa* ATCC 27853 and MRSA ATCC 43300) were grown in LB broth at 37°C.

Evaluation of hydrolytic enzyme activity

Amylase production was checked on starch media. Amylase producing bacteria showed starch hydrolysis as clear zone on starch ½ R2A agar plates (Kumar et al., 2012).

To check cellulase activity, CMC agar (carboxy methyl cellulose agar) media was used. Bacteria were streaked on plates and incubated at 28°C for 2 days. After, these plates were flooded with solution 0.1% Congo red and put on orbital shaker for 15min and washed with 1MNaCl (Hendricks et al., 1995). Positive activity was seen as halo zone around bacterial colonies on CMC agar. Protease activity was checked using skim milk ½ R2A agar plates. Bacteria producing protease made clear zone on skim milk agar plates.

For lipolytic activity, tributyrin ½ R2A agar media was used. After 48 h of incubation at 28°C clear zone was detected around bacteria after hydrolysis of tributyrin.

Bacterial DNA extraction and 16S rRNA gene analysis

Bacteria isolated from two mangrove plants were used for genomic DNA extraction using DNA extraction kit (Thermo Scientific, Waltham, USA). To identify bacterial strain 16S rRNA gene, full gene sequencing was performed. Using bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), the 16S rRNA gene fragment was amplified. Amplifications were performed under the following conditions: one cycle of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, an annealing of 58°C for 50s and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min.

Using PCR purification kit (Thermo Scientific, Waltham, USA) PCR products were purified and were sequenced commercially by Macrogen (Seoul, Korea). Sequences obtained after 16S rRNA gene similarity were blast using EzTaxon server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012), for identification of bacteria. To determine the phylogenetic placement of antagonistic bacteria and related type strains, the 16S rRNA gene sequences of related type strains sequences were obtained from National Centre for Biotechnology Information (NCBI).

Phylogenetic analysis

For phylogenetic analysis using CLUSTALX (Thompson et al., 1997), multiple alignments of the sequences were performed and BioEdit software (Hall, 1999) was used for editing of gaps.

The neighbour-joining method in a MEGA6 Program with bootstrap values based on 1000 replications was used for construction of phylogenetic tree based on 16S rRNA gene sequences (Tamura et al., 2013).

Nucleotide sequence numbers

All nucleotide sequences of antagonistic strains have been deposited in the GenBank database under accession numbers KY234238- KY234262.

RESULTS

Isolation of rhizo and endophytic enzyme producing bacteria

A total of 317 rhizo and endophytic antagonistic bacteria were isolated from two mangrove plants *C. conglomerates* and *H. perfoliata*. All bacterial isolates were isolated from soil, roots and leaves tissues of plants. All bacterial strains isolated from different parts of the plants mentioned above were cultured on four different media ½ TSA and ½ R2A, MA and NA.

Bacterial number was high on ½ TSA and MA as compared to ½ R2A and ½ NA. But more antagonistic bacteria were recovered from ½ R2A culture media. As different parts of plants were used, more number of antagonistic rhizobacteria was isolated from *C. conglomerates* while endophytes were more in number from *H. perfoliata* (Table 1).

Screening for antimicrobial potential

Further these bacteria were used to check their antifungal potential against two different pathogenic fungi, *Pythium ultimum* and *Phytophthora capsici*. From these total 317 bacteria, when screened against these two fungi, 25 (7.8%) isolates were active against *Py. ultimum* and 21 (6.6%) were active against *P. capsici*.

These rhizo and endophytic antagonistic bacteria were checked for their antifungal potential against three different fungi that is *Magnaporthe grisea*, *A. malli,* and *Fusarium oxysporum.* Fourteen isolates (4.4%) showed inhibition against *M. grisea.* Only six bacterial isolates (1.8%) were active against *A. malli* while 8 showed

Table 1. Antimicrobial activity of bacteria isolated from two mangroves plants against different pathogenic fungi and bacteria.

Lab no.	Accession no.	Type strain[a]	% identity[b]	Antifungal activity against[c]					Antibacterial activity[d]				
				P. ultimum	*P. capsici*	*M. grisea*	*A. mali*	*F. oxysporum*	*E. coli*	*E. faecalis*	*E. faceium*	*P. aeruginosa*	*S. aureus*
				Haplopeplis perfoliata									
				Soil									
EA216	KY234238	*Bacillus sonorensis* NBRC 101234 T	99.4	+++	+++	+++	+++	+++	+++	++++	++++	-	-
EA217	KY234239	*Halomonas smyrnensis* AAD6(T)	97.5	++	++	-	-	++	-	-	-	-	-
				Roots									
EA218	KY234240	*Bacillus amyloliquefaciens subsp. plantarum* FZB42 T	99.7	+++	+++	+++	+++	+++	+	-	-	+	-
EA219	KY234241	*Labrenzia aggregata* IAM 12614 T	99	++	++	-	-	-	-	-	-	-	-
EA220	KY234242	*Labrenzia alexandrii* DFL-11 T	98.1	+	+	+	-	-	-	+	-	-	+
EA221	KY234243	*Mycobacterium bacteremicum* ATCC 25791 T	99.7	+	+	+	-	-	-	-	-	-	-
EA222	KY234244	*Celeribacter halophilus* ZXM137 T	98	+	+	-	-	-	-	-	-	-	-
EA223	KY234245	*Chromohalobacter israelensis* ATCC 43985 T	98	++	+++	-	-	-	+	-	-	-	-
				Leaves									
EA225	KY234246	*Bacillus subterraneus* DSM 13966 T	99.4	+	++	-	-	-	-	-	-	-	-
EA226	KY234247	*Roseovarius indicus* B108 T	98	+	++	-	-	-	-	-	-	-	-
				Cyperus conglomeratus									
				Soil									
EA200	KY234248	*Halomonas anticariensis* FP35(T)	97.8	+	-	-	-	-	-	-	-	-	-
EA201	KY234249	*Pseudoalteromonas flavipulchra* NCIMB 2033(T)	99.9	W	+	W	-	-	-	-	-	-	-
EA202	KY234250	*Microbulbifer halophilus* YIM91118(T)	97.2	++	+++	-	-	-	-	-	-	-	-
EA203	KY234251	*Salinicola halophilus* CG 4.1(T)	97.8	+	W	-	-	-	-	-	-	-	-
EA204	KY234252	*Pseudoalteromonas ruthenica* KMM 300(T)	99.1	+	W	W	-	-	-	-	-	-	-
EA205	KY234253	*Alteromonas australica* H 17(T)	99.5	++	++++	W	++	-		+	-	-	-
EA206	KY234254	*Halomonas stenophila* N12(T)	98.7	+	++	-	-	-	-	-	-	-	-
EA207	KY234255	*Marinobacter mobilis* CN46(T)	97.9	+	+	-	-	+	-	-	-	-	-
EA208	KY234256	*Bacillus licheniformis* ATCC 14580(T)	99.1	+++	+++	+++	++	+	-	+	-	+	+
EA209	KY234257	*Pseudomonas pachastrellae* KMM 330(T)	99.7	++	++++	+++	+	+	-	-	-	-	-
EA210	KY234258	*Sinomicrobium pectinilyticum* 5DNS001(T)	94.8	+++	++++	++++	+	+	-	-	-	-	-
EA211	KY234259	*Marinobacter mobilis* CN46(T)	97.1	+	+	++++	-	-	-	-	-	-	-

Table 1. Contd.

				Roots									
EA213	KY234260	*Microbulbifer halophilus* YIM91118(T)	99	+	-	+	-	-	-	-	-	-	-
EA214	KY234261	*Microbulbifer variabilis* Ni-2088(T)	92.8	+	+	+	-	W	-	-	-	-	-
EA215	KY234262	*Salinimonas lutimaris* DPSR-4(T)	98.7	+	-	W	-	-	-	-	-	-	-

[a]Identification of strain based on 16S rRNA gene sequence analyses. [b]Percentage similarity of strain with closely related type strain. [c]Antifungal activity of bacteria against different pathogenic fungi. The activity was measured after 4-5 days after incubating at 28°C by measuring the clear zone of mycelial growth inhibition: w, weak activity; +, 3 mm; ++, between 4 to 6mm; +++, between 7 to 9mm, ++++, between 10 to 11 mm. [d]Antibacterial activity against different human pathogenic bacteria was determined by soft agar assay. The activity was measured after 2-4 days incubation at 28°C by measuring the clear zone: w, weak activity less than 3mm, +, 3 mm; ++, between 5 to 7 mm; +++, between 8 to 9 mm, ++++, between 10 to 11 mm.

inhibition to *F. oxysporum* (2.5%).

Inhibition pattern against different fungi was different where strongest inhibition was seen against oomycetes (Figure 1). Rhizobacteria EA208 from *C. conglomerates* showed strong activity with 7 to 9 mm mycelial inhibition against both pathogenic fungi tested. This strain has similarity to *Bacillus licheniformis* ATCC 14580(T). Another rhizobacteria EA216 from *Halopeplis perfoliata* showed strong inhibition against all fungi tested.

An endophytic isolate EA218 from same host plant has similar strong inhibition against pathogenic fungi. This strain belongs to class Firmicutes and has closest 16S rRNA similarity to *Bacillus amyloliquefaciens subsp. plantarum* FZB42 (T) (Table 1). Some bacteria have strong inhibition only against oomycetes pathogens which have weak or no inhibition against other fungi tested. Strain EA202, EA205, EA210 and EA223 showed strong inhibition for oomycetes which are weak or inactive against other fungi.

Antagonistic bacteria were further checked for their antibacterial activity using agar overlay method. Only few bacteria showed inhibition against human pathogenic bacteria. From them, 4 isolates were active against *Enterococcus faecalis,* 3 for *E. coli* following 2 against *P. aeruginosa* and MRSA and only 1 was active against *Ent. faecium.*

The antagonistic isolate EA208 and EA216 showed inhibition with three different test pathogenic bacteria. Where EA216 had strong inhibition against *E. coli*, *Ent. faecium* and *Ent. faecalis.* Both these isolates belong to genus *Bacillus* (Table 1).

Enzymatic activities of antagonistic bacteria

Of 25 antagonistic bacteria tested, only few were positive for enzymatic activities tested (Table 2). Protease activity was higher than other hydrolytic enzymatic activities. More number of bacteria were able to excrete protease enzyme (n=10; 40%). Strong activity was detected in strain EA202, EA205, EA206, EA218 and EA226. Eight bacteria were able to show amylase activity (32%).

Most of the rhizobacteria exhibit amylase activity. Strain EA200, EA202, EA215 and EA216 showed strong amylase activity (Figure 2). Lipase production was observed in 7 (28%) isolates where strong lipase production was seen in EA208, EA209 and EA216. Both strains belong to genus *Bacillus* but only 2 strains EA208 and EA213 were able to exhibit cellulase activity in an *in vitro* assay. Both of them had moderate activity. Most of these antagonistic bacteria produce different hydrolytic enzymes belonging to *γ-Proteobacteria.*

Phylogenetic analysis of endophytic bacteria on the basis of 16S rRNA gene sequence

All antagonitsic bacteria were then identified by partial 16S rRNA gene sequence analysis. Seventeen different genera were encountered which in turn belong to four major classes: *γ-Proteobacteria* (n=15; 60%), *α-Proteobacteria* (n=4; 16%), *Firmicutes* (n=4; 16%), *Bacteroidetes* (n=1; 4%) and *Actinobacteria* (n=1; 4%) (Figure 3).

Phylogenetic analysis was performed and tree was generated from the distance data using, the neighbor-joining method with the Jukes and Cantor model in a MEGA6 Program (Figure 4).

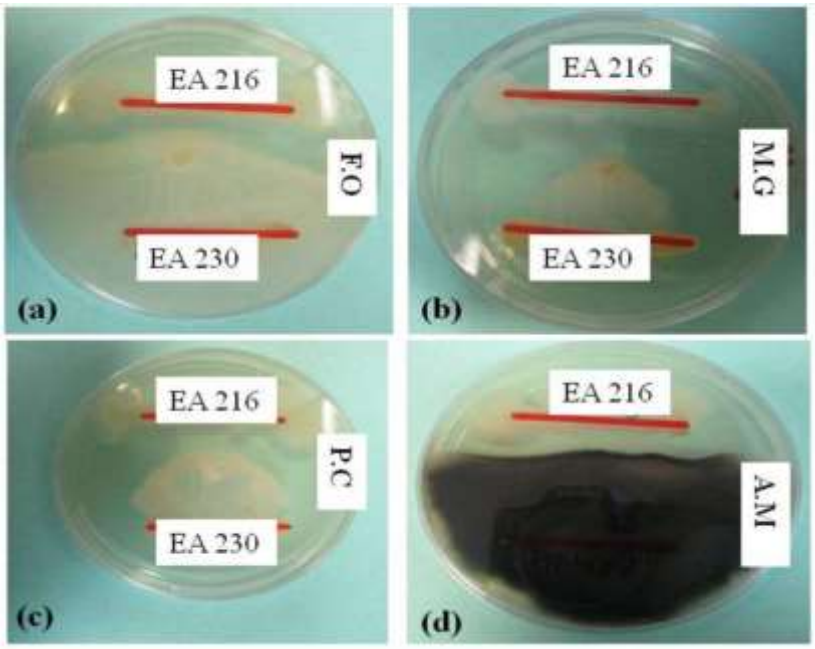

Figure 1. Antifungal activity of bacteria isolated from mangroves against pathogenic fungi. (a) *Fusarium oxysporum* (F.O), (b) *Magnaporthe grisea* (M.G), (c) *Phytophthora capsici* (P.C), and (d) *Altenaria malli* (A.M).

Table 2. Enzyme production of different bacteria isolated from mangrove plants.

			Enzymatic activities [a]			
Lab No	**Accession No**	**Type strain**	**Amylase**	**Cellulase**	**Lipase**	**Protease**
		Haplopeplis perfoliata				
		Soil				
EA216	KY234238	*Bacillus sonorensis* NBRC 101234 [T]	+++	+++	+++	++
EA217	KY234239	*Halomonas smyrnensis* AAD6(T)	-	-	++	-
		Roots				
EA218	KY234240	*Bacillus amyloliquefaciens subsp. plantarum* FZB42 [T]	++++	-	-	++++
EA219	KY234241	*Labrenzia aggregata* IAM 12614 [T]	-	+	-	-
EA220	KY234242	*Labrenzia alexandrii* DFL-11 [T]	-	+	-	-
EA221	KY234243	*Mycobacterium bacteremicum* ATCC 25791 [T]	-	-	++	++++
EA222	KY234244	*Celeribacter halophilus* ZXM137 [T]	-	-	-	-
EA223	KY234245	*Chromohalobacter israelensis* ATCC 43985 [T]	-	-	-	-
		Leaves				
EA225	KY234246	*Bacillus subterraneus* DSM 13966 [T]	-	-	++	++
EA226	KY234247	*Roseovarius indicus* B108 [T]	++	-	-	-
		Cyperus conglomeratus				
		Soil				
EA200	KY234248	*Halomonas anticariensis* FP35(T)	+++	-	-	-
EA201	KY234249	*Pseudoalteromonas flavipulchra* NCIMB 2033(T)	-	-	-	-
EA202	KY234250	*Microbulbifer halophilus* YIM91118(T)	+++	-	+++	++++
EA203	KY234251	*Salinicola halophilus* CG 4.1(T)	-	-	-	-
EA204	KY234252	*Pseudoalteromonas ruthenica* KMM 300(T)	-	-	-	-

Table 2. Contd.

EA205	KY234253	*Alteromonas australica* H 17(T)	++	-	-	+++
EA206	KY234254	*Halomonas stenophila* N12(T)	++	-	-	++++
EA207	KY234255	*Marinobacter mobilis* CN46(T)	-	-	-	-
EA208	KY234256	*Bacillus licheniformis* ATCC 14580(T)	-	++	++++	+++
EA209	KY234257	*Pseudomonas pachastrellae* KMM 330(T)	-	-	++++	-
EA210	KY234258	*Sinomicrobium pectinilyticum* 5DNS001(T)	-	-	-	-
EA211	KY234259	*Marinobacter mobilis* CN46(T)	-	-	-	-
		Roots				
EA213	KY234260	*Microbulbifer halophilus* YIM91118(T)	-	-	-	++
EA214	KY234261	*Microbulbifer variabilis* Ni-2088(T)	-	-	-	-
EA215	KY234262	*Salinimonas lutimaris* DPSR-4(T)	-	-	-	-

[a]Enzymatic activity of bacteria isolated in this study on different enzymatic media. Production of amylase, cellulase, lipase and protease was determined by plate assay. The activity was measured after 2-4 days incubation at 28°C by measuring the clear zone: +, 3 mm; ++, between 5 to 7 mm; +++, between 8 to 9 mm, ++++, between 10 to 11 mm.

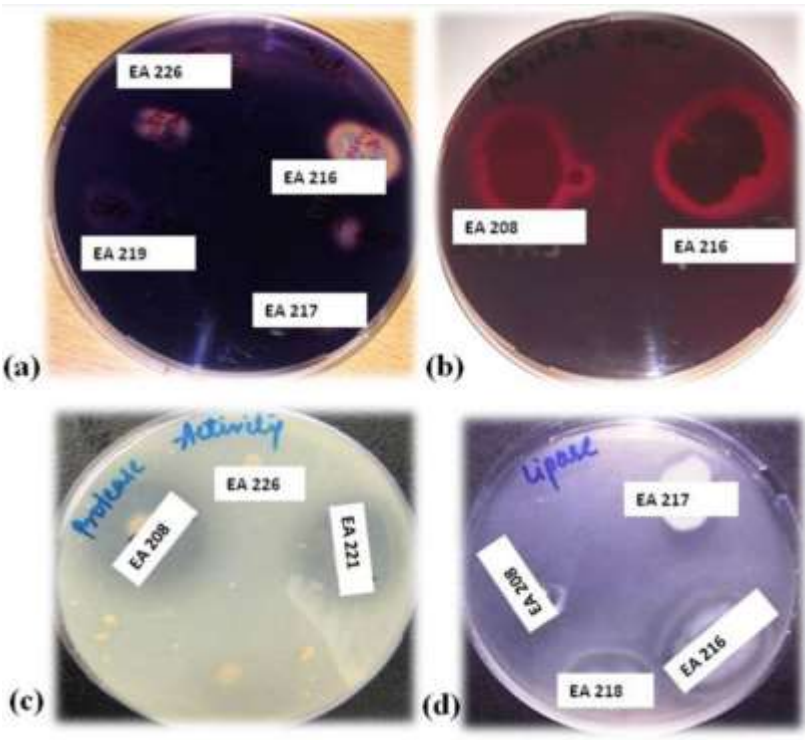

Figure 2. Screening of antagonistic bacteria for enzymatic activities. (a) Amylase activity (b) Cellulase activity (c) Protease activity and (d) Lipase activity.

High bootstrap values have been seen which resulted in significant branching points of phylogenetic tree. Phylogenetic analysis on the basis of 16S rRNA gene sequences has revealed five main clusters. First include species of class *γ-Proteobacteria,* second include species related to class *α-Proteobacteria*, third include species from class *Firmicutes* and fourth and fifth cluster comprises of only one spp. which belongs to both class *Bacteroidetes* and *Actinobacteria,* respectively.

Bacterial isolates showed 16S rRNA gene sequence

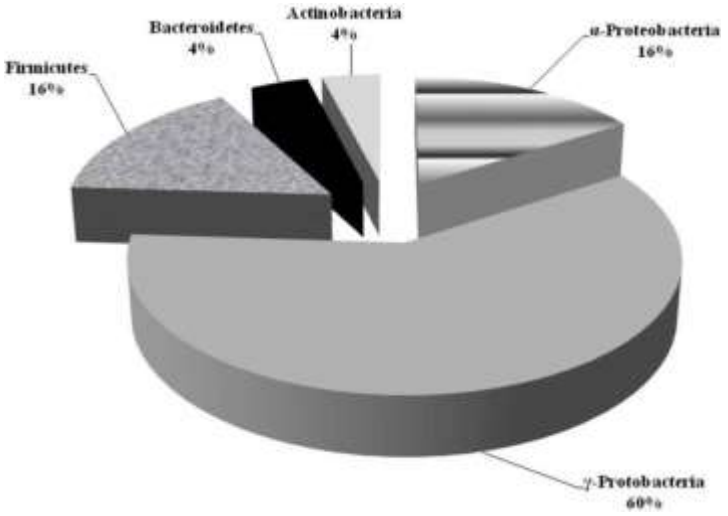

Figure 3. Percentage composition of different phyla of antagonistic rhizo and endophytic bacteria isolated from mangroves on the basis of 16S rRNA gene sequence similarity.

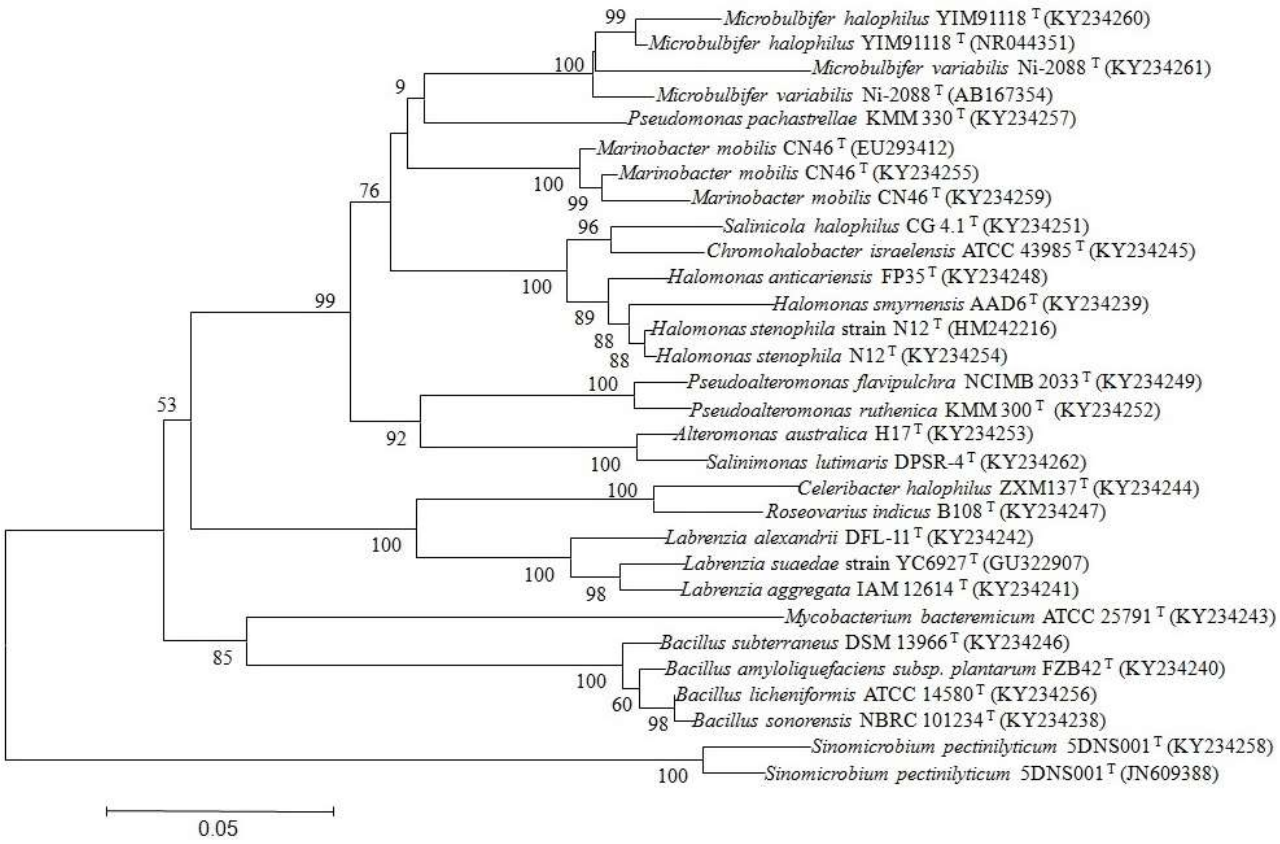

Figure 4. Phylogenetic placement of antagonistic bacteria isolated from mangroves on the basis of 16S rRNA gene sequences and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene by using the neighbor-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar, 0.01 accumulated changes per nucleotide.

similarity of 92.8 to 99.9% with their closest type strain. High bootstrap values have been observed for all species making four different clusters. *γ-Proteobacteria* comprised of 10 different genera, clearly identified as separate cluster with bootstrap values of 89 to 100% (Figure 4). Representative of class *α-Proteobacteria* comprised of four different genera with high bootstrap values (98 to 100%). *Bacteroidetes* comprise only one strain EA210 *Sinomicrobium pectinilyticum* 5DNS001 (T) with low 16S rRNA gene sequence similarity (94%), which were also recovered. *Actinobacteria* consist of one strain (EA221) which is an endophyte, *Celeribacter halophilus* ZXM137 (T).

DISCUSSION

Mangrove plants are an excellent source of chemical compounds of medicinal and agricultural use (Miles et al., 1998). However, studies related to isolation and screening of antagonistic rhizo and endophytic bacteria from mangrove plants, from Saudi Arabia is not done before. To our knowledge, our study is the first report regarding isolation, screening and identification of rhizo and endophytic bacteria from *H. perfoliata* and *C. conglomeratus* in Saudi Arabia.

In microbial antagonism, different types of metabolites are secreted by microbes especially growth inhibitor due to competition for nutrients and living space (Whipps et al., 2001; Riley and Wertz, 2002). Endophytic bacteria produce such metabolites in defence of host plants against different plant pathogens (Strobel and Daisy, 2003). We have used different combinations of culturing media for isolation of bacteria. Great diversity have been seen in bacteria as well as their biological activities isolated on different culturing media. This variation in growth of bacteria on different media is also seen in previous studies, where specific group of bacteria recover on specific media and conditions (Vieira and Nahas, 2005; Chang et al., 2015).

317 rhizo and endophytic bacteria have been isolated and screen for their antifungal potential against five different pathogenic fungi mentioned above. Several previous studies also reported isolation of antagonistic bacteria from mangroves. Mainly endophytic bacteria, which produce secondary metabolites were isolated (Hu and Wu, 2010; Hu et al., 2010; Ding et al., 2012; Eldeen et al., 2015). These active bacterial isolates were further checked for their antibacterial activity where only few bacteria were active against human pathogenic bacteria. Mostly, strains of *Bacillus* in our study showed antibacterial activity which is similar to finding reported previously (Hu et al., 2010; Eldeen et al., 2015), where strain of *Bacillus* isolated from mangrove plant exhibit antibacterial activity against different pathogenic bacteria.

Microorganisms from mangrove plants are capable of producing various groups of enzymes of industrial and biotechnological significance (Thatoi et al., 2013; Saravanakumar et al., 2016). Antagonistic bacteria from two mangroves were checked for ability to prodcue hydrolytic enzymes.

In a recent study from Thailand, endophytic bacteria isolated from mangrove plants were screened for hydrolytic enzymes production. Many bacteria were positive for production of proteases, lipases, amylases or cellulases where one strain of *Bacillus safensis* was able to produce all enzymes (Khianngam et al., 2013). In another study, enzymatic potential of the bacteria from mangroves have been evaluated, where four strains of *Bacillus* sp. exhibited strong enzyme production (Tabao and Moasalud, 2010).

In this study, *Bacillus* sp. showed strong enzymatic activities. From Saudi Arabia, mangroves were used for isolation of bacteria producing a polymer Polyhydroxybutyrate (PHB) (Alarfaj et al., 2015). A strain of *B. thuringiensis* from this study showed maximum production of PHB. In another study, 12 different fungi have been isolated from mangroves growing on Red Sea Coast of Saudi Arabia. These fungal isolates were able to produce different enzymes and help in biodegradation of diesel fuel (Ameen et al., 2016). This study is the first report on isolation, screening on the basis of antimicrobial activity and enzyme characterization of bacteria from *C. conglomerates and H. perfoliata* in Saudi Arabia.

In this study, *Bacillus* was the dominant genus that is similar to many previous studies where different strains of *Bacillus* were isolated from mangrove plants during screening for their antifungal potential (Liu et al., 2010; Ando et al., 2001). Two endophytic strains of *Bacillus*, *B. thuringiensis* and *Bacillus pumilus* were isolated from mangrove plants from India (Ravikumar et al., 2010). These two endophytic strains of *Bacillus* were able to inhibit many bacterial and fungal pathogens. Feng et al. (2009) have evaluated bacterial communities from mangroves for their antagonism. One endophytic strain *Bacillus amyloliquefaciens* was able to control Phytophthora blight caused by *Phytophthora capsici* in *capsicum* in an *in vitro* assay. In our work, we have many strains from *α-Proteobacteria* and *Firmicutes,* showing strong antagonistic activity against *Py.ultimum* and *P.capsici* suggest their application as biocontrol agent in future.

Twenty-five antagonistic rhizo and endophytic bacteria from mangroves exhibit both antifungal and antibacterial activities. Furthermore, bacteria exhibited different types of enzymatic activities of industrial importance. It is concluded from the study that rhizo and endophytic bacteria isolated from soil, roots and leaves of mangrove plants produce enzymes, antibacterial and antifungal metabolites, pointing their significant role in host plant.

Further study is in progress to identify these active metabolites responsible for antimicrobial activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Characterisation and evaluation of thiol-releasing and lower volatile acidity forming intra-genus and inter-genus hybrid yeast strains for Sauvignon Blanc wine

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Wine yeast expressed proteins are influential during the production of varietal aromatic Sauvignon Blanc wines as they release or mediate aroma compounds and undesirable volatile acidity (VA). As *Torulaspora delbrueckii* in conjunction with *Saccharomyces cerevisiae* as well as a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid were previously shown to produce white wine with enhanced aroma and/or lower VA, intra- and novel inter-genus hybrids were trialled for the production of aromatic Sauvignon Blanc with lower VA. The inter-genus hybrid NH 07/1 produced wine with a more positive association with the aroma compound 3-mercaptohexylacetate (3MHA) than two commercial thiol-releasing wine yeast (TRWY) strains, Zymaflore X5 and Zymaflore VL3. The wine also had a negative association with VA, and a positive association with floral and tropical fruit aromas. Three intra-genus hybrids, NH 56, NH 57 and NH 88, produced wines with a negative association with VA, and a positive association with tropical fruit aroma. These wines also had a stronger association with the aroma compound, 3-mercaptohexanol (3MH) than wines produced with all commercial TRWY. The hybrid NH 07/1 and Zymaflore VL3 also over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol, 4-mercapto-4-methyl-pentan-2-one (4MMP) by cleaving its carbon-sulphur bonds. Therefore, lactoylglutathione lyase is a potential biomarker for 3MH-release, as this thiol also contains a carbon-sulphur-bond. Dehydrogenase proteins might also be useful biomarkers for VA formation by fermenting wine yeasts. Three intra- and one inter-genus hybrids with the abiliy to produce aromatic Sauvignon Blanc wines with lower VA compared to commercial TRWY references were identified.

Key words: Acetic acid, isobaric tags for relative and absolute quantitation (iTRAQ), metabolomic, Orbitrap liquid chromatography tandem mass spectrometry (LC-MS/MS), proteomic, solid phase extraction-gas chromatography coupled to tandem mass spectrometry (SPE GC-MS/MS), volatile thiols.

INTRODUCTION

Sauvignon Blanc wines are associated world-wide with either vegetative (herbaceous) or tropical fruit and/or floral aromas (Marais, 1994; Von Mollendorf, 2013; Hart et al., 2016). Key to the production of high quality

Sauvignon Blanc wines with the desired properties are wine yeasts, namely *Saccharomyces cerevisiae* that can convert relatively "neutral" grape must lacking varietal aromas into varietal-typical aromatic wines through their metabolic activity (Swiegers et al., 2006a; 2007a). Sauvignon Blanc wine aroma and flavour are the result of grape derived compounds (metabolites), *e.g.* methoxipyrazines, *de novo* synthesised metabolites or compounds released from aroma-inactive, non-volatile grape-derived precursors by wine yeast during fermentation (Bovo et al., 2015; Pinu et al., 2015). However, yeast also produces undesirable metabolites, for example, acetic acid the main contributor to volatile acidity (VA). These compounds are responsible for vinegar-like off-flavours that are detrimental to overall wine organoleptic quality (Du Toit and Pretorius, 2000; Swiegers et al., 2005). Such wines will have negative financial implications as expensive reverse osmosis techniques have to be used to remove the excessive VA. Commercial yeast strains implicated in the production of wines with higher VA values will create negative perceptions for the yeast manufacturer and result in loss of revenue due to lower yeast sales (Margaret Fundira, Personal communication, 2016).

Wine yeast expressed enzymes (proteins) during winemaking were previously reported to be key effectors of wine aroma and flavour compounds present in wines (Holt et al., 2011; Roncoroni et al., 2011). Furthermore, Holt et al. (2012) and Pretorius (2016) reported that yeast expressed proteins with carbon-sulphur *β*-lyase activity are involved in the release of the aroma enhancing volatile thiol, that is, 4-mercapto-4-methyl-pentan-2-one (4MMP). Dehydrogenase enzymes were also reported to be involved in the production of acetic acid, the main contributor to total fatty acids (Varela et al., 2012; Walkey et al., 2012). Additionally, it was reported that over-expression of dehydrogenase enzymes by wine yeast during fermentation of Sauvignon Blanc grape must resulted in wines with elevated total fatty acids (Hart et al., 2016, 2017).

The use of the yeast *Torulaspora delbrueckii* was shown to produce wines with lower VA levels, and enhancing varietal aromas when inoculated singly or sequentially with *S. cerevisiae* (Albertin et al., 2014; Renault et al., 2016). *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids also produced wine with enhanced aroma and flavour upon completion of fermentation (Santos et al., 2008). Therefore, *T. delbrueckii* can be advantageous for the development of new hybrid strains with the ability to produce aromatic white wines with lower VA. For that reason, the aims of this study were to breed *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids using classical mating which is naturally occuring phenomenon, characterise and evaluate these inter-genus hybrids for their fermentation potential, thiol-releasing abilities and low VA formation during the production of Sauvignon Blanc wines. Promising *S. cerevisiae* intra-genus hybrids previously identified by Hart et al. (2016) for their ability to produce wines with enhanced tropical fruit aroma (henceforth referred to as TFPH) and lower VA (henceforth referred to as LVPH) compared to commercial 'thiol-releasing' wine yeasts (TRWY) were included in this study. Additionally, wine yeast regulated proteins and aroma compounds, especially volatile thiols *viz*. 3-mercaptohexanol (3MH) and 3-mercaptohexylacetate (3MHA) as well as volatile acidity *viz*. acetic acid present at the end of fermentation and their association with final wine aroma and flavour were investigated. It is envisioned that potential protein biomarkers associated with aroma-enhancing metabolites and VA will be identified.

MATERIALS AND METHODS

Origin of yeast strains

Reference yeast strains: The following commercial *S. cerevisiae* hybrid strains, namely NT 112 and NT 116 (Anchor Yeast, South Africa) served as references for the laboratory-scale fermentations, whilst the commercial thiol-releasing wine yeast (TRWY) strains, VIN 7 and VIN 13 (Anchor Yeast, South Africa), Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands), were included as references for the small-scale fermentations. All TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Anonymous, Personal communication, 2005a, b, 2017a, b, c). Another commercial strain, N 96 (Anchor Yeast, South Africa) and an experimental strain, P 35 (ARC Infruitec-Nietvoorbij, South Africa) used in hybrid breeding programmes, were also included in this study as references. The latter strains have the ability to produce wine with tropical fruit aromas (henceforth abbreviated as TFPP).

Intra-genus hybrids: Ten *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145, previously characterised as TFPH and LVPH were included in this study (Hart et al., 2016).

Inter-genus hybrids: Two inter-genus hybrids, NH 07/1 and NH 07/2, were generated through classical mating by fusing protoplasts originating from a *S. cerevisiae* strain MCB C6, isolated from Madeba cellar winery equipment, Robertson, South Africa and *T. delbrueckii* strain M2/1 (Van Breda et al., 2013), resulting in inter-genus hybrids. Briefly, freeze cultures containing diploid *S. cerevisiae* strain MCB C6 and the haploid *T. delbrueckii* (Sasaki and Ohshima, 1987; Kurtzman et al., 2011) strain M2/1 were

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thawed and streaked onto yeast extract peptone dextrose (YPD) agar (Biolab, Merck, South Africa). Agar plates were incubated at 28°C for at least 48 h until single yeast colonies were visible. A single colony from the diploid (2n) *S. cerevisiae* yeast strain was aseptically transferred onto plates containing nitrogen-limiting growth media (0.25% [w/v] yeast extract, 0.1% [w/v] dextrose, 1% [v/v] potassium acetate, and 1% [w/v] agar) and incubated for 72 h at 28°C until asci, each containing four haploid (n) spores could be observed. Thereafter, a single colony from the sporulated *S. cerevisiae* MCB C6 culture and the *T. delbrueckii* M2/1 culture were transferred into separate tubes containing 10% (w/v) *β*-d-glucuronidase enzyme, and mixed until the suspension appeared milky. Protoplasts were generated by incubation of suspensions at 30°C for 30 min. Thereafter, sterile water was added to each micro-centrifuge tube to rinse cell residue from protoplasts. Respective supernatants were gently removed and transferred into new tubes. Thereafter, 100 µL of each protoplast-containing supernatant were streaked onto different sections of a YPDA-plate, and placed on a Singer MSM system series 200 micro-manipulator (Singer Instruments, Watched, Somerset, UK) as described (Morin et al., 2009). Protoplasts were physically disrupted using a micro-fine needle; where after haploid spores from the two parental strains were placed in close proximity on the YPDA. Thereafter, the plates were incubated at 28°C for at least 48 h to allow haploids to fuse (karyogamy) to form diploid (2n) inter-genus hybrids.

Characterisation techniques

Contour clamped homogeneous electric field (CHEF) DNA karyotyping

The CHEF DNA karyotyping was conducted according to the embedded agarose procedure used for commercial TRWY and intra-genus hybrids described by Hart et al. (2016). A Bio-Rad image analyser (Bio-Rad, Madrid, Spain) was used to visualise chromosomal banding patterns on 0.01% (v/v) ethidium bromide-stained agarose gels.

Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Yeast strains were characterised by MALDI biotyping using a Bruker UltrafleXtreme MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) used for commercial TRWY and intra-genus hybrids described by Hart et al. (2016).

Evaluation techniques

Laboratory-scale fermentation trials

Fermentation potential of wet culture inter-genus hybrids was evaluated in laboratory-scale vinifications of Chardonnay clarified grape must (juice) (total sugar 21.3°B; total acidity (TA) 8.1 g/L; pH 3.10), similar to vinifications with TRWY and intra-genus hybrids as described by Hart et al. (2016). Commercial yeast strains, NT 112 and NT 116 (Anchor Yeast, South Africa) were included in the trials as references. All fermentations were conducted in triplicate in a completely randomised order (Addelman, 1970) at 15°C, whilst gently shaking on an orbital shaker. Fermentations were monitored by CO_2 weight loss. Subsequently, both inter-genus hybrids were trialled in small-scale winemaking after it was established that they fermented the grape must (juice) to dryness (residual sugar <5 g/L) using a portable DMA 35 density meter (Anton Paar, Southern Africa).

Small-scale winemaking trials

Small-scale Sauvignon Blanc wines were made in triplicate using commercial TRWY, intra- and inter-genus hybrids according to a standardised cellar method as described by Hart and Jolly (2008). For each treatment replicate, nine litres Sauvignon Blanc grape must (total sugar 21.9°B; TA 9.3 g/L; pH 3.28) were dispensed into 10 L stainless steel canisters with fermentation caps, and inoculated with the respective wine yeast starter cultures. The method was adjusted by having the respective yeast inoculums cultured for 24 h in 600 mL YPD broth (Biolab, Merck, South Africa) medium. Subsequently, 180 mL of the 24 h cultures (optical density at 600 nm = 0.92 ± 0.05; cfu/mL = 10^7 ± 10^6; viability = 97.93% ± 1.67) were used to inoculate clarified Sauvignon Blanc grape must (2% inoculum). Fermenting must was sampled every 48 h to measure residual glucose/fructose (R/S), ethanol, VA, TA and pH, using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) until fermentations went to dryness. This was repeated until the R/S concentrations were below 5 g/L, where after the free-SO_2 of the wines was adjusted to 35 mg/L, following racking. Wines were cold stabilised at 0°C for at least two weeks prior to bottling.

Gas chromatography (GC) analysis

Wine aroma metabolites, namely esters, total fatty acids and higher alcohols (fusel oils), were analysed by gas chromatography (GC) on wine samples (50 mL) taken on day 15 of fermentation as described by Hart et al. (2016, 2017).

Solid-phase extraction (SPE) and GC-MS/MS analysis

The main wine volatile thiols, 3MH and 3MHA, were pre-concentrated by deploying solid-phase extraction (SPE) as described by Hart et al. (2016, 2017). Subsequently, GC coupled to tandem mass spectrometry (GC-MS/MS) as described by Mattivi et al. (2012) was used to quantify volatile aromatic thiols. The GC-MS/MS system used in this study comprised of a GC Trace 1300/TSQ8000 mass selective detector equipped with an AI 1310 auto sampler (Thermo Scientific™ Inc, USA). Aroma compounds were separated using a 30 m × 0.25 mm × 0.25 µm Zebron WAX plus column (Phenomenex Inc., Torrance, CA, USA).

Sensory evaluation

An experienced panel consisting of 14 members conducted descriptive sensory evaluation of bottled wines. The panel was requested to indicate the intensity of aroma descriptors on a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]). Panel members also had to specify the most prominent aromas associated with Sauvignon Blanc wines *viz.* 'tropical fruit' (*e.g.* banana, guava, peach, passion fruit and citrus); 'vegetative' (*e.g.* asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry); or 'floral' (*e.g.* rose, orange blossom), they perceived.

Quantitative LC-based iTRAQ proteomic analysis

Based on chemical (lower VA and total fatty acids) and sensory (tropical fruit aroma) analyses of final wines, yeast-containing ferments sampled (50 mL) in triplicate on day 15 of fermentation were selected for quantitative proteomic analysis using an iTRAQ 8-

plex reagent kit (AB Sciex, USA) in conjunction with LC-MS/MS at the mass spectroscopy unit, Proteomics laboratory, Central Analytical Facility (CAF), University of Stellenbosch (US). Briefly, proteins were extracted from the different strains, followed by alkylation in methylthiosulphonate (MMTS) and digestion at 37°C using 1 µg/µL trypsin solution (Promega, Madison, WI, USA) as described by Boutureira and Bernardes (2015). Tryptic digests originating from the eight yeasts (TRWY:VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9; the intra-genus TFPH and LVPH:NH 84; two promising natural isolates; MCB C6 and M 2/1; and one inter-genus hybrids:NH 07/1), were tagged according to manufacturer's recommendations with iTRAQ labels 113, 114, 115, 116, 117, 118, 119 and 121, respectively, as described by Kim et al. (2012). The TRWY VIN 7 served as reference as the yeast was reported to be a high 'thiol-releaser' used for the production varietal aromatic Sauvignon Blanc wines with enhanced tropical fruit aroma (Swiegers et al., 2006b; Howe, 2016). Subsequently, proteins were characterised using a mass spectrometer equipped with a nanospray flex ionisation source (Thermo Scientific™ Inc, USA) in conjunction with Mascot algorithm (Matrix DiffScience, London, UK), and SequestHT algorithm included in Proteome Discoverer v1.4. Isobaric tags for relative and absolute quantitation algorithm were used for protein quantitation. Only proteins with more than 2 peptides, but less than 20% variation, and iTRAQ ratios below 0.5 and above 2 were considered down-regulated and over-expressed, respectively. Differentially expressed proteins were also subjected to Protein ANalysis through Evolutionary Relationships (PANTHER, www.pantherdb.org/) to establish their involvement in biological processes, molecular function and protein classes (Sharma et al., 2014).

Statistical analyses

Analysis of variance (ANOVA) and principal component analysis (PCA) were conducted on data from chemical, sensory and metabolomic analyses data (Pearson, 1896, 1901; Zou et al., 2006). The linear relationship between the chemical, sensory and metabolomic variables was analysed by means of a Pearson's correlation using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (that is, F1 and F2).

RESULTS AND DISCUSSION

Characterisation of yeast strains

Contour clamped homogeneous electric field DNA karyotyping

The CHEF DNA karyotyping technique was previously used to successfully differentiate between *S. cerevisiae* and *T. delbrueckii* yeast strains (van Breda et al., 2013). Additionally, CHEF could also differentiate between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Van der Westhuizen and Pretorius, 1992; Hoff, 2012; Hart et al., 2016). Subsequently, CHEF successfully differentiated *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 parental strains from inter-genus hybrids, NH 07/1 and NH 07/2 during this investigation.

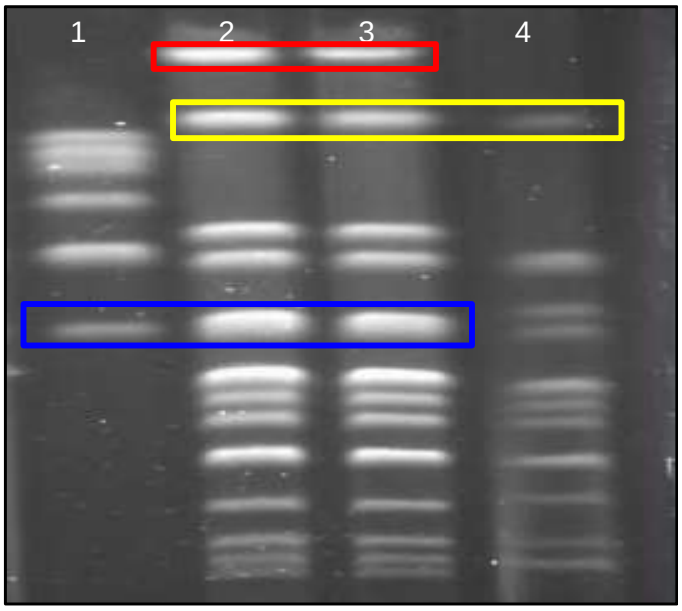

Figure 1. Contour clamped homogeneous electric field (CHEF) DNA karyotypes of parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection (ARC Inf-Nvbij CC). Lane 1, M2/1; Lanes 2 and 3, NH 07/1 and NH 07/2; Lane 4, MCB C6.

The inter-genus hybrid strains shared similar (yellow and blue text box) and different (red text box) chromosomes in terms of size with both parental strains (Figure 1). Both inter-genus hybrids had matching DNA karyotypes, so they may be the same strain, hence MALDI biotyping was deployed as a complementary characterisation tool.

Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Biotyping successfully differentiated between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Hart et al., 2016). Ribosomal proteins extracted from *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 were matched to that of a database described by Hart et al. (2016, 2017). Strains MCB C6, NH 07/1 and NH 07/2 were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens and Lodder, 1942; Kurtzman et al., 2011), whilst strain M2/1 was identified as *C. collucilosa,* the anamorph to *T. delburueckii* (Table 1) (Van Breda et al., 2013; Jolly et al., 2014). It can tentatively be speculated that inter-genus hybdrids were classified as *C. robusta,* as the database does not have inter-genus reference accessions.

Table 1. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) real time classification of parental strains, *Saccharomyces cerevisiae* MCB C6 and *Torulaspora delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 used for the production of varietal Sauvignon Blanc wines.

Mass spectra number	Yeast strain	MALDI-TOF MS log (score) value	Identification
1	MCB C6[3]	1.7	*Candida robusta*[1]
2	M2/1[3]	2.0	*C. colliculosa*[2]
3	NH 07/1[3]	2.1	*Candida robusta*[1]
4	NH 07/2[3]	1.9	*C. robusta*[1]

[1]***C. robusta*** *(**anamorph*** of ***S. cerevisiae***); [2]***C. colliculosa*** *(**anamorph*** of ***T. delbrueckii)***; [3]Experimental yeast (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). *It can tentatively be speculated that inter-genus hybdrids were classified as *C. robusta,* as the database does not have inter-genus reference accessions.

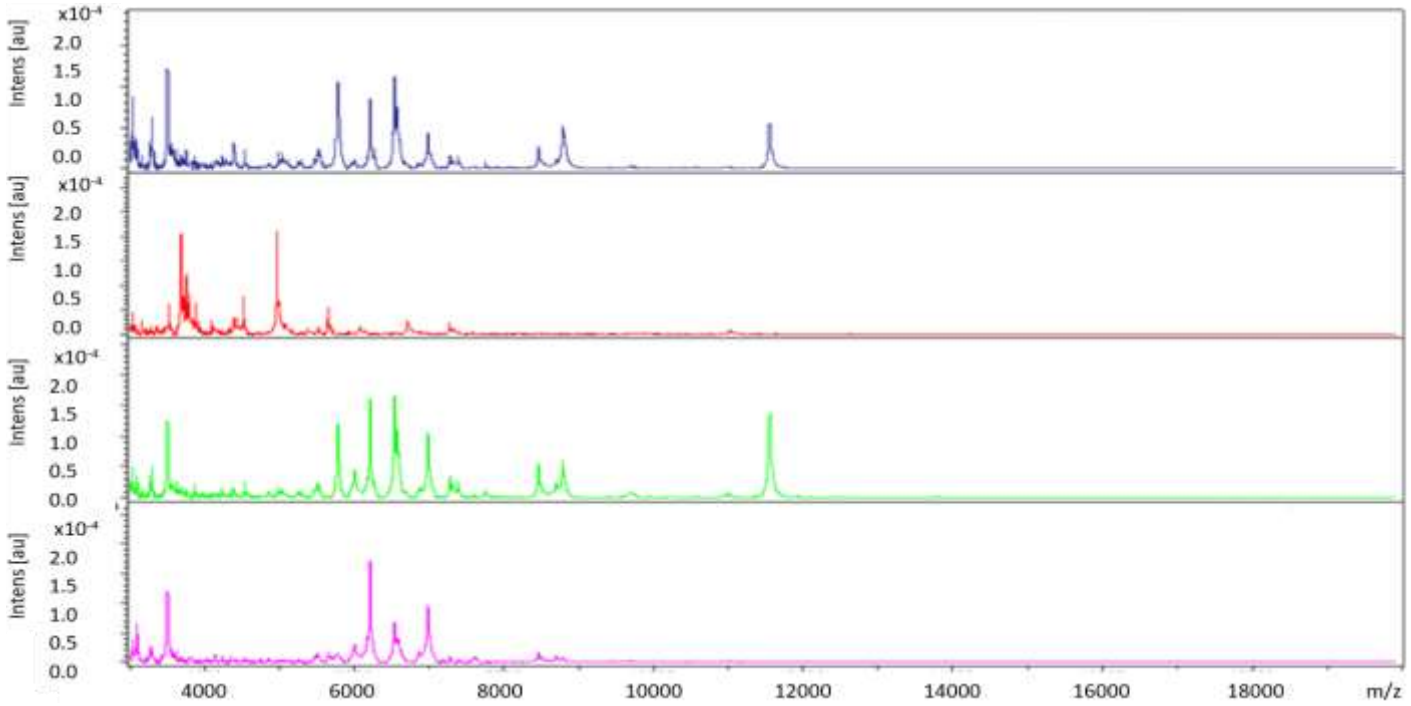

Figure 2. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of parental strains, *S. cerevisiae* MCB C6 (blue-coloured spectrum) and *T. delbrueckii* M2/1 (red-coloured spectrum) and inter-genus hybrids, NH 07/1 (green-coloured spectrum) and NH 07/2 (purple-coloured spectrum) conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Inter-genus hybrids were selected for the production of aromatic white wine, especially Sauvignon Blanc. The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

It is envisioned that the database will be extended by including spectral data of both novel inter-genus hybrids. Parental strains MCB C6 and M2/1 and inter-genus hybrids, that is, NH 07/1 and NH 07/2 also had distinctive mass spectra (Figure 2). Therefore, MALDI-TOF/MS biotyping proved more reliable to distinguish closely related inter-genus hybrids compared to CHEF karyotyping. Nonetheless, the two methods were complementary, as inter-genus hybrids were distinguished from parental strains.

Evaluation of yeast strains

Laboratory-scale fermentation trials

Hart et al. (2016, 2017) previously reported on the fermentation potential of intra-genus hybrids NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 compared to commercial TRWY references used in this study. Laboratory-scale white wine fermentations showed that both inter-genus hybrids,

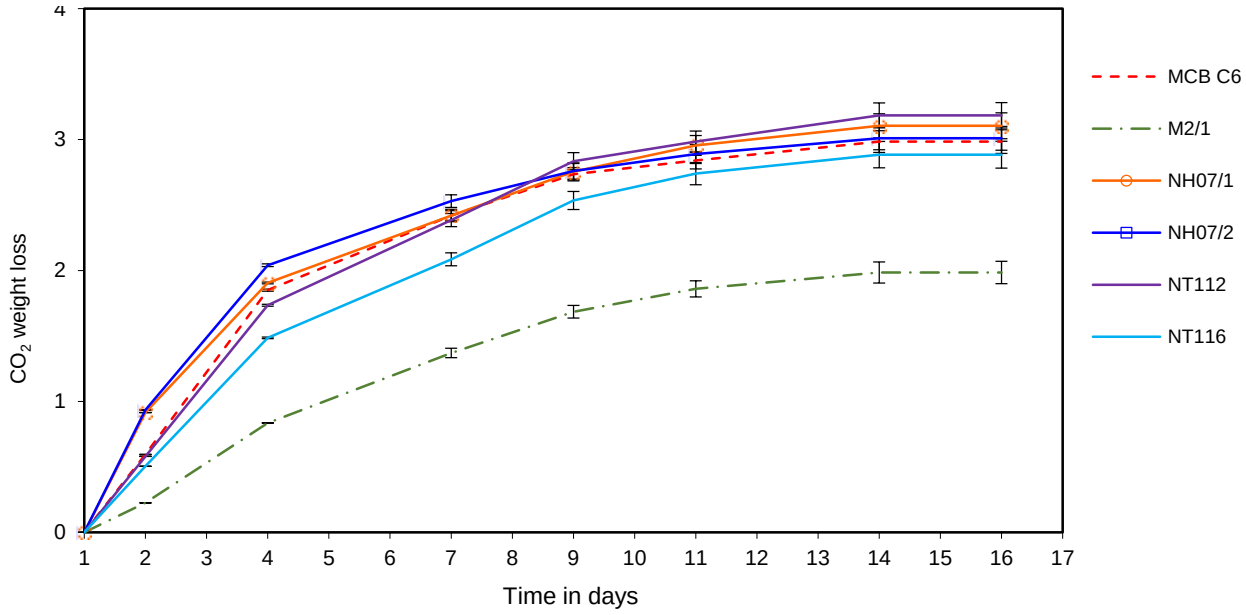

Figure 3. CO_2 weight loss of Chardonnay grape must (juice) fermented at an ambient temperature of 15°C using *S. cerevisiae* strain MCB C6, *T. delbrueckii* strain M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 in laboratory-scale vinifications.

NH 07/1 and NH 07/2 were also able to ferment grape must at a similar rate to commercial references, NT 112 and NT 116 as well as the *S. cerevisiae* strain MCB C6 parental yeast (Figure 3). The parental strain MCB C6 and inter-genus hybrids fermented at a similar rate, whilst the *T. delbrueckii* parental strain M2/1 fermented at a slower rate. Both hybrids also fermented the grape must to dryness (<5 g/L). Nonetheless, the latter was chosen as parental strain for its lower VA formation as reported by Van Breda et al. (2013). Subsequently, both inter-genus strains were compared to intra-genus hybrids and commercial TRWY references for small-scale production of varietal aromatic Sauvignon Blanc wines with lower VA.

Small-scale winemaking

Fourier transform infra-red (FTIR) spectroscopy

Principle component analysis biplot of standard wine chemical parameters showed that both parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids NH 07/1 and NH 07/2 produced final Sauvignon Blanc with a negative association with VA (Figure 4). This observation with regard to *T. delbrueckii* M2/1 complements observations made by Jolly et al. (2003) and Van Breda et al. (2013). The inter-genus hybrids can also provisionally be classified as LVPH a trait inherited from the non-*Saccharomyces* parental strain. Intra-genus hybrid strains provisionally characterised as LVPH, NH 48, NH 57, NH 143, and NH 145 were positioned in the left quadrants (Figure 4), and the wines also had a negative association with VA. The yeast Zymaflore VL3, positioned in the top-right quadrant, was the only commercial TRWY reference that produced wine with a positive association with VA (Figure 4).

Sensory evaluation

Overall, none of the wines were perceived to be undesirable during descriptive sensory evaluation, but differences were evident regarding expression of tropical fruit, floral and vegetative aroma notes (Figure 5). The PCA biplot of descriptive sensory evaluation data showed that two commercial TRWY references, VIN 7 and Zymaflore X5 positioned in the bottom left quadrant, produced Sauvignon Blanc wines with a positive association with tropical fruit aromas, thereby supporting recommendations by yeast manufacturers for their use in the production of aromatic white wines, especially Sauvignon Blanc (Figure 5). The commercial TRWY, Zymaflore VL3 and Fermicru 4F9 positioned in the top quadrants, on the other hand produced wines that is positively associated with floral and vegetative aromas. The commercial TRWY VIN 13, two intra-genus TFPH and LVPH, NH 56 and NH 97 as well as the intra-genus hybrid parental strains, N 96 and P 35 positioned in the

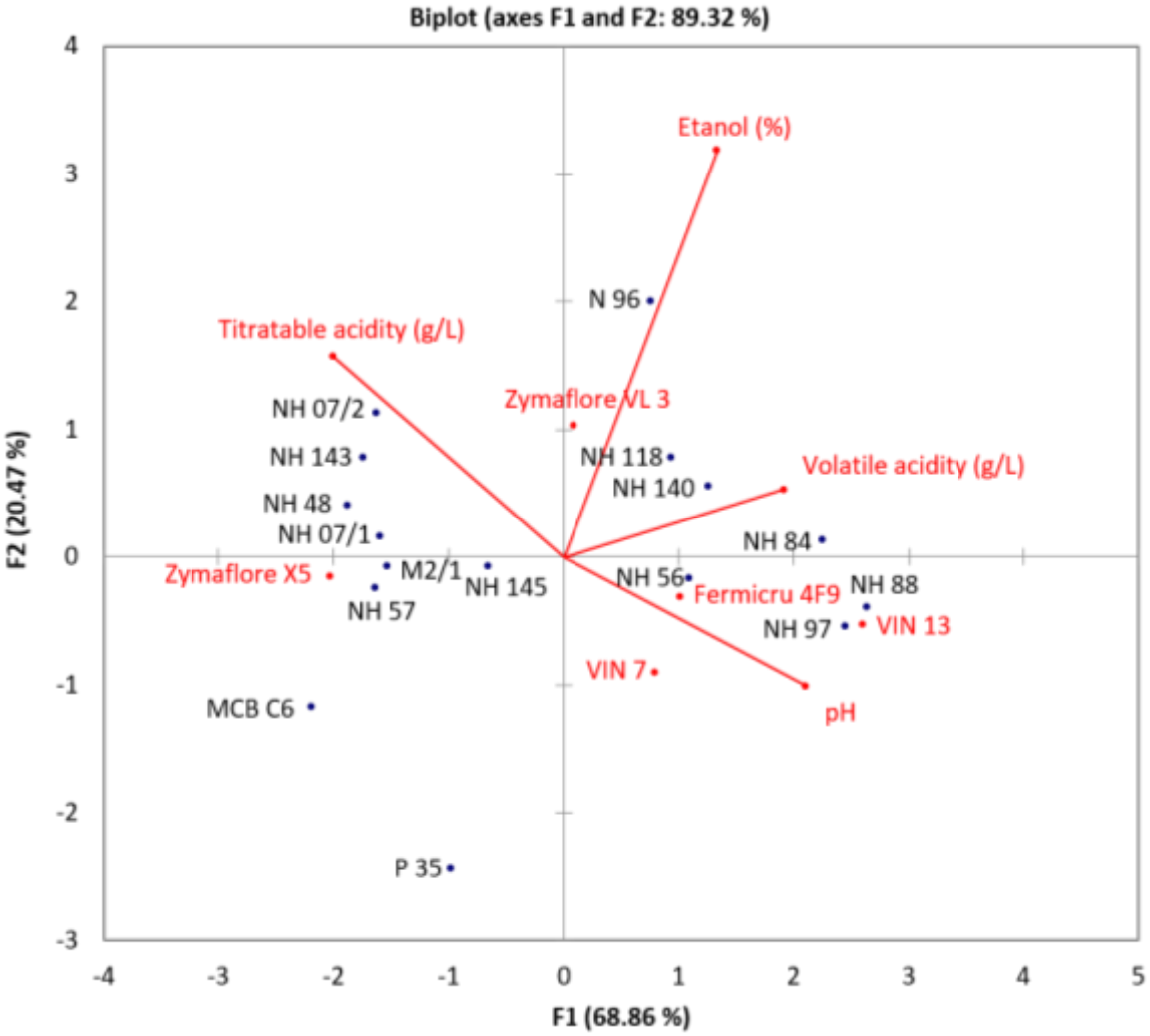

Figure 4. Biplot of basic chemical parameters of small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.

top left quadrant, produced wines that positively associated with both vegetative and tropical fruit aromas. Vegetative aromas associated with Sauvignon Blanc wines can be attributed to grape-derived aroma compounds for example, 2-isobutyl-3-methoxypyrazine (IBMP), especially when grapes were harvested and processed under cooler conditions (Marais, 1994; Lapalus, 2016). It can, therefore, be concluded that these compounds masked the effect of the sought-after volatile thiols (Marais, 1994) associated with tropical fruit aroma, as VIN 13 is a known TRWY strain (Swiegers et al., 2009; Von Mollendorf, 2013).

Both inter-genus hybrids, NH 07/1 and NH 07/2 as well as two intra-genus TFPH, NH 118 and NH 145 produced wines with a positive association with floral aromas (Figure 5). These hybrids can provisionally be characterised as having the ability to produce wines with floral aroma (henceforth referred to as FLPH). Both MCB C6 and M2/1, positioned in the bottom quadrants, produced wines that is associated with floral and tropical fruit aromas. This supports previous observations that a *T. delbrueckii* strain (Belda et al., 2015; Renault et al., 2016) as well as an *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid (Santos et al., 2008) produced aromatic

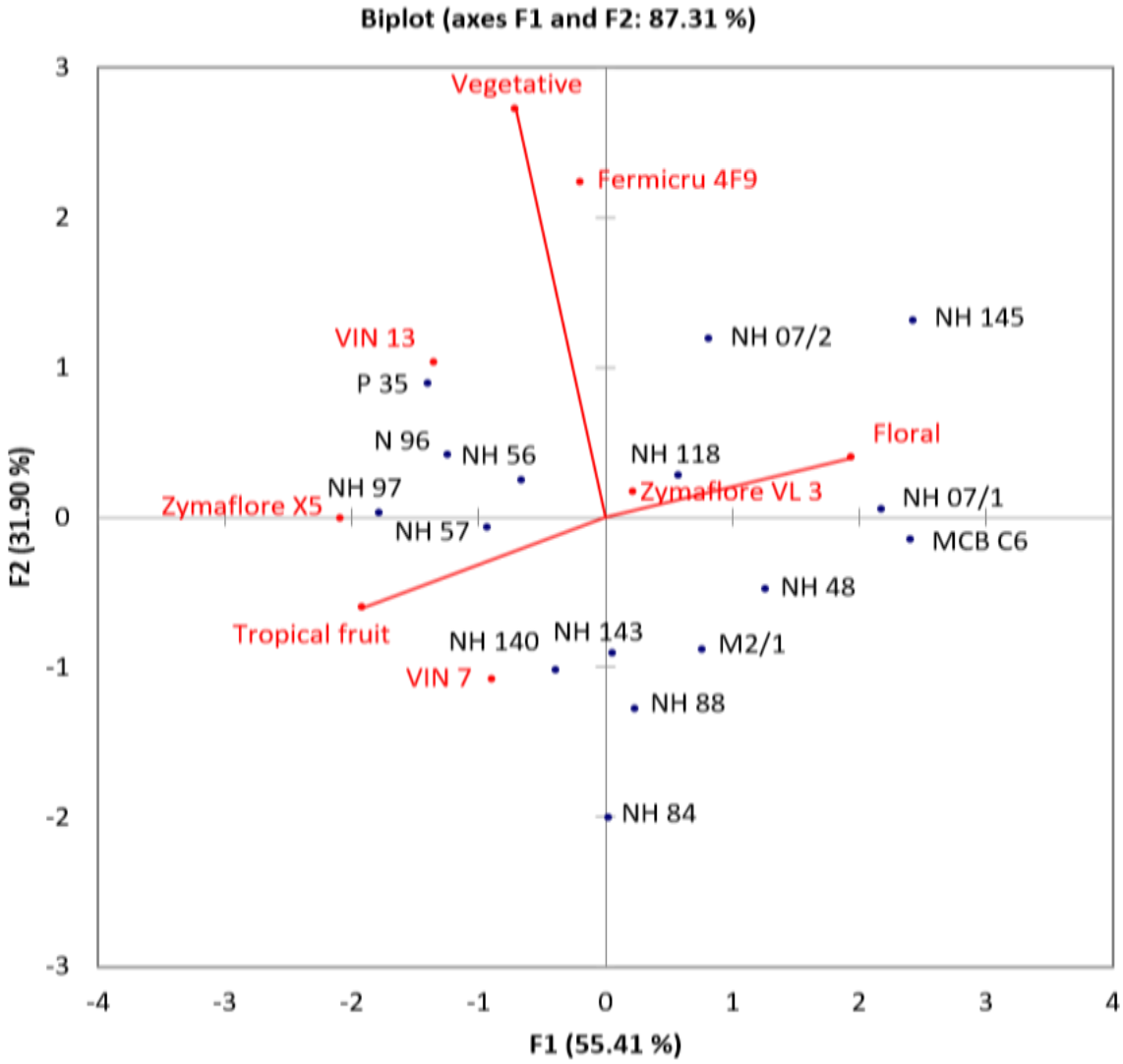

Figure 5. Biplot of descriptive sensory evaluation of small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

wines. Floral aromas, frequently associated with Sauvignon Blanc wines, are the result of yeast-mediated metabolites, namely monoterpenes produced from pre-cursors present in grape must (Von Mollendorf, 2013; Hart et al., 2017). It is apparent that the inter-genus hybrids inherited the ability to release monoterpenes from the parental strains. The intra-genus TFPH, NH 48, NH 84, NH 88 and NH 143 some of which were also shown to be LVPH (Figure 4), produced wines with a positive association with tropical fruit and floral aromas. These TFPH also produced wines with a negative association with vegetative aromas. Both inter-genus FLPH, NH 07/1 and NH 07/2, also produced Sauvignon Blanc wines with a negative association with VA (Figure 4) and can provisionally be characterised as LVPH. Two intra-genus TFPH and LVPH, NH 57 and NH 145 produced wines with a negative association with VA (Figure 4), and a positive association with tropical fruit aroma (Figure 5). Therefore, these intra- and interspecfic TFPH, FLPH and LVPH yeasts showed promise for the production of typical varietal aromatic Sauvignon Blanc wines with lower VA.

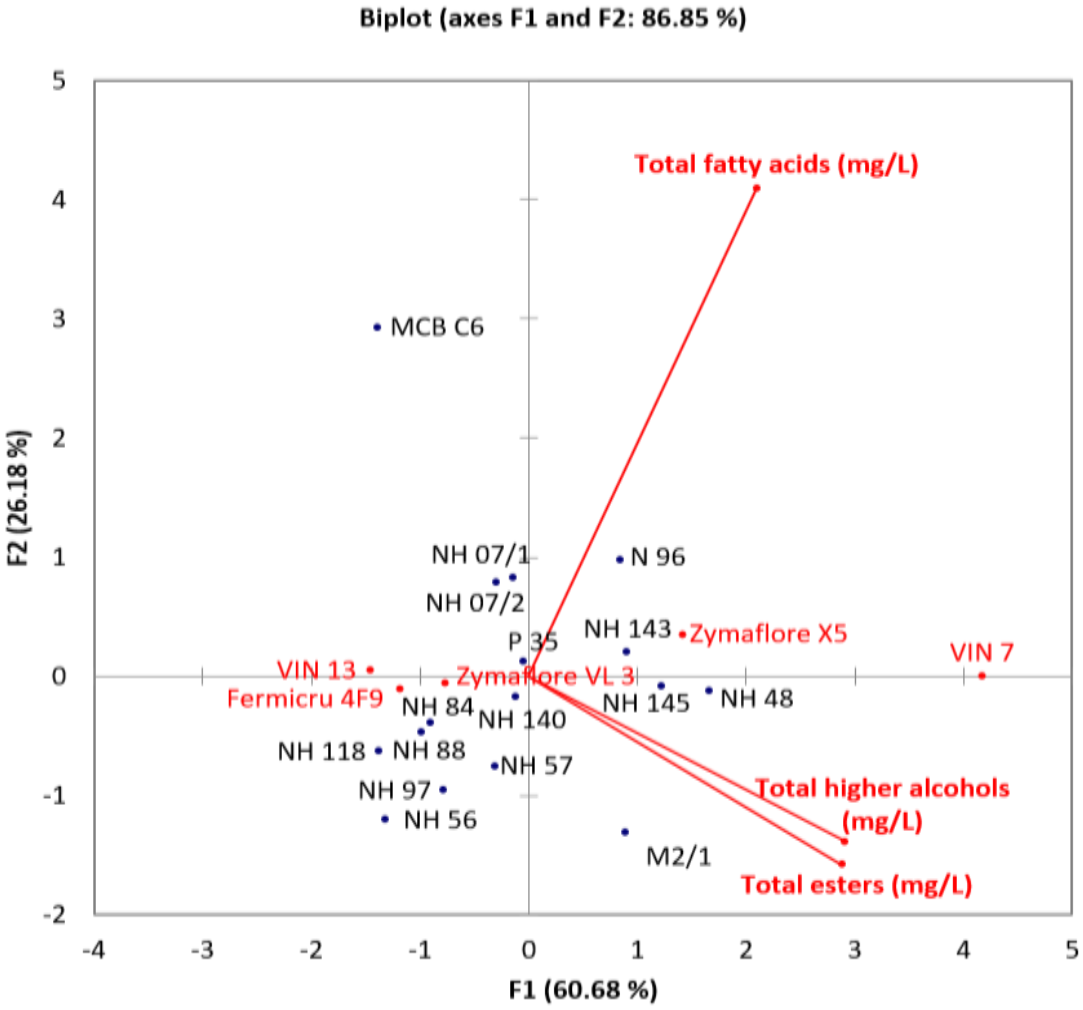

Figure 6. Biplot of aroma compounds, esters, higher alcohols and fatty acids in small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

Gas chromatography (GC) analysis

Gas chromatography was deployed to quantify wine aroma compounds, namely esters, total fatty acids and higher alcohols, most of which are associated with wine 'fermentation bouquet' and/or 'fruitiness' (Lambrechts and Pretorius, 2000; Coetzee and du Toit, 2015). The PCA biplot showed that strain *T. delbrueckii* M2/1 produced wines with a positive association with esters and higher alcohols compared to the remaining *S. cerevisiae* and hybrid yeast strains (Figure 6). Strains of *T. delbrueckii* were previously reported to produce wines with enhanced aroma (Van Breda et al., 2013; Renault et al., 2016). The TRWY reference VIN 7 and the intra-genus hybrids, NH 48 and NH 145, positioned in the right quadrant, also produced wines with a positive association with esters and higher alcohols, which imparts fruity aromas and complexity. These wines had positive associations with, amongst others, tropical fruit and floral aromas (Figure 5).

The TRWY reference, Zymaflore X5, the two TFPP, N 96 (Figure 5) as well as the intra-genus hybrid NH 143, positioned in the top right quadrant, produced wines with a positive association with total fatty acids also referred to as volatile fatty acids. Some volatile fatty acids (for example, octanoic acid, decanoic acid) were reported to be associated with faint fruity and citrus wine aromas (Lambrechts and Pretorius, 2000). However, acetic acid, responsible for vinegar-like off-flavours at higher concen-

trations still remains the main contributor to total fatty acids (Swiegers et al., 2005; Ugliano et al., 2009; Vilela-Moura et al., 2011). Nevertheless, Zymaflore X5 still produced wines with a positive association with fruity aroma, specifically tropical fruit (Figure 5). The TRWY references VIN 13, Zymaflore VL3 and Fermicru 4F9, positioned in the left quadrants, produced wines with a negative association with total fatty acids (Figure 6). These wines also had a positive association with, amongst others, tropical fruit and floral aromas (Figure 5).

Both inter-genus FLPH, NH 07/1 and NH 07/2 also produced wines with a negative association with volatile fatty acids. This can also be seen in Figures 4 and 5, there was a negative association with VA (Figure 4) and positive association with floral aroma (Figure 5). The lower production of VA by the inter-genus hybrids can be attributed to inheritance from the *T. delbrueckii* parent strain.

Seven intra-genus hybrids provisionally characterised as TFPH and LVPH, namely NH 56, NH 57, NH 84, NH 88, NH 97, NH 118 and NH 140 also produced wines with a negative association with volatile fatty acids, including acetic acid (Figure 6). Two of these intra-genus hybrids were also shown to produce Sauvignon Blanc wines with a negative association with VA (Figure 4) and positive association with floral aroma (Figure 5). These intra-genus hybrids also produced wines with a postive association with esters and higher alcohols (Figure 6). Yeast strains, namely VIN 7, M2/1, NH 48, NH 57, NH 84, NH 88, NH 140 and NH 143 produced wines with a positive association with 'fruitiness' (tropical fruit aroma) (Figure 5) and higher alcohols (Figure 6). Therefore, this observation compliments a previous study that showed higher alcohols to be the key precursors involved in ester formation (Patrianakou and Roussis, 2013). Based on this data set, intra- and inter-genus TFPH, FPH and LVPH have great potential for the production of varietal aromatic Sauvignon Blanc wines with lower VA, as they comply with yeast selection criteria set forth in the objectives.

Solid-phase extraction (SPE) and GC-MS/MS analysis

Volatile aromatic thiols; for example, 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), primarily responsible for passion fruit, tropical fruit and citrus aromas in Sauvignon Blanc wines, are released by fermenting wine yeasts from aroma-inactive, bound precursors present in grape juice (Swiegers et al., 2007b; Roland et al., 2011; Harsch et al., 2013). The PCA biplot of volatile thiol analyses showed that the inter-genus hybrid NH 07/1 produced wine with a more positive association with volatile thiols, 3MHA in particular, than either parental strain as well as known two commercial TRWY yeasts, Zymaflore X5 and Zymaflore VL3 (Figure 7). It is noteworthy that NH 07/1 also produced wines with a negative association with VA (Figure 4) and acetic acid (Figure 5), whilst having a positive association with floral aroma (Figure 6). These wines also had hints of tropical fruit aroma, and data suggests that inter-genus hybrids can be applied for the production of varietal aromatic Sauvignon Blanc wines with lower VA. The inter-genus hybrid NH 07/2, on the other hand, produced wines with a negative association with 3MH and 3MHA (Figure 7). This observation compliments the descriptive sensory evaluation, as NH 07/2 produced wines with a positive association with vegetative aroma (Figure 5). Nonetheless, NH 07/2 produced wines with chemically detectable 3MH and 3MHA levels, albeit lowest of all yeast strains included in this study. It can, therefore, be speculated that the tropical fruit aroma and effect of volatile thiols were masked by methoxypyrazines, another aroma compound naturally associated with this cultivar (Marais, 1994; Lapalus, 2016).

Three intra-genus hybrids, NH 56, NH 84 and NH 88 produced wines with stronger association with the volatile thiol 3MH than the commercial TRWY VIN 7 reference. The latter was also reported to be the highest producer of another volatile thiol, 4MMP, associated with 'fruity' aroma in wine during previous studies (Swiegers et al., 2009; Borneman et al., 2012). Five more intra-genus yeasts, namely NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3 (Dubourdieu, 2006; Bowyer et al., 2008). These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 also produced wines with a negative association with VA (Figure 4) and acetic acid (Figure 5), whilst having a positive association with tropical fruit aroma (Figure 6). Therefore, observations made during this study is indicative that these intra-genus hybrids can be used for the production of varietal aromatic Sauvignon Blanc wines with low VA. It is noteworthy that the intra-genus TFPH, namely NH 84 produced wines with 3MH levels that were significantly higher than its sensory detection threshold (Van Wyngaard, 2013). The 3MH levels in these wines were also discernibly higher compared to wines produced by the best commercial TRWY reference VIN 7 in this study. The TFPP N 96, considered to be a 'neutral' yeast by the manufacturer (Anchor Yeast, South Africa - N 96 product data sheet), produced wines with a more positive association with 3MH (Figure 7) than the *T. delbrueckii* M2/1 previously shown to produce aromatic wines (Jolly et al., 2003; Van Breda et al., 2013).

Indications, therefore, are that intra-genus TFPH inherited the 'thiol-releasing' abilities from both *S. cerevisiae* parental strains, that is, N 96 and P 35. The latter produced wines with a stronger association with 3MH than all commercial TRWY references included in this study (Figure 7).

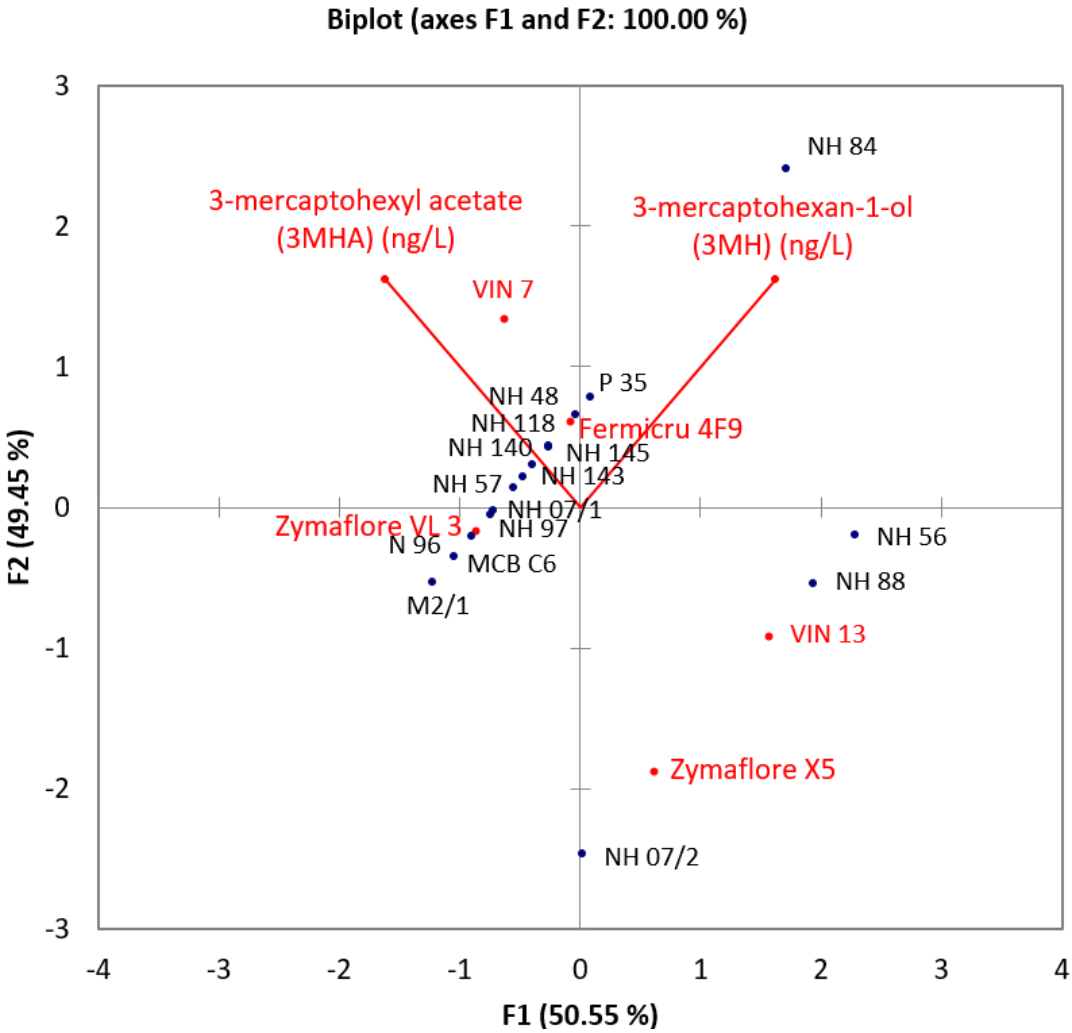

Figure 7. Biplot of volatile thiols, 3MH and 3MHA in small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

Quantitative LC-based iTRAQ proteomic analysis

Yeast-expressed enzymes (proteins) during fermentation regulate wine aroma compounds (metabolites) responsible for wine aroma and flavour (organoleptic quality) (Moreno-García et al., 2015). Analysis of the combined datasets in conjunction with Uniprot *S. cerevisiae* database identified a total of 998 yeast derived proteins (Table 2) on day 15 of fermentation when fermentations stabilised and/or were dry. Commercial TRWY (VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9), naturally isolated parental strains (MCB C6 and M2/1), as well as both intra- and inter-genus hybrids (NH 84, NH 07/1) were shown to vary in their up- and down-regulated proteins compared to the TRWY VIN 7 reference expressed proteins. Overall 25 proteins (2.51%) were down-regulated and 122 proteins (12.22%) were overexpressed. Properties and relative expression of down-regulated and overexpressed proteins of yeast strains, amongst others, the intra-genus TFPH and LVPH NH 84 and inter-genus FLPH and LVPH, NH 07/1 were established by using quantitative LC-based iTRAQ proteomic analysis (all data can be obtained from the Agricultural research council (ARC) Infruitec-Nietvoorbij, Microbiology Department, Stellenbosch, South Africa).

Proteomic analyses showed that the TRWY Zymaflore

Table 2. Number of differentially expressed proteins originating from fermenting commercial 'thiol-releasing' wine yeasts (TRWY), with ability to produce wine with tropical fruit aromas, naturally isolated parental strains, intra- and inter-genus hybrid yeast strains with the ability to produce wines with enhanced tropical fruit aroma (abbreviated as TFPH) and lower VA (abbreviated as LVPH) during the fermentation of 2013 Sauvignon Blanc grape juice.

Yeast strain	Proteomic analysis	
	Down-regulated	Over-expressed
VIN 7 TRWY Reference	998 proteins characterised	
VL3 TRWY	2	27
X5 TRWY	4	9
4F9 TRWY	1	60
NH 84 TFPH and LVPH	0	6
MCB C6	6	9
M2/1	11	3
NH 07/1 FLPH and LVPH	1	8

VL3 reference over-expressed the lactoylglutathione lyase protein, an enzyme responsible for cleaving a carbon-sulphur bond to release the volatile thiol 4MMP from its bound aroma-inactive precursor (Howell et al., 2005). Unfortunately, 4MMP was not quantified during this study. However, the TRWY produced wine with a moderate association with 3MH. It can, therefore, be speculated that aforementioned carbon-sulphur lyase enzyme is also involved in the release of 3MH from its carbon-sulphur-containing precursor (Swiegers et al., 2007a). A gene encoding for *β*-lyase involved in the release of volatile thiols, 3MH and 4MMP was previously reported (Holt et al., 2011). Therefore, lactoylglutathione lyase might be used as a protein biomarker for volatile thiol release during production of varietal Sauvignon Blanc wines. Additionally, Zymaflore VL3 produced wines with a positive association with floral aroma that is influenced by yeast-mediated released monoterpenes, which essentially are hydrocarbons and/or glycoconjugates, from its bound aroma-inactive precursors in grape juice (Von Mollendorf, 2013). Monoterpenes was shown to be released in abundance by using genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase (Pardo et al., 2015). Nevertheless, non-GM *S. cerevisiae* can release moderate geraniol and linalool levels (Lambrechts and Pretorius, 2000). The TRWY was also shown to produce wines with a moderate association with VA (Figure 6), which comprise acetic acid, an intermediate of long chain fatty acid production catalysed by fatty acid synthases (Tehlivets et al., 2007). However, the yeast did not regulate any synthases. Nonetheless, the association between regulated proteins, especially synthases, and their effect on VA formation should be further investigated.

The TRWY Zymaflore X5 was shown not to regulate any lyases and synthases (Table 2), which complements FTIR analyses (Figure 4), descriptive sensory evaluation (Figure 5), GC-analyses (Figure 6) and SPE-GC/MS analyses (Figure 7), as the yeast produced wines with a negative association with VA and total fatty acids (comprised mainly of acetic acid), a positive association with tropical fruit aroma, and a negative association with volatile thiols, respectively. On the other hand, the TRWY Fermicru 4F9 was shown to regulate dehydrogenases. Proteins in the same class were previously implicated in excessive acetic acid production (Varela et al., 2012; Walkey et al., 2012). However, regulated dehydrogenases by Fermicru 4F9 do not seem to have stimulated VA formation as the yeast produced wines with a negative association with VA and total fatty acids (Figures 4 and 6). The yeast also did not regulate any carbon-sulphur lyases responsible for volatile 'thiol-release' and, therefore, complements descriptive sensory evaluation as the yeast produce wines with a negative association with tropical fruit aroma (Figure 5). SPE-GC/MS analyses revealed that the TRWY produced wines with a positive association with volatile thiols 3MH and 3MHA (Figure 7). This therefore, implies that more proteins might be involved in volatile thiol-release.

Proteomic analyses further showed that the intra-genus TFPH and LVPH NH 84 was the only strain not to have down-regulated any proteins, whilst the remaining strains down-regulated from one to 11 proteins. Furthermore, NH 84 only overexpressed six proteins classed as nucleic acid 'binders', hydrolases and transporters, some of which are associated with cell proliferation and protein synthesis. As NH 84 was the only strain to have regulated these proteins, they could also be associated with higher 3MH released by this strain. These proteins will in future be further investigated as potential biomarkers, as the yeast also produced wines with a positive association with tropical fruit aroma (Figure 5) and volatile thiols, especially 3MH (Figure 7).

Both inter-genus parental strains, *S. cerevisiae* MCB

C6 and *T. delbrueckii* M2/1 were shown not to regulate the lactoylglutathione lyase protein. Nevertheless, the inter-genus hybrid, NH 07/1 provisionally characterised as a FLPH and LVPH, as observed with the TRWY Zymaflore VL3, over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP (Howell et al., 2005). As previously mentioned, 4MMP was not quantified during this study. The inter-genus hybrid not only produced wine with a positive association with 3MH, it was more pronounced than wines produced with both parental strains MCB C6 and M2/1 and TRWY Zymaflore VL3 and Zymaflore X5. As 3MH release also involves enzymatic cleavage by a carbon-sulphur lyase, there is a possibility that this over-expressed protein could also be involved in the release of 3MH from its carbon-sulphur-containing precursor (Howell et al., 2005; Swiegers et al., 2007a). This observation further supports the notion that lactoylglutathione lyase might be a useful biomarker for volatile thiol release by NH 07/1 during production of varietal Sauvignon Blanc wines.

The inter-genus FLPH and LVPH, NH 07/1 also produced wines with a positive association with floral aroma that is influenced by yeast-released mono-terpenes, a metabolite that was released in abundance by a genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase as mentioned previously (Von Mollendorf, 2013; Pardo et al., 2015). However, the inter-genus FLPH did not regulate any synthases, which suggests that other proteins are involved in monoterpene release. Strains belonging to the same species of parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 are known to produce monoterpenes (King and Dickinson, 2000). This warrants further investigation to identify protein biomarkers associated with floral wine aroma and monoterpene release.

Differentially expressed proteins by the intra-genus TFPH and LVPH, NH 84 and inter-genus FLPH and LVPH, NH 07/1 during the stationary phase of Sauvignon Blanc grape must fermentation were classified according to molecular function, biological process and protein class using PANTHER (Sharma et al., 2014). Classification of proteins according to molecular function showed that NH 84 regulated proteins were linked to translation regulator activity, catalytic activity and transporter activity, whilst that of NH 07/1 were linked to binding activity, structural molecule activity, catalytic activity and antioxidant activity (Figure 8a and b). Classification of proteins according to biological processes showed that NH 84 regulated proteins were linked to cellular processes, metabolic processes and localisation, whilst those of NH 07/1 were linked to response to stimuli, cellular processes, metabolic processes and cellular biogenesis (Figure 8c and d). Furthermore, NH 84 regulated proteins clustered into three protein classes *viz.* nucleic acid binding, hydrolase and transporter, whilst those of NH 07/1 also clustered into different protein classes *i.a.* hydrolases, and oxidoreductases (Figure 8e and f). It is evident that regulated proteins differed between intra-genus and inter-genus strains, explaining the production of wines with different chemical (Figure 4) and sensory (Figure 5) properties, as well as differences in aroma and off-flavour compound levels (Figures 6 and 7).

Conclusions

The inter-genus FLPH and LVPH, NH 07/1, produced wine with a more positive association with volatile thiols, 3MHA in particular, than both parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, as well as commercial TRWY, Zymaflore X5 and Zymaflore VL3. This hybrid also produced wines with a negative association with VA and acetic acid, but a positive association with floral aroma with hints of tropical fruit aroma. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 produced wines with a negative association with VA and acetic acid, but with a positive association with tropical fruit aroma. These intra-genus hybrids also produced Sauvignon Blanc wines with a stronger association with 3MH than the commercial reference. Five more intra-genus yeasts, NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3. These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Proteomic analyses showed that NH 07/1 and Zymaflore VL3 over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP by cleaving its carbon-sulphur bonds. Since 3MH release also involves enzymatic cleavage by a carbon-sulphur lyase, there is a possibility that the aforementioned over-expressed protein could also be involved in the release of 3MH from its carbon-sulphur-containing precursor. Lactoylglutathione lyase might be a useful protein biomarker for volatile thiol release by especially NH 07/1 during production of varietal Sauvignon Blanc wines. As dehydrogenases were previously implicated in VA formation, these proteins might also be useful biomarkers for VA and/or acetic acid formation by fermenting wine yeasts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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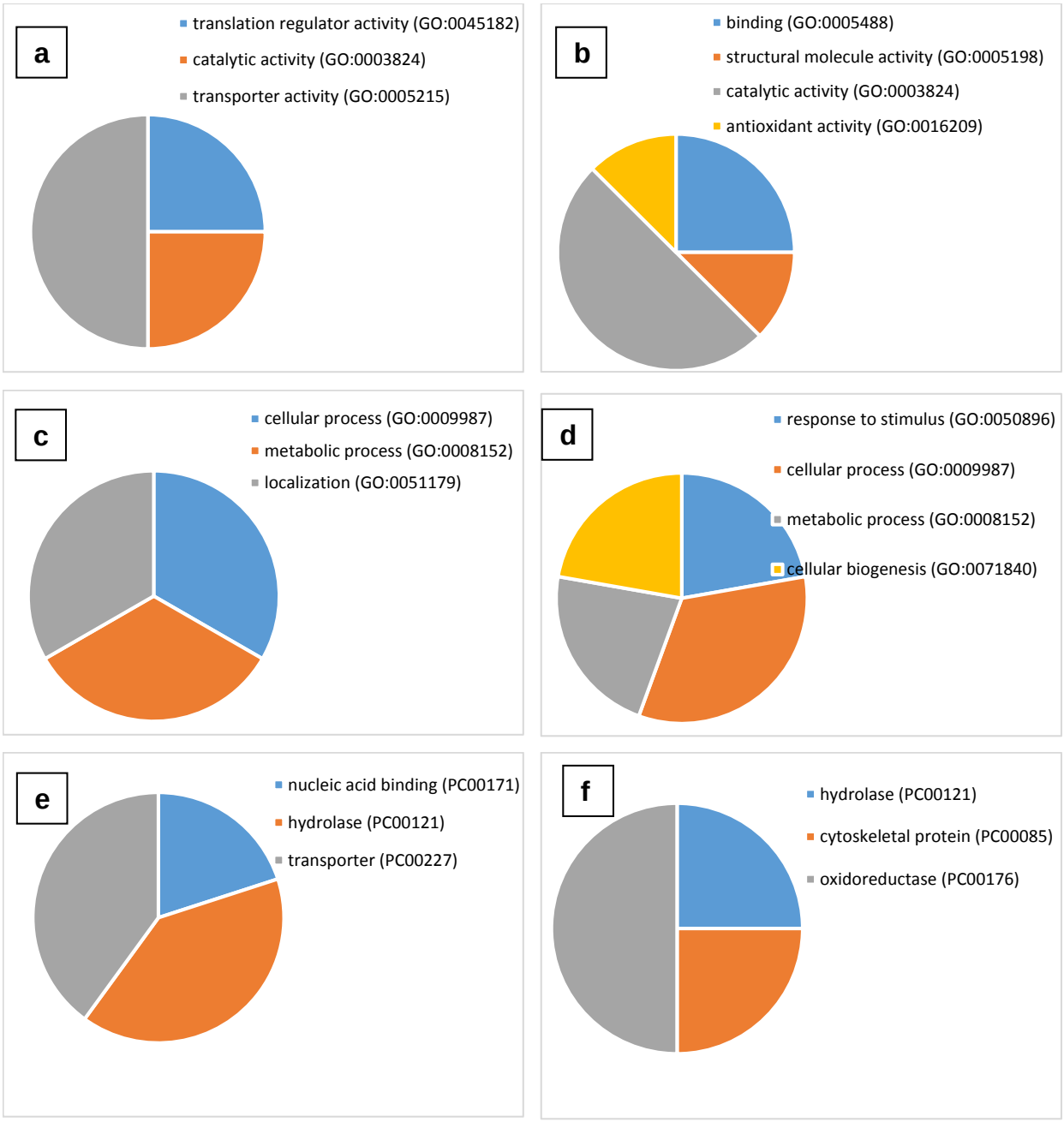

Figure 8. Classification of differentially expressed proteins by intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma (abbreviated as TFPH) and low VA (abbreviated as LVPH), NH 84 and the inter-genus hybrid, NH 07/1 during the end of Sauvignon Blanc grape must fermentation according to (a and b) Molecular function, (c and d) Biological process, and (e and f) Protein class using Protein ANalysis THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/).

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Full Length Research Paper

Colorimetric gold nanoparticles-based assay for direct detection of *Clostridium difficile* in clinical isolates from Qatar

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***Clostridium difficile* infection (CDI) is a significant health problem worldwide. Control and prevention strategies of *C. difficile* horizontal transmission require assays with fast detection with high specificity and sensitivity. Conventional diagnostic methods are time consuming and costly for clinical field settings. This study aims to develop gold nanoparticles (AuNPs)-based assay for direct qualitative detection of the nucleic acid of *C. difficile* and its toxins. A colloidal solution of AuNPs with a diameter of 13±1 nm was prepared and characterized. The qualitative colorimetric AuNPs assay was developed for restricted genomic *C. difficile* DNA detection, and results were confirmed by PCR. One hundred and five positive *C. difficile* isolates were collected from patients with diarrheal diseases and tested using AuNPs based-assay. Ninety-six samples (91.4%) were detected positive using AuNPs based assay, as indicated by the color change from red to blue within 1 min. All ninety-six positive samples were positive for toxin B. In conclusion, nano-gold assay prototype was developed for direct and inexpensive detection of *C. difficile*. The developed prototypes are simple, sensitive, rapid and can substitute PCR-based detection. The developed assay may show potential in the clinical diagnosis of *C. difficile*, especially in developing countries as it is less costly as compared to the commercially available assays.**

Key words: Gold nanoparticles, *Clostridium difficile,* colorimetric assay, polymerase chain reaction (PCR).

INTRODUCTION

Clostridium difficile is a Gram-positive, strictly anaerobic, spore-forming bacterium (Chankhamhaengdecha et al., 2013), first recognized in the late 1970s (Burnham and Carroll, 2013). It is documented as the causative agent of

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a broad spectrum of intestinal diseases ranging from mild self-limiting diarrhea to more serious and potentially life threatening manifestations such as *Pseudomembranous colitis* and is responsible for most cases of antibiotic-associated diarrhea (Carter et al., 2007; Goncalves and Decre, 2004). Pathogenicity of *C. difficile* is linked to two major toxins produced by the bacteria: toxins A (enterotoxin) and B (cytotoxin). Toxins A and B are encoded by the genes *tcdA* and *tcdB*, respectively, and are located in the 19.6-kb pathogenicity locus (PaLoc) of the *C. difficile* chromosome (Persson et al., 2011). Some strains of *C. difficile* also secrete binary toxin. CdtA and cdtB genes that are located outside the PaLoc (Goncalves and Decre, 2004) encode these binary toxins.

Rapid and reliable identification of toxigenic *C. difficile* is essential for appropriate patient management and implementation of timely infection control measures due to the rapidly increasing infection rate of *C. difficile* in health care facilities. Current laboratory diagnosis remains challenging with many limitations, as rapid test procedures relying on enzyme immunoassays (EIAs) show limited sensitivity. On the other hand, the gold standard toxigenic culture and cytotoxicity assays, which are considered as reference standard, are time-consuming (Dalpke et al., 2013). A two-step algorithms consisting of sensitive detection of glutamate dehydrogenase enzyme (GDH) followed by a confirmatory test using PCR have been proposed by Ticehurst et al. (2006) to increase sensitivity and specificity of the detection.

Gold has been used as a vital material in nanotechnology and incorporated in diagnostic procedures. Distinctive size-dependent optical properties of AuNPs, their inertness and strength make them one of the most robust materials utilized in nano diagnostics technology (Syed, 2014). They are spherical in shape with a typical diameter of approximately 2 to 100 nm that exhibit a unique phenomenon known as “localized surface plasmon resonance” or LSPR, which is responsible for their intense red color. Upon aggregation, AuNPs change color and that is easily detected visually without the aid of any instrumentation. Addition of salt (NaCl) during hybridization shields the surface charge on the AuNPs, which are typically negatively charged due to reduced citrate ions on their surfaces, leading to aggregation of AuNPs and thus change in color from red to blue (Hussain et al., 2013; Syed, 2014). This property is especially useful in colorimetric detection based assay, which is evaluated in this study (Azzazy et al., 2006; Jennings and Strouse, 2007; West and Halas, 2003). The principle of colorimetric AuNPs-based assay in microbial identification is illustrated in Figure 1.

To the best of the authors’ knowledge, this is the first study aimed to develop unmodified AuNPs - based assay for direct qualitative detection of the nucleic acid of *C. difficile* and its toxins. Additionally, it evaluated AuNPs assay sensitivity and specificity as compared to RT-PCR (GeneXpert, Cepheid, CA, USA).

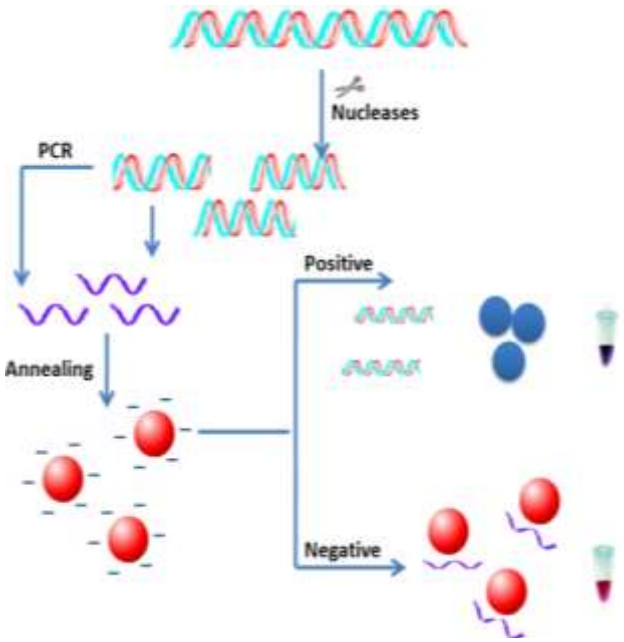

Figure 1. Schematic diagram illustrating the principle of colorimetric AuNPs-based assay in microbial identification. In positive samples, the primer is complementary to the DNA/RNA target, thus not available in the hybridization buffer, containing NaCl to protect and stabilize AuNp, leading to aggregation of AuNPs and blue color formation. On the other hand, in the absence of the target or non-specific DNA, the primer will be free in the reaction mixture and binds to AuNPs preventing aggregation and the color remains unchanged.

MATERIALS AND METHODS

Synthesis of AuNPs

Spherical AuNPs were prepared by citrate reduction of gold 111 chloride trihydrate ($HAuCl_4.3H_2O$). Synthesis was carried out as previously prescribed by Grabar et al. (1995). Briefly, the reflux system was cleaned by aqua regia and then rinsed with ultrapure water and left to dry. An aqueous solution of 1 Mm $HAuCl_4.3H_2O$ (Sigma Aldrich) was brought to boil under reflux conditions while stirring. When gold started to boil, 1% of trisodium citrate (Sigma Aldrich) was rapidly added. This resulted in a subsequent change in solution color from yellow - clear - black - purple - deep red. Afterwards, the solution was then refluxed for an additional 15 min and subsequently allowed to cool to room temperature. The colloidal solution was then stored into a clean dark storage glass bottle at 4°C until further use.

Characterization of AuNPs

The absorbance of the prepared AuNPs solution was measured by Agilent 8453 UV-visible spectrophotometer at wavelength 400 to 700 nm. The size and distribution was characterized using Zeta sizer (Malvern, Nano ZSP, UK) based on light scattering principle and scanning electron microscope, SEM (FE1 NOVA-NanoSEM, 450, USA). The recommended AuNPs size ranges from 12 to 15

nm with the absorbance of the visible range 400 to 700nm (Grabar et al., 1995).

Collection of bacterial isolates

A total of 105 *C. difficile* clinical isolates used in this study were, originally provided for routine laboratory diagnosis at Hamad Medical Corporation (HMC) - Al Khor Hospital, Qatar during the period of 2011 to 2012. Cryopreserved *C. difficile* isolates were revived and sub-cultured onto blood agar enrichment medium, and incubated anaerobically. Samples were transported to Qatar University (Biomedical Research Center) for DNA extraction and testing with gold nanoparticles-based assay. Isolates were confirmed to be *C. difficile* using RT-polymerase chain reaction, RT-PCR, (GeneXpert, Cepheid, CA, USA). According to RT- PCR results, all *C. difficile* isolates (105) were positive for toxin B. Twenty nine additional ATCC bacterial isolates other than *C. difficile* were used to assess specificity of the assay. The Research Ethical Committee of Hamad Medical Corporation, Qatar, approved the study.

DNA extraction

Genomic DNA was extracted from bacterial cultures following manufacturer's instructions of QIAamp DNA Mini kit (Qiagen; Cat. No. 51306).

Restriction of genomic *C. diff* DNA

The entire extracted genomic *C. diff* DNA was digested using *Bam* HI, according to the conditions recommended by the supplier of the enzyme kit (Promega). Briefly, 17.3 µl of the extracted genomic DNA were restricted by addition of 0.5 µl of the enzyme, 2 µl of the buffer, 0.2 µl of the acetylated bovine serum albumin. Then incubated at 37°C for 1 h after that inactivated at 65°C for 20 min.

Colorimetric AuNPs assay for detection of *C. difficile* DNA: Development and optimization

The colorimetric qualitative AuNPs assay for *C. difficile* was optimized through adjustment of the assay parameters such as annealing temperature, salt concentration and targeting oligonucleotide sequences. Different concentrations of NaCl and primer concentrations were tested to determine the optimum concentrations for performing the assay (data not shown). Hybridization buffer was prepared using 0.50 M NaCl and 10 µM primer. Different volumes of the AuNPs were tested, and 25 µL of the prepared AuNPs (12 to 15 nm) was selected for use in the final assay. Forward CD-R 5'- CCC TGC ACC CTT AAT AAC TTG ACC-3' (Integrated DNA Technologies, Inc. Belgium) primer was used in the assay due to its high specificity to all *C. difficile*. The assay was performed as follows: 22 µL of the extracted DNA (1.7ng/µl), were placed in a sterile PCR tube. Then, 13 µL of the hybridization buffer were added and mixed well (final concentration of the primer and NaCl after addition of AuNPs were 0.9 µM and 0.04 M, respectively) to obtain a final volume of 35 µl per PCR tube. The mixture was then heated at 95°C for 30 s, and annealed at 50°C for 30 s and then cooled to room temperature for 10 min. 25 µl of colloidal AuNPs were then added to the mixture, and the color was observed within 1 min (Shawky et al., 2010).

Colorimetric AuNPs assay for detection of *C. difficile* toxin B

All *C. difficile* positive samples were further tested for toxin B following similar methods for detection of *C. diff* DNA by AuNPs but using specific toxin B (Ted B) primer (5-CAC GCC TGG AGA ATC TAT ATT TGT AGA AA-3).

RESULTS

Characterization of AuNPs

The SEM image (Figure 2) showed that the synthesized AuNPs prepared in our laboratory were well dispersed and spherical in shape. The extinction spectrum of the prepared AuNPs demonstrated a single peak in the visible region (400 to 700nm) with λ_{max} at 519 to 521 nm (Figure 3). The diameter of AuNPs was found to be 13±1 nm (Figure 4), as characterized by both SEM and Zeta sizer.

C. difficile gold nanoparticles assay prototype

Optimization of the AuNPs-based assay is affected by four main factors namely, concentrations of NaCl, AuNPs size, primer and the assay annealing temperature. In the positive samples, blue color indicates the presence of primers complementary to the *C. difficile* DNA sequence and this leads to the aggregation of AuNPs. On the other hand, red color indicates that the primer is free in the mixture that leads to stabilization of AuNPs and prevents their aggregation. Any minimal change in color from red to blue or purple is considered as a positive result (Figure 5).

Ninety six (96) out of 105 (91.4%) *C. diff* positive samples showed blue and 9 out of 105 (8.6%) showed a red color. The nine negative samples were retested and yielded same results (Figure 6). Reference strain *E. coli* ATCC 25922, *Bacteroides fragilis*, *Peptostreptococcus anaerobious* and *Campylobacter jejuni* tested negvative with our assay as expected (Figure 6B). Additionally, all twenty-nine ATCC strains of *Clostridium* species other than *C. diff* were also tested negative with AuNPs-based assay (red color) (Figures 6A, B and C) indicating high specificity.Control

Assay performance assessment

No cross-reactivity was observed between *C. difficile* and other bacteria tested (Figure 6). The AuNPs-based assay performance was then compared with commercially available RT-PCR (GeneXpert, Cepheid, USA), and yielded 91.4% positivity, all samples were positive with RT-PCR.

Gold nanoparticles assay to detect *C. diff* toxin B

The assay was then optimized to detect toxin B gene(*tcdB*) of *C. difficile*. All ninety-six samples positive

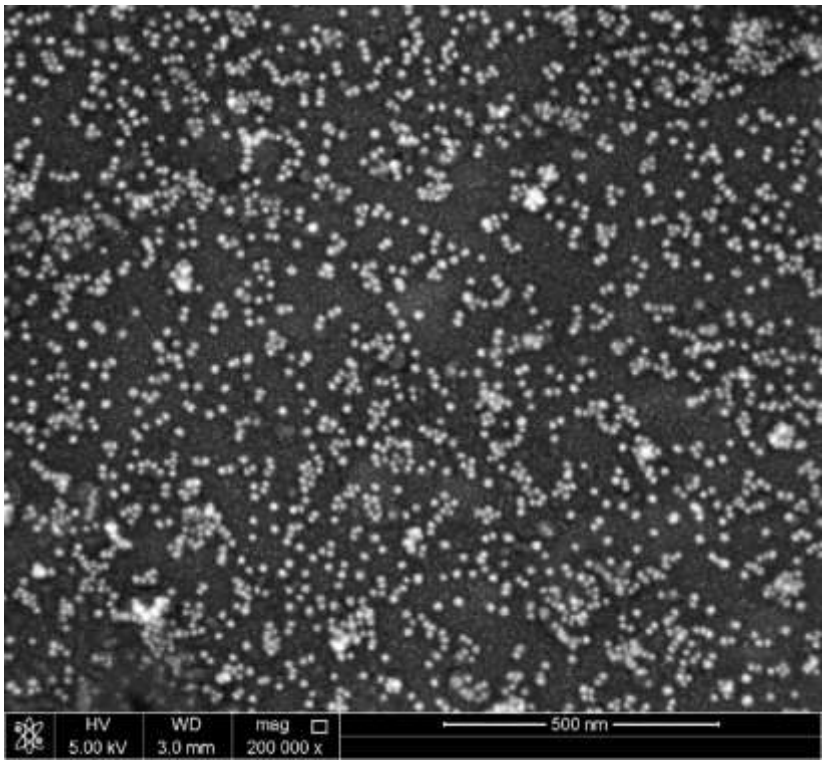

Figure 2. SEM image showing spherical AuNPs, with a size of 13±1 nm, prepared using citrate reduction method. The image was analyzed using the professional image analysis software (Clemex vision P.E 4).

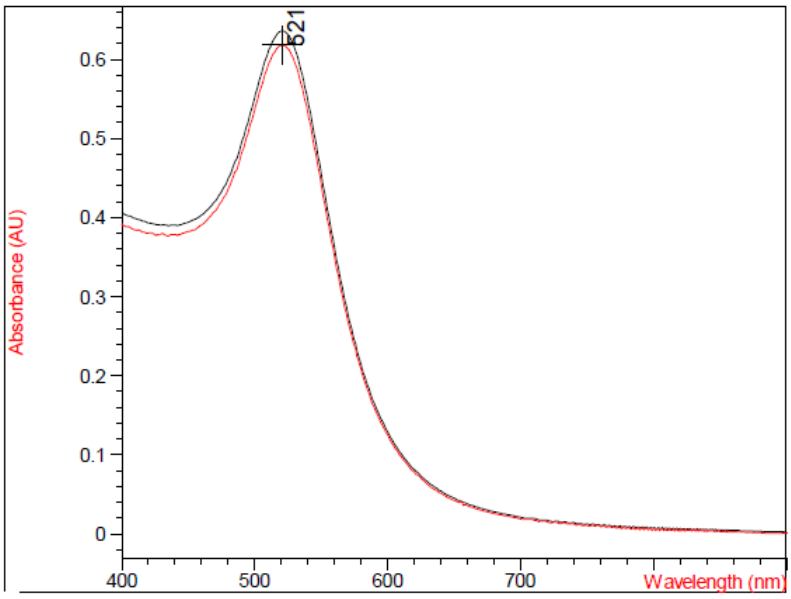

Figure 3. Extinction spectra of the prepared AuNPs solution. The peak is displayed in the visible light region with λ_{max} 518-521 nm.

with *C. difficile* prototype detection assay were also positive for toxin B gene (Figure 7). Equivalent result was obtained when using RT-PCR method. *C. perfringens* ATCC 13124 was used as a negative control in toxin B

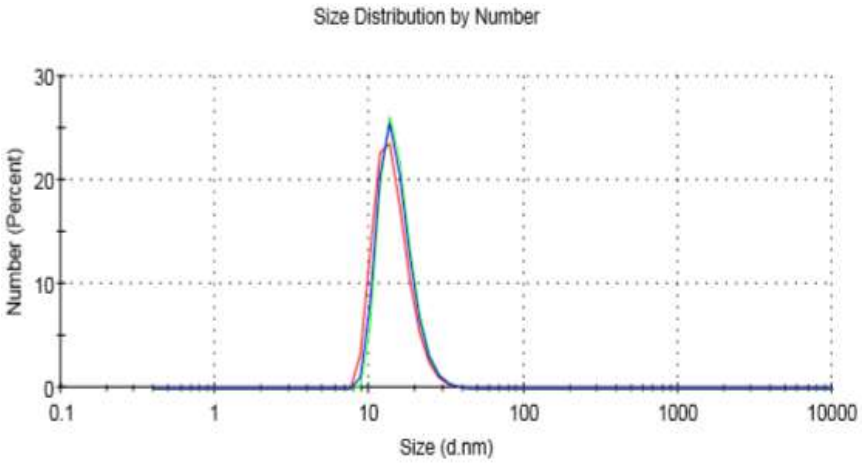

Figure 4. Dynamic light scattering analysis of size distribution of the prepared AuNPs using Zetasizer Nano ZSP (Malvern, UK). The peak represents the number (percent) of AuNPs that have size between 12 and 15 nm.

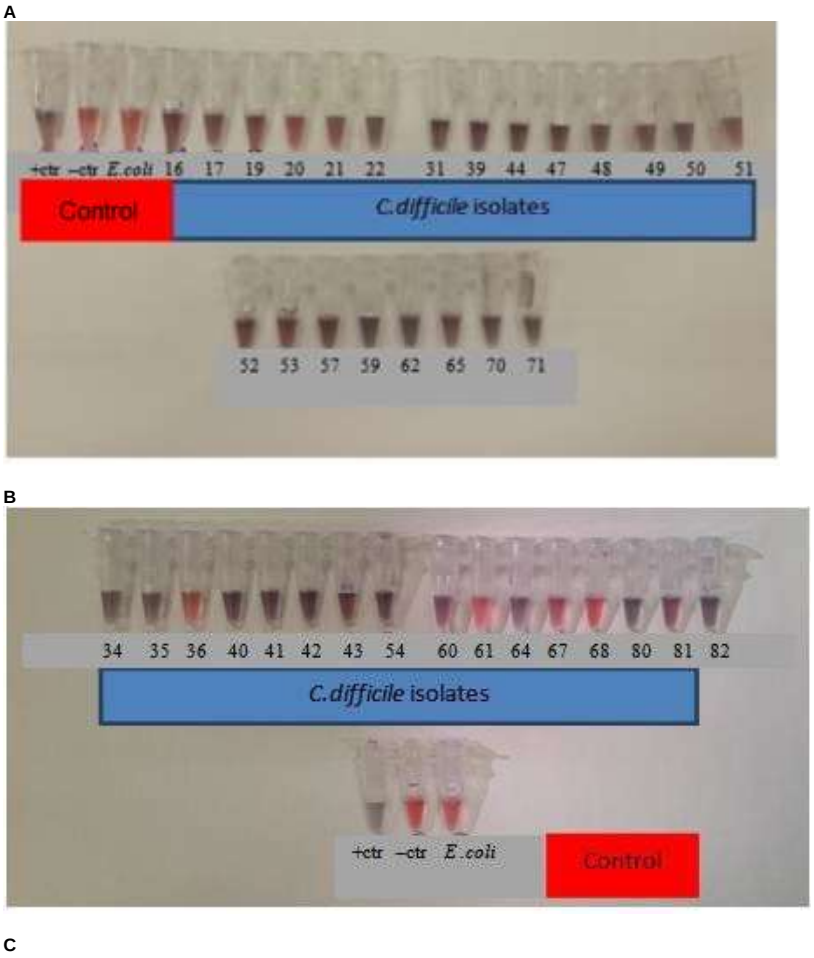

Figure 5. Qualitative AuNPs-based assay for *C. difficile* detection. Positive control (+ctr) = blue, Negative control (-ctr) = red. *E.coli* ATCC 25922 as negative control = red. A) All *C. difficile* samples were positive (change in color from red to blue). B) All *C. difficile* samples were positive (blue color) except 36, 61 and 68. C) Negative samples were retested and yielded the same results.

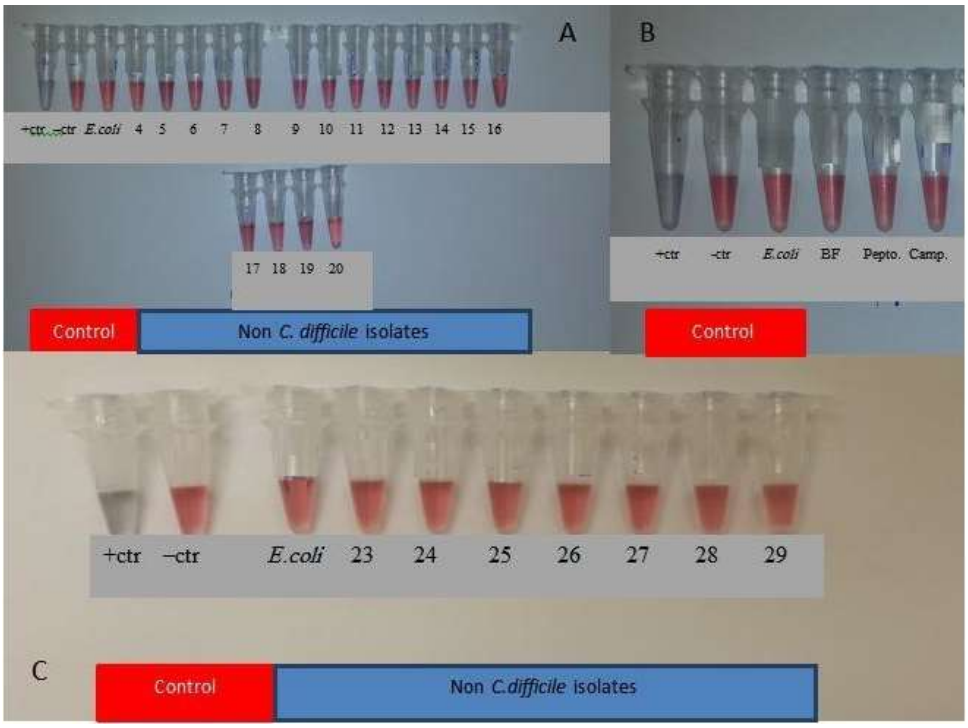

Figure 6. Qualitative AuNPs based assay for isolates other than *C. difficile* and other *Clostridium* species. Positive control (+ctr) = blue, Negative control (-ctr) = red. All samples were negative (red color). *E.coli* ATCC 25922 as negative control = red. BF: *Bacteroides fragilis*, Pepto: *Peptostreptococcus anaerobious* and Camp: *Campylobacter jejuni.*

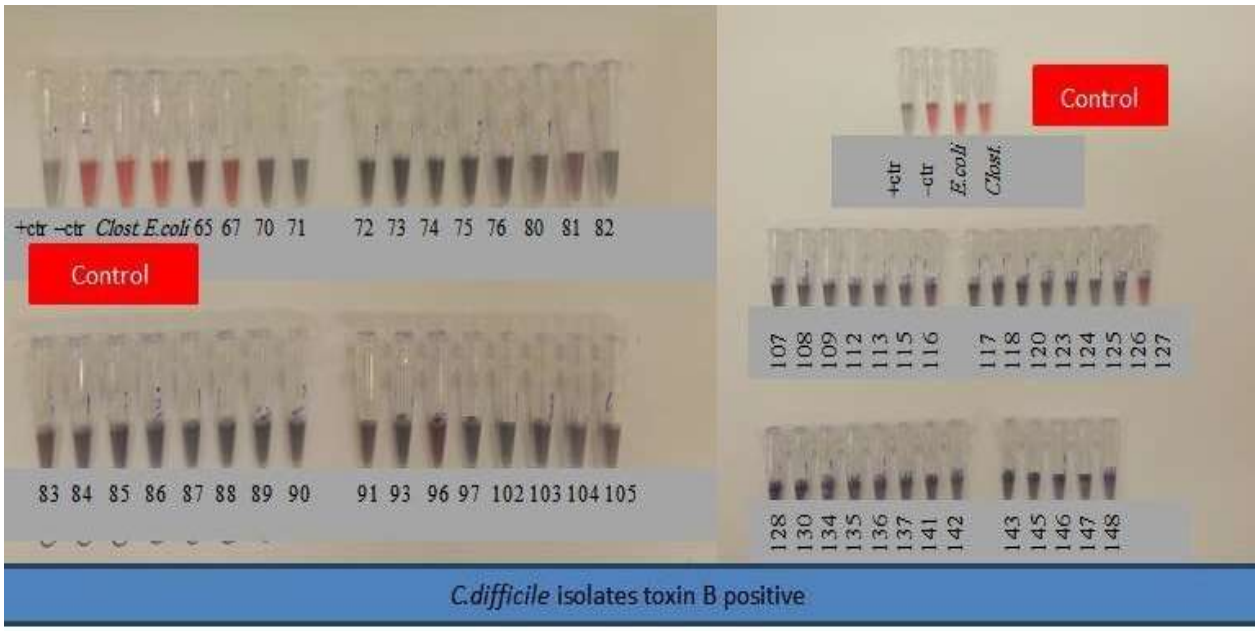

Figure 7. Qualitative AuNPs based assay for *C. difficile* toxin B detection positive control (+ctr) = blue, Negative control (-ctr) = red, Clost.: *C. perfringens* ATCC 13124 and *E. coli* ATCC 25922 as negative control= red. All samples were positive (blue color). AuNP: Gold nanoparticles; +ctr: Positive control; -ctr: Negative control; BF: *Bacteroides fragilis*; Pepto.: *Peptostreptococcus anaerobious*; Camp: *Campylobacter jejuni*; cdtA & cdtB: Binary toxins; Clost.: *Clostridum. Perfringens*; *C. diff*: *Clostridium difficile*

detection with AuNPs.

DISCUSSION

AuNPs-based methods have been established for detection of several pathogenic organisms such as *Mycobacterium tuberculosis* (MTB), Hepatitis C virus (HCV), methicillin resistant *Staphylococcus aureus* (MRSA) and others, owing to the high sensitivity and specificity of AuNPs- based assays for detection of nucleic acid targets (Shawky et al., 2010; Hussain et al., 2013). AuNPs study carried out by Shawky et al. (2010), showed sensitivity and specificity of 93.3 and 88.9%, respectively, in detecting HCV. Another study by Hussain et al. (2013). indicated 96.6% sensitivity and 98.9% specificity for the detection of *M. tuberculosis* complex (MTBC) and 94.7% sensitivity and 99.6% specificity for the detection of MTB. These results are consistent with our findings in this study which, demonstrated 91.4% sensitivity and 100% specificity as compared to RT-PCR. PCR assays include amplification steps and has always been considered more sensitive than enzymetic/cloromertic assays. Additionaly, the lower sensitivity of AuNPs- assay (91.4%) could be clarified by the following reasons, 1) possible loss of the targeted genes due to long cryoperservation period, 2) PCR does not cover the same region used to design the primers for PCR.

Different gold nanoparticles approaches have been explored to detect *C. difficile* and its toxins, such as using an aptamer biosensor with gold nanoparticles synthesized by *Bacillus stearothermophilus* (Luo et al., 2013). Additionally, Zhu et al. (2015) considered the use of single domain antibody coated gold nanoparticles as enhancer for *C. difficile* toxin detection by electro chemical impedance immunosensors. This confirms that using AuNPs with different methodologies resulted in excellent performance with high sensitivity and is in concordance with our findings.

This study revealed that AuNPs-based assay could be used to detect *C. difficile* and *C. difficile* toxin B from unamplified genomic DNA with detection limit of 35.5 ng DNA. The assay was highly sensitive, specific, rapid, simple and minimized the need for expensive and complex equipment. Cost effectiveness is one of the advantages of synthesis of AuNPs that was explored in this study. The approximate cost of 1 g of gold chloride is 220 US Dollars which is enough to conduct 40,000.00 assays, as compared to ~ 55 USD per test for RT-PCR, (GeneXpert, Cepheid, CA, USA). Additionally, the prepared gold nanoparticles have a long shelf life and can be stored at 4°C for one year or more. Moreover, this method has short turnaround time, only 15 to 20 min, after DNA extraction; consequently, the method can be utilized by many laboratories especially in low-income countries with low resources. This developed assay may improve the management of *C. difficile* infection by early isolation of infected patient to prevent horizontal transmission in health care facilities, and may lead to a more rational use of antibiotics, as the clinicians will rapidly obtain the clinical microbiology results.

Conclusions

In conclusion, this assay will have a crucial and great impact on clinical diagnosis in low-resources countries in terms of patient's management and infection control measures. Moreover, the assay can compete with RT-PCR since it has comparable performance results.

ABBREVIATIONS

CDI, *Clostridium difficile* Infection; **AuNPs,** gold nanoparticles; **EIAs,** enzyme immunoassays; **GDH,** glutamate dehydrogenase enzyme; **LSPR,** localized surface plasmon resonance; **SEM,** scanning electron microscope; **RT-PCR,** reverse transcription-polymerase chain reaction.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diversity and distribution of fungal communities within the hot springs of soda lakes in the Kenyan rift valley

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Fungi are highly diverse and versatile, with members growing under different environmental conditions including extreme environments. Although fungal communities in some extreme environments have been investigated in recent years, little is known about their structure and richness within the hot springs of soda lakes in Kenya. The aim of the study was to determine the biogeography and diversity of fungi from the hot springs of four Soda lakes. Water, sediment and microbial mat samples were collected in triplicates from Lakes Bogoria, Magadi, Elmenteita and Little Magadi in Kenya. 454-Pyrosequencing was used to sequence amplicons of Internal Transcribed Spacer (ITS) gene region of the total community DNA in order to explore the fungal community composition in twenty four samples collected. Sequences were analyzed using QIIME pipeline Version 1.8.0, while hierarchical clustering, non-metric dimensional scaling (NMDS) and diversity indices were carried out using the R programming language and the Vegan package. A total of 139,023 quality sequence reads were obtained from which, 2,179 operational taxonomic units (OTUs) were realized at 3% genetic distance. Three known phyla (*Ascomycota* [83.3%], *Basidiomycota* [15.8%], *Glomeromycota* [0.02%]) were identified. Richness, abundance and taxonomic analyses identified *Agaricomycetes* as the most abundant and diverse class within Basidiomycota. Sequences matching with *Ascomycota* had high affinities with seven known classes, with *Sordariomycetes* and *Dothideomycetes* being the most abundant and diverse classes. The most abundant OTUs showed the highest sequence similarity to *Cladosporium* sp., *Cladosporium cladosporioides, Pleosporales* sp., *Aureobasidium pullulans* and *Aspergillus oryzae.*

Key words: Hot springs, fungi, 454 pyrosequencing, diversity, extreme environments.

INTRODUCTION

Extreme environments like the hot springs, saline and/or alkaline lakes, deserts and the ocean beds are found in nature. They are believed to have harsh conditions unfit for normal life to exist (Satyanarayana et al., 2005).

Hypersaline environments are found in all continents such as Great Salt Lake, Utah, the alkaline soda lakes of Egypt (Wadi El-Natrun), the Dead Sea, the soda lakes of Antarctica, Big Soda Lake and Mono Lake in California (Cantrell et al., 2006; Grant and Sorokin, 2011). In Kenya, the soda lakes (Bogoria, Magadi and Elmenteita) found in the East African Rift Valley represent the major type of naturally occurring highly alkaline environments.

Soda lakes are alkaline with pH values often ranging between 9 and 12. They are characterized by high concentrations of carbonate salts, especially sodium carbonate and related salt complexes. Many soda lakes also contain high concentrations of sodium chloride and other dissolved salts, making them saline or hypersaline lakes (Gunde-Cimerman et al., 2000; Litchfield and Gillevet, 2002). These hypersaline and highly alkaline soda lakes are considered some of the most extreme aquatic environments on earth. Hot springs are scattered all over the globe. They are produced by geo-thermally heated groundwater (Kauze et al., 2006) with extreme temperatures ranging about 45°C and above (Bhavesh et al., 2004). Temperature is one of the most important factor controlling the activity and evolution of microorganisms.

Microbial communities can be found in the most diverse conditions of temperature, pressure, salinity and pH (Kumar et al., 2010), as they are not limited to specific environments. Fungi have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations (Vaupotic et al., 2008) or ionizing radiation (Dadachova et al., 2007), as well as in deep sea sediments. Fungi play vital roles in the ecosystem. They are essentially decomposers, symbionts and pathogens that live closely with bacteria, plants and animals. Despite their functional importance, diversity, distribution and ecology of fungi is much less studied compared to bacteria (Desprez-Loustau et al., 2007). Traditional culturing methods that rely on morphological and other phenotypic characteristics as the main criteria for fungal classification (Bartnicki-Garcia, 1987) are heavily biased towards fast-growing species. Many fungi have specialized growth requirements, so this approach recovers only a small proportion of the community sampled (O'Brien et al., 2005). Similarly, fruiting body collection is limited to the detection of species that frequently reproduce sexually unless long-term studies are conducted **(**Straatsma and Egli, 2001). Therefore, these traditional methods alone do not enable a reliable identification of fungi at lower taxonomic levels (Feau et al., 2009). Molecular taxonomy has partially solved this problem, allowing better classification of fungi species (Fávaro et al., 2011; Gehlot et al., 2012). Recent studies carried out using illumina sequencing revealed that the phyla Ascomycota and Basidiomycota were the dominant and diverse groups of fungi within the sediments and water samples collected from Lake Magadi and Little Magadi (Kambura et al., 2016). In order to comprehensively determine the fungal diversity within the hot springs of Kenyan soda lakes, the 454 amplicon pyrosequencing approach was used (Bates et al., 2011; Dumbrell et al., 2011), that is not selective and biased for specific microbial growth like the previously used traditional methods.

MATERIALS AND METHODS

Authority to conduct research

Permission to conduct research in Kenya was granted by the National Commission for Science, Technology and Innovation (NACOSTI). All other necessary documents to access and collect samples from the soda lakes were obtained from the National Environment and Management Authority (NEMA) and the Kenya Wildlife Services (KWS).

Study sites

This study was conducted on four hot springs of the Kenyan soda lakes. Lake Elmenteita is situated at 0º 27'S, 36° 15'E on the floor of the Kenyan Rift Valley at 1,776 m above sea level, some 20 km south-east of Nakuru town and has no direct outlet (Melack, 1988). The water temperatures in the lake range between 30 and 40°C, the alkalinity is high (pH above 9) with a high concentration of carbonates, chlorides and sulphates (Mwaura, 1999). Lake Bogoria is located at 0° 13′ 33″ N, 36° 05′ 41″ E and lies at altitude of 1,000 m above sea level. The lake waters are alkaline (pH 10.5) and saline (up to 100 g/L total dissolved salts). Around Lake Bogoria are some 200 hot springs with water temperatures ranging from 39 to 98.5°C. Nearly all these springs are very close to the lake or even inside the lake. Lake Magadi (saline and alkaline lake) is situated at 1° 52′S 36° 16′E and is approximately 100 km^2 in size, lying in a catchment of faulted volcanic rocks, north of Tanzania's Lake Natron. The hot springs with temperatures up to 86°C, discharge saline alkaline waters into the lake (Behr, 2002). Little Magadi (*Nasikie eng'ida*) is located at 1° 45' 00" S, 36° 17' 00" E and is about 40 km south of the Lake Magadi. Temperatures of the springs at Little Magadi measure between 81 and 83.6°C.

Measurement of physicochemical parameters

The geographical position of each site in terms of longitude, latitude and elevation was taken using Global Positioning System (GARMIN eTrex 20). During sampling, the temperature, electrical conductivity (EC), total dissolved solids (TDS) and dissolved oxygen (DO) of each sampling point were measured on site using Electrical Chemical Analyzer (Jenway - 3405), whereas the pH was measured with a portable pH-meter (Oakton pH 110, Eutech Instruments Pty. Ltd) and confirmed with indicator strips (Merck, range 5-10). Temperature was recorded at three distinctive

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Table 1. Summary of samples collected from the four soda lakes and their parameters.

Soda Lake	Latitude ºS	Longitude ºE	Elevation (m)	Sampling point	Sample label	Sample type	Temperature (°C)	pH	Dissolved oxygen (Mg/l)	EC (mS/cm)	TDS (mg/l)
Little Magadi	1° 43'08.9"	36° 16'15.8"	606	1	LM1	Mats	81.90	8.5	0.29	1	1
				3	LM3	Mats	76.30	8.5	0.01		
				2	LMb	Sediment	67.90	8.6	0.98		
				3	LMc	Sediment	76.30	8.5	0.01		
				1	LMd	Water	81.90	8.6	0.29		
Lake Magadi	2° 00′08.1 S	36° 13′53.0″	616	1	MM1	Mats	43.80	8.8	1.17		
				2	MM2	Mats	41.00	8.8	6.04		
				3	MM3	Mats	37.90	8.8	4.80		
				1	MMa	Sediment	43.80	8.8	1.17		
				2	MMb	Sediment	41.00	8.8	6.04		
				3	MMc	Sediment	37.90	8.8	4.80		
				1	MMd	Water	43.80	8.8	1.17		
Lake Bogoria	0°13′ 46.1″ N	36° 05′34.8″	1,000	1	B1	Mats	84.60	8.2	0.10		
				2	B2	Mats	77.70	8.3	0.23		
				3	B3	Mats	54.00	8.7	0.53		
				1	Ba	Sediment	84.60	8.2	0.10		
				2	Bb	Sediment	77.70	8.3	0.23		
				1	Bd	Water	84.60	9	0.10		
Lake Elmenteita	0º28'21.6″ S	36°15'27.9″	1,789	1	E1	Mats	45.00	8.7	2.67		
				2	E2	Mats	44.70	8.7	2.45		
				3	E3	Mats	33.80	8.8	3.12		
				1	Ea	Sediment	45.00	8.7	2.67		
				2	Eb	Sediment	44.70	8.7	2.45		
				3	Ec	Sediment	33.80	8.8	3.12		

points along the rivulets of each hot spring and assigned to all the sample types for that site.

Sample collection

Sampling was carried out on 8th to 11th July, 2014. Water samples were collected from the mouth of each hot spring (L. Magadi at 43.8ºC, pH 8.8, Little Magadi at 81.9°C, pH 8.6, L. Bogoria at 84.6ºC, pH 9.0 and L. Elmenteita at 45ºC, pH 8.7) in triplicates using one liter sterile containers. Wet sediments (500 g) and microbial mats (500 g) were collected from the floor of each rivulet in triplicates using sterile jam jars at three distinct points (rivulet point 1, 2 and 3) shown in Table 1 that differed in temperature (L. Magadi: 43.9, 41 and 37.9ºC; Little Magadi: 81.9, 76.3, and 67.9ºC; L. Bogoria 84.6, 77.7 and 54ºC, and L. Elmenteita: 45, 44.7 and 33.8ºC).

The samples were labeled properly and transported on dry ice in cool boxes to the laboratory at the Jomo Kenyatta University of Agriculture and Technology. Water for DNA extraction was filtered through a 0.22 µM Whatman filter paper using a water pump (model Sartorius 16824) and stored at -80°C. Pellets for DNA extraction were obtained from water samples by re-suspending the filter papers in phosphate buffer solution (pH 7.5), and centrifuging 5 mL of the suspension at 13000 rpm for

10 min.

DNA extraction

Total microbial DNA was extracted from 0.4 g of each sample of wet sediments and microbial mats in triplicates using phenol chloroform DNA extraction protocol as described by Sambrook et al. (1989). The microbial DNA extracted from triplicate samples were pooled during the precipitation stage, washed, air dried and stored at -20°C prior to PCR.

PCR amplification and 454 pyrosequencing

PCR amplification of the fungal ITS gene region of 18S rDNA from the microbial DNA was performed using ITS1 (5´TCCGTAGGTGAACCTTGCGG3´) and ITS4 (5´TCCTCCGCTTATTGATATGC3´) primers (White et al., 1990) with barcodes. Amplification proceeded in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with initial heating at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s and extension at 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. PCR products were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) and visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their DNA concentrations. The pooled and purified PCR products were used to prepare DNA library, according to the pyrosequencing protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA, MR DNA (www.mrdnalab.com, MR DNA Shallowater, TX, USA) utilizing the Roche 454 FLX titanium sequencing platform and reagents following the manufacturer's guidelines.

Processing of pyrosequencing data

Sequences were depleted of barcodes and primers using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Low quality sequences were identified by denoising and filtered out of the dataset (Reeder and Knight, 2010). Sequences that were < 200 base pairs after phred20- based quality trimming, sequences with ambiguous base calls and those with homopolymer runs exceeding 6 bp were eliminated. Sequences were analyzed by a script optimized for high-throughput data to identify possible chimeras in the sequence files, and all definite chimeras were depleted (Gontcharova et al., 2010). *De novo* Operational Taxonomic Unit (OTU) clustering was done with standard UCLUST method using the default settings as implemented in QIIME pipeline Version 1.8.0 at 3% genetic distance (Caporaso et al., 2010). Taxonomy was assigned to each OTU using nucleotide Basic local Alignment Tool (BLASTn) against SILVA SSU Reference 119 database at default e-value threshold of 0.01 in QIIME (Quast et al., 2013).

The diversity within each sample (alpha diversity) was evaluated using the observed species metric (count of unique OTUs in each sample), richness (Chao1), Shannon (diversity) and evenness were calculated in QIIME. Comparisons between community dissimilarity and environmental conditions were carried out using environmentally fitted non metric dimensional scaling (NMDS) plots (Minchin, 1987) using the Vegan package in R (Oksanen et al., 2012).

Statistical analysis

Alpha diversity indices (Shannon, Simpson, richness, observed species and Evenness) in each sample were calculated using vegan package version 1.16-32 in R software version 3.1.3 (R Development Core Team, 2012; Zhao et al., 2014). Community and Environmental distances were compared using analysis of similarity (ANOSIM) test, based upon Bray-Curtis distance measurements with 999 permutations. Significance was determined at 95% confidence interval ($p = 0.05$). Non metric dimensional scaling (NMDS) and hierarchical clustering were carried out using the R programming language (DeLong et al., 2006) and the Vegan package (Oksanen et al., 2012). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to genus using the taxa_summary.txt output from QIIME pipeline Version 1.8.0.

RESULTS

Sampling

Microbial mats, sediments and water samples were randomly collected at three different locations in the hot springs of the soda lakes in Kenya. The metadata collected before sampling included the geographical position of each site in terms of latitude, longitude and elevation, temperature, pH, electrical conductivity, total dissolved solids and dissolved oxygen. The various samples collected from the four soda lakes and their parameters are summarized in Table 1.

Estimators for diversity and species richness of fungal communities

For the 24 sequenced samples, five from Little Magadi, seven from L. Magadi and six each from both L. Bogoria and L. Elmenteita (Table 1), exclusion of low-quality and short sequence reads yielded 139,023 fungal ITS reads. Out of these, L. Elmenteita recorded the highest reads (51,913) while little Magadi had the lowest reads (25,958). Lake Magadi recorded 34,718 reads and L. Bogoria had 26,434 reads. The wet sediments, sample Ea at 45°C from L. Elmenteita, had the highest reads (14,929). Among the microbial mats, sample MM2 at 41°C from L. Magadi recorded the highest reads (10,574) followed by sample B1 at 84.6°C from L. Bogoria that had 8,204 reads while sample LM1 at 81.9°C from Little Magadi had 7,678 reads. Richness (S) estimated the sediments at L. Magadi, sample MMb at 41°C, to be the richest site with 633 species. The evenness (J′) value was closer to 0 with the highest value (0.258) recorded in sample MM1 at 43.80°C which revealed less distribution in abundance among species. Simpson (1/D) and Shannon's index (H') indicated the sediments in sample MMc at 37.90°C to harbor the most diverse taxa with 11.66 and 3.739 values respectively (Table 2).

Comparison of fungal communities between different sampling sites

Using a 3% dissimilarity cut-off for clustering, the reads

Table 2. Diversity indices computed on all OTU-based fungal taxonomic units.

Sample	No. of sequence reads		No. of OTUs per site	Richness (S)	Shannon (H')	Simpson (I/D)	Evenness (J')
	Before quality check	After quality check					
LM1	8285	7678	295	114	2.23	4.76	0.082
LM3	5088	4733		97	1.794	4.12	0.062
LMb	3922	3534		21	0.2	1.06	0.058
LMc	6281	5792		21	1.025	2.28	0.133
LMd	4498	4221		74	1.486	3.02	0.06
MM1	6490	5924	394	50	2.558	9.47	0.258
MM2	11385	10575		154	2.108	3.92	0.053
MM3	3005	2904		159	1.587	1.98	0.031
MMa	3883	3598		262	1.658	2.1	0.02
MMb	194	184		633	2.629	2.79	0.022
MMc	7463	6926		404	3.739	11.66	0.104
MMd	4944	4607		37	1.407	2.13	0.11
B1	8709	8204	294	116	2.342	4.13	0.09
B2	7168	6729		63	1.787	4.21	0.095
B3	2295	2106		26	0.938	2.02	0.098
Ba	1695	1590		83	1.911	5.2	0.081
Bb	6487	6004		41	0.409	1.14	0.037
Bd	1936	1801		52	0.817	1.4	0.044
E1	7535	6868	1196	172	2.248	3.77	0.055
E2	8864	8165		31	0.795	1.44	0.071
E3	14251	13084		51	1.469	2.66	0.085
Ea	15874	14929		6	0.192	1.07	0.202
Eb	6862	6422		125	2.082	4.23	0.064
Ec	2601	2445		35	1.013	1.87	0.079

were grouped into different operational taxonomic units (OTUs). The Fungal OTUs common to the four sampling sites were then presented using a Venn diagram to compare the relationships between the four communities (Figure 1). The numbers of fungal OTUs obtained for each prefecture were as follows: The hot spring at L. Elmenteita recorded the highest OTUs (1,196) while the one at L. Bogoria had 294, the least OTUs in this study. Lake Magadi hot spring had 394 OTUs and that of little Magadi had 295 OTUs. The hot springs at L. Bogoria and Little Magadi shared 87 OTUs, L. Elmenteita and L. Magadi shared 82 OTUs, L. Bogoria and L. Elmenteita shared 71 OTUs and Little Magadi and L. Magadi shared 95 OTUs. All the four sites (hot springs) shared 31 OTUs (Figure 1).

Fungal community composition and structure analysis

All analyzed sequences were classified into three known fungal phyla namely *Ascomycota*, *Basidiomycota*, *Glomeromycota* and *unclassified fungi* phylum. *Ascomycota* represented the most dominant and diverse phyla while *Glomeromycota* was the least dominant (Figure 2).

Ascomycota in hot springs of soda lakes

The phylum *Ascomycota* had a relative abundance of 100% in samples B3 at 54°C, LMc at 67.9°C, MM1 at 43.9°C and MMd at 43.9°C, 99% in samples LM3 at 67.9°C, LMd at 81.9°C, MM2 at 41°C, MM3 at 37.9°C, B1 at 84.6°C, Bb at 77.7°C and Eb at 44.7°C. Sample Ec at 33.8°C had the lowest relative abundance of 29.9%. In addition, all samples had relative abundances above 50% apart from Ec at 33.8°C (29%), E3 at 33.8°C (28%) and MMb at 41°C (0%). Seven classes were identified namely; *Dothideomycetes*, *Eurotiomycetes*, *Saccharomycetes*, *Sordariomycetes*, Pezizomycetes, *Leotiomycetes* and *Lichinomycetes* (Figure 3). *Dothideomycetes* and *Eurotiomycetes* were the most dominant (91.7 and 70.8%, respectively) and diverse as

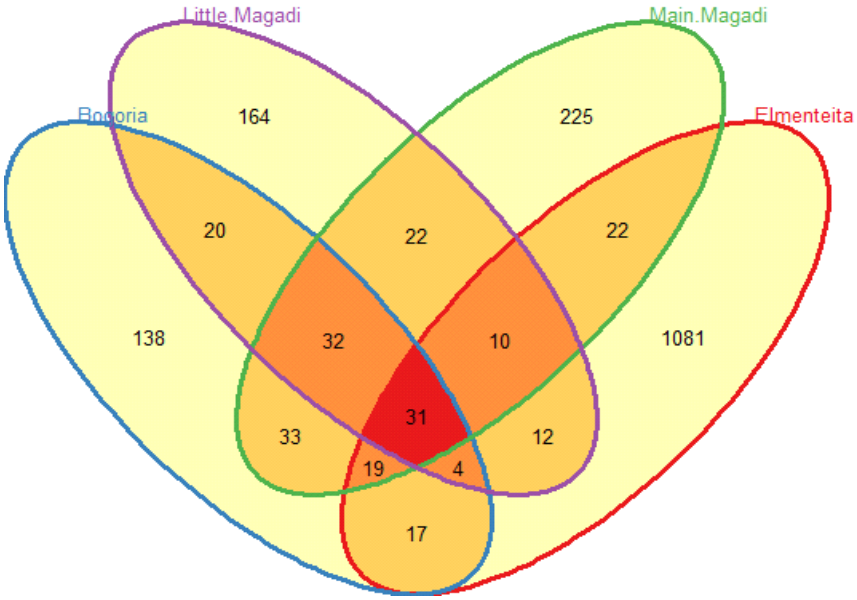

Figure 1. Venn diagram representing the number of fungal OTUs that are unique and shared between the samples from 4 different sampling sites. Main Magadi stands for Lake Magadi.

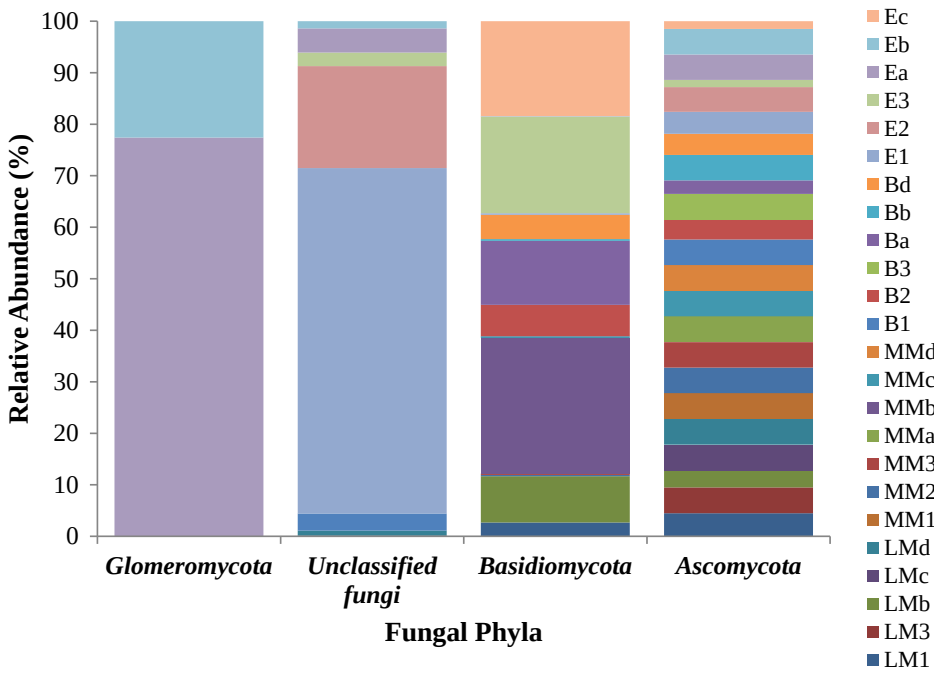

Figure 2. Taxonomic composition Analysis at phylum level.

they were present in 22 and 17 samples respectively, out of the 24 samples analyzed. The class *Dothideomycetes* was the most dominant in the water samples, representing 99.7% relative abundance in LMd at 81.9°C, 81.5% in MMd at 43.9°C and 37.3% in Bd at 84.6°C. In the microbial mats the class *Dothideomycetes* accounted for 54% in sample LM3 at 76.3°C, 98% in MM3 at 37.9°C, 89% in MM1 at 43.8°C, 98% in B3 at 54°C, 70% in B1 at

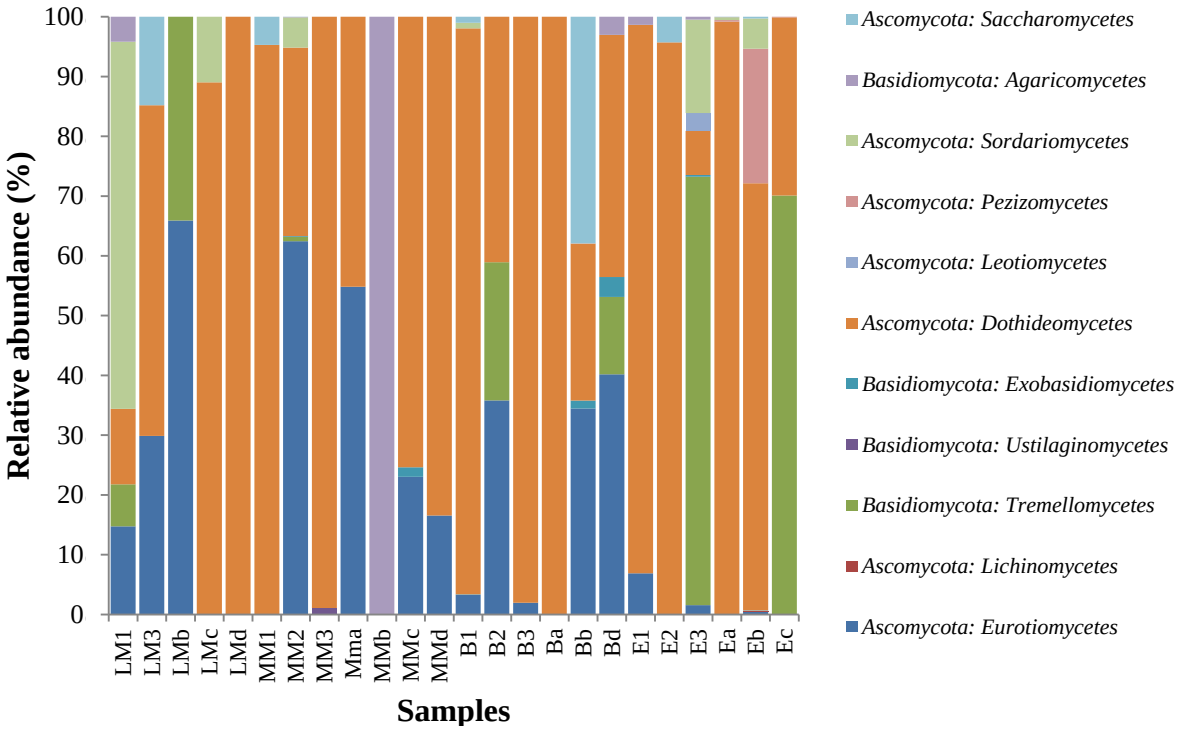

Figure 3. Taxonomic composition analysis at class level.

84.6°C, 91% in E2 at 44.7°C and 76% in sample E1 at 45°C. The percentages of the class *Dothideomycetes* in the wet sediments also varied. The highest value was recorded in sample Ea at 45°C with 85.6%. LMc recorded 69.9% at 76.3°C, Eb 60% at 44.7°C, 56% in sample MMc at 37.9°C and 52% in sample Ba at 84.6°C**.** The class *Eurotiomycetes* was also present in the water samples at 16% in MMd at 43.8°C, and 37% in Bd at 84.6°C (Figure 3).

Sequences from *Ascomycota* matched 16 known orders, with *Pleosporales* being the most diverse as it had six families affiliated to it. The most abundant fungal order was *Capnodiales* in little Magadi and lake Bogoria accounting for 99.8% in sample LMd at 81.9°C and 52.7% in sample Ba at 84.6°C respectively. The order *Dothideales* accounted for 95 % in sample MM1 at 43.9°C in lake Magadi and the order *Pleosporales* had a percentage of 91.6% in sample E2 at 44.7°C. Ascomycota phyla had twenty seven (27) families with *Davidiellaceae* being the most abundant and diverse family (99.8%) in sample LMd at 81.9°C, *Dothioraceae* (95%) in sample MM1 at 43.8°C, *Sporormiaceae* (91%) in sample E2 at 44.7°C, *Didymellaceae* (68 %) in sample MMd at 43.8°C, and *Onygenaceae* (55%) in sample MM2 at 41°C.

Out of the 62 genera detected in this study, the dominant genera were *Cladosporium* (99.79% in LMd, 52.79% in Ba), *Aureobasidium* (79.88% in MM1), *Aspergillus* (34.37% in Bb), *Penicillium* (65.90% in LMd), *Westerdykella* (91.62% in E2 and 79.40% in Ea), *Epicoccum* (42.76% in B1), *Debaryomyces* (12.67% in LM3), *Auxarthron (*16.18% in MMd) and among other varied percentages in many of the samples. Most genera were recorded from Lake Elmenteita in samples Ea at 45°C, Eb at 44.7°C and E3 at 33.8°C with varying percentages. The common fungal species were *Cladosporium* sp. (83.08% in LMd), *Cladosporium cladosporioides* (17.90% in Ba), *Pleosporales* sp. (86.07% in MM3), *Aureobasidium pullulans* (79.88% in MM1) and *Aspergillus oryzae* (35.02% in B2). *Cladosporium* sp. was present in 17 samples, *C. cladosporioides* in 14 samples, *Pleosporales* sp. in 13 samples A. *pullulans* in 13 samples and *Aspergillus oryzae* in 9 samples out of the 24 samples sequenced in this study.

Basidiomycota in hot springs of soda lakes

The distribution of *Basidiomycota phylum* was diverse within the samples, accounting for 100% in sample MMb at 41°C, 71% in sample E3 at 33.8°C and 70% in sample Ec at 33.8°C while the rest of the samples had relative abundances of below 50%. Sequences matching with *Basidiomycota* were affiliated to the following classes; *Agaricomycetes*, *Exobasidiomycetes*, *Tremellomycetes* and *Ustilaginomycetes* (Figure 3). *Tremellomycetes* was the most abundant and diverse, representing 70.6%

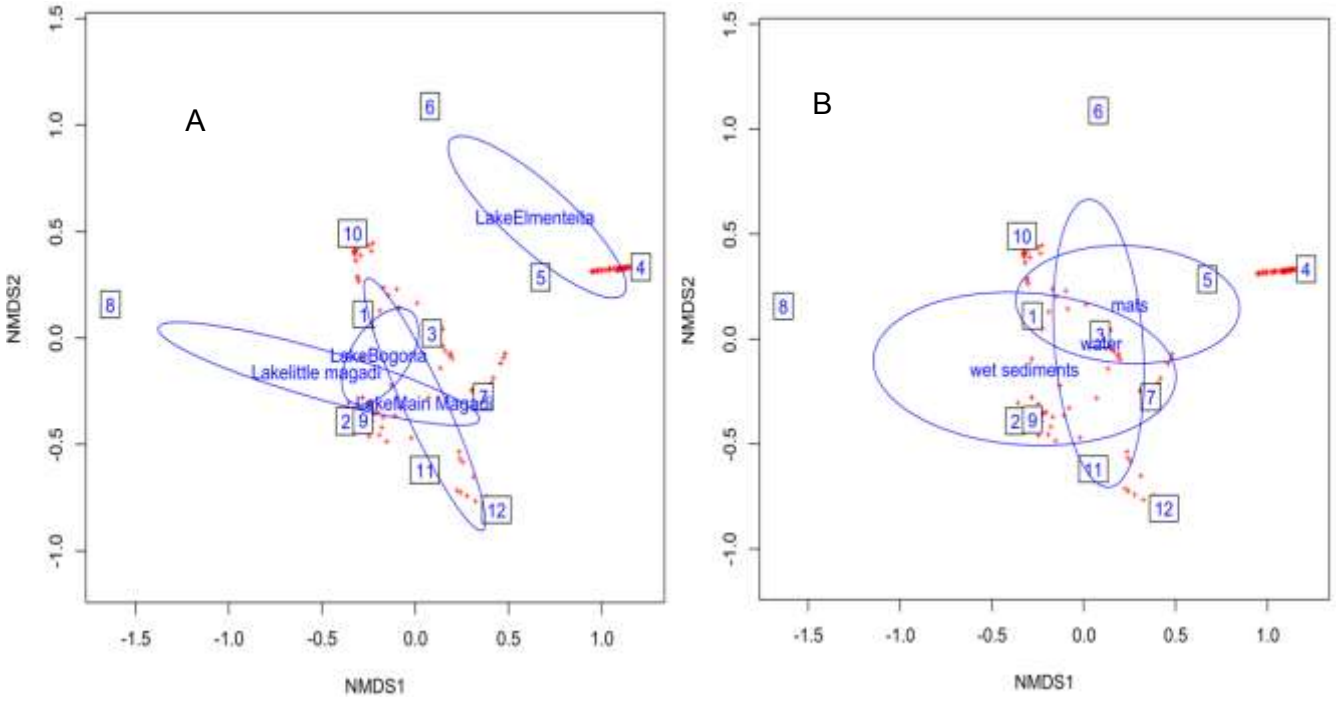

Figure 4. Non-metric dimensional scaling (NMDS) based on Bray Curtis dissimilarities between microbial compositions within various samples. A and B represents different lakes and sample types of each site respectively. References 1, 2 and 3; 4, 5 and 6; 7, 8 and 9; and 10, 11 and 12 represents mats, sediments and water samples from lake Bogoria, Elmenteita, Little Magadi and Magadi, respectively.

relative abundance in E3 at 33.8°C and 69.9% in sample Ec at the same temperature. *Agaricomycetes* recorded a relative abundance of 100% in sample MMb at 41°C. Sequences from *Basidiomycota* matched 9 known orders, with *Agaricales* being the most abundant recording 100% in sample MMb at 41°C. *Malasseziales* and *Tremellales* were the diverse orders as they were present in seven samples out of the possible 24 samples analyzed in this study. *Malasseziales* recorded 3.1% in sample Bd at 84.6°C while *Tremellales* had 70.5% in sample E3 and 70.05% in sample Ec both at 33.8°C.

At family level, OTUs were distributed in seven (7) fungal families with the most abundant (100%) belonging to *Lyophyllaceae* in sample MMb at 41°C, *Tremellaceae* (33%) in sample LMb at 67.9°C, *Lachnocladiaceae* (2.8%) in sample Bd at 84.6°C and *Malasseziaceae* (1.3%) in sample Bb at 77.7°C. Out of the 62 genera in this study, few were affiliated to *Basiodiomycota* phyla. This included *Termitomyces* (100%) in sample MMb at 41°C, *Rhodotorula* (47.2%) in sample Ba at 84.6°C, *Tremella* (32.95 %) in sample LMb at 67.9°C and *Malassezia* (1.3 %) in sample Bb at 77.7°C. Among the dominant species were; *Termitomyces* sp. (100 %) in sample MMb at 41°C, *Tremella aurantialba* (25%) and *Tremella encephala* (7.95%) both in sample LMb at 67.9°C, *Dioszegia hungarica* (1.7%) in sample MM2 at 41°C and *Malassezia globosa* (1.25%) in sample MMc at 37.9°C.

Glomeromycota and *unclassified fungi* in hot springs of soda Lakes

The phylum *Glomeromycota* was present only in samples Ea at 45°C and Eb at 44.7°C with 0.4 and 0.1% relative abundances respectively. The proportion of unclassified fungi was relatively small and was recorded in eight samples only with the highest relative abundance in sample E1 (13% at 45°C) and sample E2 (4% at 44.7°C) with the rest having relative abundances below 1% (Figure 2). Notably was the presence of order *Mortierellales* (0.07%) from the subkingdom *Incertae sedis* in sample Eb at 44.7°C.

Relationships between fungal communities and environmental variables

To test which environmental or geographical parameters correlated with community dissimilarity, a non-metric dimensional scaling (NMDS) plot was drawn for the sampling sites or lakes (Figure 4A) and sampling types (Figure 4B). These showed that the sample types had less influence on the fungal communities than temperature and/or the sampling sites, supporting environmental variation as the major determinant of fungal community structure. The Bray-Curtis clustering indicated a tendency of the communities to group by sample types

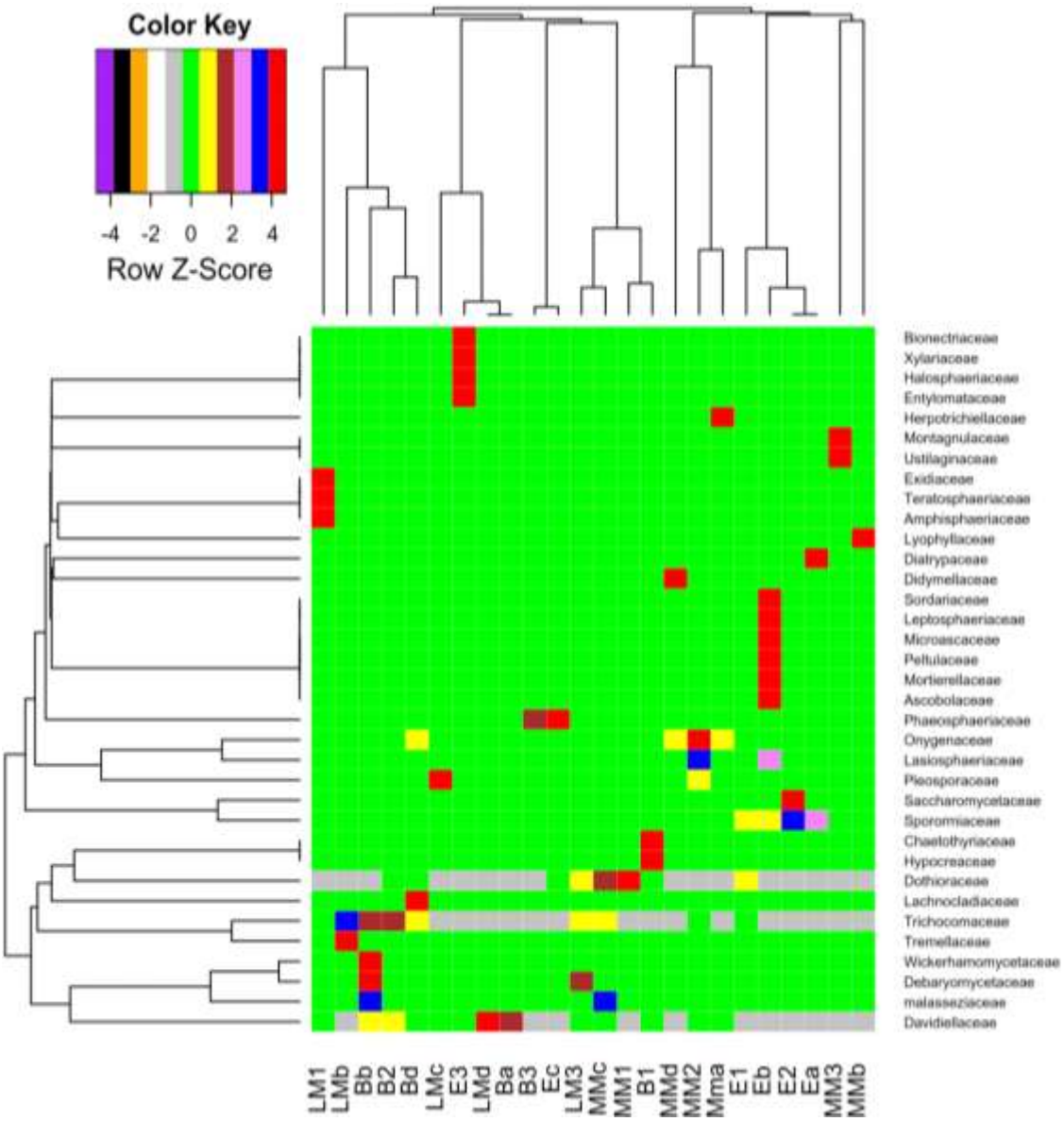

Figure 5. Hierarchical clustering of 18S rDNA samples collected from the four hot springs under investigation. Family level was chosen to be used in hierarchical clustering to assess the relationships between samples and taxa.

but differed in temperature and sampling sites. As the sampling sites were in different climatic regions, the changes in salinity, temperature and latitude appeared to have contributed to these differences.

Hierarchical clustering of various samples and fungal taxa at family level revealed the most dominant families to be *Entylomataceae*, *Halosphaeriaceae*, *Xylariaceae* and *Bionectriaceae* in microbial mats (Sample E3) at lake Elmenteita at 33.8°C and the families *Sordariaceae*, *Leptosphaeriaceae*, *Microascaceae*, *Peltulaceae*, *Mortierellaceae* and *Ascobalaceae* in wet sediments (Sample Eb) at the same lake at a temperature of 44.7°C. Lake Elmenteita was found to harbor the most dominant fungal taxa as compared to the other three. This could be attributed to the lower temperatures recorded during sampling that were favorable for the growth of fungi (Figure 5).

The phylogenetic diversity of fungi revealed in this study is relatively low compared to that of studies done on terrestrial or marine habitats like soils, plants and mangroves. Although a total of 2,179 OTUs were recorded in this study there was a distinct discrepancy in the number of OTUs per sampling site. Notably lake Elmenteita had the highest number of fungal OTUs at 1,196 followed distantly far by lake Magadi with 394 OTUs (Table 2).

DISCUSSION

There has been significant interest in finding life in extreme environments with high temperatures, salinity and pH like hot springs and soda lakes. To the best of our knowledge, this is the first report on the use of 454-pyrosequencing approach to investigate fungal diversity and community structure at different temperature gradients along the flow of hot springs of four Kenyan soda lakes. This ecological study aimed at examining fungal indicators of life in such extreme environments and being the first study to be done on hot springs of soda lakes, results here were compared to soils, hypersaline environments and sediments and not necessarily from soda lakes.

Sequences analyzed in this study revealed that the majority of recovered fungal sequences belonged to the Domain *Eukaryota* and comprised of the phyla *Ascomycota* (83.3%), *Basidiomycota* (15.8%), *Glomeromycota* (0.02%) and unclassified *fungi* (0.9%) which represented only a small proportion of the fungal communities. These results are in agreement with findings from Schadt et al. (2003) that found a large proportion of the members of the phylum *Ascomycota* in 125 cloned fungal sequences from Tundra soils. Similar studies on hypersaline environments done by Santini et al. (2015) on fungal communities found that 73% of the total OTUs were dominated by members of the phylum *Ascomycota* (52 to 100%) with minor contributions from the phylum *Basidiomycota*. Contrary to many reports on hypersaline environments, Singh et al. (2011) and Bass et al. (2007) found Basidiomycete yeasts to be the most dominant fungal forms in deep-sea environments. Generally members of the phylum *Ascomycota* occur naturally in all land ecosystems worldwide. Chytridiomycota, a phylum of fungi distinguished by having zoospores were evidently missing from the sequences obtained in this study. This observation is similar to the findings of a previous study on frequency and distribution of zoosporic fungi from moss covered and exposed forest soils that reported members of the phylum *Chytridiomycota* could also be found in freshwater or wet soils, with most species being infrequent and scarce to rare (Letcher and Powell, 2001; Letcher et al., 2004). Members of *Chytridiomycota* phylum are the simplest and most primitive Eumycota. Although many ecotypes are adapted to extreme environmental conditions, most members are ubiquitous in many ecosystems, especially in cool, moist soils and freshwater habitats that are rich in organic matter. This explains why the phylum *Chytridiomycota* could not be detected in saline environments with high temperatures and pH as these conditions proved harsh for their survival. However, a previous study that applied illumina DNA sequencing analysis of samples collected from the hot springs of Lake Magadi and Little Magadi showed members of Chytridiomycota to represent only a small proportion of the hot spring fungal communities (Kambura et al., 2016).

In this study, the most commonly identified classes within the phylum *Ascomycota* were *Sordariomycetes* and *Dothideomycetes*. The class *Sordariomycetes* had seven orders and nine families affiliated to it while the class *Dothideomycetes* had 3 orders and nine families. These data are consistent with a previous study on fungal communities in the deep-sea sediments of the Pacific Ocean (Xu and Luo, 2014) and that of diversity and distribution of fungal communities in marine sediments of Kongsfjorden, Svalbard, (Tao et al., 2015) that found the two classes *Sordariomycetes* and *Dothideomycetes* to be the most diverse and abundant. The classes *Sordariomycetes* and *Dothideomycetes* are so far the largest and most phylogenetically diverse classes within the phylum, *Ascomycota* (Kirk et al., 2008). The members are a heterogeneous group of fungi that subsist in majority of niches where fungi can be found.

The dominant genera were *Cladosporium, Aureobasidium, Aspergillus*, *Penicillium*, *Westerdykella, Epicoccum, Debaryomyces*, *Auxarthron* and *Malassezia*. The common fungal species were *Cladosporium* sp., *C. cladosporioides, Pleosporales* sp., *A. pullulans* and *A. oryzae*. Kambura et al. (2016) also found that *C. cladosporioides* species were unique to sediment samples that were collected at 83.6°C from the hot springs of Little Magadi. In the same study, Kambura et al. (2016) observed that sediment samples collected at 81°C had *Aspergillus* within the phylum *Ascomycota* as the most abundant species. This is also similar to previous studies in hypersaline waters of salterns that revealed different species of *Aspergillus, Penicillium* and diverse non-melanised yeasts (Gunde-Cimerman et al., 2005). Studies done by Razieh et al. (2015) indicated that most strains isolated from coastal waters of the southern Caspian Sea, belonged to the genus *Cladosporium*. Also, Damare et al. (2006) showed that the genera *Penicillium*, *Aspergillus* and *Cladosporium* were the most abundant in aquatic environments. Jaouani et al. (2014) isolated fungi belonging to the genera *Cladosporium, Alternaria, Aspergillus, Penicillium, Ulocladium, Engyodontium* and *Cladosporium cladosporioides* that were able to grow in media containing 10% of salt with an initial pH 10 from Sebkha El Melah, a Saharan Salt Flat in Southern Tunisia. A study done by Purnima et al. (2011) on the phylogenetic diversity of culturable fungi from the deep-sea sediments of the Central Indian Basin grouped the fungal microorganisms into seven (7) clusters belonging to *Aspergillus*, *Sagenomella* sp, *Exophiala* sp, *Capronia* sp, *Cladosporium*, *Acremonium* sp. and *Tritirachium* sp. Another study that used morphological and molecular techniques to identify a series of halotolerant fungi from hypersaline environments of solar salterns revealed 86 isolates of 26 species from salt ponds, which were identified as *C. cladosporioides*, nine *Aspergillus* sp, five *Penicillium* sp. and the black yeast *Hortaea werneckii* (Cantrell et al., 2006). In this study, most of the fungal

taxa such as *Aspergillus*, *Cladosporium* and *Penicillium* species are derived from terrestrial habitats like soils. This could be attributed to previous run off waters from adjacent areas that may have brought large numbers of terrestrial fungi in form of spores and fungal hyphae into the hot spring rivulets. Therefore, the fungi detected in this study may have originated from other environments and adapted to saline conditions, high temperatures and alkaline pH by developing effective strategies to tolerate stress in the hot springs. Due to this adaptability, the fungal groups could be candidates for exploitation in search for potential industrial products.

Conclusion

Study of fungi has been given little attention compared to other microorganisms like bacteria and archaea. Therefore, the findings in this study will be of high significance in the field of mycology. The results obtained using high-throughput analysis indicate that sediments, mats and water from the studied hot springs of soda lakes in Kenya are important niches that harbor unexpectedly high richness of fungal species, most of which possibly originated from terrestrial environments. However, the ecological roles of these fungi and their adaptive mechanism remain poorly investigated. It is also unclear if these fungi are actively growing in these environments or being dormant propagules (spores) that are washed into the sediments, microbial mats and water during the rainy seasons. Therefore, a combination of different technologies including traditional culture-based method, metagenomics, metatranscriptomics and meta-proteomics may help to answer these pending questions and may reveal the functions of the genes present in these extreme environments. The use of these technologies will have a huge impact on the functions of fungal communities in the ecosystem of Kenyan soda lakes and may also serve as a useful community model for further ecological and evolutionary study of fungi in these extreme environments.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ITS, Internal transcribed spacer; **OTUs,** operational taxonomic units; **DNA,** deoxyribonucleic acid; **NMDS,** non-metric dimensional scaling**; QIIME,** Quantitative Insights into Microbial Ecology.

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