High-throughput sequence analysis of bacterial communities in commercial biofertiliser products marketed in South Africa: an independent snapshot quality assessment

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Abstract

The genetic and predicted functional diversity of bacterial communities in 12 commercial biofertiliser products were evaluated using high-throughput sequencing of the 16S rRNA gene. Proteobacteria, Firmicutes and Bacteroides dominated the bacterial communities, with the genera *Pseudomonas*, *Lactobacillus*, *Bacillus*, *Bradyrhizobium* and *Rhizobium* being prevalent. The manufacturer-specified species were detected in relatively high abundance in two of the products while a few or none of the specified species were detected in some products. A number of unspecified microbes were detected, including potential human and crop pathogens such as *Alcaligenes*, *Clostridium*, *Escherichia–Shigella* and *Proteus*. The functional prediction unravelled high prevalence of enzyme-coding genes such as nitrogenase, NifT, alkaline phosphatase and reductases of nitric oxide, nitrate and nitrite which contribute to nitrogen-fixation, phosphorus solubilisation and degradation of nitrates and nitrites. In addition, toxins such as leukocidin/hemolysin and colicin V protein that cause product quality damage were highly predicted in over 67% of the products. Overall, high-throughput sequence analysis of bacterial communities in biofertiliser products revealed that majority of the products were of poor quality. This result justifies the need for regular quality assessment and improvement in quality control systems during biofertiliser formulation.

Keywords Bacterial diversity · Biofertiliser · High-throughput sequencing · Predicted functional diversity · Quality assessment

Introduction

The increasing demand for a safer environment, cleaner technologies and organic farm produce has necessitated a shift from the use of chemical fertilisers to a more sustainable

and ecologically friendly soil nutrient management system (Bashan 1998; Malusà et al. 2016). Lately, the application and development of biofertilisers, as a sustainable alternative to chemical fertilisers, have gained prominence (Bhardwaj et al. 2014).

Biofertilisers are composites of various functionally beneficial microbes, including nitrogen-fixing *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium*, as well as phosphate-solubilising *Pseudomonas*, *Bacillus* and *Enterobacter* (Raimi et al. 2017). Species of *Azotobacter*, *Azospirillum*, *Serratia* and *Alcaligenes* have also been incorporated into several biofertiliser products. The microbial species in biofertilisers improve the availability of soil nutrients, including phosphorous, zinc, iron and copper, thereby improving plant-nutrient uptake (Han and Lee 2005). Some of these microbial species also produce phytohormones such as indole acetic acid (IAA), cytokinin, and gibberellin, which enhance plant growth and development (Adesemoye et al. 2008; Mehta et al. 2016). In addition, the microbial species in biofertiliser produce antimicrobial metabolites,

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toxins and siderophore that suppress soil-borne pathogens which compete for soil nutrients or niches (Herrmann et al. 2015; Sayyed et al. 2010).

However, product quality parameters such as high level of contamination, low shelf life and low-quality microbial content have contributed to the low economic advantage and poor perception of biofertilisers by end users—the farmers (Malusá and Vassilev 2014). The quality of biofertiliser products is a crucial determinant of its effectiveness when applied in the field. To a great extent, field effectiveness of a biofertiliser product is a function of the genetic diversity and functional attributes of its microbial consortium. According to Herridge et al. (2002), if the biofertiliser quality is poor, then everything about its application is inconsequential. Several biofertiliser-quality-assessment studies have observed that the specified microbial contents of biofertiliser products are either not present or when present, the viable cell counts are below the industry standard (Lupwayi et al. 2000; Olsen et al. 1995). For example, Olsen et al. (1995) reported about 98% of the biofertiliser tested in North America had more microbial contaminants than the stipulated rhizobia cells; in particular, three of the products contained approximately 1000 times more microbial contaminants than the rhizobia contents. Unfortunately, most microbial contaminants found in biofertiliser products are known pathogens of humans and plants. In a study by Herrmann et al. (2015), over 84% of contaminated biofertilisers had microbial contaminants that were either human, animal or plant pathogens. Some of the pathogenic contaminants such as *Agrobacterium* sp., *Staphylococcus* sp., *Stenotrophomonas* sp., *Cuprivadus* sp., *Enterobacter* sp., have been widely reported in different studies (Olsen et al. 1996; Herrmann et al. 2015). Therefore, the assessment of microbial communities of biofertiliser products is important for ascertaining quality and potential field efficiency.

The assessment of bacterial communities of biofertilisers has been carried out in different studies using culture-based techniques (Deaker et al. 2011; Herrmann and Lesueur 2013) that are well-known to underestimate community diversity. Given the limitations of microbial cultivation and the estimated 99% of yet uncultivable microbial species, culture-based techniques are highly inadequate for analysing and processing complex environmental samples, especially for large-scale studies (Christine 2004; Sanger et al. 1977). In recent years, the advent of next-generation sequencing (NGS) technologies has enabled the in-depth analysis of microbial communities without a need for a cultivation step (Ezeokoli et al. 2018; Mashiane et al. 2017). Next-generation sequencing is a reliable and widely used technique in analysing microbial communities of several environments (Mashiane et al. 2017; Van Wyk et al. 2017; Tyx et al. 2016).

Presently, several biofertiliser products are available commercially in South Africa, including imported and locally

manufactured products. Although regulatory frameworks and quality guidelines are still being drafted by the South African department of Agriculture, Forestry and Fisheries (DAFF)—the organisation with a mandate to regulate agro-based products—there are presently no comprehensive quality control guidelines to ascertain the integrity of commercial biofertiliser products in South Africa. As an independent assessment of biofertiliser quality, this study was supported by DAFF to investigate in a snapshot (one-time), the quality of biofertiliser products in South Africa. For the assessment, high-throughput sequencing of the 16S rRNA gene on the Illumina MiSeq next-generation sequencer was employed. It is envisaged that information obtained from this study will contribute to the growing empirical evidence for a need to improve quality control systems for commercial biofertiliser production in South Africa and beyond.

Materials and methods

Biofertiliser samples

Biofertiliser products commercially available in the South African market were randomly sampled ($N=12$) for this study. The sample pool included both single-strain and mixed-consortium biofertiliser types. Information on the types and form of the biofertiliser products are provided in supplementary materials (Table S1). Samples were collected without compromising the integrity of the contents and all analyses were performed aseptically. Samples were stored at 4 °C or under room temperature as stipulated by the manufacturer prior to high-throughput sequence analysis of bacterial communities. Samples were analysed within a week of collection.

DNA extraction

Total genomic DNA of microorganisms in biofertiliser samples was extracted from 0.25 g (for solids) or 200 µL (for liquids, after centrifugation at 10000 rpm for 5 min) aliquots using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., California, USA) according to the manufacturer's protocol. Integrity and quantification of extracted DNA were verified by agarose gel electrophoresis and fluorometric quantification (Qubit 2.0 fluorometer, Invitrogen, CA, USA), respectively.

16S rRNA gene library preparation and high-throughput sequencing

The hypervariable V3–V4 region (approximately 460 bp) of the 16S rRNA gene was amplified in a polymerase chain reaction (PCR) using region-specific primers 341F

(5'-CCTAC GGGNGGCWGCAG-3') and 805R (5'-GAC TACHVGGGTATCTAATCC-3') (Klindworth et al. 2013) and following the method described by Van Wyk et al. (2017). The 5'-end of forward and reverse primer sequences contained Illumina forward and the reverse adapters (Illumina Inc., California, USA), respectively. Partial 16S rRNA amplicons were further processed and sequenced on an Illumina MiSeq sequencer by following the steps described in the MiSeq 16S library preparation guide (Illumina n.d.).

Bioinformatics analyses

Demultiplexed sequence reads were analysed for quality using FastQC (Babraham Bioinformatics, UK; <https://www.bioinformatics.babraham.ac.uk/index.html>) and PANDAseq was used to assemble forward and reverse reads, and eliminate reads with ambiguous bases and spurious read length (Masella et al. 2012). Thereafter, assembled reads were binned into operational taxonomic units (OTUs) at 97% 16S rRNA gene sequence similarities using the open-reference OTU-picking strategy in the Quantitative Insight into Microbial Ecology (QIIME) software (Caporaso et al. 2010). For OTU picking and taxonomic assignment, sequences were aligned against the SILVA rRNA database (SILVA 128 release) (Quast et al. 2012) with usearch61 (Edgar et al. 2011). Because sample characteristics varied for each biofertiliser product and comparisons were meaningless, the OTU count table was not rarefied (normalised) to even depth prior to subsequent analyses. The generated OTU count table was taxonomically summarised in QIIME, while the computation of the alpha (α) and beta (β) diversity measures was done using a combination of the vegan and ape packages of R software version 3.4.4 (R Core Team 2013).

Predicted functional analysis of bacterial communities

The functional abilities of the bacterial community in biofertiliser samples were predicted using the Tax4Fun package (Abhauer et al. 2015) in R software (R Core Team 2013). In summary, the obtained OTU table from the SILVA rRNA database alignment was transformed into a functional or metabolic profiles by the Tax4Fun package with the aid of a pre-computed association matrix of prokaryotic Kyoto Encyclopedia for Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) (Abhauer et al. 2015; Kanehisa et al. 2013). BRENDA (<https://www.brenda-enzymes.org/>) (Placzek et al. 2016) and KEGG orthology expression (Kanehisa et al. 2013) were used to obtain information on predicted enzyme-coding genes. For this study, the focus was on selected genes involved in the cycling and mineralization of nutrients in the soil.

Data accessibility

The raw sequence reads obtained in the present study have been deposited in the Sequence Read Archive of the National Centre for Biotechnology Information. (<https://www.ncbi.nlm.nih.gov/sra>) under the BioProject accession number PRJNA483344.

Results

Operational taxonomic units diversity

The number of sequence reads generated and selected for alpha-diversity measures are summarised in Supplementary Table S2. A total of 2,647,214 sequence reads were initially obtained from all samples (Table S2). After quality filtering, 2,186,464 high-quality reads were obtained and subsequently assigned to operational taxonomic units (OTUs). The rarefaction analysis of detected sequences showed sufficient sampling efforts (Supplementary Fig. S1). In total, 5791 OTUs were detected from all samples (data not shown). Amongst samples, CB1L had the highest number of observed OTUs, while CB2S had the least number of observed OTUs (Table S2). Similar trends (as with observed OTUs) in estimated richness (chao1), Shannon–Weiner and Simpson's indices were observed (Table S2). In general, OTU abundances detected in all the biofertiliser samples were a close estimation of the true OTUs diversity as indicated by the calculated goods coverage of approximately 1 (100%).

The diversity of bacterial phylotypes

Bacterial phylotypes detected in all samples spanned 35 phyla, 92 classes, 222 orders, 453 families and 1030 genera with a classifiable read (at each taxa level) of 100%, 88%, 79%, 69% and 66%, respectively. The relative abundance of phyla taxa in the biofertiliser samples differed across the different products with Proteobacteria being the major dominant phylum in all the products except in samples CB3L and CB4L where Firmicutes occurred at 98% and 97%, respectively (Fig. 1). Other dominant phyla above 1% relative abundance included Acidobacteria, Actinobacteria, Bacteroides and Chloroflexi. Proteobacteria was more predominant in the single-strain products than the consortium products, occurring with the highest abundance of 96% in CB12L (Fig. 1).

At the family taxa level, consortium-strain products were more diverse than the single-strain products, with an average of 11 families per consortium product and 6 families per single-strain products. The most dominant families were Lactobacillaceae, Pseudomonadaceae, Rhizobiaceae,

Fig. 1 Relative abundance of bacterial phyla in biofertiliser products

Bradyrhizobiaceae, Enterobacteriaceae and Clostridiaceae (Fig. S2). A high dominance of Lactobacillaceae was observed in samples CB3L and CB4L, while Enterobacteriaceae was the most abundant family detected in CB10L, CB11L and CB12L. Samples CB8L, CB9L and CB1L were dominated by Pseudomonadaceae, Clostridiaceae and Methylocystaceae, respectively, while samples CB5S and CB2S, which are carrier-based products predominantly contain Bradyrhizobiaceae and Rhizobiaceae, respectively (Fig. S2).

Of the total 1030 detectable genera, only 63 genera occurred at a minimum of 1% relative abundance in at least one sample (Fig. 2). Of these, the genera *Methylocystis*, *Parvibaculum*, *Pusillimonas*, *Pelagibacterium*, *Paucimonas*, *Salinihabitans*, *Pigmentiphaga*, *Devosia*, *Thioalkalispira*, *Proteiniphilum*, *Flavobacterium*, *Sphingomonas*, *Chryseobacterium* and *Petrimonas* were detectable only in sample CB1L, *Cellulosimicrobium* and *Rhizobium* occurred in CB2S, *Brevibacillus* and *Stenotrophomonas* were observed in CB4L while *Providencia*, *Serratia*, *Rahnella* and *Ewingella* occurred in only CB12L (Fig. 2). Other genera detected in a single product included *Pediococcus* (sample CB3L), *Bradyrhizobium* (sample CB5S), *Lactococcus* (sample CB8L), *Kluyvera* (sample CB10L) and *Enterococcus* (sample CB8L). Similarly, *Cronobacter*, *Pantoea* and *Tyzzerella* 3 were observed only in sample CB11L. Of all the genera, *Bradyrhizobium* had the highest relative abundance of 94%, followed by *Lactobacillus* with 91% and 73% relative abundance in samples CB3L and CB4L, respectively (Fig. 2). Similarly, *Rhizobium* had a high relative abundance of 76%

in sample CB2S while *Pseudomonas*, though occurring in all the samples, had high relative abundance in only samples CB8L (69%) and CB12L (30%), respectively (Fig. 2).

Quality assessment: manufacturer-specified composition versus detectable phylotypes

The detectability of manufacturer-specified and -unspecified bacteria in biofertiliser samples was used to infer product quality. The manufacturer specified species and those detected (based on $\geq 1\%$ relative abundance per sample) in biofertiliser products are presented in Table 1. Taking into cognizance the criteria for biofertiliser quality and limitations of the NGS technique, we devised a somewhat relaxed operational scoring system in which products were categorised as high quality (all the specified species and total relative abundance of unspecified species $\leq 10\%$ detected), medium quality ($\geq 50\%$ of the specified species and a total relative abundance of unspecified species $\leq 50\%$ detected), low quality ($< 50\%$ of specified species and a relative abundance of unspecified species $> 50\%$ detected) and poor quality (specified species not detected). The genus taxa level was used to define a “species” because the taxonomic assignment of NGS analysis beyond this taxonomic rank may not be absolutely reliable. The products quality ranged from high (8.3%), medium (8.3%), low (25%) and poor-quality (58.3%) (Table 1). In addition, one or more unspecified bacteria herein referred to as microbial contaminants were detected in all the samples (Table 1). For instance, sample CB5S,

Fig. 2 Relative abundance of bacterial genera in biofertiliser products

had the specified species, *Bradyrhizobium* at 94% relative abundance and only one unspecified species, *Nosocomiicoccus*, at 1.2% relative abundance. Similarly, CB2S had the specified species, *Rhizobium*, at approximately 76% relative abundance with unspecified microbes: *Cellulosimicrobium*, *Nocardioides* and *Promicromonospora* detected at a relative abundance of 11.4%, 7.2% and 1.0%, respectively. Therefore, CB5S and CB2S (rhizobial products, see Table S1) were regarded as high and medium quality products, respectively (as defined in the present study), because they contained the specified bacteria at higher relative abundance but with a relatively lower abundance of unspecified species. Aside from being carrier-based products, samples CB5S and CB2S are also imported products. On the other hand, sample CB4L a plant growth promoting rhizobacteria (PGPR) product was regarded as a low-quality product because it had the expected microbe, *Bacillus*, at a lower relative abundance (14.7%) than the unspecified species (83.8%) (*Lactobacillus*, *Brevibacillus* and *Stenotromonas*).

Also, consortium (mixed) products CB1L and CB3L were categorised as low-quality biofertilisers. In sample CB1L with five specified bacteria genera, only phylotypes *Pseudomonas* (1.4%) was detectable at $\geq 1\%$ relative abundance while the unspecified species were observed at a much higher prevalence. Similarly, of the four bacterial species specified in CB3L, only one species including (relative abundance in parenthesis) *Lactobacillus* spp. (91%) were observed, while unspecified species including *Nosocomiicoccus* spp. (1.7%) and *Pediococcus* spp. (4.3%) were also detected (Table 1). Other samples including CB6L, CB7L,

CB8L, CB9L, CB10L, CB11L and CB12L, which have none of the specified species detected but containing only the unspecified microorganisms were regarded as poor-quality products.

Generally, the results showed the detection of unspecified microbes at different levels of abundance in the samples. Of all the 125 unspecified phylotypes recorded at $\geq 1\%$ relative abundance (at the genus taxa level), an average of 9 and 11 species occurred in single-strain and consortium-strain products, respectively. The high and medium-quality products being a rhizobia single-strain product had less of the unspecified species while the poor-quality products, especially the other PGPR and the free-living nitrogen-fixing bacteria products recorded high unspecified species.

Predicted functional profile of the biofertiliser bacterial community

A total of 6564 KEGG Orthology groups (KO functional orthologs) were obtained from the imputed metagenomes of all the biofertiliser samples (data not shown). Of these, only genes coding for enzymes directly involved with microbial growth and metabolism as well as potential product field functions were focused on. Generally, predicted N-fixation genes such as Nif-specific regulatory protein, nitrogenase molybdenum-iron protein alpha- and beta-chain, and nitrogenase iron protein NifH were relatively high in most of the samples except in samples CB3L and CB4L, while nitrogenase fixation protein NifT and nitrogenase were less abundant in all the samples (Fig. 3). In addition, denitrification

Table 1 Expected and detected bacterial species in biofertiliser products based on partial 16S rRNA gene analysis

Sample code	Manufacturer-specified bacterial genus ^a	No. of genus expected ^a	Observed NGS sequences (OTUs) at above 1% relative abundance	No. of unspecified genera	Quality score ^c
CB1L	<i>Enterobacter</i> , <i>Stenotomonas</i> , <i>Bacillus</i> , <i>Rhizobium</i> , <i>Pseudomonas</i>	5	<i>Pseudomonas</i> ^b , <i>Chryseobacterium</i> , <i>Pigmentiphaga</i> , <i>Proteiniphilum</i> , <i>Devosia</i> , <i>Pusillimonas</i> , <i>Dysgonomonas</i> , <i>Salinhabitans</i> , <i>Flavobacterium</i> , <i>Paucimonas</i> , <i>Sphigomonas</i> , <i>Methylocystis</i> , <i>Thioalkalispira</i> , <i>Parvibaculum</i> , <i>Pertimonas</i> , <i>Pelagibacterium</i>	15	Low
CB2S	<i>Rhizobium</i>	1	<i>Rhizobium</i> ^b , <i>Cellulosimicrobium</i> , <i>Nocardioideis</i> , <i>Promicromonospora</i>	3	Medium
CB3L	<i>Bacillus</i> , <i>Lactobacillus</i> , <i>Pseudomonas</i> , <i>Azotobacter</i>	4	<i>Lactobacillus</i> ^b , <i>Nosocomiicoccus</i> , <i>Pediococcus</i>	2	Low
CB4L	<i>Bacillus</i>	1	<i>Bacillus</i> ^b , <i>Brevibacillus</i> , <i>Lactobacillus</i> , <i>Stenotomonas</i>	4	Low
CB5S	<i>Bradyrhizobium</i>	1	<i>Bradyrhizobium</i> ^b , <i>Nosocomiicoccus</i>	1	High
CB6S	<i>Azospirillum</i>	1	<i>Macellibacteroides</i> , <i>Alcaligenes</i> , <i>Pseudomonas</i> , <i>Hafnia</i> , <i>Clostridium sensu stricto</i> 1, 12 & 5, <i>Proteus</i> , <i>Dysgonomonas</i> , <i>Microvirgula</i> , <i>Morganella</i>	11	Poor
CB7S	<i>Azospirillum</i> , <i>Azotobacter</i>	2	<i>Microvirgula</i> , <i>Clostridium sensu stricto</i> 1, 12, 2 & 5, <i>Macellibacteroides</i> , <i>Morganella</i> , <i>Phascolarctobacterium</i> , <i>Alcaligenes</i> , <i>Ruminiclostridium</i> 5, <i>Pseudomonas</i> , <i>Proteus</i> , <i>Dysgonomonas</i>	13	Poor
CB8S	<i>Bradyrhizobium</i>	1	<i>Alcaligenes</i> , <i>Pseudomonas</i> , <i>Enterobacter</i> , <i>Enterococcus</i> , <i>Proteus</i> , <i>Lactobacillus</i> , <i>Lactococcus</i> , <i>Microvirgula</i>	8	Poor
CB9L	<i>Pseudomonas</i>	1	<i>Alcaligenes</i> , <i>Clostridium sensu stricto</i> 1, 12, 2 & 5, <i>Dysgonomonas</i> , <i>Macellibacteroides</i> , <i>Microvirgula</i> , <i>Morganella</i> , <i>Enterobacter</i> , <i>Ruminiclostridium</i> , <i>Hafnia</i> , <i>Escherichia–Shigella</i> , <i>Desulfovibrio</i> , <i>Proteus</i>	15	Poor
CB10L	<i>Brevibacillus</i> , <i>Paenibacillus</i> , <i>Lysinibacillus</i> , <i>Sporolactobacillus</i>	4	<i>Citrobacter</i> , <i>Clostridium sensu stricto</i> 2 & 5, <i>Desulfovibrio</i> , <i>Dysgonomonas</i> , <i>Escherichia–Shigella</i> , <i>Proteus</i> , <i>Kluyvera</i> , <i>Enterobacter</i> , <i>Macellibacteroides</i> , <i>Morganella</i> , <i>Microvirgula</i> , <i>Phascolarctobacterium</i> , <i>Ruminiclostridium</i>	14	Poor
CB11L	<i>Bacillus</i>	1	<i>Cronobacter</i> , <i>Clostridium sensu stricto</i> 1&5, <i>Alcaligenes</i> , <i>Pantoea</i> , <i>Ruminiclostridium</i> 5, <i>Microvirgula</i> , <i>Dysgonomonas</i> , <i>Enterobacter</i> , <i>Tyzzerella</i> , <i>Escherichia–Shigella</i> , <i>Citrobacter</i> , <i>Morganella</i> , <i>Desulfovibrio</i> , <i>Desulfovibrio</i> , <i>Proteus</i> , <i>Macellibacteroides</i>	16	Poor
CB12L	<i>Rhizobium phaseolus</i>	1	<i>Enterobacter</i> , <i>Ewingella</i> , <i>Providencia</i> , <i>Morganella</i> , <i>Proteus</i> , <i>Pseudomonas</i> , <i>Serratia</i> , <i>Rahnella</i> , <i>Hafnia</i>	9	Poor

^aBased on information provided by the manufacturer. Only genus classification was used

^bSpecified species

^cQuality score: high quality, all the specified species and total relative abundance of unspecified species $\leq 10\%$ detected; medium quality, $\geq 50\%$ of the specified species and a total relative abundance of unspecified species $\leq 50\%$ detected; low quality, $< 50\%$ of specified species and a relative abundance of unspecified species $> 50\%$ detected; poor quality, specified species not detected

Fig. 3 Heatmap showing several enzyme-coding genes of interest in biofertiliser products. Predicted metagenomic functional profile was performed using Tax4Fun package in R software. Heatmap was con-

structed using the gplot package of R software. KEGG orthology of genes are in parenthesis

genes including nitric oxide reductase subunit B and nitrous-oxide reductase had medium predicted occurrence amongst the samples while nitric oxide subunit C was less abundant across the samples. Other predicted genes which are important to N mineralization included the nitrate and nitrite reductase genes. The nitrite reductase (NAD(P)H) large subunit and small subunit had a high prevalence in all the samples while nitrite reductase (NADH) and ferredoxin-nitrite reductases had a relatively low abundance. Similarly, all the predicted nitrate reductases (alpha, beta, delta and gamma subunits) were in high abundance in the samples. However, alpha and beta subunits had higher abundance than other nitrate reductase genes.

Based on functional prediction, alkaline phosphatase was more abundant than acid phosphatases across all the samples. In addition, phosphoserine phosphatase and 4-nitrophenylphosphatase were predicted in all the samples. Sulphur is as necessary as phosphorous for the formation of important enzymes in microorganisms and plants. Of the four sulphur-degrading enzymes predicted, arylsulphatase was the most prevalent followed by sulphate adenylyltransferase subunit 1 and subunit 2, while sulphate adenylyltransferase occurred in low abundance. Other essential genes investigated included

the iron uptake and glucose-degrading genes. Ferric uptake regulator catecholate siderophore receptor and high-affinity iron transporter were relatively dominant in all the samples, with the highest abundance in samples CB4L and CB8L. The ferric-chelate reductase was observed to be less abundant across all the samples. Furthermore, toxin genes such as Shiga, leukocidin and haemolysin were predicted, while the leukocidin/haemolysin toxin family protein had a higher prevalence amongst five samples (CB2L, CB3L, CB4L, CB8L and CB12L), Shiga toxin genes had a very low abundance in all the samples. Similarly, membrane protein for colicin V production was less prevalent while gene encoding for glucosidase was widely predicted in high abundance in all the samples.

Discussion

Bacterial diversity in biofertiliser products

The quality of a biofertiliser product is determined by its microbial consortium, function and field effectiveness (Malusá et al. 2012; Balume 2013). Invariably, if bacterial

species in a biofertiliser are of low potency or missing, its (biofertiliser) effectiveness is affected. Therefore, towards improving crop productivity, the quality assessment of microbial components of biofertilisers has become increasingly necessary. This concern is born of the need to improve the quality of products available to farmers, especially small-holder farmers. Few studies have assessed the quality of commercial biofertiliser products using Sanger sequencing technique (Herrmann et al. 2015; Olsen et al. 1995). However, recent high-throughput next-generation sequencing (HT-NGS) offers a more (compared to culture-dependent and pre-HT-NGS techniques) in-depth and accurate analysis of the microbial diversity of any given environment or matrix (Tyx et al. 2016; Zhang et al. 2017). Therefore, this study utilised high-throughput sequencing of the partial 16S rRNA gene in biofertiliser products available to farmers in South Africa.

In the present study, the relative abundance of OTUs revealed major bacterial genera in the biofertiliser samples to be *Lactobacillus*, *Bradyrhizobium*, *Pseudomonas*, *Alcaligenes*, *Bacillus* and *Rhizobium*. These are predominantly efficient PGPR that have been previously identified in different biofertiliser samples (Hasan et al. 2014; Hatayama et al. 2005; Malusá et al. 2012). *Rhizobium* and *Bradyrhizobium* belong to the group; rhizobia, which contributes significantly to biological nitrogen fixation (BNF), especially in legume-cultivated agricultural soils (Raimi et al. 2017; Sutherland et al. 2000). Similarly, *Pseudomonas*, *Bacillus* and *Alcaligenes* have been widely used as biofertilisers, which make phosphorous available for crop use by solubilising insoluble phosphorous (Behera et al. 2017; Fitriatin et al. 2011). Some of these bacteria also produce plant-growth-promoting substances such as indole acetic acid (IAA), gibberellin, 1-aminocyclopropane-1-carboxylic acid (ACC) and siderophores (Raimi et al. 2017; Khan et al. 2016).

Several factors contribute to microbial diversity in biofertiliser products. Some of these factors include the microbial species formulation by the manufacturers (either single or consortium) and a good quality control system that reduces or eliminates unwanted bacterial species in the products (Lupwayi et al. 2000; Olsen et al. 1995; Yadav and Chandra 2014). Formulation of microbial consortium with a better competitive advantage over native species, in diverse ecological conditions, is crucial for high field efficiency (Faye et al. 2013; Kyei-Boahen et al. 2002). This is because the diverse indigenous microbes have heterogeneous functionalities and adaptive traits to metabolise a broad range of compounds and survive under different field conditions, respectively. Results obtained in this study suggest microbial formulation may have influenced the OTU richness and diversity of the products. However, consortium product formulation is very challenging because of the less selective carrier material which must support an array of microbial

species. Consequently, this less selective carrier material encourages the growth of unwanted species in the biofertiliser products (Stephens and Rask 2000). On the other hand, single-species products are selectively formulated to support a particular species. Perhaps, the foregoing explanation contributed to the higher species diversity of unwanted species observed in consortium products as opposed to the single-species products. Similarly, the influence of formulation type may explain the low diversity of unwanted species and thus, 'good quality' observed in the rhizobial products (CB2S and CB5S). Most rhizobial biofertilisers had high rhizobial density and less unwanted bacterial species. This observation could be due to carrier materials that selectively supported the optimal growth of rhizobia while preventing the proliferation of unwanted species (Balume 2013; Olsen et al. 1995). Nonetheless, with respect to efficiency and crop productivity, the application of single-species products in the field may be economically risky. This is because the likelihood of bacteria to establish an effective plant–microbe relationship in the rhizosphere is subject to different ecological factors; and no species is effective under all ecological conditions (Herrmann et al. 2015; Sutherland et al. 2000).

Higher OTU diversities (based on observed OTUs, Shannon–Weiner and Simpson diversity indices) were observed in liquid biofertiliser products compared to the carrier-based counterparts. Previous studies have reported that product form—liquid or carrier-based—influences the growth and development of bacteria in biofertiliser products (Herrmann et al. 2015; Pindi and Satyanarayana 2012). The observation of higher OTUs' diversities in liquid products may be attributed to the readily available nutrients and water contents in the liquid products, which aid substantial bacterial growth (Pindi and Satyanarayana 2012). Additionally, samples CB9L and CB11L containing other PGPR such as *Bacillus* and *Pseudomonas*, as well as samples CB6L and CB7L containing *Azotobacter* and *Azospirillum* were found to have higher species diversities compared to other products. This observation may also be due to the formulation type. Similarly, Herrmann et al. (2015) reported a higher species diversity in free-living nitrogen-fixing biofertilisers containing species of *Azotobacter* and *Azospirillum*.

Comparing specified and observed bacterial species in biofertiliser products

The disparities between the observed bacterial OTUs and the specified bacterial species (as informed by the manufacturers' labelling information) showed that the products had varying levels of unspecified bacteria. Similar to other reported studies, observed bacterial diversity not listed by the manufacturers are referred to as contaminants (Herrmann et al. 2015; Olsen et al. 1995; Deaker et al. 2011). Different levels of contamination and the consequential

effects on product quality have been reported (Herridge et al. 2002; Lupwayi et al. 2000; Olsen et al. 1995). In a study by El-Fattah et al. (2013), it was observed that diverse contaminants adversely affected the quality of biofertiliser products. Similarly, the present study showed different levels of contaminants with major ones found in CB1L, CB6L, CB7L, CB8L, CB9L, CB10L, CB11L, and CB12L. Interestingly, five of these products were produced by the same manufacturer. The analogous level of contamination between products from the same manufacturer could imply the use of similar sources of materials or production processes. This observation is similar to the work of Herrmann et al. (2015) who reported the same levels of contamination in products from the same manufacturer.

Several biofertiliser-associated contaminants have been reported to be potentially dangerous to the environment, humans and crop health (Herridge et al. 2002; Olsen et al. 1996). Akin to what was obtained in this study, Herrmann et al. (2015) reported that 53% of tested inoculants had contaminants with diverse opportunistic pathogenic effect. In this study, contaminants such as *Acinetobacter*, *Arthrobacter* and *Alcaligenes* have been reported in various studies to cause diseases in humans and animals (Goodfellow et al. 2012; Liu et al. 2002; Saffarian et al. 2017; Tille 2013). Similarly, several species of *Brevibacillus*, including *B. laterosprus*, are pathogens of invertebrates (Ruii 2013) while the genera *Proteus*, *Clostridium*, *Escherichia-Shigella*, *Enterococcus*, *Staphylococcus* and *Pediococcus* have many species that are known pathogens of humans and animals (Chaves et al. 2005; Corcoran et al. 1991; Kim et al. 1981; Rivas et al. 2015; Teixeira et al. 2001). Some of these pathogenic contaminants also inhibit the growth of beneficial microbes such as rhizobia in biofertiliser products (Gomez et al. 1997). Considering these adverse effects, the incidence of contaminants in biofertilisers should be considered more seriously beyond being mere contaminants (Catroux et al. 2001).

Predictive metagenomics profiling of 16S rRNA gene

The predictive functional profiling analysis revealed significantly diverse enzyme-coding genes amongst the bacterial communities. It is important to note that the presence of these genes do not mean that they will be expressed under field conditions. Hence, the predicted functions are discussed herein on the premise of mere potential functions. The functions of some of these extracellular enzymes in microbial metabolism and biogeochemical cycles have been widely studied (Bhardwaj et al. 2014; Malusà et al. 2016; De Bruijn 2015). For example, the predicted N-fixation genes are involved in biological nitrogen fixation (BNF). BNF is a natural process whereby essential atmospheric nitrogen is

converted to usable forms of ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) in the soil for rhizosphere microflora and plant use (De Bruijn 2015). The N-fixation genes predicted in the PGPR-products containing *Pseudomonas*, *Enterobacter* and *Citrobacter* suggest that the bacterial communities can possibly participate in BNF (Desnoues et al. 2003; Hatayama et al. 2005; Neilson and Sparell 1976). Additionally, N-cycling pathways such as denitrification, which involves nitrate and nitrite degradation, are essential in converting nitrate, nitrite or ammonia to N gas. The presence of these genes suggests the prominence of denitrification ability among the bacterial (Šimek and Cooper 2002).

Furthermore, in oxygen-deficient conditions, such as during long-term product storage, respiratory nitrate reductase is often expressed where nitrate is present. Hence, the predicted nitrate reductase genes in the bacterial community suggest the use of nitrate as an alternative to oxygen to gain electrons for maintaining the proton-motive force in microbial cells (Tyx et al. 2016). However, the build up of extracellular nitrite during the respiratory process is toxic to microbial cells. To overcome this situation, nitrite-exporting enzymes are expressed, which establish the reason for the presence of nitrite genes in some of the bacterial communities. Most bacteria with assimilation and denitrification ability can further use the produced nitrite (Kraft et al. 2014; Luque-Almagro et al. 2011). If the aforementioned genes are absent, microbial cell death may occur after a long period of storage. This may have contributed to the low-quality or total product damage observed in some of the products.

Furthermore, the alkaline phosphatase gene, an important enzyme in phosphate mineralisation was predicted with a high abundance amongst some of the bacterial communities. This suggests the ability of bacterial communities to potentially mineralise phosphate when applied in the field, especially in alkaline pH environments (Behera et al. 2017; Fitriatin et al. 2011). Similarly, the predicted sulphur-degrading genes, especially arylsulphatase, may indicate the potential role of biofertiliser communities in sulphur cycling and mineralisation in the soil. Likewise, the presence of the ferric uptake regulator suggests potentials for the uptake of iron in micro-organisms. The ferric uptake regulator gene improves crop growth and development through direct or indirect mechanisms of iron uptake by the bacterial communities (Sayyed et al. 2010). The presence of gene encoding for glucosidase suggests microbial communities could use different sources of carbohydrates, such as glucose. Other important predicted genes such as Shiga, leucocidin and haemolysin toxin genes have great potential in causing human diseases and product damage. These toxins are frequently cytotoxic, destroying cells by creating unregulated pores in the membranes of the host cells (Gouaux et al. 1997; Laohachai et al. 2003). *Escherichia-Shigella*, a major genus responsible for the production of Shiga toxin was observed in some of the products (Laohachai

et al. 2003). Microbial communities producing these toxins may suffer from loss of viable cells due to the damaging effect of the toxins on the beneficial bacterial cells, thereby causing poor-quality products (Gomez et al. 1997).

Conclusions

Of concern in the present study is the abundant population of undesired microbes and the presence of toxins that contribute to biofertiliser product damage. These contaminants and toxins are dangerous to human and plant health. Therefore, eliminating or reducing microbial contaminants and their damaging effects in biofertiliser products should be pursued through improved quality control management involving regular product-quality evaluation. A rapid technique of evaluation such as NGS technology used in this study is, therefore, suggested as a suitable technique for assessing the microbial quality of biofertiliser products to establish product quality and potential efficiency before field application.

Moreover, the development of an appropriate quality control system is essential for a successful production of a quality product that will improve crop productivity and farmers profitability. This is also a step towards environmental sustainability. Nevertheless, further studies are required to investigate other factors such as physicochemical properties and storage conditions of products that may influence the microbial quality of biofertiliser products. Toxicity and pathogenicity of the microbes contained in biofertiliser formulations should be further investigated as well as gene expression studies to validate the activities of the predicted functional genes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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