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Evaluation of denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS) in combination with enrichment culture techniques to identify bacteria in commercial microbial-based products

R.M. Subasinghe, A.D. Samarajeewa, R. Scroggins, L.A. Beaudette*

Biological Assessment and Standardization Section, Environment and Climate Change Canada, 335 River Road, Ottawa, Ontario K1V 1C7, Canada

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ABSTRACT

Identification of bacteria in new or existing commercial microbial-based products (MBPs) is important for compliance with government regulations and for human and environmental risk assessment. Research was performed to develop effective methods to identify bacteria present in a MBP using a combined approach of conventional enrichment culture technique and denaturing gradient gel electrophoresis (DGGE) followed by clonal sequencing or next generation sequencing (NGS). Genomic DNA obtained from un-enriched or enriched MBP in MacConkey broth, Azide Dextrose broth, Peptone Water mixed with Polymixine B and Gram Negative (GN) media under three different temperatures (22 °C, 28 °C and 37 °C) were sequenced in two methods for the V3 and V6 hypersensitive regions of 16S ribosomal DNA (rDNA) and compared.

Enrichment followed by DGGE and clonal sequence analysis identified 20 bacterial genera in all enriched and un-enriched media. In contrast, NGS was able to identify 114 bacterial families and 134 genera both in V3 and V6 regions. In clonal sequence analysis, in comparison to the un-enriched MBP, the MacConkey broth enriched for Escherichia or Shigella and Morganella species, GN medium enriched for Proteus and Morganella species and Azide Dextrose broth enriched for Vagococcus and Enterococcus species at both 28 °C and 37 °C. Moreover, the enrichment facilitated NGS to record higher numbers of families and genera in all enrichment cultures, comparatively higher variations in V3 region than in V6. More prominently, NGS identified 14 genera and 9 species in the family Enterobacteriaceae compared to only 5 genera identified in the un-enriched control using V6 region variance in MacConkey broth at 28 °C. Increasing the temperature without enrichment identified specific families by V3 and V6 regions.

This study indicates that the polyphasic approach with appropriate enrichment and incubation at different temperatures followed by NGS analysis is a promising method for the identification of viable, non-pathogenic or potential pathogenic bacteria in complex MBPs.

1. Introduction

Commercial microbial-based products (MBPs) are widely used in bioremediation to degrade pollutants in soil contamination events, in septic tank treatments and in water purification and composting treatments (Hoff, 1993; Zhu et al., 2004). The microorganisms used in these products are commonly isolated from natural environments. Due to the highly diverse and complex microbial populations in natural environments, isolation of all single bacterial species is impossible using conventional culturing techniques (Amann et al., 1995, Hugenholtz and Pace, 1996, Rondon et al., 2000, Liu et al., 2007, Porter and Hajibabaei, 2018). As such, not all of the bacteria can be identified. Assessment of these products for all the bacteria is important for environmental protection and compliance of government regulations. With the lack of an assessment method for MBPs, compliance of these products by regulatory agencies is difficult (Spiegelman et al., 2005; Arvanitakis et al., 2018).

Culture-independent genomic techniques to characterize a single bacterium or a bacterial consortium are known to have significant advantages over standard biochemical and microbiological techniques (Amann et al., 1995; Spiegelman et al., 2005). Genomic characterization is commonly based on the variability of the hypersensitive regions of the highly conserved 16S rRNA gene in bacteria (Muyzer et al., 1993; Rondon et al., 2000; Boon et al., 2002; Temudo et al., 2008; Stephan et al., 2011; Vasileiadis et al., 2012; Subasinghe et al., 2017). PCR amplification of the variable 16S rRNA regions followed by denaturing

* Corresponding author.
E-mail address: lee.beaudette@canada.ca (L.A. Beaudette).

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gradient gel electrophoresis (DGGE) (Boon et al., 2002; Dubois et al., 2004; Nowak et al., 2008; Masson et al., 2011; Samarajeewa et al., 2015), clonal restriction fragment length polymorphism (C/RFLP) (Masson et al., 2011; Samarajeewa et al., 2015; Xiang et al., 2010), clonal-sequencing (Samarajeewa et al., 2015) or microarray methods (Dubois et al., 2004, Nowak et al., 2008, Masson et al., 2011,) are among the common genomic methods used to identify bacteria in a microbial consortia. Each of these methods have some inherent limitations, including amplification biases in the polymerase chain reaction based (PCR-based) methods. In addition, the clonal-sequencing method only sequences individual species, and therefore is unable to process complex environmental samples, which consists of DNA from numerous bacterial species in the product (Hajibabaei et al., 2011; Shokralla et al., 2012).

High-throughput next generation sequencing (NGS) has proven to be a reliable technique to overcome the limitations of the clonal-sequencing method, which can sequence the DNA directly isolated from environmental samples at unprecedented speed (Claesson et al., 2010; Petrosino et al., 2009; Samarajeewa et al., 2015; Schuster, 2008; Sogin et al., 2006; Whiteley et al., 2012; Porter and Hajibabaei, 2018). For example, an assessment of bacteria present in a MBP using NGS was able to detect bacterial species that were not detected using DGGE, C/RFLP or clonal-Sanger sequencing (PRACS) (Subasinghe et al., 2017; Samarajeewa et al., 2015). However, it was noted that the combined use of two or more genomic methods provided greater coverage for bacterial identification (Masson et al., 2011; Samarajeewa et al., 2015).

In recent studies, enrichment culture techniques were used to investigate specific microbial species and foodborne pathogens (Park et al., 2011; Margot et al., 2015; Jarvis et al., 2015; Hill et al., 2017) and microbes in natural habitats (Durso, 2013; Lee et al., 2013; Gao et al., 2014; Cross et al., 2015). This method identified novel bacterial species in microbial communities including saline-alkaline soils (Panosyan et al., 2018), oil reservoirs under anaerobic conditions (Wang et al., 2011), Freeze–thaw stressed soils (Wilson et al., 2012), Panosyan diesel contaminated Antarctic soils (Mills et al., 2003; Vázquez et al., 2013), and sludge compost (Ueno et al., 2001). The use of enrichment media allowed for specific bacteria to be isolated based on their diverse metabolic and catabolic activities (Entcheva et al., 2001, Ueno et al., 2001, Wang et al., 2011, Conrad et al., 2016). After enrichment, further development of large-insert libraries by direct cloning of DNA could be achieved, which would then be used to identify a large number of diverse bacterial species with similar metabolic gene activities (Entcheva et al., 2001; Knietsch et al., 2003).

In the present study, research was performed to further enhance our ability to directly identify bacterial species from commercial MBP products using DGGE and Ion Torrent NGS combined with enrichment culture techniques.

2. Materials and methods

2.1. Commercial microbial-based product

A commercially formulated liquid form of MBP, which is used for the bioremediation of sites contaminated with petroleum hydrocarbon, was used in this study. The product was purchased in a 50 US gallon (189.27 L) bulk container and stored in room temperature (22 °C) upon arrival keeping the lid tightly closed. After thoroughly mixing the product manually using a drum dolly, two homogeneous samples of 2 L each were collected aseptically while stirring the contents with a sterile steel rod. Two sample collections were carried out; one within 24 h of receiving the product and the second two weeks later. A sterile transparent silicone tube connected to a vacuum aspirator pump was used to collect each sample. The collection bottles, tubes and steel rod were sterilized by autoclaving for 15 min at 121 °C (15 psi).

2.2. Enrichment culture establishment and genomic DNA isolation

To enhance the growth of bacteria, 25 mL of enrichment media were prepared in each 125 mL Erlenmeyer flasks with two replicates of 5% final concentration of MBP in single strength MacConkey broth (M), Azide Dextrose broth (AZ), Gram-negative broth (GN) and peptone water + Polymyxine B (P) (5 $\mu g\,L^{-1}$). The enrichment media were incubated separately at 22 °C, 28 °C and 37 °C continuously shaking at 150 rpm for 16–20 h. In addition to the enrichment media, 25 mL of the MBP was incubated at the same temperature and incubation conditions as above without any additives. After incubation, the contents of each flask were transferred into 50 mL centrifuge tubes and centrifuged at 7500 x*g* for 10 min. The pellet was re-suspended in 4.5 mL of 1 X PBS buffer (1 X phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The resuspended pellet was then pipetted (1.5 mL) into three micro centrifuge tubes and the bacterial genomic DNA (gDNA) extracted using the Wizard® genomic DNA purification Kit (Promega) as per the manufacturer's instructions. Additionally, bacterial gDNA was directly extracted from the MBP on each of the sampling days. To achieve this, 10 mL of the product was filtered through a 0.45 µm nitrocellulose membrane filter using a vacuum pump and the gDNA extracted using the PowerWater® DNA Isolation Kit (MoBio) following the manufacture's instruction. Combinations of the enrichment media and incubation temperatures used in the DGGE analysis are shown in Table 1.

2.3. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

Genomic DNA (gDNA) from two different extraction times were mixed and the bacterial V3 and V6 hypersensitive regions of 16S rDNA were PCR amplified separately by universal primers after testing the quantity and quality of extracted gDNA on a NanoDrop 2000c UV–Vis spectrophotometer. To amplify 203 bp from the V3 region, the following forward primer 5'-CCAGACTCCTACGGGAGGCAG-3′ (334–354) and reverse primer 5′- CGTATTACCGCGGCTGCTG-3′ (519–537) (Chakravorty et al., 2007) were used. For the V6 region (440 bp), the forward primer F984 (968–984) 5'-AACGCGAAGAACCTTAC-3′ and reverse primer R1378 (1378–1401), 5'-CGGTGTGTACAAGGCCCGGGAACG-3′ (Novinscak et al., 2009) were used. Both forward primers contained a 40 bp GC clamp (5 -CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′). 50–100 $ng\,\mu L^{-1}$ of gDNA from each

Table 1
Summary of treatments used in DGGE and NGS.

Control and enrichment media	Incubation temperature (C°)	Treatment ID	DGGE	V regions in NGS analyzed	
				V3	V6
Un-enriched Control (C)	Un-incubated	C	√	√	√
	22	C-22	√	-	-
	28	C-28	√	-	-
	37	C-37	√	√	√
MacConkey broth (M)	22	M-22	√		- -
	28	M-28	√	√	√
	37	M-37	√	√	√
Azide Dextrose broth (AZ)	22	AZ-22	√	-	-
	28	AZ-28	√	-	-
	37	AZ-37	√	-	√
Gram-negative broth (GN)	22	GN-22	√	-	-
	28	GN-28	√	-	-
				-	-
	37	GN-37	√		
Peptone water + polymyxine B (P)	22	P-22	√	-	-
	28	P-28	√	-	-
	37	P-37	√	√	√

treatment was amplified in a final volume of a 25 μL PCR reaction mixture containing 5 μL of 5× PCR reaction buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.5 μmol L^{-1} of forward and reverse primers specific to the V3 or V6 region, 200 μmol L^{-1} dNTPs, 1.25 units of Taq polymerase and sterile DNA and RNA free H_2O. The amplicons were synthesized in a thermocycler at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min and finally at 72 °C for 5 min.

The resulting PCR products were tested on 1% agarose gel and the replicates of the treatments were combined and run on the DGGE for each treatment; this was performed three times. The DGGE was performed using a denaturing gradient of 38–70% prepared by combining the denaturant (7 M urea, 40% (*v/v*) formamide, and 8% (*w/v*) polyacrylamide in 0.5× Tris acetate/EDTA buffer) with 0% buffer (8% (w/v) polyacrylamide in 0.5× Tris-acetate/EDTA buffer) using a gradient maker. Electrophoresis was performed in 1× Tris-acetate/EDTA buffer at 61 °C at a constant voltage of 75 V for 16 h using a DCode universal mutation detection system (BioRad). The DGGE gels were then stained in 1:10000-diluted SYBR Safe for 45 min on an orbit shaker at 45 rpm. Gel images were captured using an Alpha Imager 2200 system.

2.4. PCR cloning, sequencing and analysis of sequencing data

Distinct DNA bands from the DGGE were excised using an X-tracta™ - Agarose Gel Extraction Tool (LifeScience Products) and the DNA were eluted. The gel piece was crushed using a sterilized glass rod in a 1.5 mL microcentrifuge tube and 20 μL of elution buffer from the UltraClean GelSpin® DNA Extraction Kit (MoBio). The tube was mixed frequently while being kept at 37 °C for 5 h. The gel pieces were separated by centrifuging at 10,000 × *g* for 2 min at 4 °C. Eluted DNA (50–100 ng μL^{-1}) was re-amplified using the same primers and PCR program as mentioned above. The PCR products were then purified using a Gen Elute ™ PCR Clean-up Kit (Sigma).

Clone libraries were constructed by ligating purified-PCR products from each band into a pCR 2.1 TOPO vector (Invitrogen) and transforming them into DH5α ™ $T1^R$ chemical competent cells following the manufacturer's instructions. The transformed cells were screened on LB-Agar plates containing 100 mg mL^{-1} ampicillin, 50 mg mL^{-1} kanamycin, 300 mg L^{-1} S-Gal (Sigma) and 500 mg L^{-1} ferric ammonium citrate. A total of four colonies from each band were randomly selected for sequencing. The selected colonies were grown in LB medium containing 50 mg mL^{-1} kanamycin at 37 °C. The clones were sent for one-directional Sanger sequencing with T7 primers to Backman Coulter Genomics (MA, USA).

The sequences obtained were quality trimmed and analyzed using the SeqMatch program of the RDP pipeline pyrosequencing database (at KNN = 3, Version 03) to identify the bacteria based on the DNA sequence from the DGGE band.

2.5. Ion torrent sequencing

2.5.1. Ion amplicon library preparation by the fusion method

Specific forward and reverse primers were designed to amplify the V3 and V6 hypersensitive regions of 16S rDNA to facilitate unidirectional genomic fusion primer library construction. The V3 and V6 specific forward primers (described in the DGGE section) were designed to contain an overhang 'A-adaptor' sequence (5'-CCATCTCATCCCTGC GTGTCTCCGAC-3′) followed by a key sequence ('5-TCAG-3′) and a distinct IonExpress barcode sequence for each treatment. The reverse primers of the V3 and V6 target sequences were redesigned with a P1 adaptor sequence (5'-CCTCTCTATGGGCAGTCGGTGAT-3′) as described in the Ion Amplicon Library Preparation user guide (Fusion Method, Life Technologies). The gDNA from an un-enriched MBP (C), MBP grown at 37 °C (C-37), in MacConkey broth at 28 °C (M-28) and at 37 °C (M-37), in Peptone water + polymixine B at 37 °C (P-37) and in azide dextrose broth at 37 °C (AZ-37) were only selected for multiplexing in the genomic barcoded-library using V6 primers. gDNA was also used for the V3 primers with the exception of AZ-37. Therefore, 5 treatments (including the control) were tested with V3 primer and 6 treatments with V6 primer. Combinations of the enrichment media and incubation temperatures used in NGS analysis are shown in Table 1. The number of treatments/primer was decided based on the results of DGGE (treatments provided higher bacterial identification) and the limitation in the number of treatments that can be added in a genomic library. Combinations of the enrichment media and incubation temperatures used in the NGS analysis are shown in Table 1. The PCR was carried out separately for each treatment. 50–100 ng μL^{-1} of template gDNA was amplified in 25 μL of the PCR reaction mixture containing the same concentrations of dNTPs, Taq polymerase, and primers as previously described. Also, the same DGGE PCR thermocycler program was used.

Following the PCR, products were run on a 1% agarose gel and the corresponding bands were excised from the gel and the DNA purified using a QIAquick® Gel Extraction Kit (Qiagen). The DNA was quantified on a Bioanalyzer using an Agilent® High Sensitivity 1000 DNA Kit (Agilent Technologies).

2.5.2. Ion torrent PGM

Two different fusion libraries from the V3 and V6 hypervariable regions of 16S rRNA were prepared at 26 pM L^{-1} final concentrations. The preparation included using the Ion PGM™ Template OT2 400 Kit (Life Technologies) on the Ion OneTouch™ 2 system (Life Technologies), and enrichment on the Ion OneTouch™ ES (Life Technologies) instrument to facilitate the attachment of the DNA to the surface of the Ion Sphere™ particles (ISPs). Once completed, the percentage of ISPs was determined using a Qubit®2 fluorometer (Life Technologies). It was important to maintain the polyclonal percentage at 20–30%. The templated-ISPs from the two libraries were loaded onto a 316 v2 chip and sequenced on the Ion Torrent Personal Genome Machine (Ion PGM™ System, Life Technologies) using a PGM™ Sequencing 400 Kit (Life Technologies) with 850 sequencing flows on the Torrent-Suite program version 4.2.1.

2.5.3. Analysis of PGM sequence data

The resulting sequences were analyzed on the Ion Reporter™ Software using the 16S Metagenomics beta workflow (Life Technologies, Version 4.0) to identify the bacteria represented by the DNA sequences. The analytical parameters of the workflow of the Ion Reporter™ Software were adjusted to Read Length Filter = 150 bp, Minimum Alignment Coverage = 90, Read Abundance Filter (Number of unique reads needed for that read to be valid) = 10, Genus Cut-off (Percentage identity value required to make a genus ID) = 97, Species Cut-off (Percentage identity value required to make a species ID) = 99, Slash ID Reporting Percentage = 80% in all the analysis. All the counts recorded as 'slash calls' in the family or genus results were not included in the calculations.

3. Results

3.1. DGGE

Ninety nine distinct bands were excised from DGGE gels of the enriched and control treatments. DNA was extracted from the bands and PCR amplicons from the V3 and V6 regions were cloned and sequenced. A total of 19 different clones were made from the MBP product alone grown at 22 °C, 28 °C and 37 °C. Another 29, 19, 11 and 8 clones, respectively, were prepared from the enriched MBP grown in MacConkey broth, peptone water + polymixine B media, GN broth, and azide dextrose broth at different temperatues. Five clones from the un-enriched MBP were sequenced, using V6 and 8 clones from the V3 region, as controls. Four colonies from each clone were selected for sequencing.

To identify the bacteria represented by the sequences, the RDP

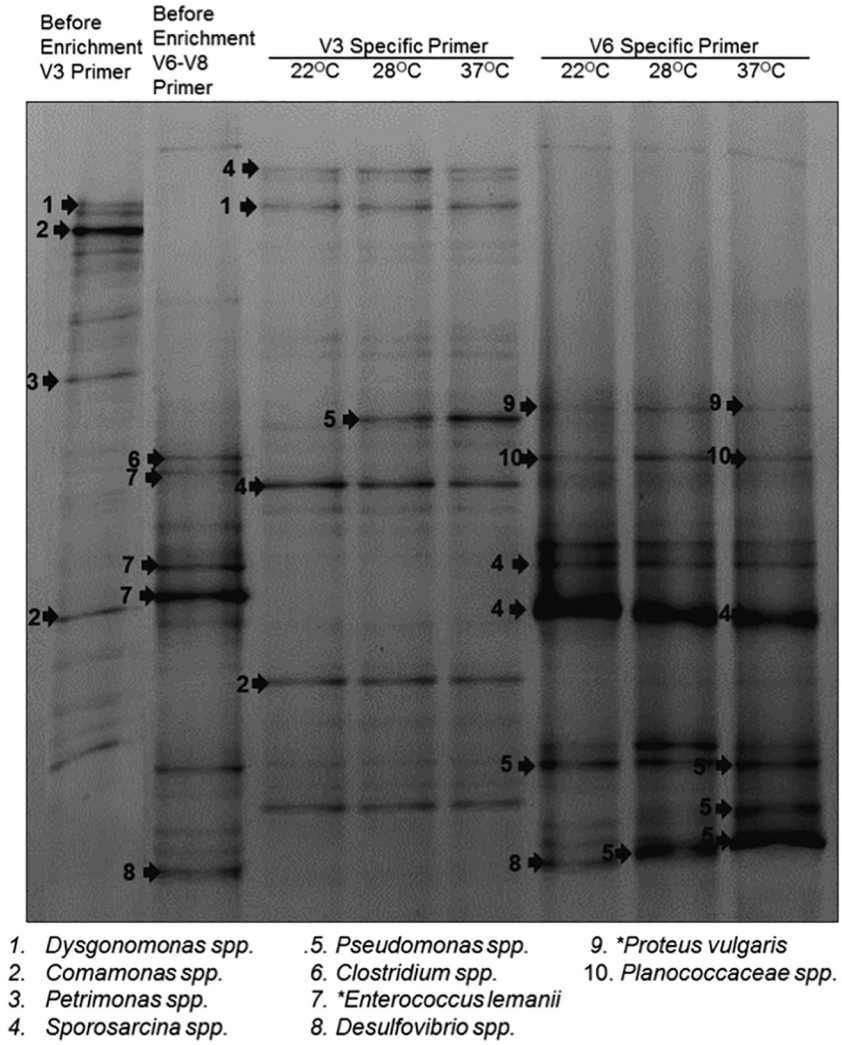

Fig. 1. DGGE banding profile of the MBP before and after incubation at 22 °C, 28 °C and 37 °C temperatures. The MBP was incubated without any enrichment media and the genera and species were identified by the sequence variance of V3 and V6 hypersensitive regions. DGGE was performed three times with the three combined PCR replicates from each treatment.

Seqmatch program was used and the Rootrank (3) match sequences were taken with the highest S_ab score (the percentage of shared 7-mers between two sequences). The top genus listed in RDP Seqmatch analysis of the individual band sequence are indicated in Figs. 1 to 5. For some sequences, the RDP Seqmatch was able to identify up to the species level. The results indicated that a single genus was represented by multiple bands on DGGE.

The DGGE banding profile of the unenriched MBP before and after incubation at 22 °C, 28 °C and 37 °C temperatures and the genera and species identified by the sequence variations of V3 and V6 hypersensitive regions are shown in Fig. 1. In the unenriched controls, genera *Dysgonomonas, Petrimonas* and *Comamona* were detected by V3 specific primers while the genera *Clostridium, Enterococcus,* and *Desulfovibrio* were detected by V6 specific primers. The MBP grown at 22 °C, 28 °C and 37 °C temperatures without any enrichment media were able to enhance the growth of *Pseudomonas spp*. (at 28 °C and 37 °C) which was not evident at 22 °C. *Sporosarcina, Proteus* and *Planococcaceae* spp. were identified only after incubation at 22 °C, 28 °C and 37 °C. However, *Proteus* spp. *and Planococcaceae spp*. were identified only by the V6 primer.

Different DGGE banding profiles and bacterial compositions were found when enriched in MacConkey broth as compared with the unenriched controls (Fig. 2). *Morganella morganii* and *Erysipelothrix rhusiopathiae* species were identified in MacConkey broth at 22 °C by the V3 primer. Interestingly, the genus *Escherichia* and *Shigella* were identified in MacConkey broth after enriched at 28 °C and 37 °C.

The MBP grown in MacConkey Broth (Fig. 2) and GN broth (Fig. 3) selectively enhanced the growth of Gram-negative *Proteus* and *Morganella* species. Also, increasing the temperature enhanced the growth of *Pseudomonas/Shigella* spp. in the MacConkey Broth (Fig. 2).

Peptone water + Polymyxine B media (Fig. 4) and Azide Dextrose Broth (Fig. 5) enhanced the growth of Gram-positive *Vagococcus* and *Enterococcus* spp. which were not identified in the un-enriched MBP.

3.2. Ion torrent sequencing

The read counts of all the sequences with the exception of the 'slash calls' were used in the data analysis. The 'slash calls' are assigned when the variations between sequence reads were not verified by the metagenomics analytical program or if the difference in percentage match between the top hit and the next hit was ≤80% (Ion Reporter™ Software Publication by Ion Torrent, Life Technologies, 2014). The only read counts that were used in the research were those that had > 10 reads. In this study, we identified 114 families, 134 genera and 72 species from all the treatments and controls in the V3 and V6 regions combined (Table 2).

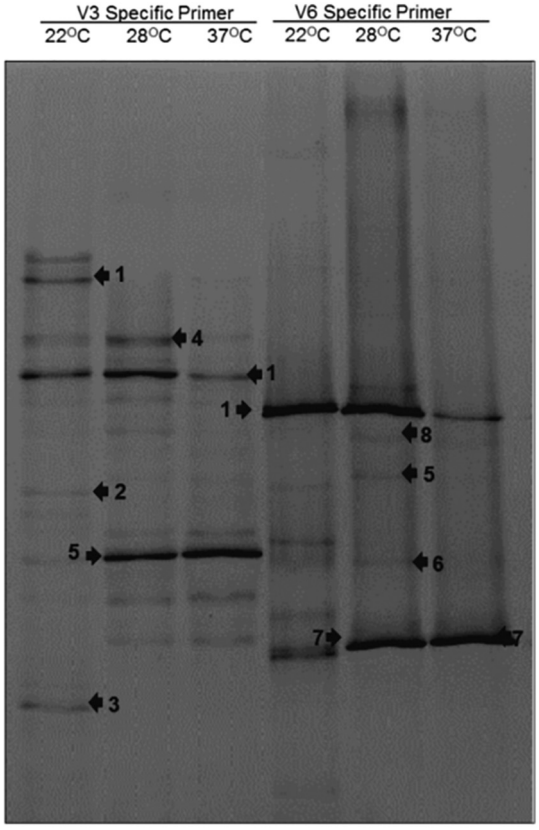

Fig. 2. DGGE banding profile of the MBP incubated in MacConkey Broth at 22 °C, 28 °C and 37 °C temperatures and the genera and species identified by the sequence variations of V3 and V6 hypersensitive regions. DGGE was performed three times with the three combined PCR replicates from each treatment.

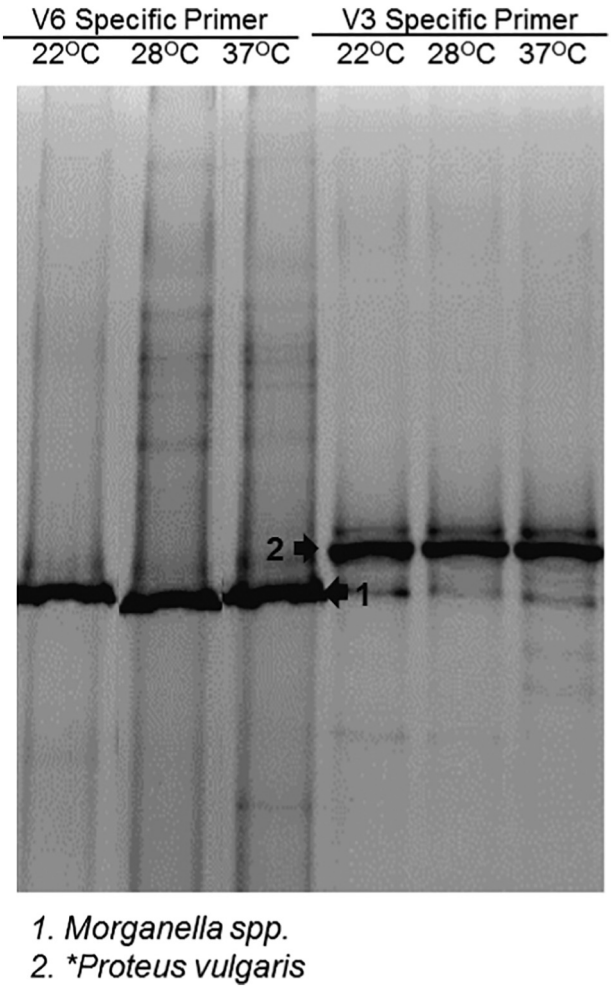

Fig. 3. DGGE banding profile of the MBP incubated in Gram-negative broth (GN) at 22 °C, 28 °C and 37 °C temperatures and the different genera identified by the sequence variations of V3 and V6 hypersensitive regions. DGGE was performed three times with the three combined PCR replicates from each treatment.

3.3. Detection based on V3 hypersensitive region

Altogether 82 families, 93 genera and 44 species were identified in controls and treatments, based on V3- region specific primers (Table 2). If one includes all the treatments and the controls, the totals of the specific numbers of the families, genera and species from the V3 region are comparatively higher than the numbers observed from the V6 region (Table 2). In addition, the read count percentages of the specific families, genera and species to the totals of both V3 and V6 regions showed a higher percentage of specific families (39.5%), genera (51.5%) and species (50%) by the V3 region in comparison to the V6 region (Table 2).

Table 3 compares the specific and total numbers of families, genera and species, which were identified in different treatments based on sequence variation of the V3 and V6 regions. A total of 94 families were identified in the control treatment (C) from which 37 were specific to the V3 region and 26 were specific to the V6 region. In addition, 37 families, 63 genera and 25 species found in the V3 region were specific to the control (C). Therefore, the families, genera and species found in the C could be seen in other treatments. In the P-37 enrichment, 14 families, 8 genera and 6 species were found specific to the V3 region when the totals from both V3 and V6 regions were 17, 12 and 7 respectively. In contrast, the M-28 and M-37 enrichments showed higher specific genera and species numbers in the V6 region. This indicated that the V3 region is much effective in detecting the bacteria, when the MBP was enriched at P-37 (Table 3). The AZ-37 was not tested for the V3 region analysis.

When the treatments are compared within the V3 region, a higher number of specific families, genera and species were shown in the control (C) and in the MBP grown at 37 °C without enrichment (C-37) (Table 4). However, the increased temperature and enrichment media resulted in the ability to identify the genera *Proteus, Providencia* and *Vagococcus* that did not appear in the control (Fig. 7), although the total number of families, genera and species detected were comparatively low in these treatments.

The family Enterococcaceae scored the highest percentage of reads (27.8%) followed by Porphyromonadaceae (23.8%) and Comamonadaceae (23.5%) in the C (Fig. 6). In comparison to the C-37, when the MBP was enriched in (M-28), (M-37) and (P-37), the family Enterococcaceae was reduced to 0.2–0.6%. The family Pseudomonadaceae recorded the highest percentage read counts in C-37 (37.9%) followed by Comamonadaceae (20%) and Planococcaceae (19.7%) (Fig. 6). From the family Enterococcaceae found in the C, we were able to confirm the presence of *Enterobactor faecium, Enterobactor raffinosus, Te45R*, and *NLAE-zl-H227* but not in any other treatments (Table 5). In addition, the genus *Vagococcus* was only found in C-37 and P-37, but not in the control (Fig. 7).

The family Pseudomonadaceae was found in the C (2.6%), C-37 (37.9%), M-37 (0.2%) and P-37 (0.1%) while the genus *Pseudomonas* was common in all treatments (Fig. 7). The species *P. denitrificans,* and

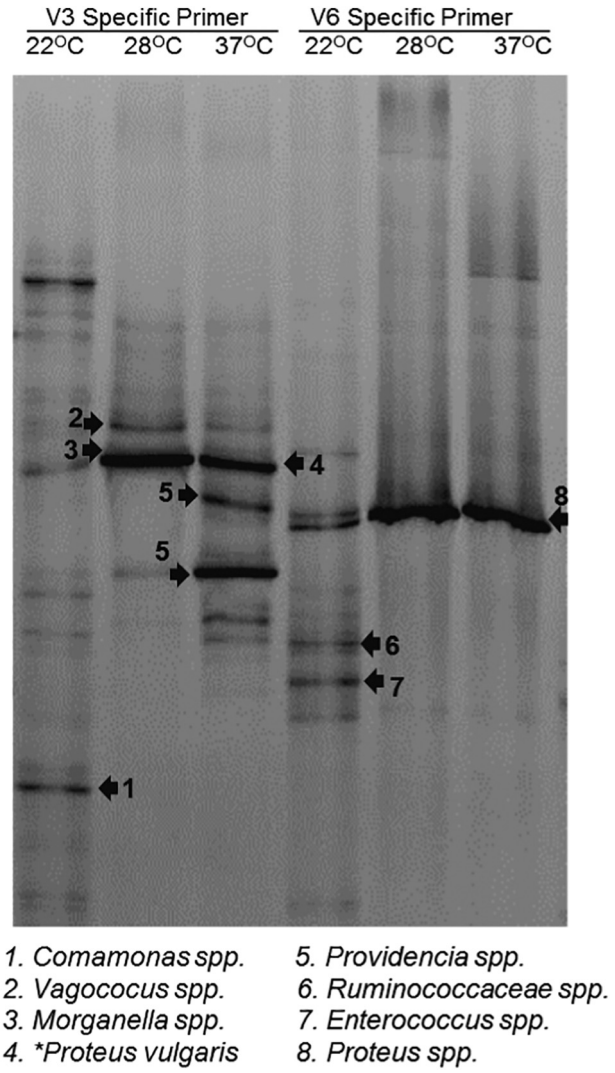

Fig. 4. DGGE banding profile of the MBP incubated in Peptone Water + Polymixine B media at 22 °C, 28 °C and 37 °C temperatures and the different genera identified by the sequence variations of V3 and V6 hypersensitive regions. DGGE was performed three times with the three combined PCR replicates from each treatment.

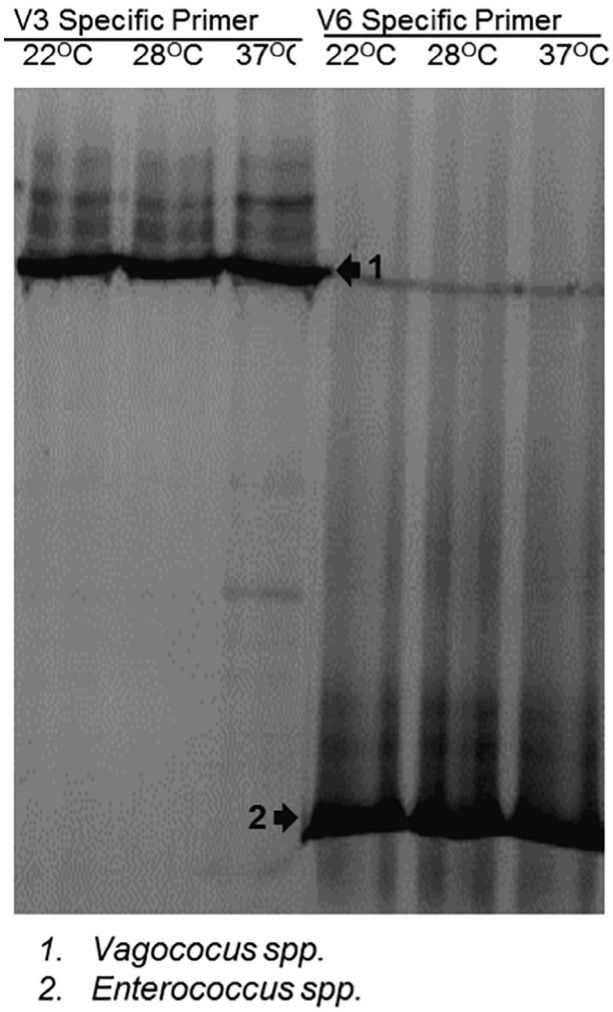

Fig. 5. DGGE banding profile of the MBP incubated in Azide Dextrose Broth at 22 °C, 28 °C and 37 °C temperatures and the different genera identified by the sequence variations of V3 and V6 hypersensitive regions. DGGE was performed three times with the three combined PCR replicates from each treatment.

P. pertucinogena were only found in C-37 (Table 5). The family Actinomycetaceae was identified only in the control C and C-37. However, the genus *Trueperella* was confirmed in C, while Actinomyces (*Actinomyces ruminicola*) and the genus *Mobiluncus* were only found in C-37. The family Alcaligenaceae was found in C and C-37. Eight different genera belonging to the family Alcaligenaceae included 4 different species (*A. faecalis, D. permanens, P. granuli and rM17* bacterium) which were found in C and C-37 (Tables 3 and 4).

The Family Porphyromonadaceae was found in C (23.8%) and C-37 (8.8%). In this family, nine different genera were found in C and seven of them were common in C-37 (Fig. 5). However, in C-37, *Petrimonas sulfuriphila and Proteiniphilum acetatigenes* spp. were specific to C-37 (Table 4). The count percentage of family Enterobacteriaceae increased from 0.4% (C) to > 99% when enriched in both M-28 and M-37 (Fig. 6). However, the genera belonging to this family included, *Citrobacter, Morganella, Proteus, Providencia and Trabulsiella* which were commonly found in both C and C-37. Enrichment in MacConkey Broth media resulted in similar genera except for *Citrobacter*. The family Planococcaceae and genus *Sporosarcina,* from the family were only found in C-37 (Figs. 6 and 7).

3.4. Detection based on V6 hypersensitive region

The V6 region analysis resulted in the identification of 69 families, 65 genera and 36 species from all treatments including the control (C) (Table 2). A higher number of specific genera and species were identified in V6 compared to V3 when the MBP was enriched in MacConkey broth (Table 3). Analysis of the total and specific numbers of families, genera and species in individual treatments are shown in Table 4. In the control treatment, 16 families were specifically identified out of 57 total families; those 16 families were not being identified by any other treatment (Table 4). Application of the V6 region resulted in less total families, genera and species in the unenriched and MBP grown at 37 °C (Table 4). The V6 region analysis was more effective in bacterial identification in the MacConkey Broth enriched MBP (Table 4).

In the V6 region, the family Enterococcaceae scored the highest read count percentage in the control (59.1%). The Families Clostridiaceae (12.15%) and Pseudomonadaceae (12.75%), respectively, were the second and third highest in the control (Fig. 8). The percentage of the family Enterococcaceae was drastically reduced in C-37 (0.17%) but increased up to 99.49% in Azide Dextrose Broth at 37 °C (AZ-37); while the counts were below 13% in M-28, M-37 and P-37 (Fig. 8). *Enterococcus* and *Vagococcus* were the two major genera found in the family Enterococcaceae in the C and AZ-37 treatments (Fig. 9). However, the species found were not identical; *Enterococcus avium, Enterococcus*

Table 2
Total number of families, genera and species identified in all unenriched and enriched treatments using the Ion Torrent™ sequence analysis based on the sequence variation of V3 and V6 hypervariable regions of 16S rRNA.

	Total from V3 and V6	Common to V3 and V6	V3 Region Analysis			V6 Region Analysis		
			Total from V3	V3 Specific	% Specific to V3	Total from V6	V6 Specific	% Specific to V6
Families	114	37	82	45	39.5%	69	32	28%
Genera	134	24	93	69	51.5%	65	41	30.6%
Species	72	8	44	36	50%	36	28	38.9%

faecalis, Enterococcus hirae, and the bacterium *NLAE-zl-G500* were found in C while *Enterococcus durans, Enterococcus faecium, Enterococcus haemoperoxidus* were in AZ-37 (Table 5).

The family Pseudomonadaceae was present only in C (8.75%) and C-37 (66.9%) (Fig. 8). The genus *Pseudomonas* had been identified in C but not the species (Table 5). In C-37, the family Pseudomonadaceae (66.9%) recorded the highest count percentage (Fig. 8). The genera *Azotobacter* and *Pseudomonas* were identified in this family with *Pseudomonas benzenivorans, Pseudomonas caeni,* and *Pseudomonas salomonii* identified as the species (Table 5).

The family Enterobacteriaceae had only 0.2% read counts in C (it was not included in the top 15 families) and 0.65% in C-37. The count increased to 98.3% in M-28, 87.52% in M-37 and 92% in P-37 (Fig. 8). In the C and C-37, the genera *Citrobacter, Morganella, Raoultella, and Salmonella* were identified in this family. After enrichment in MacConkey broth at both 28 °C and 37 °C, the genera *Enterobacter, Escherichia, Klebsiella, Morganella, Samsonia, Serratia* were confirmed. In addition, the genera *Pantoea, Proteus, Rahnella, Salmonella, Erwinia, Shigella, Trabulsiella,* and *Xenorhabdus* were specifically found in M-28. Furthermore, nine different species were identified in M-28 (Fig. 9 and Table 5).

The genus *Clostridium, Clostridiales, Comamonas, Desulfovibrio, Paracoccu, Proteiniclasticum* and *Pseudomonas* were among the top 15 genera commonly found in treatment C and C-37 (Fig. 9). Four species of the genus *Clostridium* found in the C (*C. propionicum, C. butyricum, C. intestinale, C. sartagoforme*) while only *C. propionicum* and *C. intestinale* were common to both C and C-37. *Pseudomonas* was the top genus in C (31.5%) and second highest count in C-37. No *Pseudomonas* species were confirmed in the C; however, *P. benzenivorans, P. caeni and P. salomonii* were confirmed in C-37 (Table 5).

4. Discussion

The aim of the present study was to develop a robust method to assess the bacterial composition in commercial MBP using the combination of enrichment culture techniques and DGGE based clonal sequencing or NGS.

4.1. Enrichment of commercial microbial-based product

MacConkey broth and GN media were intended to enhance the growth of Gram-negative bacteria, whereas Azide Dextrose Broth and Peptone water + Polymyxine B were to enhance the Gram-positive bacteria. However, some exceptions were observed where Peptone water + Polymyxine B enriched the Gram-negative *Morganella* and *Providencia* in addition to the common Polymyxine B resistant *Proteus spp*. (Sud and Feingold, 1970). From the DGGE-clonal sequencing analysis, the MBP grown in MacConkey Broth (Fig. 2) and GN Broth (Fig. 3) selectively enhanced the growth of Gram-negative *Proteus* and *Morganella* species. Increasing the temperature increased the growth of *Pseudomonas/Shigella* species in MacConkey Broth (Fig. 2). Peptone water + Polymyxine media (Fig. 4) and Azide Dextrose Broth (Fig. 5) enhanced the growth of Gram-positive *Vagococcus* and *Enterococcus* species. Furthermore, the MBP incubated at higher temperatures enhanced the growth of *Pseudomonas* and *Sporosarcina* species (Fig. 1). This indicated that the composition ratio of viable bacteria in the MBP can be altered by temperature changes and enrichment conditions.

The results in this study indicated that enrichment of the MBP has reduced the total numbers, but selectively enhanced the growth of some families, genera and species compared to un-enriched controls. *Pseudomonas, Shigella, Vagococcus, Enterococcus* and *Enterobacter* were

Table 4
The number of treatment-specific read counts of the families, genera and species identified based on the sequence variation of V3 and V6 hypersensitive region of 16S rRNA gene. The specific and total counts were separately analyzed only within the treatments of V3 or V6 hypervariable regions.

16S hypersensitive region	Treatments	Specific families/total families identified	Specific genera/total genera identified	Specific species/total species identified
V3 region	Control (C)	15/68	17/72	11/30
	C-37	14/71	13/71	12/31
	M-28	0/5	2/8	0/2
	M-37	0/4	0/2	0/1
	P-37	1/ 15	3/11	2/5
V6 region	Control (C)	16/60	15/44	8/18
	C-37	8/47	8/34	4/15
	M-28	1/3	7 + 5[b]/16	4[a]/10
	M-37	0/3	2 + 5[b]/9	4[a]/4
	P-37	0/2	1/4	1/1
	AZ-37	0/3	1/5	3/3

[a] All four specific species identified in MacConkey Broth at 37 °C (M-38) were common in MacConkey Broth at 28 °C (M-37) in V6 region.
[b] In addition to the specific genera found, five specific genera were commonly found in both M-28 and M-37 in V6 region analysis.

Table 3
Total and specific number of families, genera and species identified in different treatments using V3 and V6 hypersensitive regions.

Treatments	Families			Genera			Species		
	Total Families	V3 Specific	V6 Specific	Total Genera	V3 Specific	V6 Specific	Total Species	V3 Specific	V6 Specific
C	94	37	26	102	63	23	46	25	12
C-37	85	36	17	88	54	17	43	27	12
M-28	6	3	1	20	4	11	10	0	8
M-37	5	2	1	10	1	8	4	0	3
P-37	17	14	0	12	8	1	7	6	1
AZ-37	4	-	-	3	-	-	3	-	-

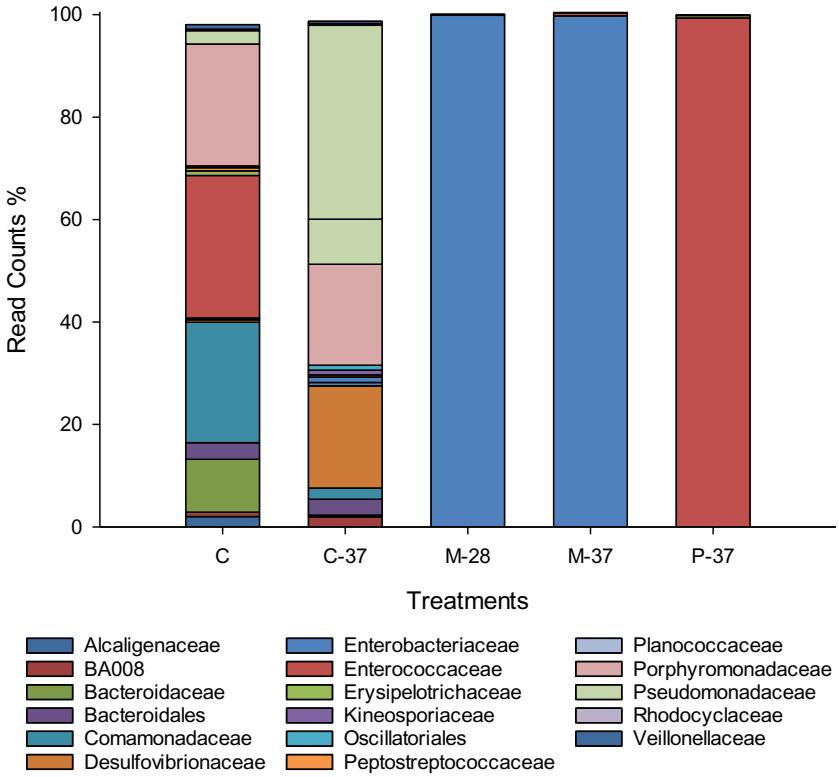

Fig. 6. Fifteen bacterial families identified with highest percentage of read counts in each unenriched and enriched treatments using the sequence variations in V3 hypersensitive regions of 16S rRNA gene. Results from a single sequencing run from two different genomic libraries sequenced are presented.

among the genera identified in the study that were not found in the controls. Some members of these genera are well-known pathogens or could be opportunistic pathogens to immunocompromised people. Some research groups have developed enrichment techniques for the identification of pathogens, including mannitol salt agar for *Staphylococcus* and *Klebsiella* (Dudley et al., 1980), Cetrimide agar for *Pseudomonas* (Dudley et al., 1980), brain heart infusion and selenite-cystine broths for Salmonella in clinical samples (Stone et al., 1994) and enrichment (FPE) media for broad-range foodborne pathogens in the food industry (Hayashi et al., 2013). Therefore, the use of enrichment media for the identification of pathogens is an essential tool in pathogen identification.

4.2. DGGE and clonal sequence analysis of enriched MBP

Analysis of sequence variance of the 16S rDNA gene has widely been used to identify bacterial species. However, no single hypervariable region of the gene is able to differentiate all bacterial species (Chakravorty et al., 2007). Because of this, two hypervariable regions were used in the study. Results from both DGGE and Ion Torrent sequencing indicated that both V3 (203pb) and V6 (440pb) hypervariable regions broaden the identification of different bacterial species.

The DGGE has widely been used as a tool to examine microbial identity and diversity in complex microbial systems (Muyzer et al., 1993). The sizes of primers recommended for DGGE are < 500 bp (Myers et al., 1985). As such, the V3 size was 203 pb and the V6 size 440pb. However, there are some inherent limitations associated with PCR-DGGE where the quality of information produced is dependent on both the number and resolution of the amplicons in the gel which are basically controlled by the primer choice and conditions applied in DGGE (Yu et al., 2004). Theoretically, each band in the DGGE represents a specific bacterial species and the relative intensity of each band represents the relative abundance of a particular species in the population (Muyzer et al., 1993). However, formation of multiple bands representing the same bacterium has been shown in this study as well as in the other studies, and it may be attributed to the drawbacks of PCR, formation of chimeric sequences or the DGGE running conditions (Nowak et al., 2008).

4.3. Identification of bacteria using V3 and V6 regions of 16S rDNA

From the Ion Torrent sequence analysis, only 32.5% of the total 114 families identified were common to both the V3 and V6 regions (Table 1). The number of specific genera and species identified by the V3 region were 69 and 36, respectively while in the V6 region were 39 and 28 respectively. This indicated that the V3 region showed a higher potential for bacterial identification than the V6 region and more specifically in the unenriched MBP incubated at 37 °C and in P-37 (Table 5). In contrast, the V6 region resulted in higher numbers of specific genera and species when enriched in MacConkey broth (Table 5). Additionally, M-28 increased the detection ability of the family Enterobacteriaceae in the V6 region. Only 5 genera were identified in the control, whereas 14 genera and 9 species were identified after enriched in M-28 which included *Escherichia coli, Serratia fonticola* and *Shigella sonnei.*

However, targeting the sequence variance in different hypervariable regions of the 16S rRNA gene for bacterial identification also has some limitations. The presence of different copy numbers in the bacterial genome, which varies from 1 to 15 or more (Klappenbach et al., 2000 and Klappenbach et al., 2001; Vétrovský and Baldrian, 2013), and sequence variation within closely related taxa or within a genome (Vétrovský and Baldrian, 2013), may cause false estimations in bacterial identification and relative abundance of the bacteria. This could result in an underestimate of bacteria with low 16S rRNA copy numbers. Additionally, the copy numbers are directly correlated with the ability to respond to favorable growth conditions (Klappenbach et al., 2000), and therefore, some bacteria may be affected by the various culture enrichment conditions.

Table 5
Species level identification of the treatments (C, C-37, M-28, M-37, P-37 and AZ-37) from both V3 and V6 after analysis of the PGM data. The eight species common to both V3 and V6 are highlighted in bold letters.

	Species	Region	Control	C-37	M-28	M-37	P-37	AZ-37		Species	Region	Control	C-37	M-28	M-37	P-37	AZ-37
1	*Acidovorax caeni*	V3	√						41	*Ignatzschineria larvae*	V3		√				
2	*Actinomyces ruminicola*	V3		√					42	*KBL009 bacterium*	V3		√				
3	*Alcaligenes faecalis*	V3	√	√					43	*Klebsiella oxytoca*	V6			√	√		
4	*Alistipes finegoldii*	V3	√	√					44	*Klebsiella pneumoniae*	V6			√			
5	*Alkalimonas amylolytica*	V6			√				45	*Kosmotoga mrcj*	V3	√					
6	*ASF500 bacterium*	V6	√						**46**	***Koukoulia aurantiaca***	V6	√	√				
7	***Atopostipes suicloacalis***	V6	√						**47**	***Koukoulia aurantiaca***	V3		√				
8	***Atopostipes suicloacalis***	V3	√						48	*MB7–1 bacterium*	V6	√	√				
9	*Bacteroides coprosuis*	V3	√	√					**49**	***Morganella morganii***	V6		√	√	√		
10	*Bdellovibrio bacteriovorus*	V6	√						**50**	***Morganella morganii***	V3	√	√	√	√	√	
11	*Brachymonas petroleovorans*	V3		√					51	*Myroides odoratimimus*	V3	√	√				
12	*Clostridium butyricum*	V6	√						52	*NLAE-zl-C320 bacterium*	V3	√	√				
13	*Clostridium intestinale*	V6	√	√					53	*NLAE-zl-C88 bacterium*	V3	√	√				
14	*Clostridium propionicum*	V6	√	√					54	*NLAE-zl-G500 bacterium*	V6	√					
15	*Clostridium sartagoforme*	V6	√						55	*NLAE-zl-H123 bacterium*	V3					√	
16	*Comamonas nitrativorans*	V3	√	√					56	*NLAE-zl-H227 bacterium*	V3	√					
17	*Curvibacter fontanus*	V3		√					57	*Pantoea agglomerans*	V6			√			
18	*Denitrobacter permanens*	V3	√	√					58	*Paracoccus solventivorans*	V6	√	√				
19	*Desulfarculus baarsii*	V3	√	√					59	*Parapedobacter soli*	V3	√					
20	*Desulfitobacterium aromaticivorans*	V3	√	√					60	*Parapusillimonas granuli*	V3	√	√				
21	*Desulfobulbus elongatus*	V3	√						61	*Petrimonas sulfuriphila*	V3		√				
22	***Desulfovibrio sulfodismutans***	V6		√					62	*Proteiniphilum acetatigenes*	V3		√				
23	***Desulfovibrio sulfodismutans***	V3	√	√					63	*Proteobacterium delta*	V3	√	√				
24	*Enterobacter cloacae*	V6		√	√				**64**	***Proteus mirabilis***	V6					√	
25	*Enterococcus avium*	V6	√						**65**	***Proteus mirabilis***	V3					√	
26	*Enterococcus durans*	V6						√	66	*Pseudomonas benzenivorans*	V6		√				
27	*Enterococcus faecalis*	V6	√	√					67	*Pseudomonas caeni*	V6		√				
28	***Enterococcus faecium***	V6						√	68	*Pseudomonas pertucinogena*	V3		√				
29	***Enterococcus faecium***	V3	√						69	*Pseudomonas salomonii*	V6		√				
30	*Enterococcus haemoperoxidus*	V6						√	70	*rM17 bacterium*	V3	√	√				
31	*Enterococcus hirae*	V6	√						71	*Rothia dentocariosa*	V3		√			√	
32	*Enterococcus raffinosus*	V3	√						72	*Salegentibacter mishustinae*	V3		√				
33	***Erysipelothrix rhusiopathiae***	V6	√	√					73	*Sedimentibacter hydroxybenzoicus*	V3		√				
34	***Erysipelothrix rhusiopathiae***	V3	√	√			√		74	*Serratia fonticola*	V6			√	√		
35	*Escherichia coli*	V6			√	√			75	*Shigella sonnei*	V6			√			
36	*Exiguobacterium artemiae*	V6	√	√					76	*Sphingobacterium mizutaii*	V3	√	√				
37	*Faecalibacterium prausnitzii*	V6	√	√					77	*Te45R bacterium*	V3	√					
38	*Fervidobacterium islandicum*	V3	√						78	*Thermomonas fusca*	V6	√	√				
39	*Geobacter lovleyi*	V3	√	√					**79**	***Trabulsiella farmeri***	V6			√			
40	*Halomonas koreensis*	V3	√						**80**	***Trabulsiella farmeri***	V3	√	√	√			

In soil bacterial diversity analysis, the V3 region was the most prominent hypervariable region whereas the V6 was the least informative compared to the other hypervariable regions (Vasileiadis et al., 2012). Additionally, previous investigations demonstrated that the regions of V3 (nucleotides 433–497) and V6 (nucleotides 986–1043) contain the maximum nucleotide heterogeneity (Chakravorty et al., 2007). Furthermore, the V3 region appears to be an excellent target for the identification of *Staphylococcal* and *Streptococcal* pathogens as well as *Clostridium* and *Neisseria* species except for closely related Enterobacteriaceae (Chakravorty et al., 2007). The V6 region (58 bp in length) was incapable of differentiating the sequence variability of closely related enterobacteriaceae *Escherichia spp., Shigella* spp. and *Salmonella spp.* (Chakravorty et al., 2007). Comparison of multiple V region sequences increased the capacity of assessing of microbial composition in complex commercial mixtures such as microbial-based cleaning products (Subasinghe et al., 2017).

DGGE followed by clonal sequence analysis indicated that diverse bacteria could be identified in different enrichment media using two different hypervariable regions. *Proteus* spp. from V3 and *Morganella* spp. from V6 were found in the GN medium at both 28 °C and 37 °C. The sequences consistent with *Vagococcus* from V3 and *Enterococcus* spp. from V6 were detected with the Azide Dextrose broth at both 28 °C and 37 °C. The MacConkey broth enrichment resulted in DNA sequences consistent with *Escherichia* or *Shigella* and *Morganella* spp. in both the V3 and V6 regions.

Temperature is an important factor in community shifting and metabolism in soil microbial communities and can account for the enrichment of sporulating microorganisms or genomes with higher G + C content (Luo et al., 2014). The results indicated that the MBP grown at different temperatures (22 °C, 28 °C and 37 °C) without any enrichment media were able to enhance the growth of *Pseudomonas* spp. at 28 °C and 37 °C, which was not evident at 22 °C in DGGE (Fig. 1). Enrichment at different temperatures helped to identify different bacteria since many organisms may have a higher optimal temperature for growth. These would not have been detected at lower temperatures.

4.4. Ion torrent sequencing of enriched MBP

High-throughput NGS is a very powerful technology as it targets the entire genetic information contained in an environmental or

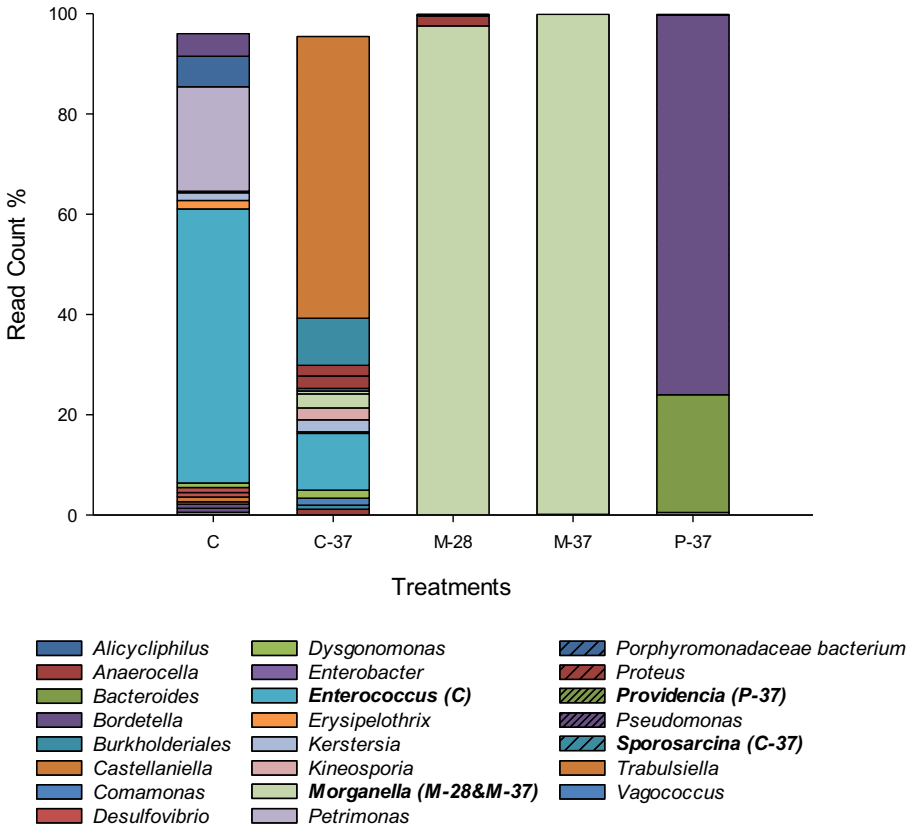

Fig. 7. Fifteen genera identified with highest percentage of read counts in each unenriched and enriched treatments using the sequence variations in V3 hypersensitive regions of 16S rRNA. The genus with the highest read count percentage in each treatment is indicated in bold letters. Results from a single sequencing run from two different genomic libraries sequenced are presented. The percentage identity value required to make a genus identification was 97%.

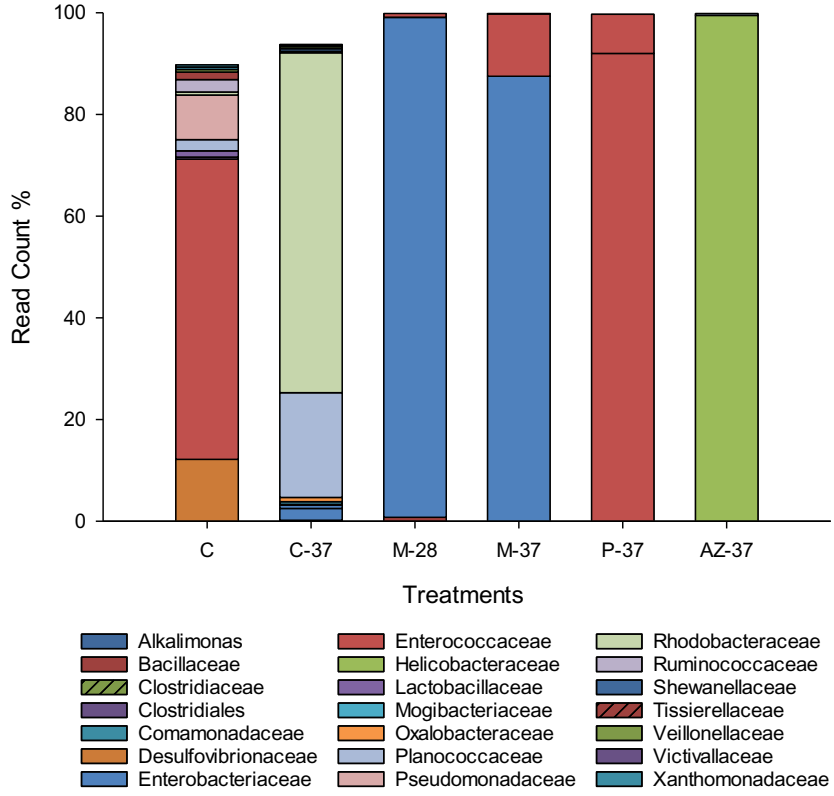

Fig. 8. Fifteen families identified with the highest read count percentages in each unenriched and enriched treatments using the sequence variations of the V6 hypersensitive region of 16S rDNA. Results from a single sequencing run from two different genomic libraries sequenced are presented.

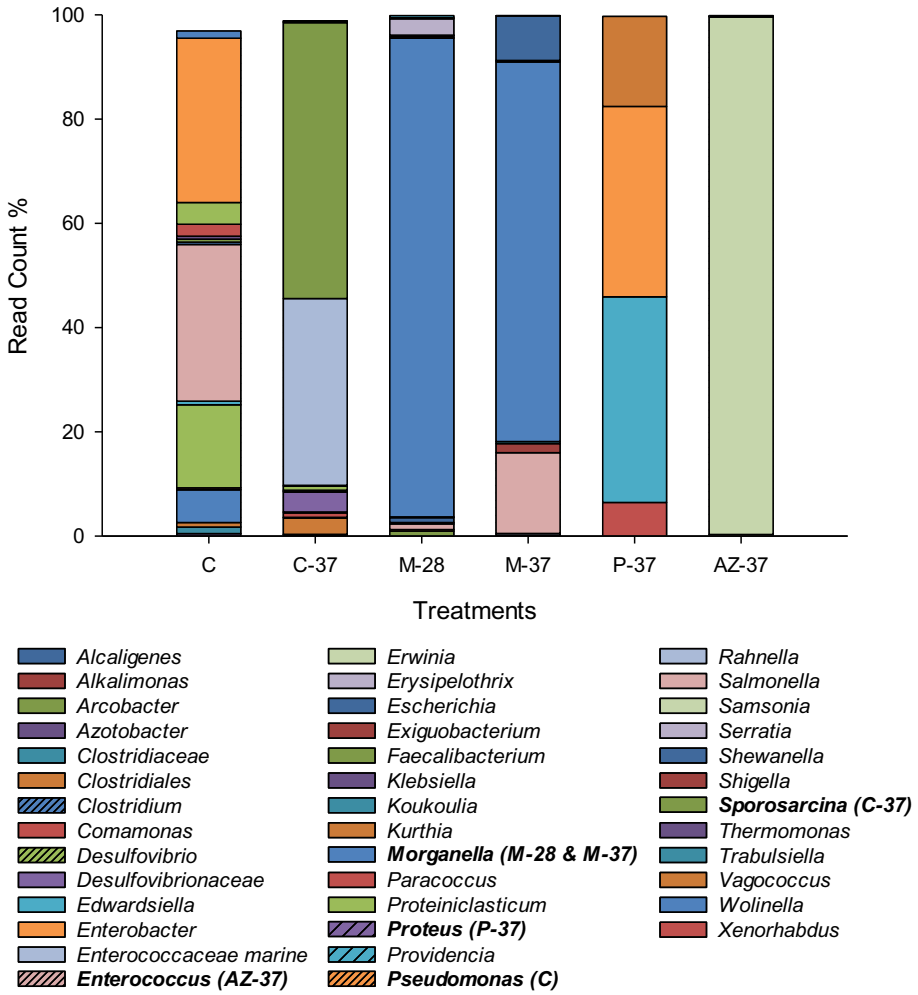

Fig. 9. Fifteen genera identified with the highest read count percentages in each unenriched and enriched treatments using the sequence variations in V6 hypersensitive region of 16S rRNA. The genus with the highest count % in each treatment is indicated bold letters. Results from a single sequencing run from two different genomic libraries sequenced are presented. The percentage identity value required to make a genus identification was 97%.

commercial MBP sample (Subasinghe et al., 2017; Samarajeewa et al., 2015; Vasileiadis et al., 2012; Whiteley et al., 2012). The number of read counts of a particular sequence in NGS can indicate the relative abundance of a specific bacterium within the community. Therefore, higher read counts of a species after enrichment clearly demonstrates the viability of enrichment. Ion Torrent PGM sequencing used in this study enhanced the identification capacity of bacteria, providing a higher number of diverse bacterial families, genera and species. However, the bacterial identification ability is limited to a maximum read length of an individual sequence (Soergel et al., 2012; Vasileiadis et al., 2012). In the current study, the sequence data analysis workflow for the Ion Reporter™ software program was standardized to the 150 bp read length depending on the mean read length of the sequences for both the V3 and V6 regions with a cut-off sequence homogeneity of 97% for genus and 99% for species. Assessing microbial community diversity using longer fragments of 16S rDNA was found to be much more reliable in identifying a bacterium to the genus and species level (Sanschagrin and Yergeau, 2014; Soergel et al., 2012). Incorporating known bacteria in the genomic library could be another option to improve the reliably of the detection method. In addition, targeting genes other than 16S rDNA, including cpn60 and rpoB genes as suggested by Sanschagrin and Yergeau (2014) and Links et al. (2012) or housekeeping genes like gyrase B subunit (*gyrB*), alpha and beta subunits of RNA polymerase (*rpoA* and *rpoB*) and *recA,* a gene encoding for an enzyme important in DNA repair (Zeigler, 2003; Emerson et al., 2008) would be helpful in further identification and confirmation of bacteria to the species level. In the current study, it was demonstrated that NGS coupled with enrichment techniques provided an added advantage in identifying a greater number of bacteria in a microbial-based product.

5. Conclusion

Due to the high microbial diversity in these products, enrichment allows for selecting bacteria with different metabolic or catabolic needs to flourish under ideal nutrient or temperature conditions and thus allows for greater detection of all species and, in particular, pathogens if present. In fact, it has recently been proposed that a polyphasic approach to microbial identification be used to support a microbial identification framework for risk assessment (MIFRA) (Bernatchez et al., 2018). Additionally, the use of enrichment techniques could minimize interferences caused by the presence of excessive naked DNA from dead bacterial cells, which is a common drawback found in microbial identification using DNA-based approaches. The research presented supports a polyphasic approach to better capture the bacteria present in MBP to ensure the safety of these products for consumers.

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