The yield and quality of cellular and bacterial DNA extracts from human oral rinse samples are variably affected by the cell lysis methodology

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Highlights

- Lysozyme-achromopeptidase cell lysis yielded the highest gDNA level
- Lysozyme-zirconium beads cell lysis extracted the highest level of bacterial DNA
- Lysozyme-zirconium beads cell lysis yielded the highest level of Firmicutes

ABSTRACT

Recent culture-independent studies have enabled detailed mapping of human microbiome that has not been hitherto achievable by culture-based methods. DNA extraction is a key element of bacterial culture-independent studies that critically impacts on the outcome of the detected microbial profile. Despite the variations in DNA extraction methods described in the literature, no standardized technique is available for the purpose of microbiome profiling. Hence, standardization of DNA extraction methods is urgently needed to yield comparable data from different studies. We examined the effect of eight different cell lysis protocols on the yield and quality of the extracted DNA from oral rinse samples. These samples were exposed to cell lysis techniques based on enzymatic, mechanical, and a combination of enzymatic-mechanical methods. The outcome measures evaluated were total bacterial population, Firmicutes levels and human DNA contamination (in terms of surrogate *GAPDH* levels). We noted that all three parameters were significantly affected by the method of cell lysis employed. Although the highest yield of gDNA was obtained using lysozyme-achromopeptidase method, the lysozyme-zirconium beads method yielded the peak quantity of total bacterial DNA and Firmicutes with a lower degree of *GAPDH* contamination compared with the other methods. Taken together our data clearly points to an urgent need for a consensus, standardized DNA extraction technique to evaluate the oral microbiome using oral rinse samples. Further, if Firmicutes levels are the focus of investigation in oral rinse microbiome analyses then the lysozyme-zirconium bead method would be the method of choice in preference to others.

Keywords: Bacterial cell lysis; DNA; Firmicutes; *GAPDH*; oral rinse sample; total bacteria

1. Introduction

Recent advances in the microbial genome sequencing technology has opened up new vistas revealing the complexities of the human microbiome. The profile of bacterial species that are detected using nucleic acid based studies can be highly dependent on the method of DNA extraction due to the susceptibility of microbial cells to the lytic activity of enzymes and chemicals, as well as mechanical cell disruption (Morita et al., 2007). For instance, the inability to detect Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* in human saliva or faecal samples in earlier studies might be explicable in terms of their resistance to lysozyme, the major cell lysis agent used in DNA extraction methods (Bera et al., 2005, Horinouchi et al., 1977). Others have noted that depending on the DNA extraction method, the diversity and the yield of Gram-negative bacteria such as Bacteroidetes may vary whilst the identical method is optimal for the quantitative and qualitative evaluation of Gram-positive bacteria such as Firmicutes (Maukonen et al., 2012).

The importance of choosing an appropriate DNA extraction method to study human and environmental microbiome has been investigated and the results indicate significant variations in the microbial profile depending on the extraction method used (Hwang et al., 2011, Maropola et al., 2015, Wesolowska-Andersen et al., 2014, Willner et al., 2012). Such variations in the detected microbial profiles, due to different DNA extraction methods, are thought to be a possible reason for the lack of Bacteroidetes 16S ribosomal RNA (16S rRNA) sequence in the random assemblies and clone libraries (Gill et al., 2006). Hence, these uncaptured bacterial species due to DNA extraction bias still remains a serious challenge on meta-analysis of different sample preparations associated with varying DNA extraction methods. Indeed, the effectiveness of cell lysis during microbial DNA extraction from either human or environmental samples has been reported as a key confounding factor in culture independent microbiome studies (Li et al., 2003).

Bacterial cell lysis, the first step in DNA extraction, could be accomplished by several methods on the basis of either enzymatic, chemical or mechanical protocols and a combination of these methods (Huang et al., 2011, Jiang et al., 2014, Niwa et al., 2005, Said et al., 2014, Schmidt et al., 2014, Xu et al., 2014). A number of workers have compared different commercial kits and non-commercial protocols using either human samples or a laboratory created bacterial community (Claassen et al., 2013, Kennedy et al., 2014, McOrist et al., 2002, Wagner Mackenzie et al., 2015, Wu et al., 2014, Yuan et al., 2012). In contrast, in the current study we attempted to evaluate the qualitative and quantitative changes of the extracted DNA consequential to various cell lysis methods described in the literature. For this purpose, we used DNA from human oral rinse samples and the methods evaluated included enzymatic, mechanical and enzymatic-mechanical cell disruption techniques.

Furthermore, quantitative real-time PCR was performed to quantify total bacteria, Firmicutes and glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) in the extracted DNA samples. Firmicutes were analysed as this phylum is a predominant constituent of the human oral microbiome (Gong et al., 2014, Segata et al., 2012). *GAPDH* was analysed as a surrogate marker of human DNA contamination. In translational terms, our aim was to define the optimal DNA extraction method for oral rinse samples, as such a bench mark technique would be valuable for comparative epidemiological studies of disparate population cohorts.

2. Materials and methods

2.1 Sample collection and preparation

Four healthy individuals (males) who have not received antibiotic therapy over a 4 months period were selected for the study. All participants provided written consent prior to sample collection. The subjects were requested to refrain from eating, drinking and brushing their

teeth before sample collection. They were asked to rinse their mouth thoroughly using 10 mL sterilized phosphate-buffered saline (pH 7.2) for 1 minute (Samaranayake et al., 1986) and expectorate the rinse into a sterile container. The collected samples were kept on ice and subjected to DNA extraction one hour after sample collection.

In order to evaluate the reproducibility of DNA extraction methods and as an internal control, we used the pooled oral rinse samples that were collected from three different individuals. For the evaluation of the effect of cell lysis methods on the yield, quality, the quantity of cellular and bacterial DNA, three samples were collected from one individual immediately after waking up over three consecutive days and subjected to DNA extraction one hour after sample collection

2.2 Cell lysis methods

Eight different bacterial cell lysis methods (methods M1-M8) were used to evaluate the effect of enzymatic, mechanical and enzymatic-mechanical cell lysis methods on the attributes of the extracted DNA (Table 1).

Table 1

Bacterial cell lysis methods used in this study

Cell lysis method	Enzymatic lysis	Mechanical lysis
M1	Lysozyme	----------
M2	Lysozyme	0.1mm glass beads
M3	Lysozyme	0.1mm zirconium beads
M4	Lysozyme-achromopeptidase	0.1mm glass beads
M5	Lysozyme-achromopeptidase	0.1mm zirconium beads
M6	Lysozyme-achromopeptidase	----------
M7	----------	0.1mm glass beads
M8	----------	0.1mm zirconium beads

The aliquot 1.5 mL oral rinse samples were centrifuged at 7500 rpm for 10 minutes and the resultant pellets were subjected to the foregoing cell lysis methods. Two enzymatic lysis buffers, TE-L lysis buffer (20 mM Tris/HCL; 2 mM EDTA; pH 8, supplemented by 15 mg/mL lysozyme, Sigma, 62970) and TE-A lysis buffer (20 mM Tris/HCL; 2 mM EDTA; pH 8, supplemented by 10000 units/ml achromopeptidase, Sigma, A3547-100KU) were prepared for enzymatic lysis. We used achromopeptidase in tandem with lysozyme for DNA extraction as this enzyme has been used to breakdown the cell wall of lysozyme resistant bacteria (Barsotti et al., 1988, Ezaki and Suzuki, 1982, Goto et al., 2007, Lloyd et al., 2013, Said et al., 2014, Ueno et al., 2011). Mechanical cell lysis was performed using 0.1 mm glass beads tubes (Sigma, Z763721) and 0.1 mm zirconium beads tubes (Pathtech, BMD1032-01). The bacterial cell lysis methods we evaluated are briefly annotated below.

Method M1. Here the pellets of oral rinse samples were subjected to a single step cell lysis using lysozyme. The latter was the sole agent of cell lysis in this method. The pellets were then re-suspended in 160 µL TE-L lysis buffer followed by 1 hour incubation at 37ºC prior to DNA purification step using kit protocol.

Methods M2 and M3. Here we used a combination of enzymatic and mechanical cell lysis. The pellets of oral rinse samples were re-suspended in 160 µL TE-L lysis buffer. The lysates were then incubated at 37ºC for 1 hour and immediately mixed with either glass beads (M2) or zirconium beads (M3). 150 µL TE buffer (20 mM Tris/HCL; 2 mM EDTA; pH 8) were added to the lysates in the tubes to make a 200 µL lysate supernatant. The tubes with beads were vortexed rigorously for 10 minutes, both vertically and horizontally prior to DNA purification step using kit protocol.

Methods M4 and M5. Enzymatic-mechanical cell lysis was performed by addition of two lytic enzymes and beads. The 160 µL TE-L lysis buffer were added to the pellets

followed by brief vortexing. The lysates were incubated at 37ºC for 1 hour. Achromopeptidase (TE-A lysis buffer) was added to the lysates to prepare a final concentration of 700 units/mL and the mixtures were then incubated at 37ºC for another 30 minutes. The lysates were added to glass beads tubes (M4) and zirconium beads tubes (M5). Finally, 150 µL TE buffer (20 mM Tris/HCL; 2 mM EDTA; pH 8) was added to the lysates in the tubes to make a 200 µL lysate supernatant. The tubes with beads were vortexed rigorously for 10 minutes, both vertically and horizontally.

Method M6. Two enzymatic cell lysis steps using lysozyme and achromopeptidase, devoid of mechanical cell disruption were used in M6. The pellets of oral rinse samples were re-suspended in 160 µL TE-L lysis buffer followed by 1 hour incubation at 37ºC. Achromopeptidase (TE-A lysis buffer) was added to the lysates for the final concentration of 700 units/mL followed by 30 minutes incubation at 37ºC.

Method M7 and M8. Mechanical cell lysis with beads alone, (i.e. devoid of an enzyme) on the yield and the quality of extracted DNA from human oral rinse samples, was analysed in M7 and M8. This was performed by adding 160 µL TE buffer (20 mM Tris/HCL; 2 mM EDTA; pH 8) to the pellets. The mixtures were added to the tubes with either glass beads (M7) or zirconium beads (M8). Another 150 µL TE buffer were added to the lysates in the tubes before vortexing. The tubes with beads were vortexed rigorously for 10 minutes both vertically and horizontally.

2.3 DNA isolation and purification

The 200 µL of the cell lysates, prepared in cell lysis step, were subjected to further DNA isolation and purification based on the ISOLATE II Genomic DNA Kit (Bioline; BIO-52066) protocol for Gram-positive bacteria. All lysates derived from (M1 to M8) were subjected to

Proteinase K (Bioloine kit provided) and RNAse (Thermo Fisher Scientific, EN0531) treatment as per the manufacturer's protocol. The extracted DNA was eluted using 100 µL elution buffer (kit provided buffer) and aliquots were stored as 20 µL elutes and stored at -20ºC for further analysis.

2.4 DNA quality and quantity

The quality of the extracted DNA and PCR amplicons was evaluated by running the template on 1% agarose gel for 45 min and 5V/cm. The DNA bands were visualized using Gel Doc™ EZ Imager (Bio-Rad). DNA concentration and purity were measured from the eluted DNA samples using a NanoDrop (spectrophotometer ND-1000).

2.5 Primers used in this study

Extracted gDNA from human oral rinse samples were amplified using Bact349F (5□-AGGCAGCAGTGGGGAAT-3□) and Bact518R (5□-ATTACCGCGGCTGCTGG-3□) for the quantification of total bacteria (Bong-Soo et al., 2011). Firmicutes were amplified and quantified using 928FirmF (5□-TGAAACTCAAAGGAATTGACG-3□) and 1040FirmR (5□-ACCATGCACCACCTGTC-3□) (Bacchetti De Gregoris et al., 2011). Human DNA was quantified by *GAPDH* amplification using *GAPDH*-F (5□- ACATCATCCCTGCCTCTAC -3□) and *GAPDH*-R (5□- TCAAAGGTGGAGGAGTGG -3□) (Karakas et al., 2006, Xu et al., 2014).

2.6 DNA standard preparation

The known DNA concentration standards were prepared from the PCR products of gDNA as previously described (Castillo et al., 2006, Fu et al., 2006). Four samples of 10-fold serially diluted DNA standards from the PCR products of *Escherichia coli* strain JM109 (Promega, P9751), *Streptococcus australis* (isolated from human saliva) and human U-937 cells (ATCC® CRL-1593.2™) were used for standard curve generation to quantify total bacteria, Firmicutes and *GAPDH*, respectively.

2.7 Quantitative real-time PCR

The Bio-Rad iQ5 thermal cycler was used to conduct quantitative real-time PCR. Three quantitative real-time PCR assays were performed to quantify the total bacteria, Firmicutes and human DNA (*GAPDH*) content of the extracted nucleic acids. Three negative control, non-template reaction mixtures (NTC), were also prepared to analyse the degree of contamination in the reaction mixtures.

Five µL iTaq universal SYBR green supermix (Bio-Rad 172-5120), 300 ng final concentration of each primer, 4 ng of DNA template and nuclease free water were mixed to make a final volume of 10 µL PCR reaction mixture. The real-time PCR was started with initial denaturation at 95ºC for three minutes for one cycle followed by 40 cycles of DNA denaturation at 95ºC for 15 seconds per cycle, annealing and extension steps for 60 seconds per cycle at 60ºC, 58.9ºC and 57.1ºC for total bacteria, Firmicutes and *GAPDH* amplification, respectively. The melting curve analysis was performed by running 81 cycles from 55ºC to 95ºC for 30 seconds for each cycle and at the range of 0.5ºC per cycle. Each real-time PCR reaction including NTC, DNA template and standard DNA were prepared in triplicates. Technical replicates were subjected to data analysis, if the Ct value (threshold cycle) difference was not more than 0.4 cycles and represented normal amplification curve.

The standard curve for the quantification of total bacteria, Firmicutes and *GAPDH* were prepared by running quantitative real-time PCR of four 10-fold serially diluted samples of known concentration DNA templates. The results were validated only when the qPCR efficiency (calculated from the slop of the standard curve; PCR efficiency = $10^{-1/slope}$ - 1) was between 90% and 110% by using at least three continuous standard concentrations (Bustin et al., 2009, Fey et al., 2004).

The copy numbers for the target PCR products were calculated from the standard curves as previously described and based on the assumption of the average weight of a base pair equal to 650 Daltons (Broberg et al., 2003, Bustin et al., 2009, Fey et al., 2004, Gruss and Sauer, 1975, Tatineni et al., 2008).

2.8 Analysis

All data were analysed statistically using GraphPad Perism (version 6.05, GraphPad Software, Inc., CA USA). One-way ANOVA (Fisher's least significant difference, LSD Post hoc) was used for the comparison analysis between DNA concentration, *GAPDH*, total bacteria and Firmicutes quantity. The mean value and standard deviations were used to express all data. *P*-value less than 0.05 was considered as a statistically significant. Microsoft excel, 2013 was used to analyse the real-time PCR data analysis, standard curve preparation and gene copy number calculations.

2.9 Ethics statement

The protocol and ethical aspects of this work were reviewed by Griffith University Human Research Ethics Committee under ethics approval certificate MSC/14/13/HREC and written informed consent were obtained from all participants.

3. Results

DNA concentration, DNA purity and the quantificative estimates of total bacteria, Firmicutes, and *GAPDH* using the extracted DNA from three oral rinse samples collected from one individual are evaluated and summarized in Table 2.

Table 2

The properties of DNA extracts derived using various cell lysis methods (M1 to M8)

Methods*	DNA concentration (ng/ µL)	A_{260}/A_{280}	A_{260}/A_{230}	Total bacterial copy number (log)**	Firmicutes copy number (log)**	*GAPDH* copy number (log)**
M1	38.13 ± 5.32	1.88 ± 0.05	2.12 ± 0.23	6.76 ± 0.07	6.8 ± 0.04	5.82 ± 0.13
M2	43.73 ± 7.60	1.86 ± 0.02	2.12 ± 0.16	6.92 ± 0.11	7.15 ± 0.12	5.90 ± 0.005
M3	41.17 ± 7.47	1.88 ± 0.05	2.25 ± 0.29	7.08 ± 0.08	7.28 ± 0.09	5.84 ± 0.10
M4	68.53 ± 30.69	1.85 ± 0.01	2.25 ± 0.14	6.85 ± 0.10	7.02 ± 0.02	5.90 ± 0.07
M5	63.27 ± 9.60	1.89 ± 0.02	2.31 ± 0.02	6.87 ± 0.07	7.10 ± 0.09	5.84 ± 0.09
M6	99.37 ± 24.71	1.88 ± 0.02	2.25 ± 0.11	6.76 ± 0.048	6.92 ± 0.11	5.71 ± 0.03
M7	57.30 ± 14.86	1.83 ± 0.01	2.33 ± 0.17	6.81 ± 0.10	6.95 ± 0.13	5.79 ± 0.14
M8	83.33 ± 15.33	1.90 ± 0.02	2.27 ± 0.13	6.74 ± 0.06	6.98 ± 0.03	5.82 ± 0.06

*Methods M1-M8 were described in methods section

**Copy numbers (log) per 4ng genomic DNA loaded in real-time PCR reaction

3.1 DNA concentration comparison

The one-way ANOVA comparison of DNA concentrations for various DNA extraction methods is shown in Fig. 1. The lowest DNA concentration/yield was obtained for M1, M2 and M3 which were 38.13 ± 5.32, 43.73 ± 7.60 and 41.17 ± 7.74 ng/µL, respectively. There was no-significant differences between M1, M2 and M3 implying that the addition of beads to lysozyme was ineffective in increasing the DNA yield.

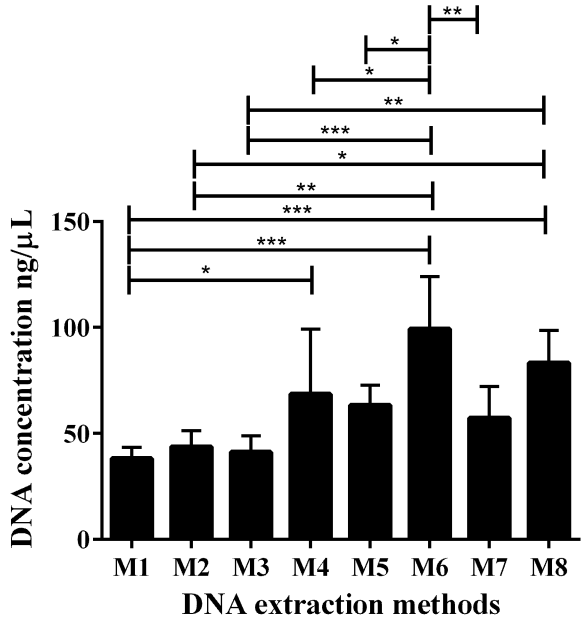

Fig. 1. The impact of using eight different cell lysis methods on the concentration of extracted DNA from three human oral rinse samples (samples were collected from one individual over the three consecutive days). The error bars are the mean of three biological replicates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

The pairwise comparison showed that there was a significant DNA yield when lysozyme-achromopeptidase was used (M6) in comparison with the yield from all the other methods except M8. The addition of beads to lysozyme-achromopeptidase (M4 and M5) marginally increased the DNA concentration in comparison with the use of only lysozyme (M1) or lysozyme-beads (M2 and M3). However, the addition of glass beads to achromopeptidase-lysozyme (M4) yielded significantly more DNA ($P < 0.05$) compared to the use of only lysozyme (M1).

We also noted a significant reduction ($P < 0.05$) in the extracted DNA yield when beads were used in the combination of lysozyme-achromopeptidase (M4 and M5) in comparison with the use of lysozyme-achromopeptidase alone (M6). In addition, the data showed that utilizing zirconium beads as a sole cell lysis agent could be effective in yielding a higher concentration of DNA (M8) compared to M1, M2 and M3 methods. Furthermore, no significant difference in the DNA yield was observed between zirconium beads (M8) and glass beads (M7) where they were used as a sole agent of cell lysis.

3.2 DNA quality comparison

The DNA gel integrity evaluation indicated a high level of DNA fragmentation when beads were used as lysis agents in M7 and M8 (Fig. 2). In addition, no obvious bands were seen in the extracted DNA using M2 (lysozyme-glass beads). Fairly well separated high molecular weight DNA bands were observed for samples from M1, M3, M4, M5 and M6. In addition, the high molecular weight DNA was separated on the gel for the first and second collected samples and no band was visualized for the third collected samples except for M4 from the same individual.

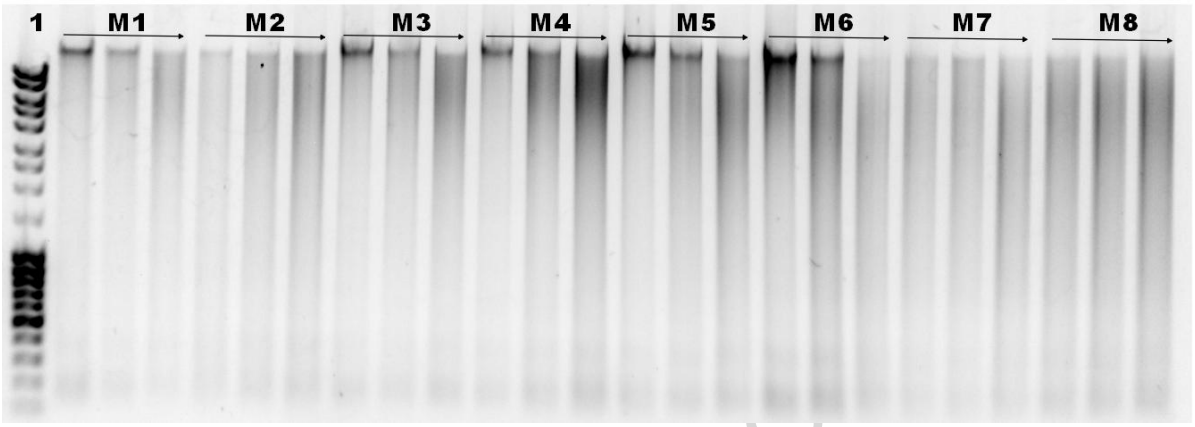

Fig. 2. The illustrative results of gel electrophoresis analysis (running on 1% agarose gel for 40 min at 5V/cm) of the isolated DNA from three oral rinse samples (samples were collected from one individual over the three consecutive days) using eight different cell lysis methods for the evaluation of DNA quality and gel integrity. **DNA ladder (**MassRuler DNA Ladder Mix (Thermo Scientific, SM0403), lane 1.

The Fig. 3 shows the purity of extracted DNA for different cell lysis methods. All A_{260}/A_{280} ratios were between 1.8 and 2 (Fig. 3A). However, there was a significant differences between the mean A_{260}/A_{280} ratios of DNA extracts between M5 and M7 as well as M7 and M8 ($P < 0.05$).

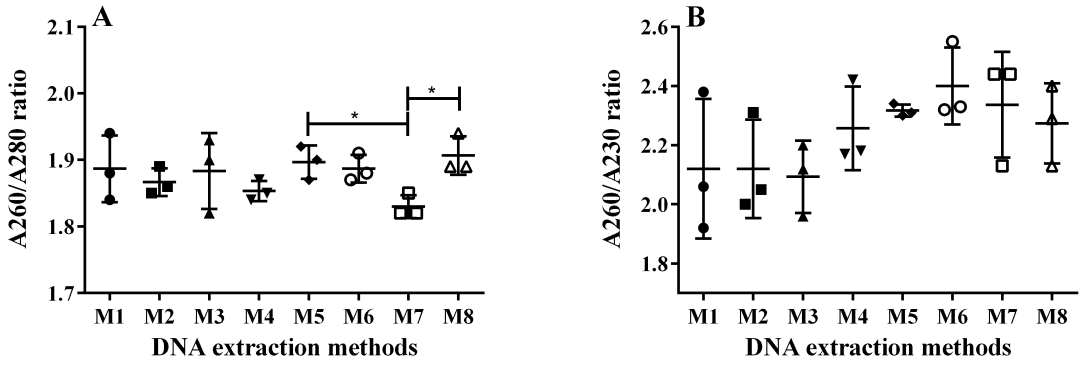

Fig. 3. The impact of using eight different bacterial cell lysis methods on the ratio of A_{260}/A_{280} (A) and A_{260}/A_{230} (B) of DNA samples extracted from human oral rinse samples (samples were collected from one individual over the three consecutive days). The error bars indicate the mean of three biological replicates (*$P < 0.05$).

The A_{260}/A_{230} ratio of the extracted DNA is shown in Fig. 3B. There was no significant difference in A_{260}/A_{230} ratios when the different cell lysis methods were used. Although the mean ratio of the A_{260}/A_{230} in methods M4 to M8 were above the accepted range of 2.2 (Olson and Morrow, 2012), the remainder of the techniques (i.e. M1 to M3) were within the accepted norm for the mean A_{260}/A_{230} ratio.

The gel electrophoresis of amplified 16S rRNA genes is shown in Fig. 4. The visualization of amplified 16S rRNA gene showed that although the ratio of A_{260}/A_{230} for extracted DNA using M4-M8 was beyond normal range (>2.2), there was no PCR inhibitors to prohibit amplification, and all samples were amplifiable to produce 1500bp amplicons using Fd1 (5□-AGAGTTTGATCCTGGCTCAG-3□) and Rd1 (5□-AAGGAGGTGATCCAGCC-3□) primers (Alves et al., 2002).

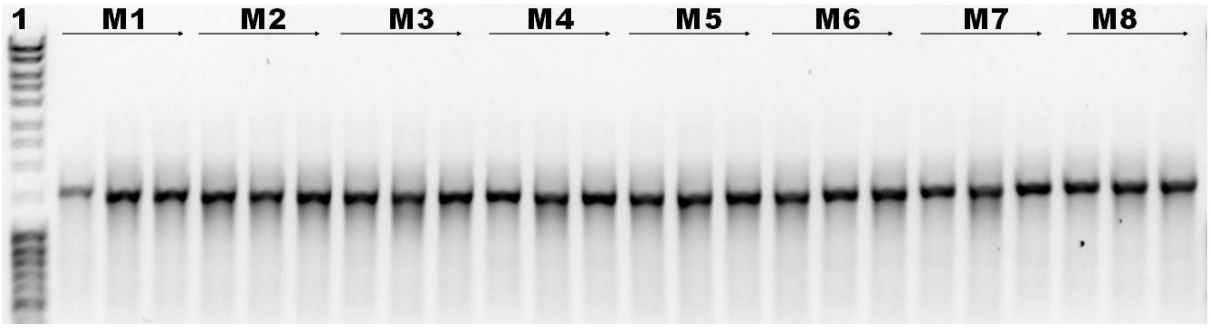

Fig. 4. The representative results of gel electrophoresis analysis (running on 1% agarose gel for 60 min at 5V/cm) for the amplified 16S rRNA using the DNA samples extracted from

human oral rinse samples (samples were collected from one individual over the three consecutive days). **DNA ladder (**MassRuler DNA Ladder Mix (Thermo Scientific, SM0403), lane 1.

3.3 DNA extraction reproducibility comparison

A single pooled oral sample from three individuals was used for the evaluation of technical reproducibility of DNA extraction methods in terms of DNA concentration and purity. Three replicates of DNA extracts from each cell lysis technique were evaluated on three different occasions for this purpose. The highest variance in DNA concentration was seen when enzymes were used as a sole source of cell lysis as shown in Fig. 5A. On the other hand, the least variations in DNA yield was noted when glass and zirconium beads were used in combination with lysozyme or when only the glass beads were used for cell lysis.

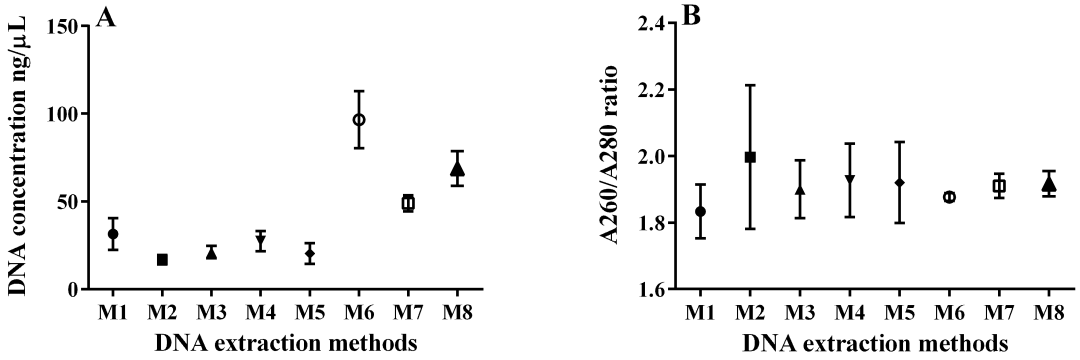

Fig. 5. The impact of applying eight different cell lysis methods on the reproducibility of DNA concentration (A) and DNA purity; A260/A280 ratio (B) of the DNA samples extracted from the pooled human oral rinse samples (samples were collected from three individuals and pooled together). The error bars indicate the mean of three technical replicates.

Although the largest variances in DNA purity (Fig. 5B) was achieved using M2 (1.99 ± 0.2), M4 (1.92 ± 0.11) and M5 (1.92 ± 0.12) methods, the one-way ANOVA comparison showed that there were no significant differences between those eight cell lysis methods in terms of DNA purity.

3.4 Quantitative real-time PCR

Each quantitative real-time PCR reaction was run in triplicates. The melting curve showed no primer dimer or any other unwanted amplifications. There were amplifications in NTC tubes in total bacterial (Ct = 26.56 ± 1.2) and Firmicutes (Ct = 33.51 ± 0.33) quantification assays. However, we discounted this low level of contamination as per the suggestions of previous investigators (Bustin et al., 2009, Bustin and Nolan, 2004, Corless et al., 2000).

There was a linear relationship between log copy numbers of target PCR products and Ct value of qPCR. The following equations were used to calculate the copy numbers of target sequences within the extracted DNA. $Ct = -3.5318\ X + 35.771$, $R^2 = 0.9998$; for the quantification of total bacteria, $Ct = -3.4312\ X + 37.735$, $R^2 = 0.9997$; for the quantification of Firmicutes and $Ct = -3.5804\ X + 41.173$, $R^2 = 0.9996$; for the quantification of *GAPDH* where X is the log copy number of target products. The efficiency calculation showed that the qPCR efficiency was 91.93%, 95.63% and 90.24% for total bacteria, Firmicutes and *GAPDH* assays, respectively.

3.5 Total bacterial quantification

The results of total bacteria quantification for the different cell lysis methods are shown in Fig. 6. The most amount of bacterial DNA was yielded using method M3. Remarkably,

addition of beads to lysozyme (M2 and M3) increased the total bacterial copy numbers significantly. However, in combination with lysozyme, zirconium beads (M3) appeared to be more effective ($P \leq 0.001$) than glass beads ($P < 0.05$) in increasing the total bacterial copy numbers. On the other hand, the use of lysozyme-achromopeptidase-beads (M4 and M5) had no impact on increasing the total bacterial copy numbers compared with other methods.

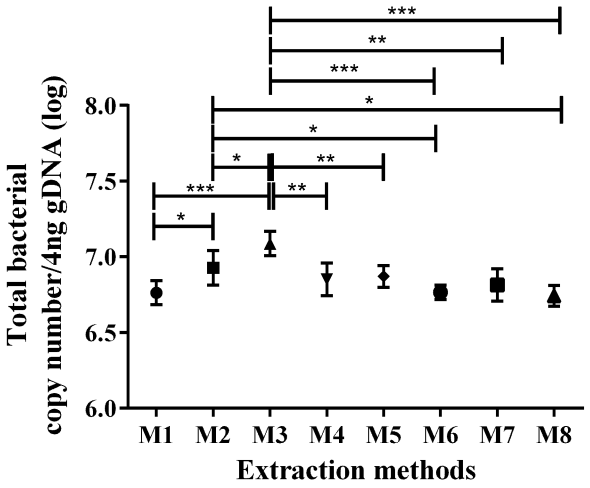

Fig. 6. The impact of using eight different cell lysis methods on the quantity of total bacterial copy numbers within the DNA samples extracted from human oral rinse samples (samples were collected from one individual over the three consecutive days). The error bars indicate the mean of three biological replicates and three technical replicates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.6 Glyceraldehyde 3-phosphate dehydrogenase quantification

Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was quantified as a surrogate marker to evaluate the effect of different bacterial cell lysis methods on the level of human DNA contamination. The results showed there was no significant differences in the quantity

of *GAPDH* within the extracted DNA except for the M6 method (Fig. 7A) which yielded significantly less human DNA in comparison to M2 and M4 ($P < 0.05$) where the glass beads were used in combination with enzymes. The ANOVA (Fig. 7B) showed a statistically significant increase ($P < 0.05$) in the ratio of bacterial DNA to human DNA in method M3 compared to M1, M4 and M8.

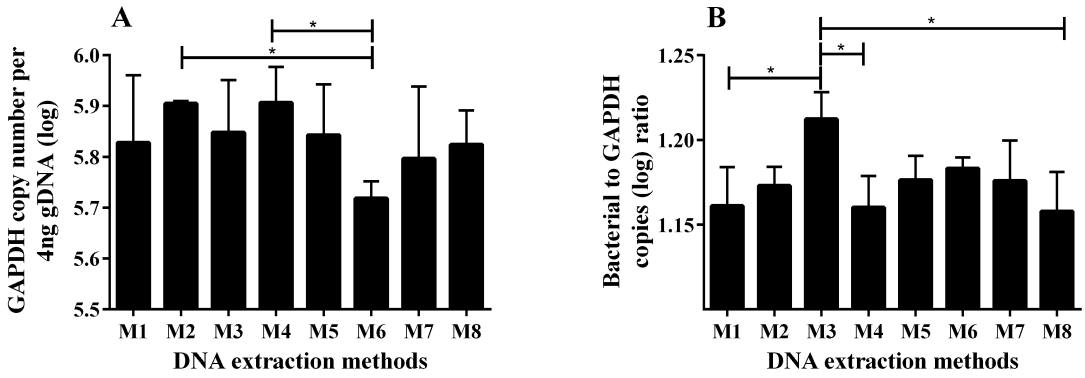

Fig. 7. The impact of using eight different cell lysis methods on the quantity of *GAPDH* copy numbers within the DNA samples extracted from human oral rinse samples (samples were collected from one individual over the three consecutive days). Boxplots show the *GAPDH* copy numbers (A) and the ratio of total bacterial to *GAPDH* (B). The error bars indicate the mean of three biological replicates and three technical replicates (*$P < 0.05$).

3.7 Firmicutes quantification

Firmicutes quantification was performed to evaluate the effect of different cell lysis methods on the yield of Firmicutes in the extracted DNA from human oral rinse samples. These results shown in Fig. 8A indicate that beads in combination with lysozyme (M2 and M3) significantly increased the Firmicutes copy numbers. This increase was more effective when

zirconium beads ($P < 0.0001$) were used instead of glass beads ($P < 0.001$). The quantity of Firmicutes from M3 also was clearly more than that obtained from other methods. The use of beads, whether zirconium or glass, as a sole agent of the cell lysis (M7 and M8) was not effective in increasing the yield of Firmicutes in the extracted DNA compared to other methods. The ratio of Firmicutes to total bacteria (Fig. 8B) showed that using M2, M5 and M8 increased the ratio of Firmicutes copy numbers to total bacteria copy numbers ($P < 0.05$) in comparison with M1.

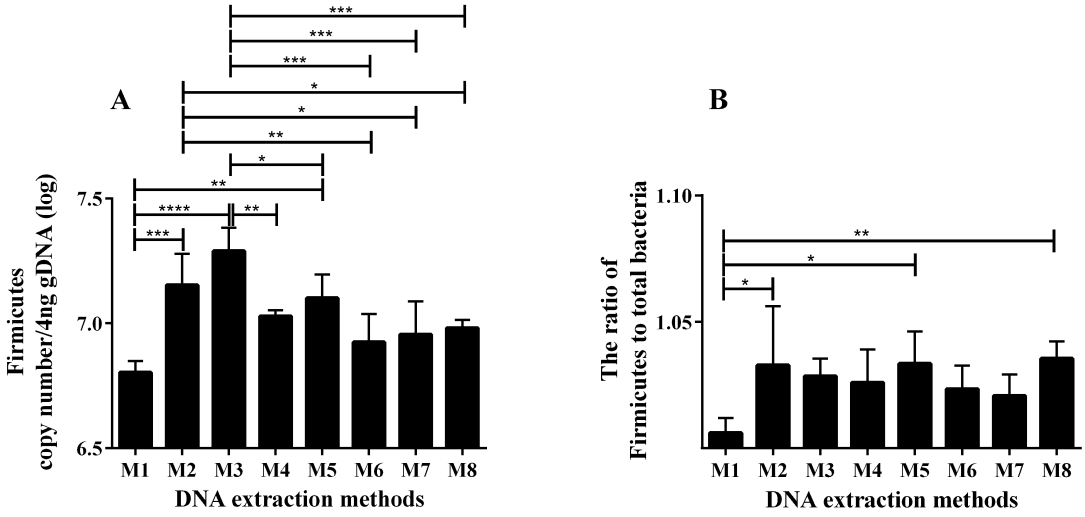

Fig. 8. The impact of using eight different cell lysis methods on the quantity of Firmicutes (A) and the ratio of Firmicutes to total bacterial (B) within the DNA samples extracted from human oral rinse samples (samples were collected from one individual over the three consecutive days). The error bars indicate the mean of three biological replicates and three technical replicates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

4. Discussion

A standardized oral rinse is a widely used, non-invasive collection method for evaluating the human oral microbiome in culture-independent studies (Ahn et al., 2011, Bassis et al., 2015, Ghannoum et al., 2010, Twigg III et al., 2014). Although wide varieties of DNA extraction methods have been used by researchers for the purpose of human oral microbiome profiling, no consistent method has emerged as a bench mark for such investigations. The variations in the resultant microbial profiles in different studies due to methodological disparities make comparisons difficult and not very meaningful (Mai and Draganov, 2009). Although the results of a few studies available to date have shown no significant effects of different DNA extraction methods on the resultant bacterial profiles of biological samples (Peng et al., 2013, Wagner Mackenzie et al., 2015, Wu et al., 2014), a number of workers have recognised and called for a consensus and a universal DNA extraction method for microbiome analyses to overcome the challenges and misrepresentation inherent in using disparate techniques (Abusleme et al., 2014, Claassen et al., 2013, Henderson et al., 2013, Kennedy et al., 2014, Lazarevic et al., 2013, Maukonen et al., 2012, Walker et al., 2015, Yuan et al., 2012).

The results of the current study clearly indicate that different cell lysis methods significantly affect the DNA yield, the yield of Firmicutes, and the total bacteria detected. We noted that of the eight different methods evaluated the highest DNA yield was achieved using a combination of two enzymes, lysozyme and achromopeptidase (M6). This concurs with the study of Ueno et al., (2011) who obtained from fecal samples, the highest DNA yield using the identical lysozyme-achromopeptidase system. Additionally, we also demonstrate here that the use of enzymatic methods such as lysozyme (M1) and lysozyme-achromopeptidase (M6) could lead to large variations in DNA concentration of technical replicates in comparison with other methods.

With regard to the gel electrophoresis of the extracted DNA, the method M6 also yielded higher molecular weight DNA from the rinse samples. In terms of visualization of

high molecular weight and non-degraded DNA, using the extracted DNA from three samples collected from the same individual, the gel electrophoresis results were clearly different between M1, M3, M4, M5, and M6. It has been reported that 10% of the DNA samples from a saline mouthwash was degraded due to individual-specific factors, and there was approximately a threefold rise in the risk of DNA degradation in subsequent samples collected from the same subjects (Zayats et al., 2009).

Our gel electrophoresis results also indicated a high level of DNA fragmentation when beads were used as a sole source of cell lysis. It appears therefore, the use of beads alone (M7 and M8) or in combination of glass beads with lysozyme (M2) may lead to the formation of chimeric products during PCR amplification possibly due to shearing of DNA into smaller molecules (Liesack et al., 1991, V. Wintzingerode et al., 1997). Taken together the available data strongly suggest that the use of any type of beads should be discouraged during DNA extraction when intact, high molecular weight nucleic acids are required as an end-product.

On the other hand, it was observed that the addition of beads to the enzymatic system could reduce the variability of DNA concentration, and yielded more consistent results from technical replicates. This suggests that a beads-enzyme mixture, especially when achromopeptidase is used, could increase the reproducibility of DNA yield besides producing high molecular weight DNA.

Purity of DNA sample is considered as a critical factor that impacts upon the downstream molecular analysis such as PCR and sequencing (McOrist et al., 2002). We performed purity assessment of the extracted DNA using spectroscopy indices A_{260}/A_{280}, as a protein and RNA contamination indicators and A_{260}/A_{230}, as a phenol, salt, protein and polysaccharide contamination indicator (Olson and Morrow, 2012). The range of 1.8 to 2 for

the ratio of A_{260}/A_{280} and 1.8 to 2.2 for the ratio of A_{260}/A_{230} is generally accepted as good quality indicators of extracted DNA (Olson and Morrow, 2012). The obtained A_{260}/A_{280} ratio showed that there were no significant differences in this parameter using all the cell lysis methods we used, and the extracted DNA was within the aforementioned range of acceptable validity. We also showed that there were no significant differences in terms of A_{260}/A_{230} ratio, in extracted DNA using different cell lysis methods. The mean value of A_{260}/A_{230} ratio in the extracted DNA using M1, M2 and M3 (<2.2) indicated the probability of extracting purer DNA using mentioned methods. Although the mean values of A_{260}/A_{230} ratio in the extracted DNA using M4-M8 was greater than 2.2, it was shown that this level of contamination was not effective to inhibit the amplification of bacterial 16S rRNA gene using universal primers.

The quantitative real-time PCR was performed to assess the effects of different cell lysis methods on the quantity of total bacteria, Firmicutes, and the ratio of extracted bacterial DNA to human DNA. Interestingly, the results of quantification showed that higher DNA yield does not necessarily lead to a parallel increase in bacterial DNA from oral rinse samples. For examples, method M6 yielded the highest amount of DNA in comparison with other methods but bacterial copy numbers (total bacteria) was the lowest compared with other methods. On the other hand, although the addition of beads to lysozyme was not effective in increasing the DNA yield, the results showed a significant impact of beads in combination with lysozyme on the total recovery of bacterial DNA. Similar result indicating the high potential of beads systems to recover more bacterial DNA and the overall low DNA yield has been reported (Abusleme et al., 2014).

This study also showed that the use of beads in combination with lysozyme was significantly more effective in improving the quantity of Firmicutes in extracted DNA from human oral rinse samples. In addition, we demonstrate that lysozyme-bead systems (M2 and M3) could improve the yield of Firmicutes significantly more than using lysozyme-

achromopeptidase-beads (M4 and M5) or using only enzymatic cell lysis (M1 and M6). Taken together our data imply that the lysozyme and zirconium bead method (M3) yields more total bacterial DNA, as well as Firmicutes relative to the other methods. Further the use of the method M3 resulted in a lower degree of human DNA contamination to the total bacteria in the extracted DNA from human oral rinse samples. It appears, therefore, If Firmicutes levels are the focus of investigation in oral rinse sample analyses then the lysozyme and zirconium bead method (M3) would be the method of choice.

5. Conclusion

Oral rinse sample collection method has been used for the study of human oral microbial analysis using both culture based and culture-independent techniques. Choosing an appropriate cell lysis method is one of the key elements that predicates the total bacterial DNA yield and significantly affects the outcome. The eight different common cell lysis methods evaluated here highlights the significant impact of each method on the DNA yield, proportion of Firmicutes and the level of human DNA contamination in bacterial DNA extraction from human oral rinse samples. This study underscores that the application of rigorous cell lysis methods (zirconium beads) together with lysozyme is more effective than using other methods to extract more bacterial DNA, more Firmicutes along with less human DNA from human oral rinse samples. Further, if Firmicutes are the focus of analysis then the lysozyme-zirconium bead method would be the method of choice. Taken together our data clearly points to an urgent need for a consensus, standardized DNA extraction technique to evaluate the oral microbiome when using oral rinse samples.

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Conflict of interest

The authors declare that they have no conflict of interests.

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The yield and quality of cellular and bacterial DNA extracts from human oral rinse samples are variably affected by the cell lysis methodology

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