Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis

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Abstract
Freezing stool samples prior to DNA extraction and downstream analysis is widely used in metagenomic studies of the human microbiota but may affect the inferred community composition. In this study, DNA was extracted either directly or following freeze storage of three homogenized human fecal samples using three different extraction methods. No consistent differences were observed in DNA yields between extractions on fresh and frozen samples; however, differences were observed between extraction methods. Quantitative PCR analysis was subsequently performed on all DNA samples using six different primer pairs targeting 16S rRNA genes of significant bacterial groups, and the community composition was evaluated by comparing specific ratios of the calculated abundances. In seven of nine cases, the Firmicutes to Bacteroidetes 16S rRNA gene ratio was significantly higher in fecal samples that had been frozen compared to identical samples that had not. This effect was further supported by qPCR analysis of bacterial groups within these two phyla. The results demonstrate that storage conditions of fecal samples may adversely affect the determined Firmicutes to Bacteroidetes ratio, which is a frequently used biomarker in gut microbiology.

Introduction
Investigating the composition of the human microbiota and correlating findings to specific clinical or physiological states such as irritable bowel diseases and obesity has demonstrated the collective importance of the bacterial community present in the gut as a regulatory factor in health and disease (Young et al., 2011). Because of the large diversity and complexity of interactions between the resident bacteria, various simplifications have been sought. An example of this is the use of the ratio between the two most predominant phyla, namely the Firmicutes and the Bacteroidetes, as a biomarker in relation to human physiology (Armougom & Raoult, 2008; Guo et al., 2008; Mariat et al., 2009). Efficient and nonbiased extraction of total genomic bacterial DNA from the complex fecal samples is a crucial first step for many molecular-based studies of the bacterial community within the gut environment. Downstream applications, such as quantitative PCR and metagenomic sequencing, ultimately require unbiased DNA input to produce accurate and creditable research results. Previous studies have assessed the effectiveness of various DNA extraction procedures based on, for example, DNA yield, extent of DNA shearing, and use as template for subsequent PCR and have often been related to detection of specific pathogens (McOrist et al., 2002; Tang et al., 2008; Ariefdjohan et al., 2010; Persson et al., 2011). The phylogenetic composition of the extracted DNA in relation to the original bacterial community has however received less attention. One major problem in assessing this is the fact that it is very difficult to know the true community composition. In two recent studies, it was shown that a mechanical bead-beating step during cell lysis resulted in increased complexity of extracted DNA as evidenced by an increased number of distinct bands in PCR-DGGE profiles (Ariefdjohan et al., 2010; Smith et al., 2011). The fact that different extraction procedures performed on the
same fecal sample may lead to different estimations of the bacterial community composition is not surprising, but may well be disturbing for comparisons between separate studies. Within a study, it is most probable that the same DNA extraction method be used throughout; however, other parameters that may affect extraction, such as storage conditions of fecal samples, may vary. It is for instance common practice, mainly for practical reasons, to freeze fecal samples immediately after sampling and then collectively extract the DNA and perform downstream analysis such as sequencing or qPCR, at some later stage (Mariat et al., 2009; Santacruz et al., 2009). In this study, the effect of freezing fecal samples prior to DNA extraction was evaluated for alterations in DNA recovery and bacterial community composition as determined by downstream quantitative PCR analysis.

**Materials and methods**

Fecal samples were obtained from three healthy adult volunteers (two women, one man), homogenized thoroughly in four volumes diluent (0.85% NaCl, 0.1% peptone), centrifuged at 300 g for 2 min to remove large debris, and finally 0.5 mL of aliquots (average 8 mg dry weight) were pelleted at 10 000 g for 5 min (Fig. 1). Extraction of DNA was performed immediately with three different extraction methods (five replicates), or samples were frozen at −20 °C for 53 ± 5 days (F) prior to extraction. Methods used for DNA extraction were M: PowerSoil® DNA Isolation kit (MO BIO Laboratories, Carlsbad), Q: QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), and B: Modified QIAamp DNA Stool Mini Kit extraction procedure with the incorporation of a bead-beating step to potentially improve cell lysis (Leser et al., 2000). Briefly, bead-beating was performed by adding 500 µL autoclaved 0.1 mm zirconia silica beads (Biospec Products Inc., Bartlesville, OK) and 30 µL of 10% sodium dodecyl sulfate and processing for 4 min. at 30 cycles per second on a Mixer Mill MM 300 (Retsch GmbH, Haan, Germany). Extractions were performed as directed by the suppliers with minor modifications, including standardized initial sample size and elution in 200 µL, 10 mM Tris, to allow better comparison of the methods. For extractions with method M, bacterial cells were treated in a Mixer Mill MM 300 (4 min at 30 cycles per second) and not the suggested Vortex adaptor. The DNA concentrations of the 90 extractions were determined fluorometrically (Qubit® dsDNA HS assay, Invitrogen).

All DNA extractions were used as template in six different quantitative PCR assays performed with the ABI Prism® 7900HT (Applied Biosystems) using optical grade 384-well plates, allowing all reactions to be performed simultaneously for each donor. The six primer pairs all target regions within the 16S rRNA gene of various groups of bacteria as specified in Table 1 and were selected to represent important bacterial groups in the gut environment. Two primer pairs targeting all bacteria within different regions of the 16S rRNA gene were included as a control and to calculate relative gene ratios. The two primer pairs targeting the Firmicutes and Bacteroidetes, respectively, were chosen to assess and compare the relative abundances of these predominant phyla of the human microbiota. Finally, primer pairs targeting the Enterococcus spp. and Bacteroides thetaiotaomicron were chosen to represent fairly low abundant but prevalent members of the above-mentioned phyla. Reactions and amplification conditions were as previously described (Vigsnæs et al., 2011). Two nanograms of DNA was used as template, and experiments were performed in duplicate. Data were baseline corrected and N0-values, representing initial concentrations of the specified 16S rRNA genes were calculated using the LinRegPCR software (Ramakers et al., 2003; Ruijter et al., 2009). The means of duplicate N0 estimations were used for further analysis. Relevant phylogenetic ratios between bacterial groups were calculated for each DNA extraction separately using the N0-values obtained for the specific bacterial groups. All statistics were performed using the GRAPHPAD PRISM software (version 5.03; GraphPad Software Inc., La Jolla, CA). Indicated P-values refer to significance in Student’s t-test.

**Results and discussion**

The yields of DNA from fecal samples from all three volunteers were significantly higher (P < 0.001) for samples extracted with method M than the two other methods (Fig. 2). No consistent difference in DNA yield was observed between the fresh and corresponding
freeze-stored samples, which indicates that freeze storage does not facilitate the release of more DNA from the fecal samples during extraction. Also, no consistent difference in DNA yield was found between extractions performed with methods Q and B, which indicates that bead-beating did not result in significantly higher yields in this setup. The apparent lack of effect of a commonly used bead-beating mechanical cell disruption step may be explained by the enrichment of the bacterial fraction in the fecal samples by differential centrifugation and the relatively low initial sample loading of the extraction kits.

The concentration of all DNA samples was adjusted to 1 ng mL\(^{-1}\) prior to qPCR analysis. The average \(C_t\)-values obtained in qPCR using universal bacterial primers (Eub2) were calculated for the three extraction methods separately and showed very little variation (\(C_{tM} = 14.43\), \(C_{tQ} = 14.83\), and \(C_{tB} = 14.60\)). This indicates similar DNA quality for the three methods based on qPCR amplification efficiency. The slightly lower \(C_t\)-value for the M extraction may be caused by the fact that the DNA concentration was initially higher for this method and thus template DNA was diluted more prior to qPCR analysis.

Further analysis of qPCR data showed that in seven of nine cases the \(\text{Firmicutes} / \text{Bacteroidetes}\) ratio was significantly higher for fecal samples that had been frozen prior to DNA extraction compared to the fresh samples extracted with the same kit (Fig. 3a). The extent of shift in the \(\text{Firmicutes} / \text{Bacteroidetes}\) ratios between frozen and fresh samples appeared to depend on both extraction method and donor in an unpredictable manner, but was on average 2.2-fold (SEM = 0.52) higher for samples that had been frozen. Analogous comparisons were made for ratios of the total bacteria as determined from two different 16S rRNA gene regions (Eub1 and Eub2) by separate qPCR assays. In this case, no significant difference was observed between the frozen and fresh samples extracted with the same kit, and the calculated average change in ratios was indeed 1.0, SEM = 0.03 (Fig. 3b). This observation strengthens the confidence of the previous finding, which may in general suggest relatively better extraction or stability of PCR amplifiable DNA from gram-positive bacteria (\(\text{Firmicutes}\)) following freeze storage. This could be caused by differences in the cellular composition of gram-positive and gram-negative bacteria. Random shearing of DNA during freeze storage is not likely to bias the qPCR-determined ratios of \(\text{Firmicutes} / \text{Bacteroidetes}\) 16S rRNA genes, because the amplification products were identical in length (Table 1). In most cases, both an increase in the overall relative abundance of

### Table 1. Group-specific primers used for qPCR analysis

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Primer sequence 5′-3′</th>
<th>Length (bp)</th>
<th>Position*</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>All bacteria (Eub1)</td>
<td>Fwd. CGGCAACGAGCGCAACCC</td>
<td>146</td>
<td>1097–1242</td>
<td>Denman &amp; McSweeney (2006)</td>
</tr>
<tr>
<td></td>
<td>Rev. CCATTGTAGACGCTGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All bacteria (Eub2)</td>
<td>Fwd. ACTCCTACGGAAGGCACAGT</td>
<td>199</td>
<td>338–536</td>
<td>Walter et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Rev. GTATTACGCCGCTGATGCGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Fwd. GGARCATGTGGTTAATTCGATG</td>
<td>126</td>
<td>944–1070</td>
<td>Guo et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Rev. AGCTGACGACAACCATGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Fwd. GGAGYATGTGGTTAATTCGAGCA</td>
<td>126</td>
<td>944–1070</td>
<td>Guo et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Rev. AGCTGACGACAAACCATGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. thetaiotaomicron</td>
<td>Fwd. GGAGCAGCTTCACTTGTGTTG</td>
<td>423</td>
<td>N.D. (^{-1})–490</td>
<td>Wang et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>Rev. GGTACATACAAAATTTCCACGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>Fwd. CCCATTTAGGTTAGGTGCCATATT</td>
<td>144</td>
<td>1112–1256</td>
<td>Rinttila et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Rev. ACTGCTTTGACTTCCATATT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fwd. Forward primer; Rev. Reverse primer.
*Positions are relative to the \(E. coli\) 16S rRNA gene sequence (accession no. U000006).
†Not determined because of low homology in the region.
Firmicutes and a corresponding decrease in relative abundance of Bacteroidetes 16S rRNA genes were observed in connection with freeze storage (Fig. 4). Also in eight of nine cases, a decrease in the relative ratio was also observed for the Bacteroidetes species B. thetaiotaomicron, which is consistent with the findings for the phylum as a whole. For the Enterococcus spp., belonging to the Firmicutes phylum, however, only a slight tendency for an increase with freezing was observed, which may be due to the near detection limit overall abundance of this genus (Fig. 4). In conclusion, the data presented in this study indicate that freeze storage of human fecal samples prior to DNA extraction affects downstream qPCR analysis of community composition and thus should be given due consideration during study design. This could be achieved by direct DNA extraction on fecal samples or, for comparisons, by ensuring that all samples have been frozen in a similar manner. Implementation of standards for DNA extraction, as previously suggested by other researchers (Turnbaugh & Gordon, 2009; Ley, 2010), would help to enable better comparisons between research projects and thus would clearly be advantageous to the field of molecular gut microbiology.

Fig. 3. Firmicutes to Bacteroidetes 16S rRNA gene ratios (a) and all bacteria (Eub1) to all bacteria (Eub2) 16S rRNA gene ratios (b) for donors 1, 2, and 3, estimated from DNA extractions on fresh fecal samples and frozen fecal sample (F) using extraction methods M, Q, and B. Each data-point (a) represents the ratio of the calculated $N_0$-values from qPCR assays on the same DNA extraction, and the means and SEM of the five replicates are indicated with bars and error bars. Significant differences between ratios on fresh or frozen fecal samples are indicated with an asterisk ($P < 0.05$, Student’s $t$-test using log-transformed data if variances differed significantly), and the calculated fold-change (frozen/fresh) for each extraction method and donor is shown.

Fig. 4. Mean fold-change (frozen/fresh) for the individual bacterial groups based on their relative abundance as determined by the calculated $N_0$-values of the 16S rRNA genes for the specific bacterial group divided by the $N_0$-value for the total bacteria (Eub2). A value of 1 represents no change. Each data-point represents the average of five estimations from DNA extraction methods M (●), Q (■), and B (▲) from each of the three donors, and the grand mean for each bacterial group is indicated with bars. Different letters indicate significant differences between the groups ($P < 0.05$).
Acknowledgements

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References


