

INTRODUCTION Studies of the microbiome have become quite popular in recent years. The gut microbiome play a role in host physiology, metabolism and nutrition, among others. Standardization of the techniques -including sample storage, DNA isolation, library preparation approach, sequencing and bioinformatics in microbiome studies - is fundamental. In recent years, dog has to be proven to be the most excellent model for the study of intestinal microbiome and complex diseases.

METHODS In this pilot project, the fecal microbiome of a 13 years old healthy dog (a Hungarian purebred dog: Pumi) was analyzed. The DNA isolated from the stool sample using four different purification kits (**Qiagen** (Q) *QIAamp Fast DNA Stool Mini Kit*, **Invitrogen** (I) *PureLink™ Microbiome DNA Purification Kit*, **Macherey-Nagel** (MN) *NucleoSpin DNA Stool Mini kit*, **ZymoResearch** (ZR) *ZymoBIOMICS DNA Miniprep Kit*). DNA samples were then used to generate libraries for short- and long-read sequencing. The 16S-based amplicon sequencing (V1-V2, V1-V3, V3-V4) was carried out on **illumina MiSeq**, and (V1-V9) on **Oxford Nanopore Technologies MinION**, as well as on **Pacific Biosciences Sequel IIe** platforms. Shotgun sequencing approach was also applied. The V1-V3 libraries were prepared with the **PerkinElmer NEXTFLEX® 16S V1-V3 Amplicon-Seq Kit. The *Quick-16S NGS Library Prep Kit* from **ZymoResearch** was applied to generate V1-V2 and V3-V4 libraries. The V1-V9 libraries for full-length 16S sequencing on long read sequencing platforms were produced by using the *16S Barcoding Kit* from **Oxford Nanopore Technologies** (ONT) and by using the *SMRTbell Express Template Prep Kit 2.0* from **Pacific Biosciences** (PacBio). The *DNA Prep Kit* (**illumina**) was used for the generation of shotgun libraries. The following software packages and tools were used for the analysis: *Kraken* (<https://github.com/jenniferlu717/KrakenTools>), *Emu* (<https://gitlab.com/treangenlab/emu>), *EPI2ME* (ONT) and *DADA2* v.1.18 (<https://benjjneb.github.io/dada2/>).**

Analysis of DNA and libraries: 1) quantity - *Qubit 4.0* and *Qubit HS DNA Assay Kit* (**Invitrogen/Thermo Scientific**); 2) quality: **Agilent TapeStation 4150**, *D1000 ScreenTape* and *Genomic DNA ScreenTape*

RESULTS DNA purification kits produce different total DNA concentrations. We also found significant effect of DNA isolation kits on DNA purity and integrity. Three from the four DNA isolation kits provided good quality DNA for high throughput sequencing with non-significant differences in the number of reads recovered, alpha, or beta diversity. One of the tested DNA purification method resulted in significantly different DNA yield in the case of the technical parallels. Our data shown that both the choice of isolation and library preparation (sequencing approaches), as well as the bioinformatics significantly affected the *Firmicutes* to *Bacteroidetes* ratio. All taxonomic levels are affected by the applied methods.

CONCLUSION Our results are in a concordance with other microbiome studies suggesting that the applied DNA extraction techniques could bias results. The choice of DNA isolation kits, library preparation and sequencing approaches, as well as bioinformatics pipelines (databases, analysis techniques) has an important effect on the results that has to be taken into account mainly in meta-analyses.

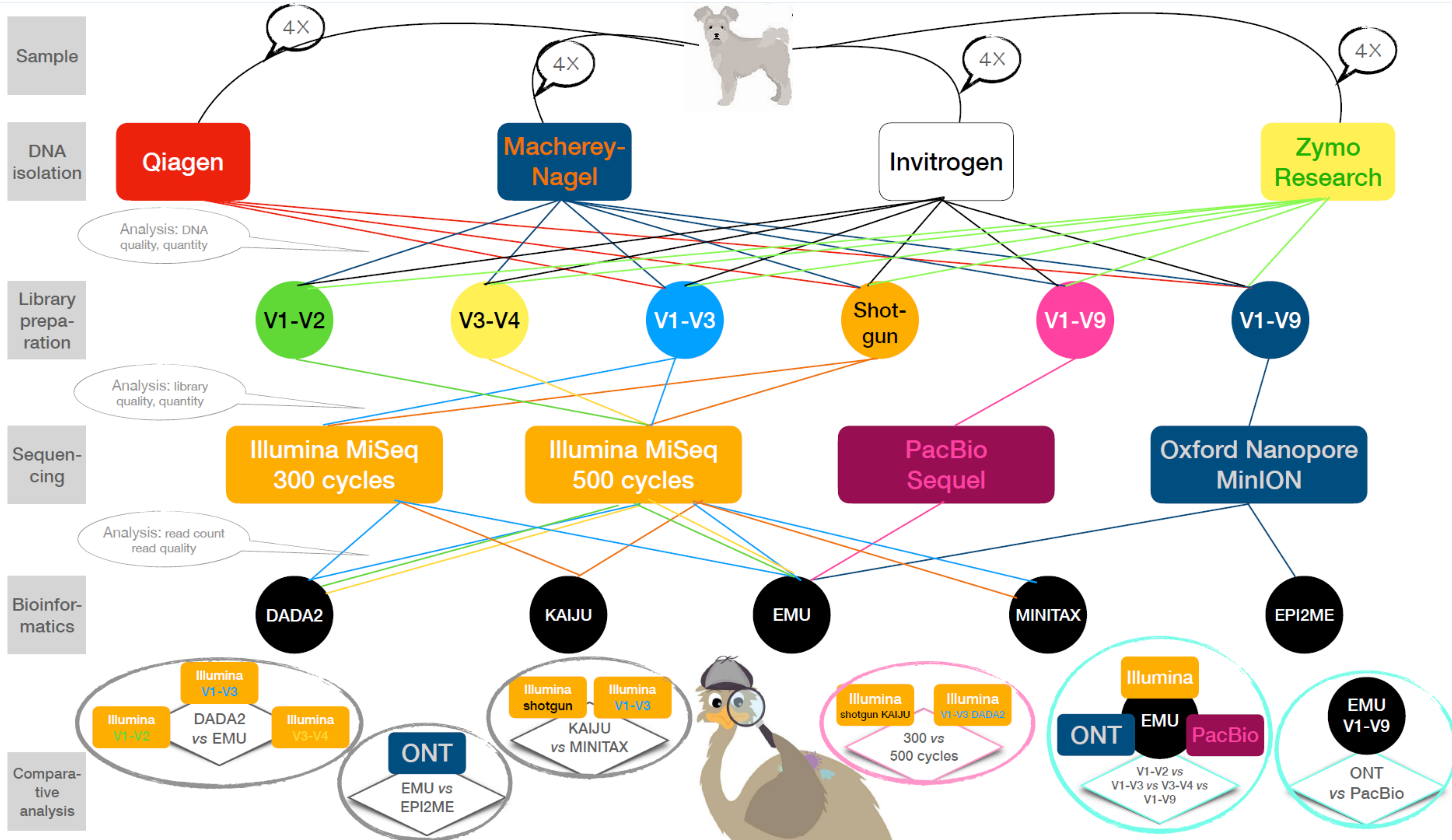


Figure 1. Schematic overview of the study design, wet-lab, bioinformatics and data analysis.

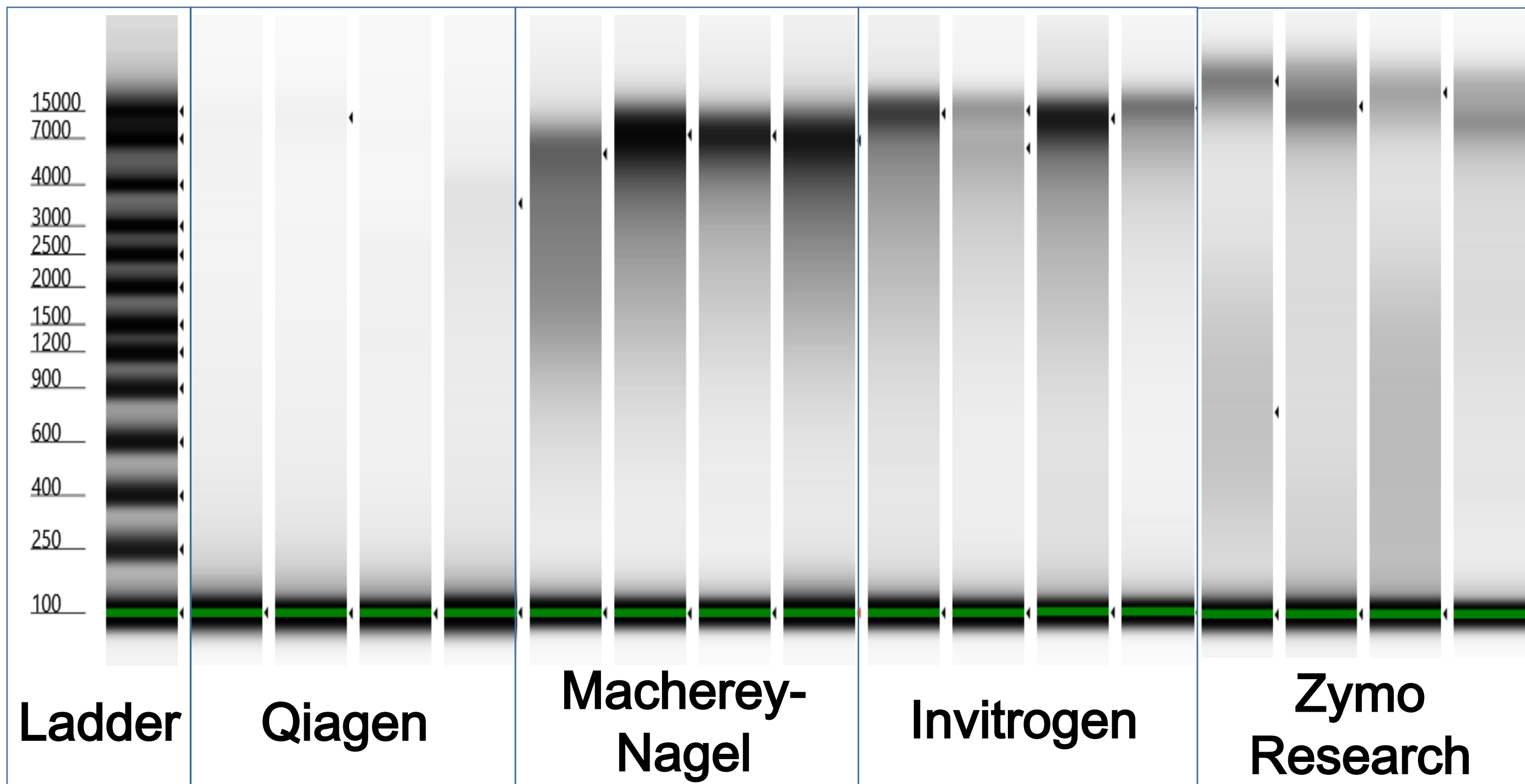
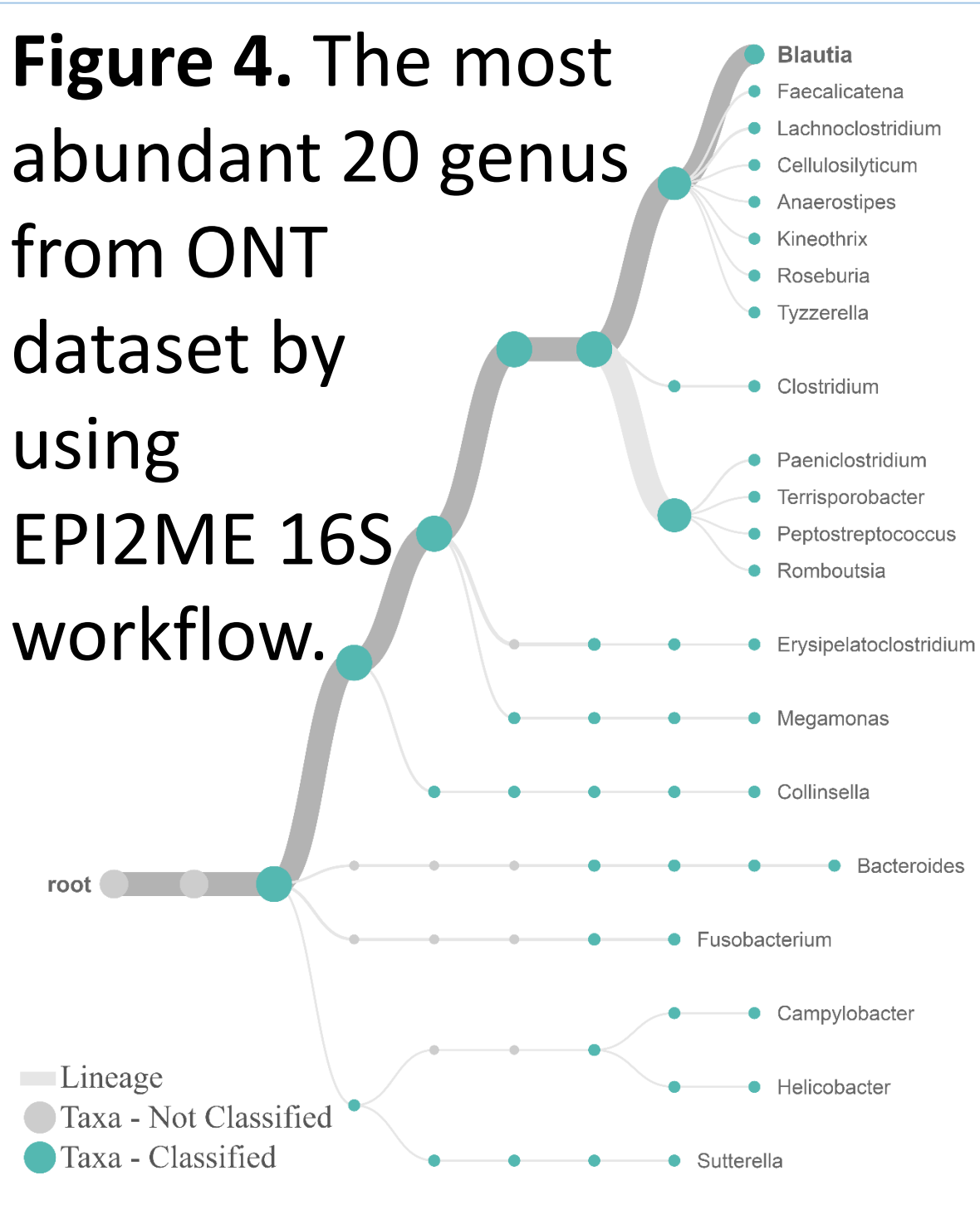


Figure 2. This figure shows the sizing analysis of DNA samples using the TapeStation 4150 system and the Genomic DNA ScreenTape assay. The longest DNA samples were generated by the ZymoResearch kit, while the Qiagen produced the shortest and worst quality samples.



Figure 3. Analysis of amplicon libraries using D1000 ScreenTape assay. The ZymoResearch kit resulted in high-quality libraries from each of the applied DNA purification methods (except Qiagen). In contrast, the PerkinElmer kit resulted in false product in many cases (Qiagen DNA: low library amount, MN and Invitrogen: aspecific products)



This analysis shows *Blautia* dominance within the sample which is inconsistent with the other approaches.

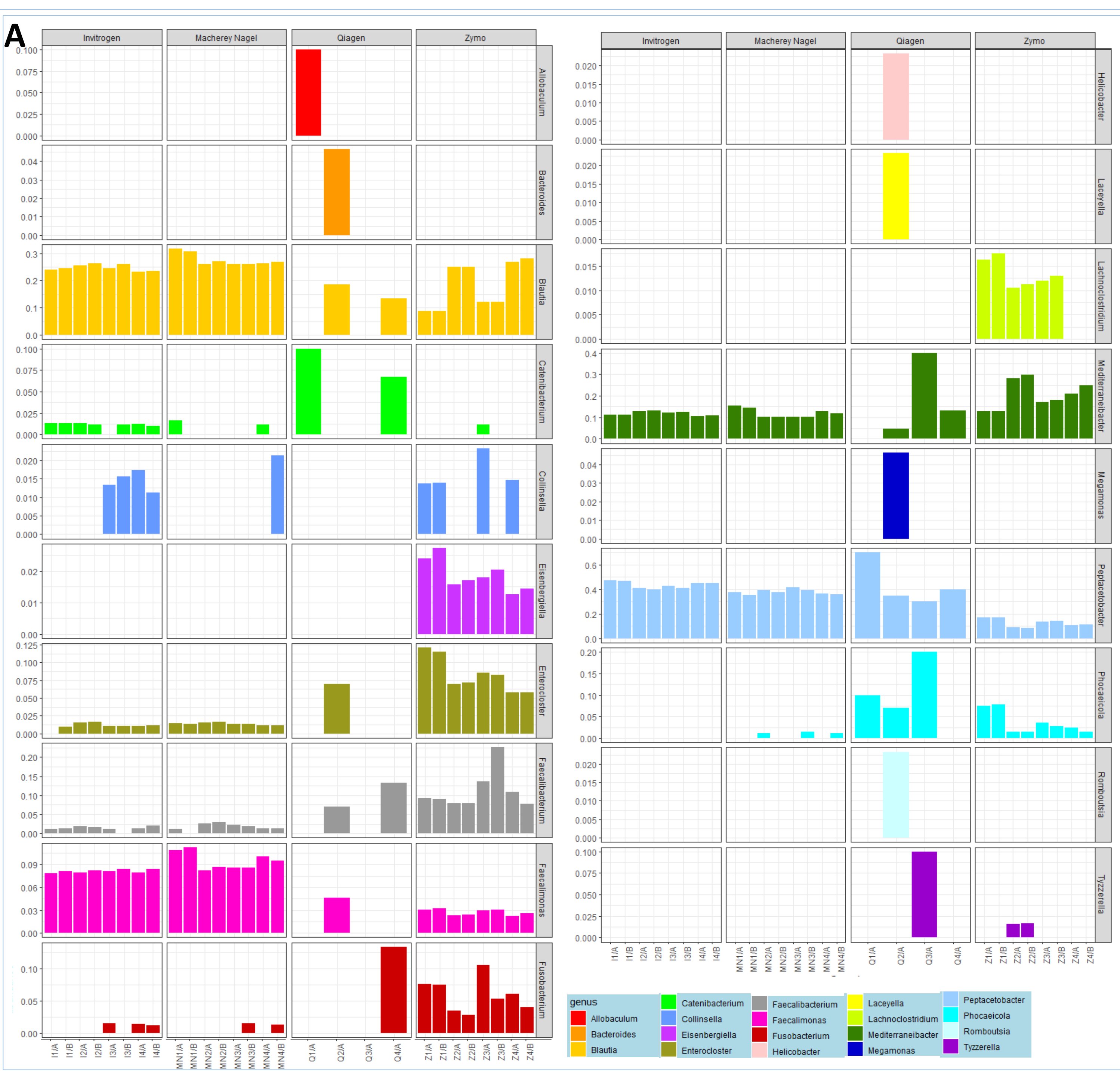


Figure 5. Bar charts represent the most abundant 20 genus, from the 16S amplicon-based long-read sequencing: **A)** ONT MinION, **B)** PacBio Sequel IIe. Both sequencing platform shows that the libraries prepared by the Invitrogen and Macherey-Nagel kits are similar. The Qiagen kit resulted in a bad DNA quality which is not suitable for LRS (see Figure 2), therefore it was run it on ONT but not on PacBio. We obtained only a few reads. Fourteen from the 26 genus are common between the two sequencing platforms. „Only PacBio genus”: *Bifidobacterium*, *Lacrimispra*, *Sutterella*, *Faecalicatena*, *Erysipelatoclostridium*, *Parasutterella*; „Only ONT genus”: *Eisenbergiella*, *Helicobacter*, *Laceyella*, *Lachnoclostridium*, *Rombutsia*. Eleven from the 14 common „ONT-PacBio genus” show the same distribution between the DNA isolation methods. The Emu tool was used for the analysis.

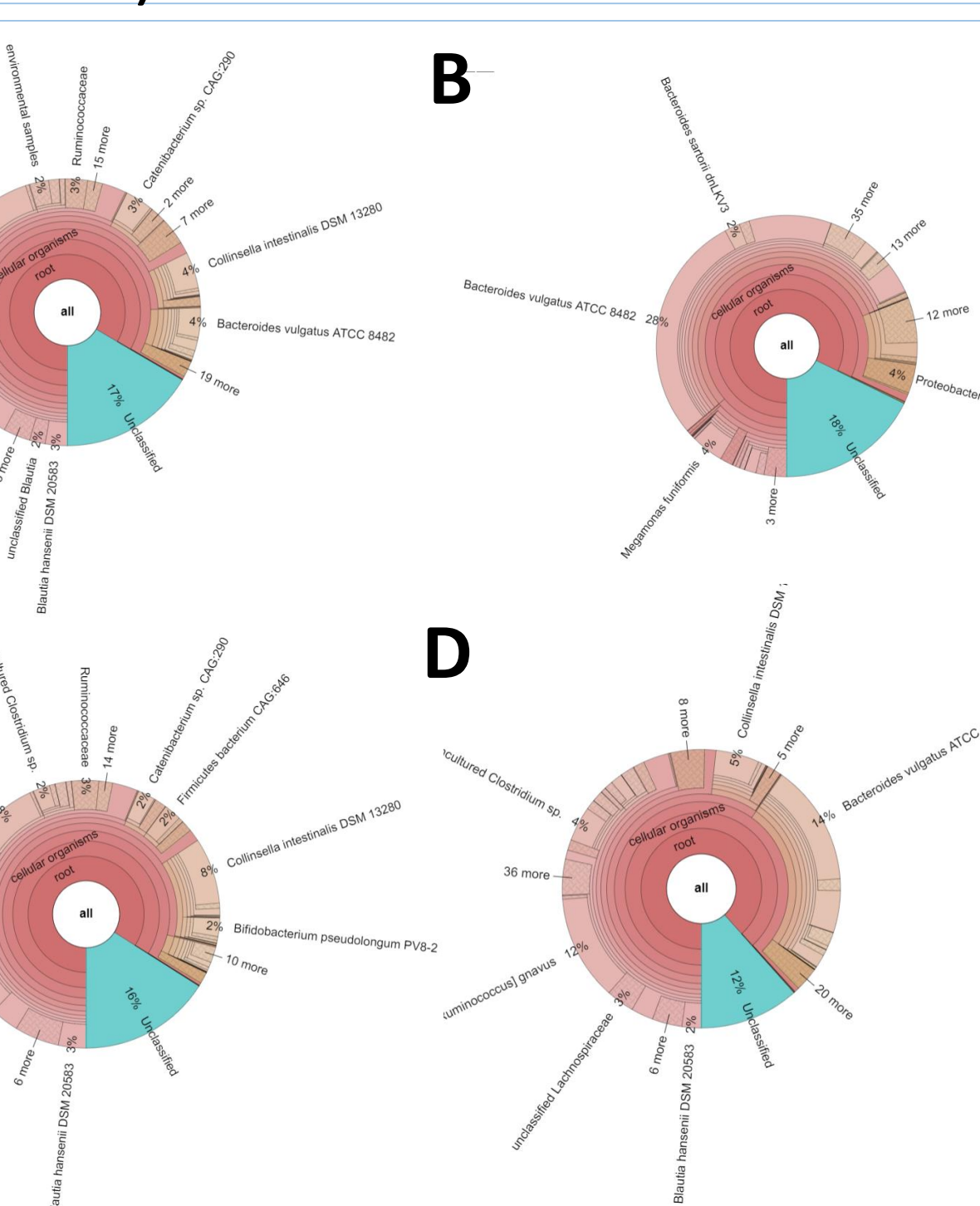


Figure 6. Krona plot showing the relative distribution of gut microbes from the 4 DNA isolation methods: A) I, B) Q, C) MN, D) ZR. (Shotgun-seq).

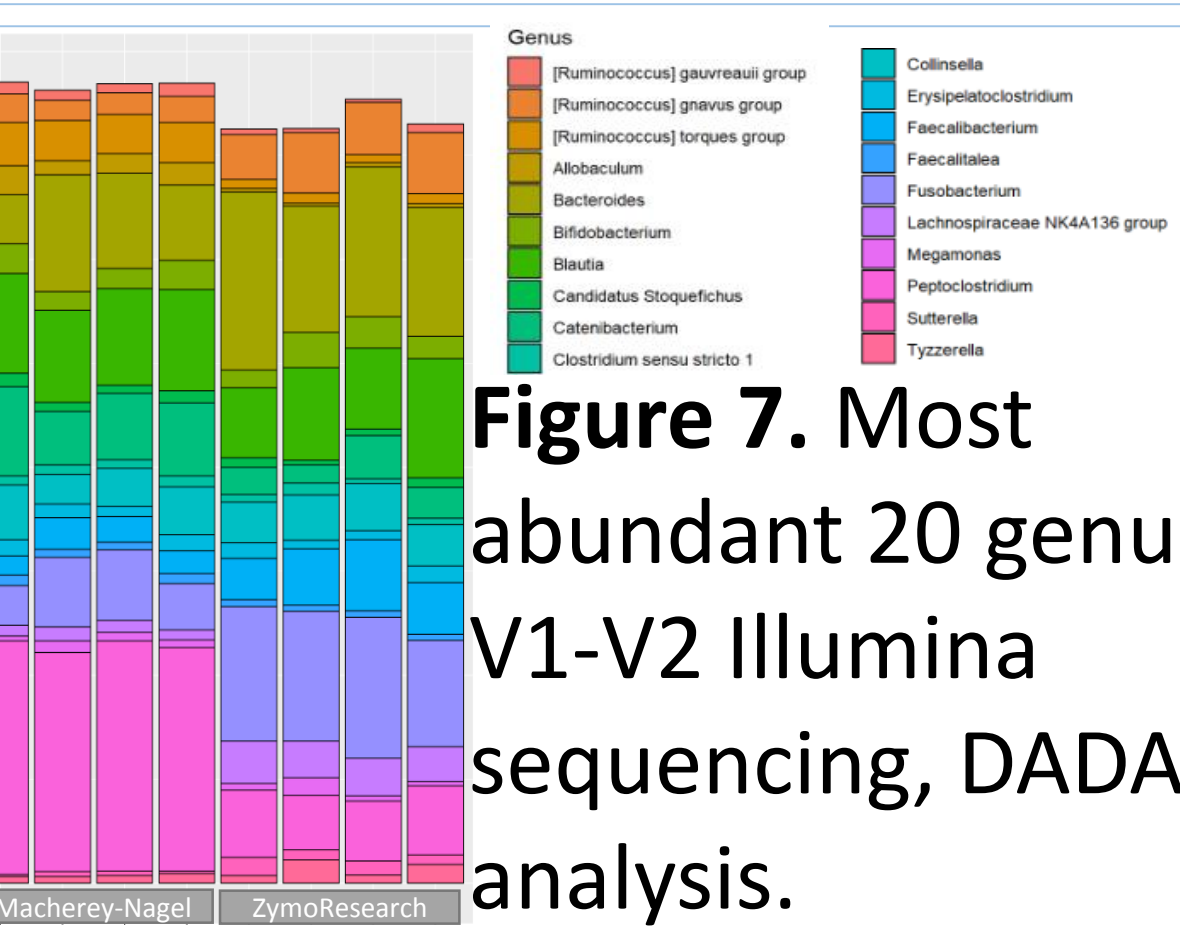


Figure 7. Most abundant 20 genus: V1-V2 Illumina sequencing, DADA2 analysis.