



A chili intervention study assessing the abundance of *Escherichia coli* in the human gut – a metagenomics-based analysis

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Introduction Chili peppers contain the active compound capsaicin. It was previously shown that capsaicin itself did not show in vitro bactericidal activity against *Escherichia coli* (*E. coli*) [1]. However, it can stimulate the release of the neuropeptide substance P (SP). SP contributes indirectly to defense mechanisms against microbes through pro-inflammatory immunomodulatory effects [2] as well as exhibits direct antimicrobial activities, especially against *E. coli* [3,4]. Substance P can be released by sensory nerve cells in the gastrointestinal tract [5,6] upon stimulation by capsaicin through the transient receptor potential vanilloid-1 (TRPV1) receptor [7]. We are testing here if the *in vitro* antimicrobial activity of substance P would be reflected in *E. coli* abundance changes before compared to after chili consumption in this *ex vivo* intervention study setup. We hypothesize that the abundance of *E. coli* is significantly lower after chili consumption compared to before and compared to healthy controls.

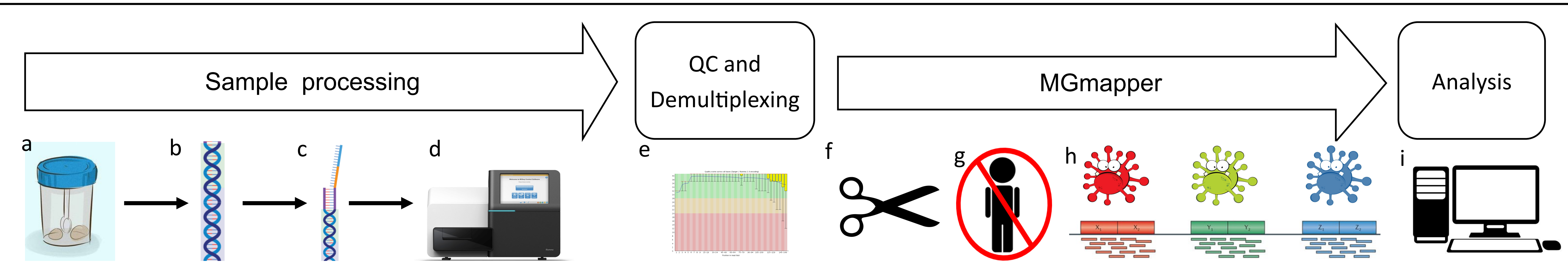
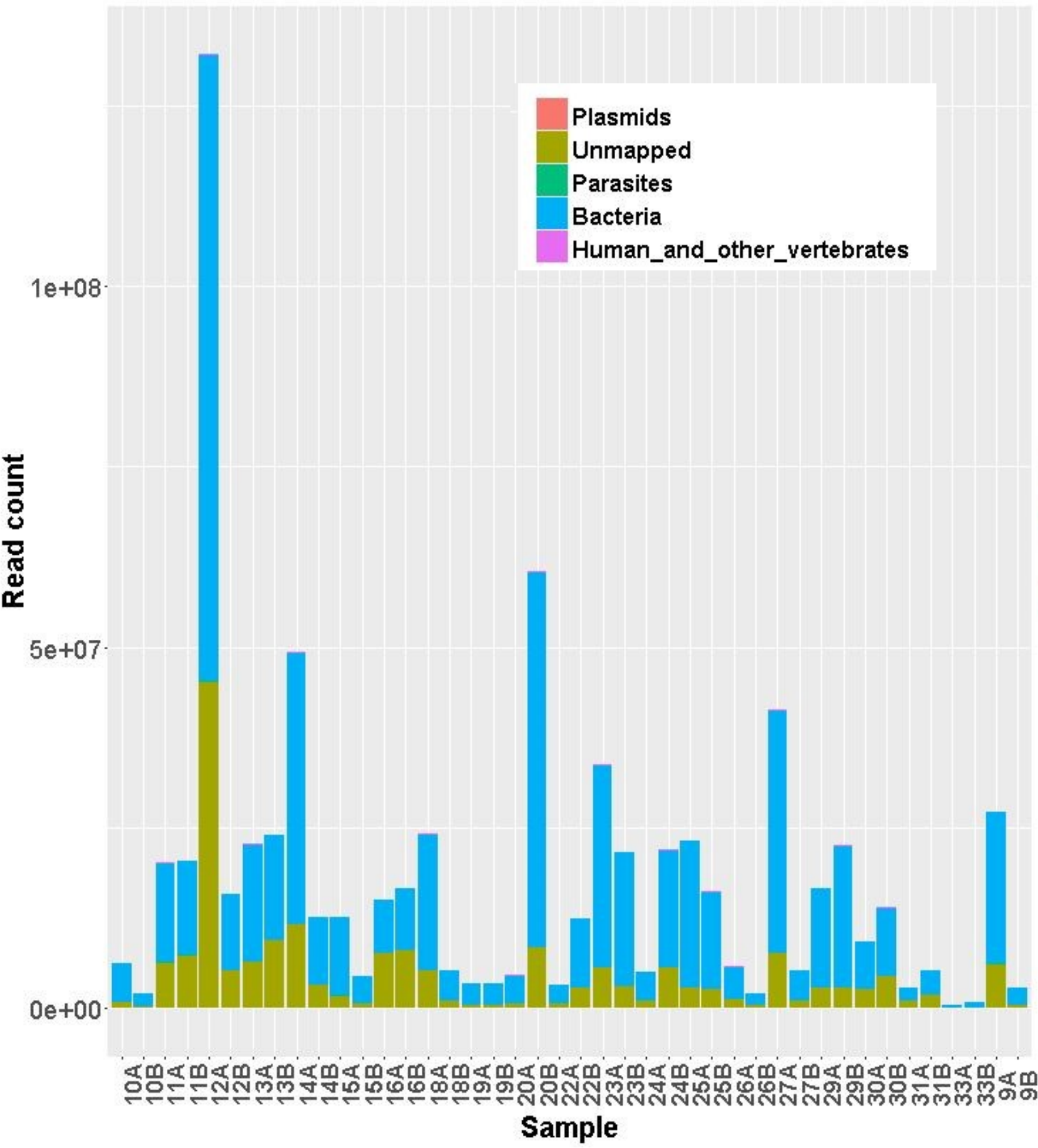


Figure 1. Flow diagram of methods. The “Sample Processing” included: sample collection (a), DNA purification (b), library preparation (c) and sequencing (d). The quality control gives an overview of the quality of the sequencing run before mapping (e). The reads were demultiplexed (e). In “MGmapper”, quality trimming was performed (f), before human and vertebrate reads were discarded (g) and the remaining reads mapped to bacterial and other databases (h). Further bioinformatics analysis included normalization, further post-processing, graphical visualization and statistical analysis of the data in R (i).

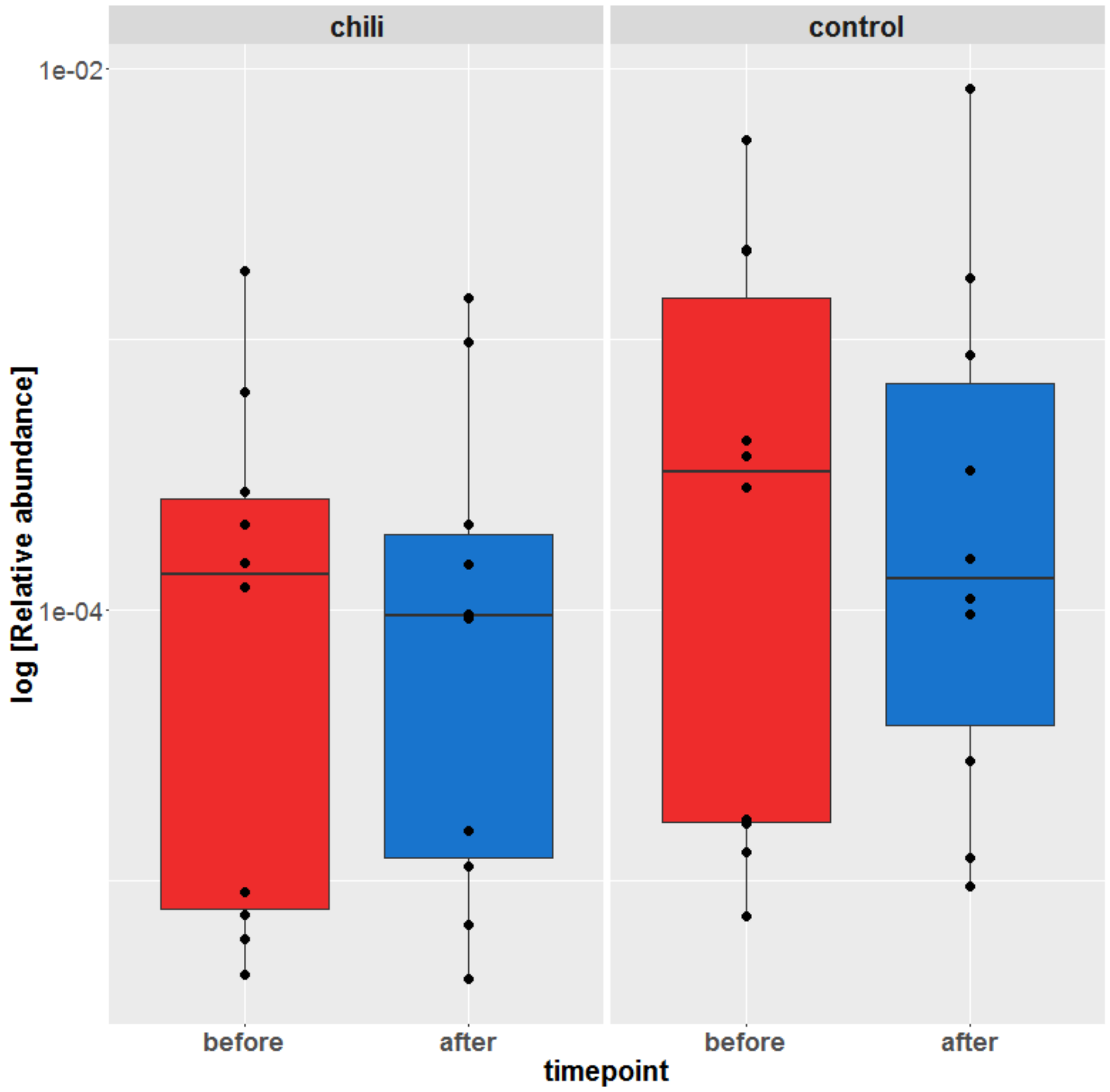
Methods Fecal samples (N=42) from 21 individuals between 20-35 years were collected on two consecutive days. The control group did not change their eating behavior, but were restricted to not eating chili. The intervention group consumed a meal with chili after the first sample was collected. Samples were stored at 5°C until transfer to the laboratory freezer (-80°C). DNA extraction was performed with the PowerSoil DNA Isolation Kit (MOBIO) according to manufacturer’s instructions including an initial bead-beating step. Libraries were prepared using the NEXTflex™ Rapid-DNA Sequencing Kit (BIOO SCIENTIFIC). Illumina’s MiSeq platform was used for paired end sequencing (2x150 bp) (Fig. 1a-d). Forward and reverse reads were uploaded to the server Padawan as two separate files. We determined the total read counts per sample based on barcode frequency. Subsequently, the demultiplexing was performed with an in-house script (Fig. 1e). Taxonomic assignment was performed with MGmapper 2.4. The processing included quality trimming with cutadapt, using a quality score cutoff of 20 and a minimum read length of 50 bp (Fig. 1f). Burrow-Wheeler alignment based mapping was performed with a (match+mismatch)/read length fraction of 0.8. Reads mapping to human and vertebrate reference databases were filtered out before mapping the remaining reads against the following databases in bestmode: “MetahitAssembly”, “Bacteria”, “Bacteria_draft”, “HumanMicrobiome”, “Plasmid”, “Parasites_vertebrates”, “Parasites_other” (Fig. 1g-h). A count abundance matrix was generated from reads mapping to the bacterial databases and subsetted to contain *E. coli* reads. Downsizing was performed to account for differences in sequencing depth (Fig. 2). Differences in relative abundance of *E. coli* between the study groups (intervention/control, before/after) were visualized in a boxplot (Fig. 3). Normality and equality of variance within groups was assessed with a Shapiro-Wilk and a Levene’s test, respectively. We performed an ANOVA on log-transformed, normalized read counts to compare group means. Furthermore, a paired t-test was performed to compare mean differences within the intervention group.



← **Figure 2. Total number of reads per sample.** The majority of reads mapped to a bacterial database or did not map to any database. Samples 9, 13, 15, 16, 22, 25, 27, 29 and 31 were from the intervention group. Samples labeled with a B were collected before the intervention and samples labeled with an A afterwards.

→ **Figure 3. Relative abundance of normalized, log-transformed reads mapping to *E. coli*** before and after the chili intervention shown for the intervention group and the control group.

Results The sequencing depth varied by more than 200-fold between samples (Fig. 2). A large degree of variation was observed for the *E. coli* reads (Fig. 3). The metadata revealed that sample 10 B was stored at elevated temperatures and samples 10 A & B were therefore excluded from the further analysis. It was not possible to detect a difference in *E. coli* abundance between the control group and intervention groups (ANOVA, $p = 0.57$). The relative pairwise differences of the *E. coli* read counts within the intervention group were also not significant (pairwise t-test, $p = 0.95$).



Discussion We found no difference in *E. coli* abundance, neither before compared to after chili consumption, nor between the intervention group and the control group. The previously described *in vitro* antimicrobial activity of SP against *E. coli* [3,4] could hence not be confirmed. This might be due to higher complexity of biological processes in an *ex vivo* setup. However, more likely the experimental design of this study is not sufficient to show a potential effect, as we faced the following limitations: (i) Sample collection and storage was not standardized, (ii) Individuals were not truly randomly assigned to either control or intervention group, (iii) The chili consumption was not standardized, (iv) The study duration might have been too short to show potential effects.

Future work Since we did not find a link between chili consumption and *E. coli* abundance we would like to broaden our analysis. The MGmapper read count table could be used to test if there is a significant change in relative abundances of other species due to the intervention. We plan to use the R-package DESeq2 to take into account the heteroscedasticity of the dataset. We would then make an MA-plot (Bland-Altman plot based) to show which organisms differed significantly due to the intervention. This could drive new hypotheses that could be tested *in vitro*, the reverse order of how we made our hypothesis.

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