The effect of an abrupt change to a four-week vegan diet on the body composition, mental health and gut microbiome

A study on the effect of veganism to the body based on five separate personal health studies

Project Ve-gang
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Abstract

Veganism is an increasingly popular phenomenon in the western world since the 2010s (Burt, 2012; Pendergrast, 2015; Talia, 2015). It was kickstarted by the vegetarian food movement of the counterculture of the 1960s in the United States, whose members were concerned about their diet and the effect of mass production of meat on the environment (Iacobbo & Iacobbo, 2004; Smith, 2011). Even though veganism is a drastic change in diet, there has been only limited study into the effect of veganism on body composition, mental health and gut microbiome (David et al., 2013; Iguacel et al., 2020; Le & Sabaté, 2014; Zimmer et al., 2012). There has been plenty of research done on long-term diet influencing the gut microbiome composition but there is substantially less research on the effect of short-term diet on the gut microbiome composition (Muegge et al., 2011; Wu et al., 2011). Here we show the effects of an abrupt change from an omnivore diet to a vegan diet on the human mental state, body composition and gut microbiome. We found that a short-time vegan diet does not significantly influence the mental health nor the body composition. Furthermore, we could not find significant evidence for a shift in the gut microbiome composition which subverted our expectations. There was expected that following a vegan diet for four weeks would be enough time to see a significant change in the gut microbiome, based on previous studies (David et al., 2014; Turnbaugh et al., 2009; Zimmer et al., 2012). Our results represent the outcomes of the collected measurement data compared between the omnivore diet and the vegan diet. There are several data visualizations created in an interactive dashboard. We anticipate our study to be a starting point to more research on the short-term effects of a vegan diet on the gut microbiome, using alternative techniques for wet- and dry laboratory work.

Abbreviations

BLASTN	Basic Local Alignment Search Tool Nucleotide
BMI	Body Mass Index
Carbs	Carbohydrates
CFU	Colony-Forming Units
gfkb	Gut Feeling Knowledge Base v4
MetaPhlAn	Metagenomic Phylogenetic Analysis tool
S.E.E.	Standard Error Estimate
S.E.M.	Standard Error of the Mean
Taxid	Taxonomic Identifier
LRS	Long-read Sequencing

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1. Introduction

The production of meat has been investigated to be one of the leading causes of the sixth mass extinction (Machovina et al., 2015; Morell, 2015). The sixth mass extinction, also referred to as the Holocene or Anthropocene extinction is the human-caused present-day mass extinction of species on earth in the current Holocene epoch (Ceballos & Ehrlich, 2018). A report from the UN proposed the switch from an animal-based diet to plant-based diet, in this way the generated environmental damage can be reduced by the lower emissions and fewer resources needed for plant-based alternatives (United Nations Environment Programme, 2010).

Veganism, defined here as the total exclusion of all animal products from the diet, has been of growing interest in the western world since the 2010s (Burt, 2012; Pendergrast, 2015; Talia, 2015). It was kickstarted by the vegetarian food movement of the counterculture of the 1960s in the United States, whose members were concerned about their diet and the effect of mass production of meat on the environment (Iacobbo & Iacobbo, 2004; Smith, 2011). Although just six per cent of the population of the United States identifies as a vegan (Neff, 2017), Reese, 2019 has predicted that by the year 2100 veganism will have completely replaced the animal-based food in our diet.

Although the benefits of veganism for the environment seem well studied, the health effects of a plant-based diet are still not fully established. A review by Dinu et al., 2017 shows some contingent evidence that a vegan diet might be correlated to a lower rate of cancer incidence. However, this result must be critically evaluated because of the low number of studies and small sample size within those studies reviewed on this topic. There is some evidence that a vegan diet can result in a deficiency of vitamin B12 and D, calcium, and omega-3 fatty acids (Craig, 2009).

While switching from an omnivore diet to a vegan diet, the goal was to maintain the same amount of consumed (macro)nutrients. To pursue this, food and beverage intakes were tracked. Also, during this study, several body composition parameters were measured. Switching from an omnivore diet to a plant-based diet can influence these parameters via a variety of mechanisms. Though, this frequently results from differences in calorie intake and energy expenditure (Najjar et al., 2019). Often, plant-based diets seem to be lower in fat content which can result in a reduced caloric intake (Kahleova et al., 2020). Since the goal of tracking food intake was to maintain the same amount of nutrients during the vegan diet as during the baseline diet, it was not expected to see any significant changes in these body composition parameters.

As previously mentioned, there are multiple studies on the health effects of veganism to the body composition, but there has only been very limited study to the mental state effect. Recently Iguacel et al., 2020 published a literature review of vegetarianism and veganism compared with mental health. This meta-analysis included data of 1249 publications and in total 17 809 individuals examined from PubMed, Scopus, ScienceDirect and ProQuest databases. Iguacel et al., 2020 concluded that vegan or vegetarian diets might be correlated to a higher risk of depression and lower anxiety scores, but that the overall quality of the studies collected was not high enough to make evident correlations.

Even though veganism is a drastic change in diet, there has been only limited study into the effect of veganism on body composition, mental health and gut microbiota (David et al., 2013; Iguacel et al., 2020; Le & Sabaté, 2014; Zimmer et al., 2012). Changes in body composition and mental health can be used as measures of well-being, and especially gut microbiota is of interest because of the drastic dependence on diet and large role in inflammatory and autoimmune conditions (Shen & Wong, 2016; Quigley, 2013).

Although there has been plenty of research done on long-term diet influencing the gut microbiota composition, there is substantially less research on the effect of short-term diet on the gut microbiota

composition (Muegge et al., 2011; Wu et al., 2011). Turnbaugh et al., 2009 has shown that switching from a "Western diet", containing high-fat and high-sugar to a low-fat, plant polysaccharide-rich diet can shift the microbiome composition within a day. However, it should be taken into consideration that this research was performed by transplanting human faecal microbial communities into germ-free mice models of the human gut ecosystem instead of direct research on humans. A study performed by David et al., 2014 looked at the effect of a short-term change from a fully animal-based to a plant-based diet. With only four days of measuring animal-based diet and four days of measuring the plant-based diet, they concluded that the gut microbiome altered significantly. In the animal-based diet, the abundance of bile-tolerant microorganisms such as *Alistipes, Bilophila* and *Bacteroides* increased significantly. They also found significantly decreased levels of *Roseburia, Eubacterium rectale* and *Ruminococcus bromii* in animal-based diet samples, all *Firmicutes* that are linked with metabolizing dietary plant polysaccharides. Based on these studies we would expect a significant change in microbiota composition between the baseline regular diet and vegan diet sample.

Profiling of gut microbiomes is influenced by numerous factors. An important factor is the DNA extraction method (Lim et al., 2020), DNA extraction methods should yield the original state of the microbiome as accurate as possible, as well as the quantity and integrity of the DNA for accurate profiling (Lim et al., 2017). According to a study performed by Gerasimidis et al., 2016 different DNA extraction methods lead to different results in downstream analysis, therefore the extraction method must be chosen with caution. Several studies claim that incorporating a bead-beating step leads to more comprehensive profiling of human gut microbiomes (Lim et al., 2017; Yuan et al., 2012; Ketchum et al., 2018). In this study, the QIAamp® PowerFecal® DNA kit (cat. no. 1104491) is used, which includes a bead-beating step.

Nanopore sequencing works by measuring the ionic current generated by DNA or RNA strands passing through a nanopore (Jain et al., 2016). These ionic currents get converted into an electrical signal, resulting in a "read" per single strand of DNA or RNA. In the case of Oxford Nanopore Technology sequencers, these reads are saved as raw data in a fast5 file format (Jain et al., 2016). Nanopore sequencing generates ultra-long reads, resulting in a more complete insight of complex genomes and fewer errors in regions of repetitive sequences and structural variation (Jain et al., 2018).

Base calling describes the process of converting the electrical signals described in fast5 files into the widely used FASTQ files. These FASTQ files contain a maximum of four thousand reads as nucleotide sequences with their corresponding Phred quality score in ASCII format (Cock et al., 2010). The Phred quality score *Q* is based on a logarithmic function taken the probability of base-called error *P*. The calculation can be found in **Formula 1**.

$$\mathrm{Q} = -10 \log_{10} \mathrm{P}$$

Formula 1.

Oxford Nanopore Technologies provides the Guppy bioinformatics toolkit for their nanopore sequencers. Guppy contains mathematical base calling models based on Recurrent Neural Networks to optimize the conversion of electrical signals to nucleotide bases.

In this study, we present five separate personal health studies on the effect of an abrupt change from a two-week animal-based to a four-week plant-based diet. Here we try to answer the question "Does an abrupt change to a four-week vegan diet influence the mental health, body and microbiota composition?". This specific research is of interest given that there are only a few studies about veganism and microbiota, few studies about short-term diet and microbiota and very few studies about the two together. We discuss the effects on body composition, mental health and microbiota presented with a web-based interactive dashboard. We expect there to be no significant change in

body composition and mental health in such a short time period. We do however expect that four weeks is enough time, and veganism is a drastic enough change in diet, to see a significant change in the microbiome composition based on previous studies (David et al., 2014; Turnbaugh et al., 2009; Zimmer et al., 2012).

2. Materials and Methods

The following chapter describes the materials and methods used in this research. It gives a detailed description of the performed analysis and used methods so they can be replicated in further research.

2.1 Data Collection

In order to have better understanding effects of a vegan diet on individuals, it was decided to collect various measurements before and during the vegan period, to help investigate this project's hypotheses. For the hypothesis, the microbiota has been the most important data to collect and measure. The remaining three data categories were food intake, body compositions and mental health. Which were collected to see whether something would change in any of these categories and investigate the remaining variables in the hypothesis. Each one of these data categories was handled in their own specific way to ensure the best results. All measurements were taken over a 6-week time period in total: two of which were the baseline and four of which were the vegan period.

Food intake data collection

Food intake data of every subject was collected by using the FatSecret application (Moses, 2007). Daily consumed food and beverages were logged by inserting every consumed product's nutritional information into the app. The app itself contains product information provided by users, which can be used to provide other users with this information. In the case of a product not being present in the FatSecret database, the subject had to add the product information themselves or use an equivalent available product to log. The full protocol containing rules and guidelines for the logging of food intake can be found in Appendix A. Measurement protocols. At the end of every week, on Sunday evening, the weekly food log was exported into a .csv formatted file and uploaded to the shared OneDrive folder (Appendix B. Shared files location). This way, every subject was provided with six food intake files.

Body composition data collection

In order to have better understanding effects of a vegan diet on individuals, we decided to collect body composition measurements before and during veganism. From a practical point of view, there are various methods for body composition analyses. These methods range from measuring weight and stature, to more complex ones such as bioelectrical impedance and dual-energy X-ray absorptiometry (Duren, 2008). In this study we used the Omron BF511 device and collected height (cm), body weight (kg), body mass index (BMI), body fat percentage, skeletal muscle percentage, resting metabolism (kcal) and visceral fat level. We took the measurements daily, in the morning, and sober, for two weeks of normal diet and four weeks of veganism.

Mental health data collection

The mental health questionnaires were filled in daily over the full six weeks. This questionnaire consisted of a total number of 27 questions. These questions considered all mental health aspects, including energy level, sleep, depression and many more. The questions can be found in Appendix A. Measurement protocols. The questions originated from an application called PsyMateTM, which was developed with the help of Maastricht University (Maastricht University, 2015). The PsyMate™ application makes use of the ESM question technique, this technique 'consists of asking individuals to provide systematic self-reports at occasions during the waking hours of a normal week' (Cerin et al., 2001) and 'this procedure makes it possible to examine the immediate effects of ever-changing cognitions' which is desirable in such a short experiment (Hektner et al., 2006). The application itself was initially supposed to be used, but after technical difficulties during the first day of the experiment, we decided to transfer the questions to OneDrive Forms.

The participants had to fill in their questionnaire daily, preferably some hours before going to bed, but no specific time had been agreed upon. The protocol entailing all the set rules for the questionnaire

can be found in Appendix A. Measurement protocols. The questionnaires were exported weekly, every Monday morning, using the OneDrive Forms web application to a csv file format and saved in the OneDrive file.

Faecal sample collection

Faecal samples were obtained twice from five participants after two weeks of the normal diet and four weeks of the plant-based diet. These stool samples were collected in the morning and stored in a container. Within two hours these containers were stored at -80°C for several weeks.

DNA extraction

To extract the bacterial genomes, DNA was extracted using the QIAamp® PowerFecal® DNA kit *(cat. no. 1104491)*. Each extraction was performed with 0,25g of faecal matter, which was added to the supplied Dry Bead Tube, bead beating was done using a vortex with a custom adapter. All subsequent steps were done following the manufacture's protocol. Elution of DNA was completed with 100µL of provided C6 elution buffer. The Invitrogen Qubit was used to determine the quantity of the extracted DNA. After elution, DNA was snap-frozen with liquid nitrogen and stored at -80°C for later use. Quality of DNA was determined using the Thermo Scientific™ NanoDrop™ *(cat.no 84027410)* a day before sequencing.

MinION sequencing

Sequencing was done using the Oxford Nanopore Technologies MinION platform. The Rapid Barcoding kit *(cat.no RBK004)* was used to multiplex the samples. This protocol was performed twice to get two libraries and was performed following the manufacturer's instructions, one library with four DNA barcoded samples and one with six barcoded DNA samples. The barcoded DNA was cleaned and concentrated with magnetic beads *(cat.no CNGS-0005)* following the manufacturer's instructions (RBK-004). These two libraries were loaded on two MinION devices with ONT R9.4 Flow Cells *(cat.no FLO-MIN106D)* and were run overnight.

Table 1: Labelling of the barcodes

Barcode	Subject	Sample
Barcode01	A	Vegan
Barcode02	B	Vegan
Barcode03	C	Vegan
Barcode04	D	Vegan
Barcode05	E	Vegan
Barcode06	A	Control
Barcode07	B	Control
Barcode08	C	Control
Barcode09	D	Control
Barcode10	E	Control

Base calling

Guppy was used to be able to base call the data generated by the MinION (Wick et al., 2019). The base calling with guppy was performed with the High accuracy (HAC) to provide a higher consensus and raw read accuracy.

To be able to classify which nucleotide sequencing belong to which sample barcoding algorithms from the Guppy bioinformatics toolkit have been applied (Wick et al., 2019). This algorithm aligns the barcodes from the Rapid barcoding kit (SQK-RBK004) against the beginning and end of every read in the FASTQ files. It was chosen to allow inferior barcodes to be able to improve the classification of

reads to their respective sample. After the barcoding was performed for a read the barcode was trimmed off to only keep the sequencing data of interest in the reads.

In order to assess the quality of the nanopore sequencing, base calling and barcoding pycoQC was used on the sequencing summary log files generated by Guppy (Leger & Leonardi, 2019).

2.2 Data pre-processing

After the necessary data has been collected by the participants and the experiment was completed, all the weekly data was combined, within their own categories, in the correct file formats and transferred to data frames. For several data sets, one more step was needed before it could be transferred to a data frame, such as translation from Dutch to English. After this process, the step to get the data into the correct formats for visualization began. This is pre-processing and different steps have been followed for each data category.

Food intake pre-processing

To make the food intake files useful for analysis, several manual adjustments were made in the files. First, when the information was not in English it needed to be translated. Also, due to file exporting, some of the files were not formatted in the proper way. The number of header rows differed per file, so we made sure every food logbook content started at the same line (line ten). The required modifications were fulfilled to retrieve equal formatting among all exported files.

Mental health pre-processing

The questionnaire answers were exported to csv files within the OneDrive Forms application which made it easy to import it using Jupyter Notebooks. Unnecessary columns, such as 'name' and 'email', which were added every time the questionnaire was filled in, were deleted as these were all empty due to the anonymized nature of this study. Pre-processing steps were executed for every single subject their final csv files. Every pre-processing step can be found in the GitHub repository (Appendix C. GitHub repository).

2.3 Data analysis

After pre-processing, the required files were ready to be further analysed. Most analyses were performed in a Jupyter Notebook (Kluyver et al., 2016). The repository containing the source code and all the created program files can be found in Appendix C. GitHub repository.

Food intake data analysis

After pre-processing, the exported food intake files could be used as input for the dashboard application. The following nutritional values were extracted: calories, carbohydrates, proteins, fats, saturated fats, fibre, sugar, sodium, cholesterol and potassium. These nutrients were loaded into five data frames, one per subject. Subsequently, three different types of graphs were created. First, four boxplots representing nutrient intakes during the baseline diet compared to the vegan diet, divided into four categories: calories, macronutrients, carbohydrates and minerals. Second, a bar plot displaying the average of every consumed nutrient during the baseline diet compared to the vegan diet. Lastly, per week there were three pie charts created that display the nutrient distributions in three categories: macronutrients, sugar and fibre, and sodium and potassium.

Body composition data analysis

For assuring body composition of subjects remain constant, we need to compare the distribution of data before and after the vegan diet. So, body composition measurements were concatenated into one data frame. Then violin plot, box plot and line plot of four measurements were developed for each subject. For making our code concise, we tried to use predefined functions from the panel library for making our plots interactive instead of building functions from scratch.

Mental health data analysis

The data frames of the mental health questionnaires are designed in three separate data frames. A data frame for the entire period, for the baseline and one more for the vegan period. First, the average scores over the total period were looked at, to see if any differences were already visible here. To find out, we made a grouped bar chart (panel Mental health, bar plot: Appendix C. GitHub repository) that compares the numerical questions next to each other, visualising the baseline vs vegan. In addition to the bar plot, an attempt was made to better analyse the total averages based on a radar plot (panel Mental health, radar plot: Appendix C. GitHub repository), in which possible shifts in feelings can be more clearly recognized. After making this radar plot, there were no clear differences in total averages. For this reason, a weekly average radar plot (panel Mental health, radar plot weekly: Appendix C. GitHub repository) has also been created that takes and displays each weekly average of each numerical question. Lastly, to investigate whether a possible trend can be found over the entire period, a line plot (see panel Mental health, line plot) was also created that shows all numerical questions, separately from each other, in a line over the entire 6 weeks. This indicates when the switch from baseline to vegan took place.

Taxonomic analysis of gut microbiota data

To infer the abundance and presence of clades in the gut microbiota data a taxonomic analysis was performed. A taxonomic analysis was performed with MetaPhlAn 3.0, a computational tool for creating a microbial composition profile from metagenomic sequencing data based on unique clade-specific marker genes identified from about a hundred thousand reference genomes (mpa_v30_CHOCOPhlAn_201901) (Beghini et al., 2020). MetaPhlAn 3.0 was chosen to classify the gut microbiota data based on its use in previous studies (McIntyre et al., 2017; Dr. Wynand Alkema & Dr. Harro Timmerman, personal communication, 2020). MetaPhlAn was run with the underlying Bowtie 2 (version: 2.4.2) very-sensitive-local flag to get a more accurate representation of the metagenomic composition (Langmead & Salzberg, 2012). It was chosen to also try to identify viruses and output the estimated abundance of unknown taxa that MetaPhlAn was not able to link to one of the seventeen thousand reference genomes. After a first taxonomic analysis it was concluded there was a need to perform MetaPhlAn with the *stat_q* and *min_mapq_val* flags set to 0.1 and -1 respectively to get a higher number of identified taxa.

A second taxonomic analysis was performed with BLASTN 2.8.1(Camacho et al., 2009). FASTQ files generated by guppy were concatenated per barcode and converted to a FASTA format. The reads stored in the FASTA files were aligned locally to the blast database (version: December 2020) with BLASTN, this tool returned ten hits per read with each the taxonomic identifier (taxid), scientific name and e-value. From this point onwards files were processed with pandas, the files were merged into one data frame and were labelled with the corresponding subject and condition (McKinney, 2010). The top hit was extracted by filtering on the lowest e-value. Reliable hits were obtained using a filtering step on e-value, reads with an e-value < 10e-10,remained in the data frame, in total $79 \cdot 10^4$ reads. This data frame, holding taxids was loaded in a script to obtain the whole lineage in taxids. A relative abundance file of each taxid was generated, and the taxids were converted to scientific names. The taxonomic profiles were visualized by a horizontal bar plot on every taxonomic level per subject. These scripts are attached in Appendix C. GitHub repository.

Interactive Dashboard

After every performed analysis and the graph creations, the several outcomes were concatenated in a final interactive dashboard. This dashboard serves multiple functionalities to view and compare the different results together.

3. Results

This chapter discusses the results of this research. Different visualizations are shown with further textual explanation of findings.

3.1 Food intake

The goal of tracking the food and liquid intake was to maintain the same nutrient intake during the vegan diet as during the baseline diet. **Figure 1** shows the caloric intake of every subject during the baseline and the vegan period in a boxplot representation. Despite some outliers, there are no noticeable changes in calories observed. This indicates that the average amount of calories is being maintained.

Though, comparing the baseline diet with the vegan diet shows some differences in macronutrient intakes. **Figure 2** shows the comparison of the macronutrients: carbohydrates, protein and fats (including saturated fats). Although the caloric intake is overall maintained, looking at subject C there is a different division between carbohydrates and fats. This subject consumed fewer carbs and more fats during the vegan period. Looking at subject B and D, there is an observable increase in carbohydrate intake during the vegan period. Observing the protein intake measurements, there is a noticeable decrease in the vegan period for every subject.

When the carbohydrate contents are being observed, there are increases in fibre intake visible for every subject. Boxplots representing the fibre and sugar intake of all subjects are shown in **Figure 3**. Despite this observing, subject D shows a noticeable increase in sugar intake during the vegan period.

More details about the food intake data are available in the interactive dashboard. A bar plot representing the averages of every consumed nutrient during the baseline diet compared to the vegan diet can be found there, as well as pie charts that represent the nutrient distributions. The created data frames containing each subject's daily nutrients intake can also be viewed in.

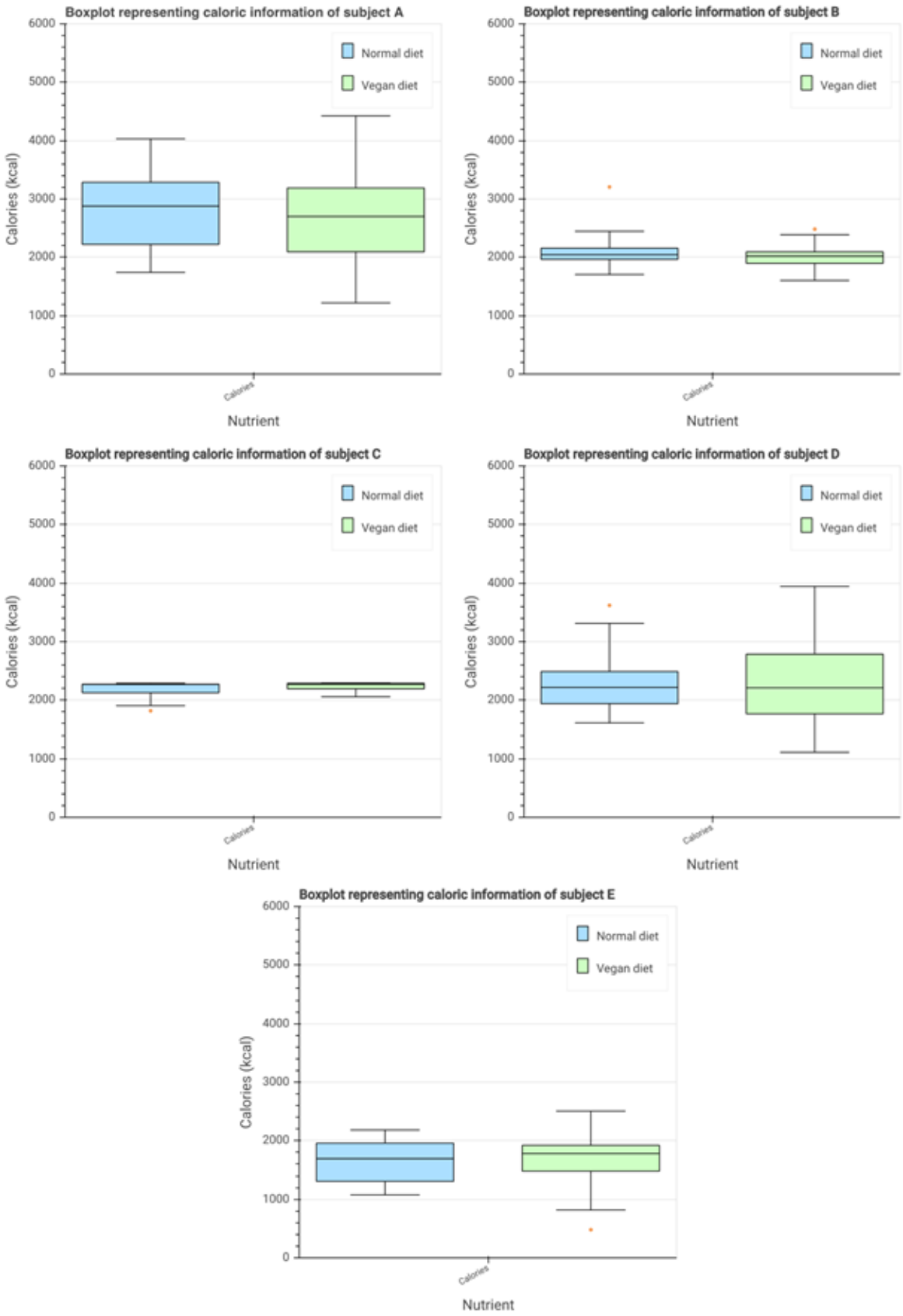

Figure1 - Boxplots displaying caloric intake of every subject during the normal and vegan diet

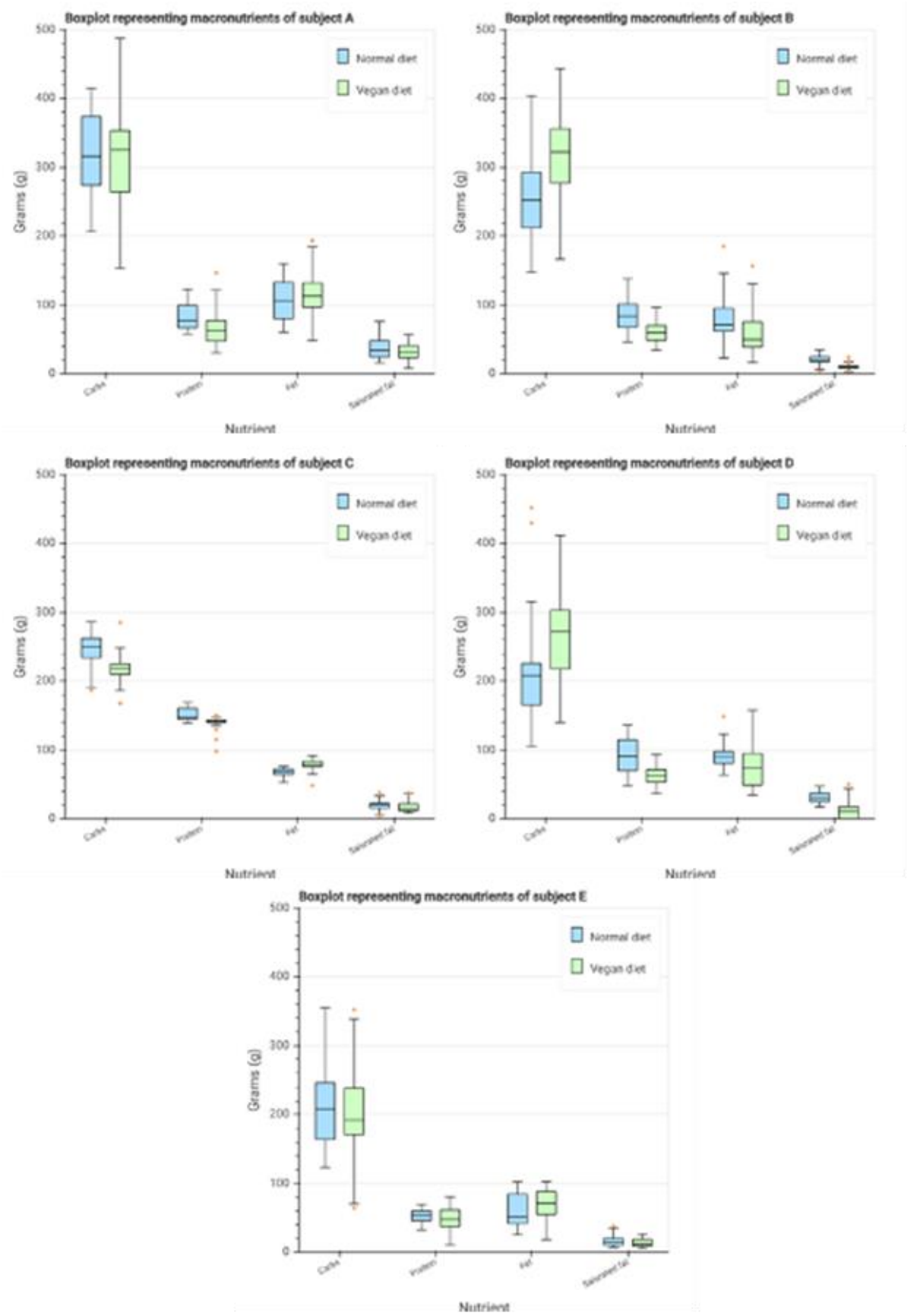

Figure2 - Boxplots displaying the intake of carbohydrates, protein, fats and saturated fats of all subjects during the normal and the vegan diet

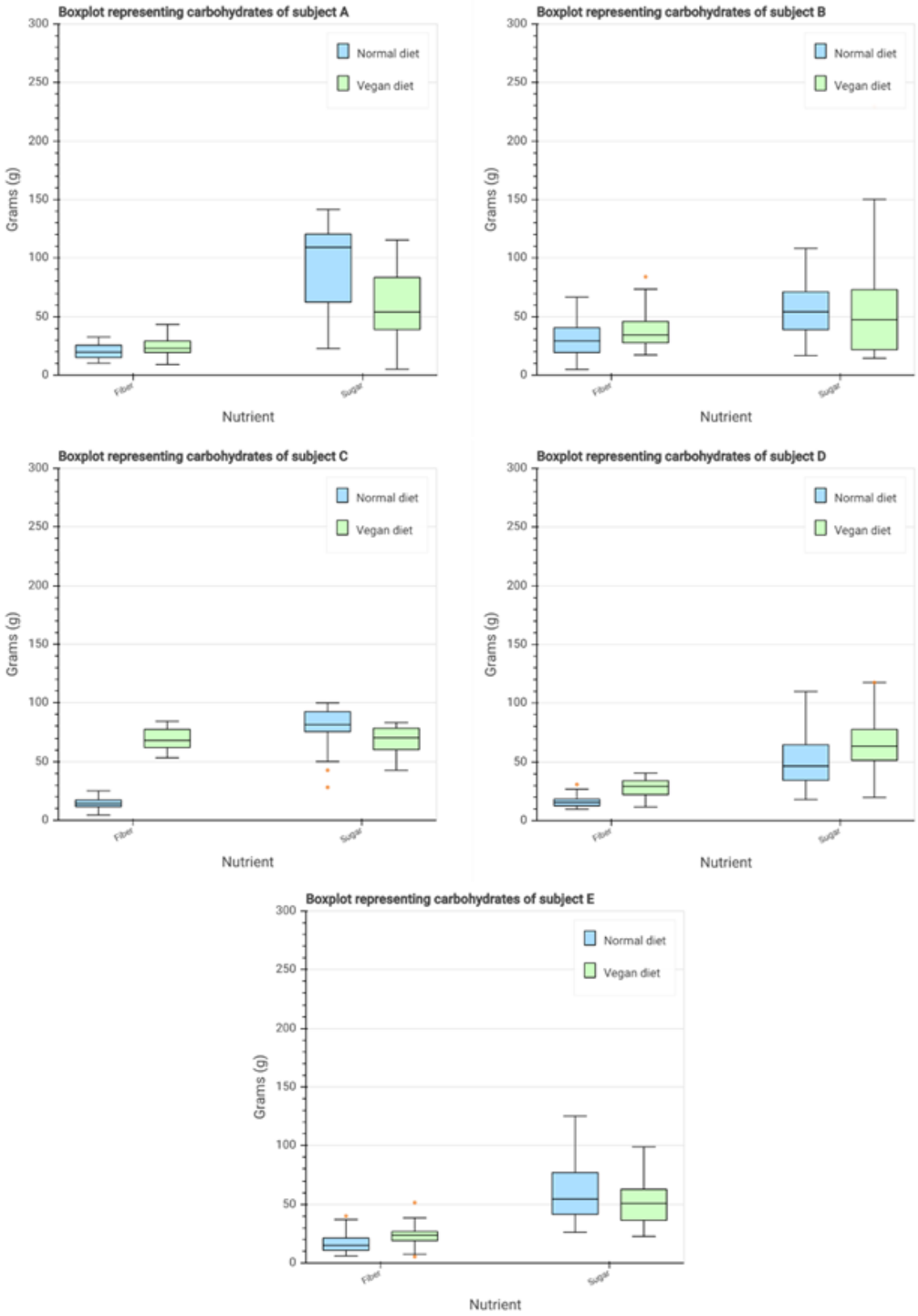

Figure3 - Boxplots displaying fibre (two leftmost boxplots) and sugar intake (two rightmost boxplots) of all subjects during the normal and vegan diet.

3.2 Body composition

We tracked body composition measurements to observe changes in weight, BMI, body fat percentage and skeletal muscle percentage of the participants. Although there is a slight negative slope in weight of subject B, C and D, it is hard to attribute these minor changes to the vegan diet.

Below are the weights, BMI, body fat and skeletal muscle percentage of the subjects during the total time period of the experiment, visualized. Although we collected resting metabolism (kcal) and visceral fat level, these two measurements were not presented in our report. Resting metabolism is a figure that shows the total number of calories burned when the body is completely at rest which is not the case in our experiment. Regarding visceral fat measurement, firstly it is better to be done by CT and MRI technologies rather than the Omron. Secondly, we collected fat percentage of the whole body and that suffices our requirement for evaluating effects of veganism on fat level of subjects.

A violin graph is used to visualise the distribution of a dataset by displaying its probability density. It is very similar to the box whisker element but provides a more faithful representation even for bi- or multimodal data. The probability density is shown by the area akin to a vertical and mirrored distribution element. The thick black bar in the centre represents the interquartile range, the thin black line extended from it represents the 95% confidence intervals, and the white dot is the median. The violin element is particularly useful to compare multiple distribution across different categories. As a simple example we can create a dataset of values with randomly assigned group and category values and compare the distributions (Violin – Holoviews, n.d.).

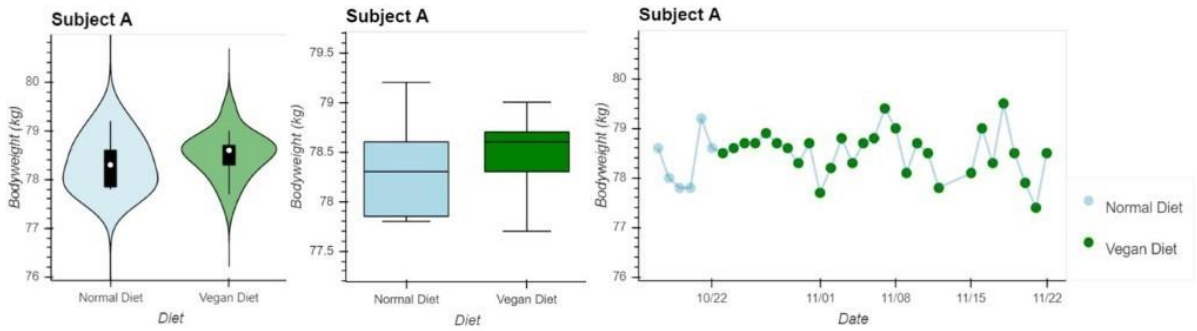

Figure 4. Bodyweight of subject A shown in violin, box and line plot

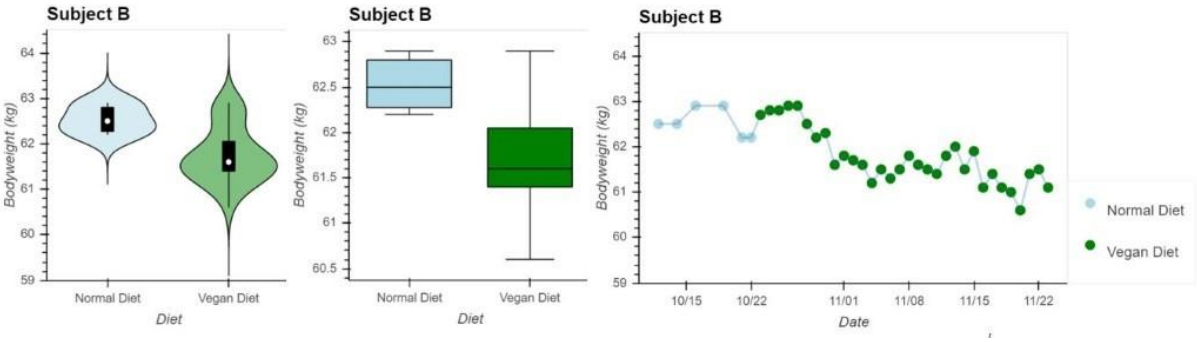

Figure 5. Bodyweight of subject B shown in violin, box and line plot

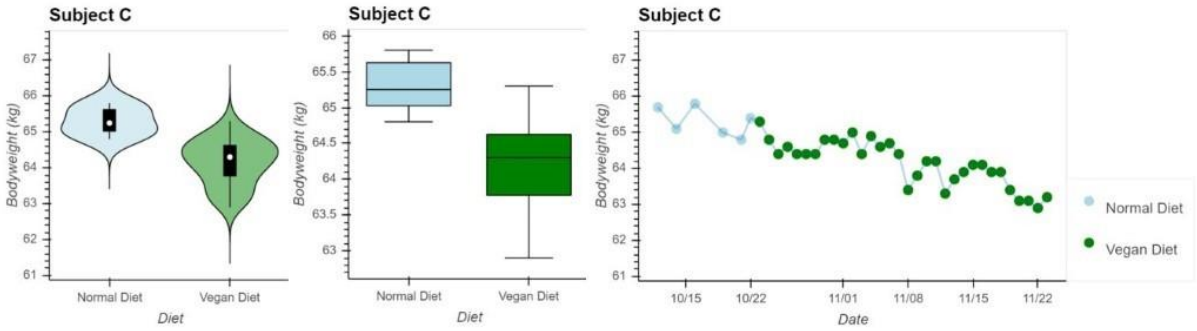

Figure 6. Bodyweight of subject C shown in violin, box and line plot

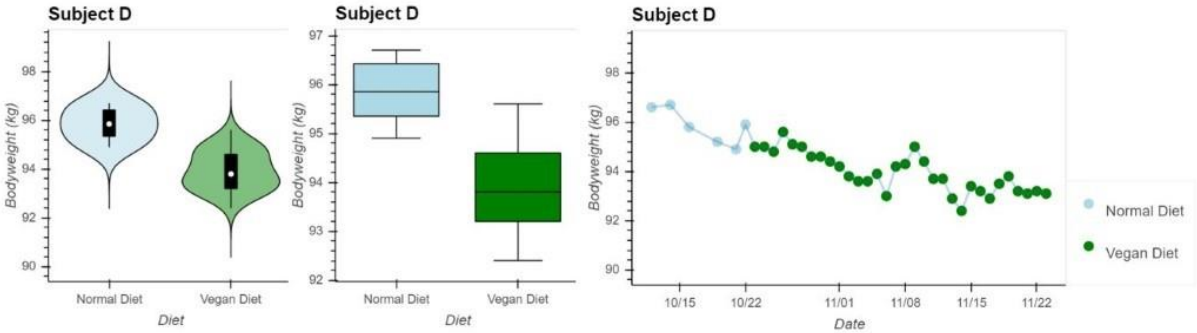

Figure 7. Bodyweight of subject D shown in violin, box and line plot

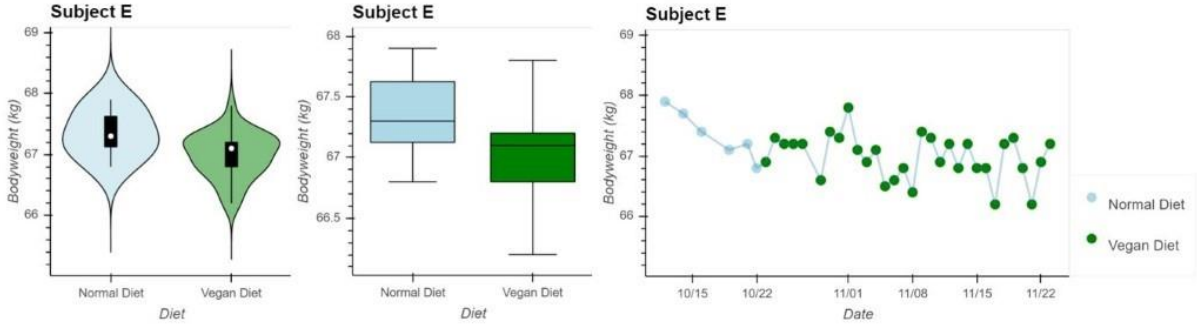

Figure 8. Bodyweight of subject E shown in violin, box and line plot

As we can see in above figures, except subject D who lost less than 2% of their average weight, others maintain their average during veganism period close to what were before the experiment. In short, four subjects have lost weights in range of 300 grams to two kilograms and one subject has gained less than 200 grams in vegan diet period.

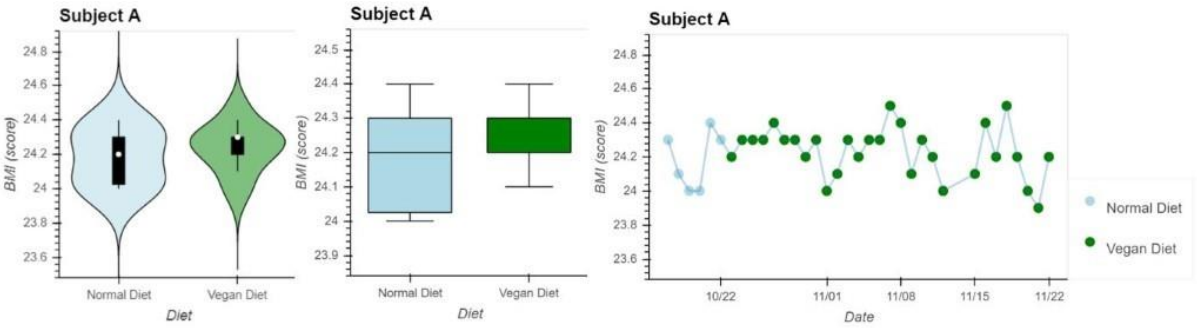

*Figure 9. BMI score of subject A shown in violin, **box** and line plot*

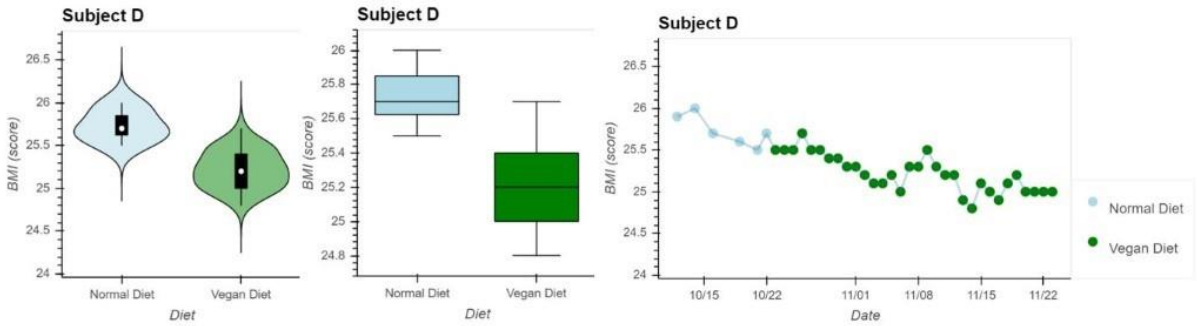

Figure 10. BMI score of subject D shown in violin, box and line plot

Five BMI figures illustrate that we have minor changes in BMI score of subjects with a maximum of +0.1 score in subject A and minimum of - 0.5 score in subject D. Totally, changes in BMI scores can be considered negligible.

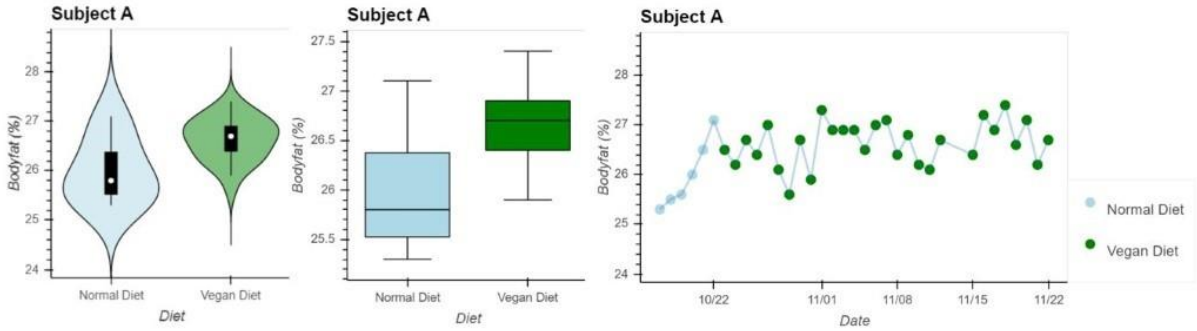

Figure 11. Body fat percentage of subject A shown in violin, box and line plot

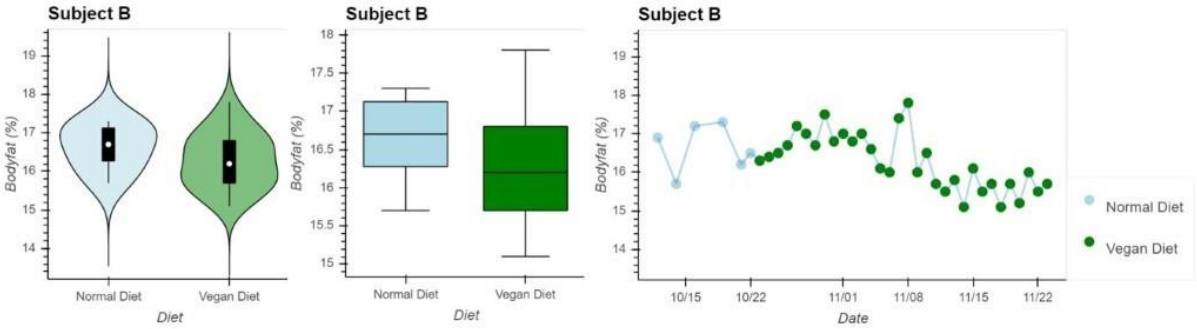

Figure 12. Body fat percentage of subject B shown in violin, box and line plot

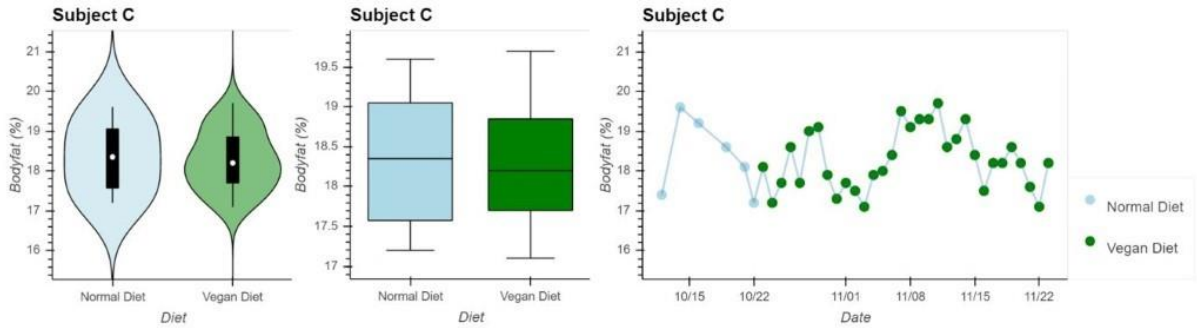

Figure 13. Body fat percentage of subject C shown in violin, box and line plot

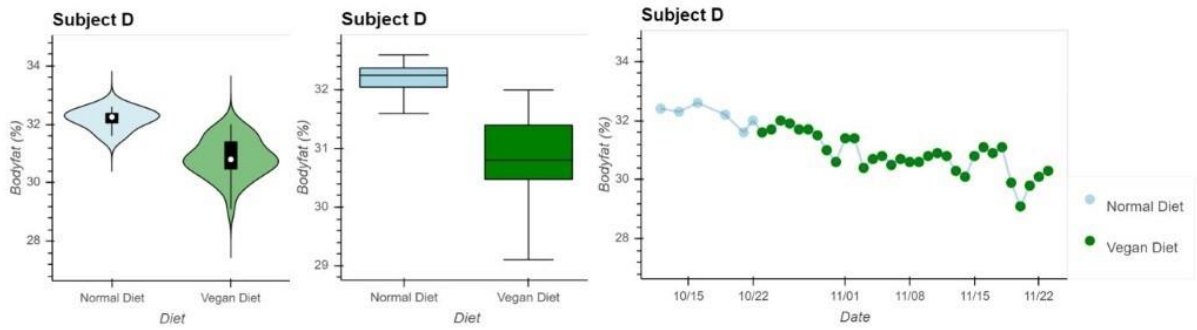

Figure 14. Body fat percentage of subject D shown in violin, box and line plot

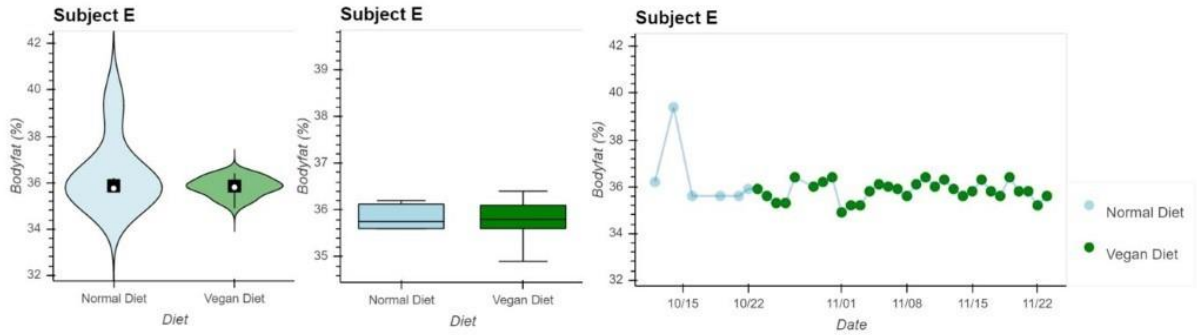

Figure 15. Body fat percentage of subject E shown in violin, box and line plot

Body fat percentage of subjects C and E remained almost constant. While subjects B and D experienced insignificant drops, subject A increased their body fat percentage with almost 1%. We can observe a sharp fluctuation in figures of subject E in their normal diet period which may stem from a mismeasurement.

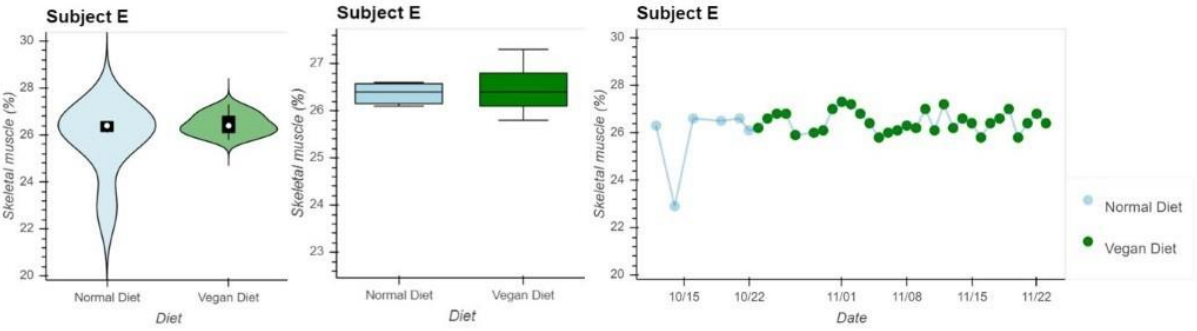

Figure 16. Skeletal muscle percentage of subject E shown in violin, box and line plot

last five figures of body composition measurements are concerned with skeletal muscle percentage of subjects. Again, both positive and negative changes in subjects are minors and no obvious pattern can be extracted from figures. As we can see in **Figure 16**, subject E had an unusual measurement in their second day which should be considered as an outlier, comparing with other data.

3.3 Mental state

Tracking the mental health of all subjects had the main purpose checking if the change in diet had any effect on the mental well-being of the subjects, mainly because there is little to no research to be found on mental health and the effect veganism has on it. All averages for subject A have been plotted in **Figure 17** (other subjects can be found in the panel, Appendix C. GitHub repository). **Figure 18** shows the score line for subject A and the mood 'relaxed' throughout the whole six-week period with a

vertical line indicating the day that was switched to the vegan diet (other subjects and moods can be found in the panel, Appendix C. GitHub repository).

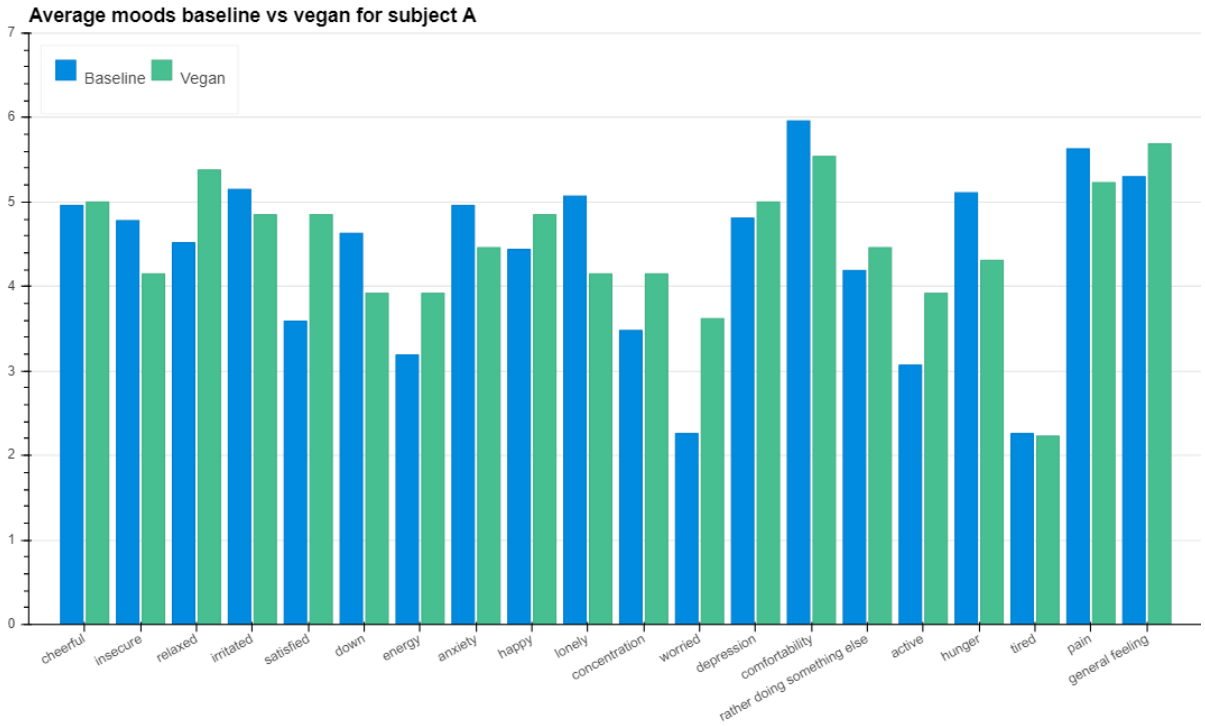

Figure 17 - Bar plot of average mood scores subject A

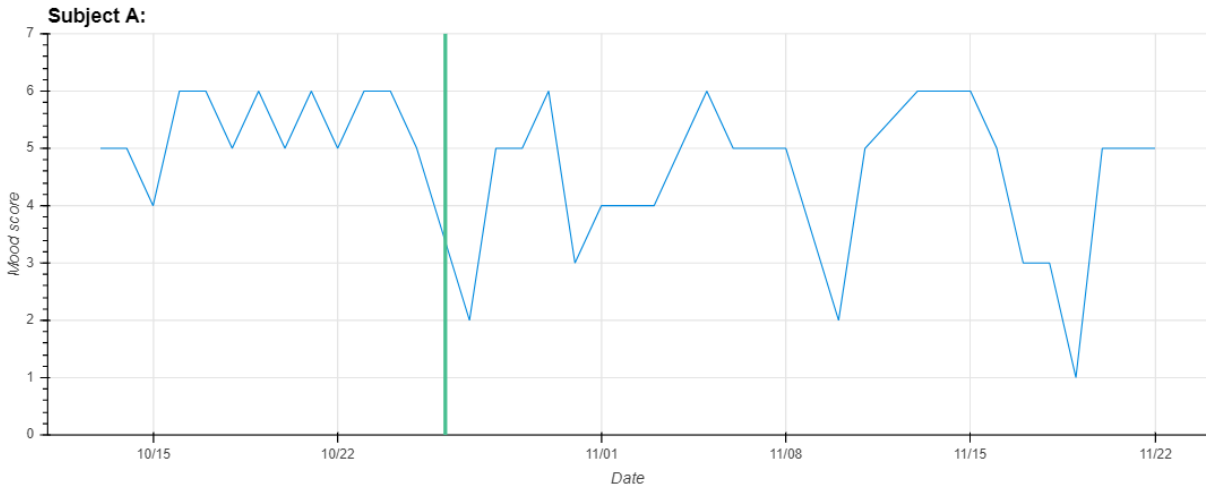

*Figure 18 - Line plot of **'relaxed'** mood for subject A*

At first glance, we can see that there were not many noticeable improvements that occurred during the vegan period. Subject B showed to be quite less anxious with an increase, meaning it improved, of 1,4 points (**Figure 19**), but as far as improvements go during the vegan period, that would be about it. There were several sudden declines in moods over the vegan period comparing to the baseline, such as 'rather doing something else' for subject C (**subject 20**) and 'anxiety' for subject E (**Figure 21**).

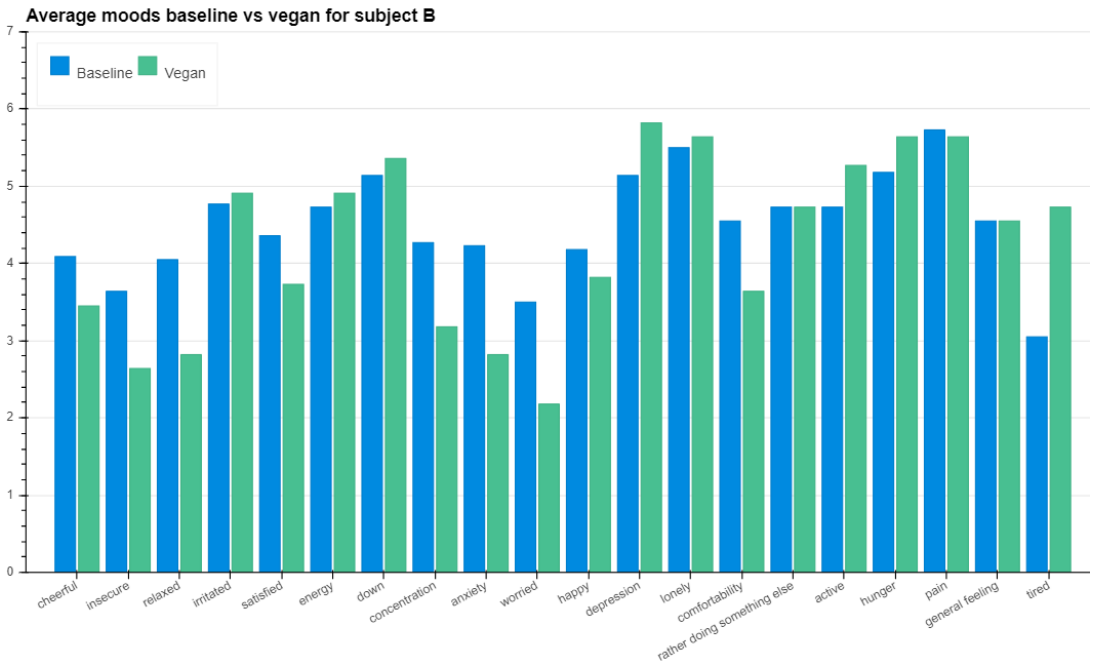

*Figure 19 - Bar plot of **average** mood scores subject B*

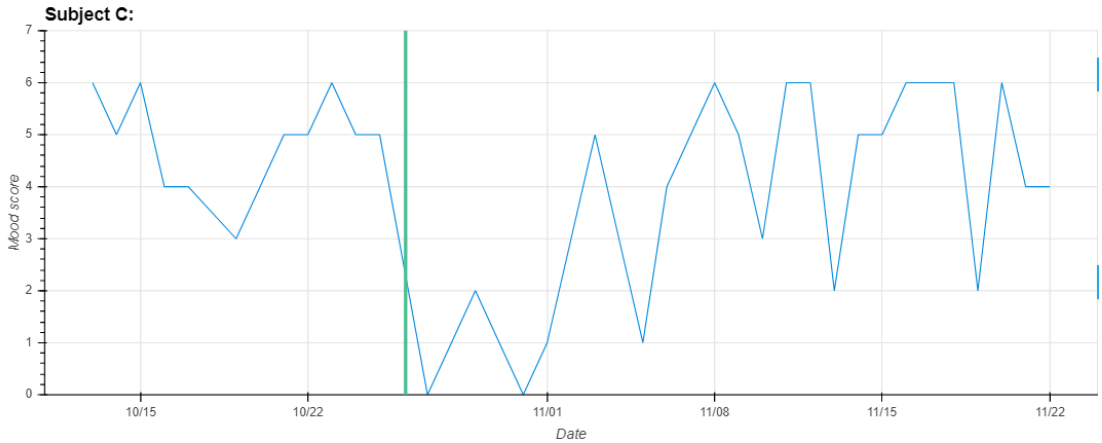

*Figure 20 - Line plot of 'rather doing **something** else' mood for subject B*

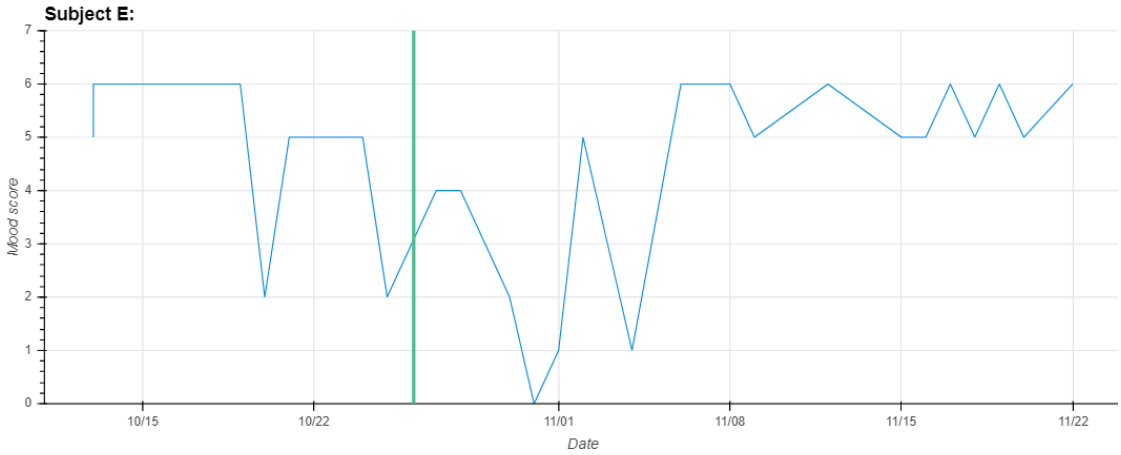

Figure 21 - Line plot of 'anxiety' mood for subject C

Overall, only four moods have increased of which three have presumably different causes. These are loneliness, concentration and comfortability. The fact that concentration has increased for most subjects during the vegan period was presumably due to the exam period, which happened in the first week of the being vegan. You can clearly see (**Figure 22**) that for subject C and E the concentration is going up around the first two weeks of the vegan period. For subject E, there is even a distinguishable drop after the exam period (which ended the 8th of November) (**Figure 23**).

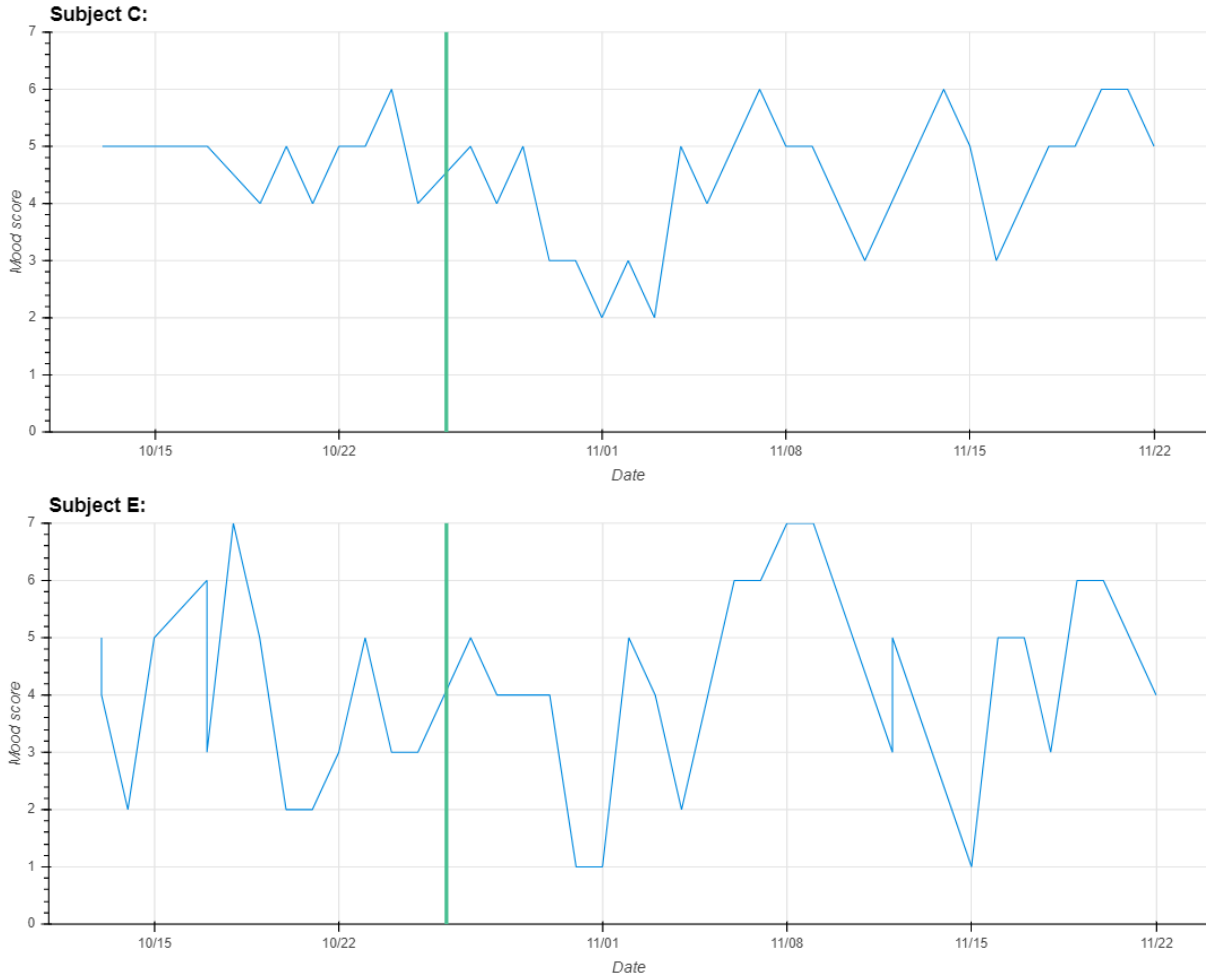

Figure 22 - Line plots of 'concentration' mood for subject C and E

3.4 Gut Microbiota

Quality of sequencing

In total, the two ONT MinION sequencers yielded a total of over one million reads (1 049 328). With the use of Guppy 84.8% (889 693) of the total reads have been successfully mapped and could be linked to their respective sample and subject (**Table** 2). The quality of the sequencing has been analysed with pycoQC (Leger, 2017/2021). The median read length is 806 base pairs, with highest values over of over forty thousand base pairs. The mean Phred quality score for all samples combined is 11.28.

Table 2. Distribution of recovered barcodes in reads.
[a]bp = base pair, Mbp = base pair 1 x 10 [6]

Barcode	Subject	Sample	Number of reads	Total bp (Mbp)[a]	Average read Length (bp)[a]
Barcode01	A	Vegan	100 191	156.6	1563
Barcode02	B	Vegan	6204	13.1	2115
Barcode03	C	Vegan	2624	6.7	2546
Barcode04	D	Vegan	302 240	475.8	1574
Barcode05	E	Vegan	12 131	28.4	2344
Barcode06	A	Control	190 740	376.8	1976
Barcode07	B	Control	110 360	107.7	976
Barcode08	C	Control	12 218	18.6	1521
Barcode09	D	Control	85 015	152.2	1790
Barcode10	E	Control	136 068	162.3	1193
Unknown	Unknown	Unknown	68 366	126.3	1847

Taxonomic analysis of microbiota data using MetaPhlan

MetaPhlAn created a taxonomic profile per barcode containing the relative abundance of found and unknown taxa. These taxonomic profiles have been processed with pandas in python into data tables per subject (Appendix B. Shared files location) (McKinney, 2010; Van Rossum & Drake, 2011). These data tables are constructed hierarchically to give the ability to get the relative abundance of taxa on all taxonomic levels before and after the intervention in an abrupt change of diet per subject. An overview of the most commonly found taxa, diversity of taxa and percentage of unknown taxa can be found in **table 3**. In this table, these most occurring taxa for pooled subjects are defined by taking the mean over the samples. Another way of defining the most commonly found taxa is by taking the sum over all samples. This would result in *Bacteroides uniformi*, *Bacteroides vulgate*, *Prevotella copri* for the vegan sample and *Bacteroides uniformi*, *Bacteroides vulgate*, *Eubacterium rectale* for the control samples in descending order.

Table 3. Information about taxonomic profiles generated by MetaPhlAn per barcode. *Most common species and percentage of unknown taxa are taken by the mean for the category "Pooled subjects". Sample sizes of pooled subjects can be found under the table.*

Barcode	Subject	Sample	Most common species (relative abundance)	Second most common species (relative abundance)	Third most common species (relative abundance)	Number of unique species	Percentage of unknown taxa
Barcode01	A	Vegan	*Prevotella copri* (24.74%)	*Alistipes putredini* (9.76%)	*Bacteroides uniformi* (9.76%)	55	0
Barcode02	B	Vegan	*Bacteroides uniformi* (2.51%)	*Alistipes putredini* (1.46%)		2	96
Barcode03	C	Vegan	*Bacteroides vulgate* (5.03%)			1	94
Barcode04	D	Vegan	*Bacteroides uniformi* (32.77%)	*Bacteroides vulgate* (20.75%)	*Bacteroides ovatu* (9.37%)	60	0
Barcode05	E	Vegan	*Bacteroides vulgate* (13.21%)	*Bacteroides stercori* (12.06%)	*Gemmata obscuriglobu* (6.41%)	11	45
Barcode01-Barcode05	**Pooled subjects**	**Vegan**	***Prevotella copri* (24.74%)[a]**	***Bacteroides vulgate* (13.0%)[a]**	***Bacteroides uniformi* (12.72%)[a]**	**83**	**47**
Barcode06	A	Control	*Bacteroides uniformi* (11.07%)	*Bifidobacterium adolescent* (7.59%)	*Alistipes putredini* (6.91%)	96	0
Barcode07	B	Control	*Eubacterium rectale* (23.78%)	*Bacteroides plebeiu* (20.47%)	*Roseburia intestinali* (18.14%)	36	0
Barcode08	C	Control	*Alistipes sp An31A* (14.93%)	*Alistipes putredini* (14.58%)	*Bacteroides uniformi* (9.56%)	7	45
Barcode09	D	Control	*Bacteroides uniformi* (30.5%)	*Bacteroides vulgate* (23.59%)	*Bacteroides ovatu* (17.17%)	26	0
Barcode10	E	Control	*Bacteroides vulgate* (14.71%)	*Bacteroides stercori* (13.44%)	*Bacteroides uniformi* (11.33%)	45	6
Barcode10-Barcode11	**Pooled subjects**	**Control**	***Bacteroides plebeiu* (20.47%)[a]**	***Roseburia intestinali* (18.14%)[a]**	***Alistipes sp An31A* (14.93%)[a]**	**112**	**10**

[a]Sample size (presence in number of barcoded samples) for *Prevotella copri* = 1, *Bacteroides vulgate* = 3, *Bacteroides uniformi* = 4, *Bacteroides plebeiu* = 1, *Roseburia intestinali* = 1 and *Alistipes sp An31A* = 1.

The relative abundance of certain taxa was plotted in a bar plot based on taxa deemed of interest by Zimmer et al. 2012. This was done per subject and by pooling the relative abundance of taxa of all subject together (taking the mean) (**figure 22**). *Clostridia* is the only one of these taxa that showed a decrease in relative abundance going from a regular diet to a vegan diet. The taxa *Bacteroides* and *Bifidobacterium* also decreased, within one standard error of the mean (S.E.M.).
were found to have a non-significant. The taxa *Escherichia coli* and *Enterobacteriaceae* were not found in the regular diet sample. All of these plots looking at these taxa are shown per subject in the dashboard (Appendix C. GitHub repository).

The same analysis has also been done for the taxa deemed of interest by David et al. 2013 (**Figure 23**). *Bilophila* was only found in the control sample of subject A. Other than the *Bacteroides* decline mentioned before, *Alistepes*, *Bacteroides, Roseburia, Eubacterium rectale* and *Ruminococcus bromii* also decreased, within one S.E.M.

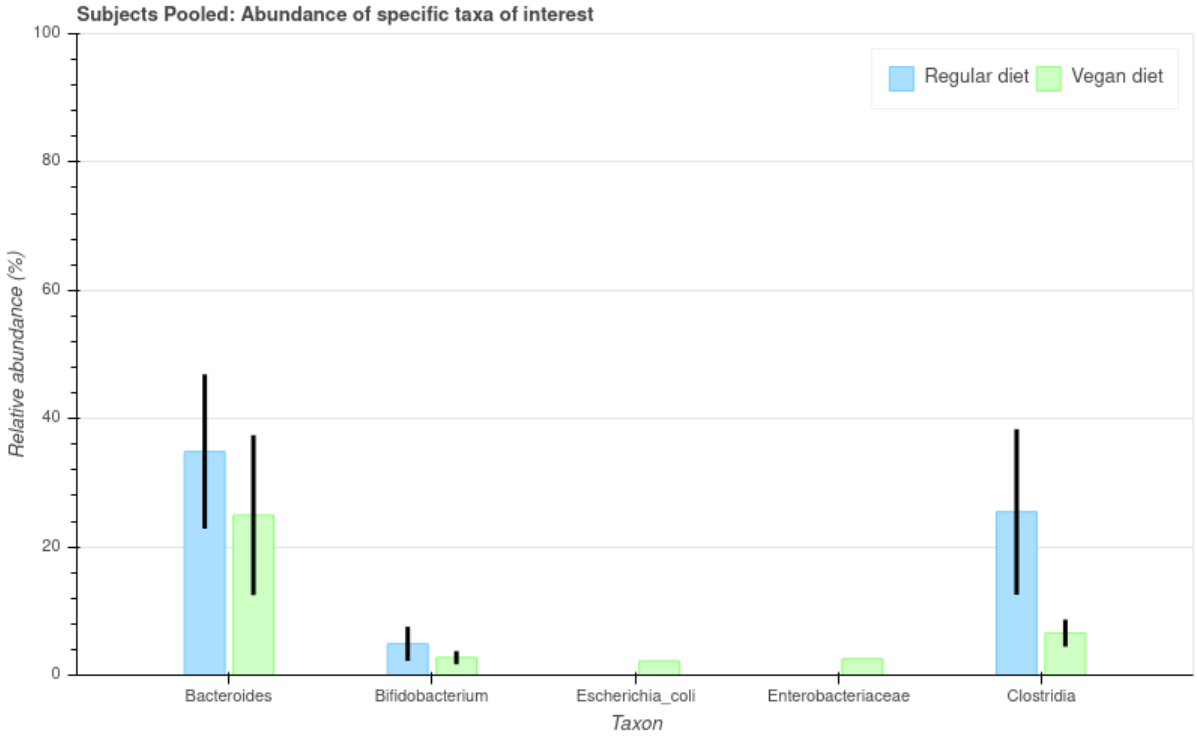

Figure 22. The abundance of specific taxa deemed of interest by Zimmer et al. 2012 of pooled subjects. *Relative abundance plotted in percentage. In light blue, the regular diet samples (control) are shown, in light green the vegan diet sample are shown. Errors bars shown as the standard error of the mean (S.E.M.). Sample size (presence in number of barcoded samples) for Bacteroides = 10 , Bifidobacterium = 5, Escherichia coli = 1, Enterobacteriaceae = 1 and Clostridia = 7.*

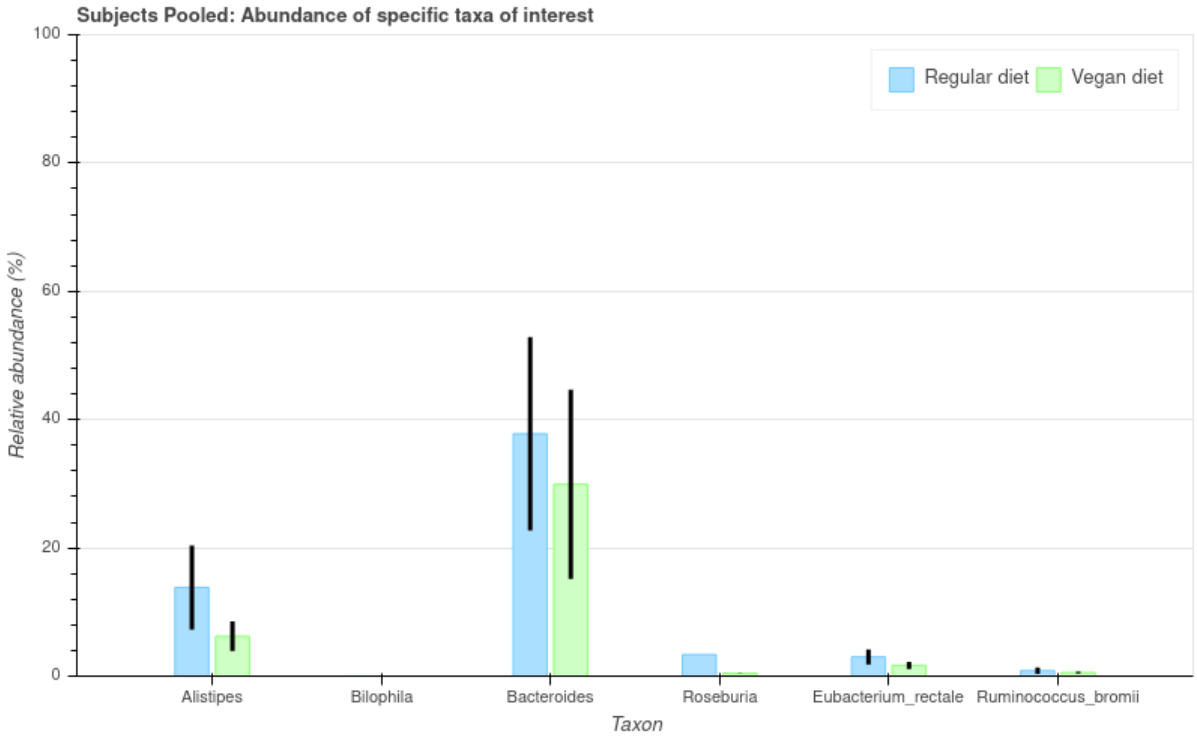

Figure 23. The abundance of specific taxa deemed of interest by David et al. 2013 of pooled subjects. *Relative abundance plotted in percentage. In light blue, the regular diet samples (control) are shown, in light green the vegan diet sample are shown. Errors bars shows as the standard error of the mean (S.E.M.). Sample size (presence in number of barcoded samples) for Alistipes = 8, Bilophila = 1, Bacteroides = 10 , Roseburia = 4, Eubacterium rectale = 6 and Ruminococcus bromii = 5.*

The relative abundance of all other taxa found is plotted in dumbbell plots. These plots have been made for all subjects individually and for all subjects pooled with a dumbbell plot per taxonomic level, of which the class, order and family level can be seen in **Figure 24**, **Figure 25** and **Figure 26** respectively.

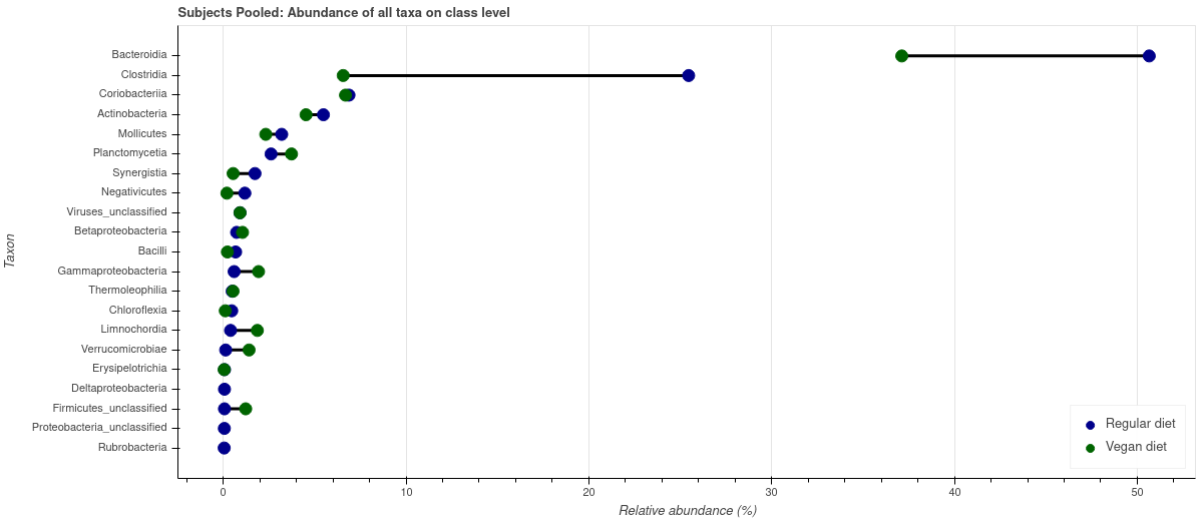

Figure 24. The abundance of all found taxa of pooled subjects on the taxonomic class level. *A line is seen as a difference between taxa (only shown for taxa found in both regular and vegan diet samples). In blue and green the mean regular diet and vegan diet samples of pooled samples are shown respectively.*

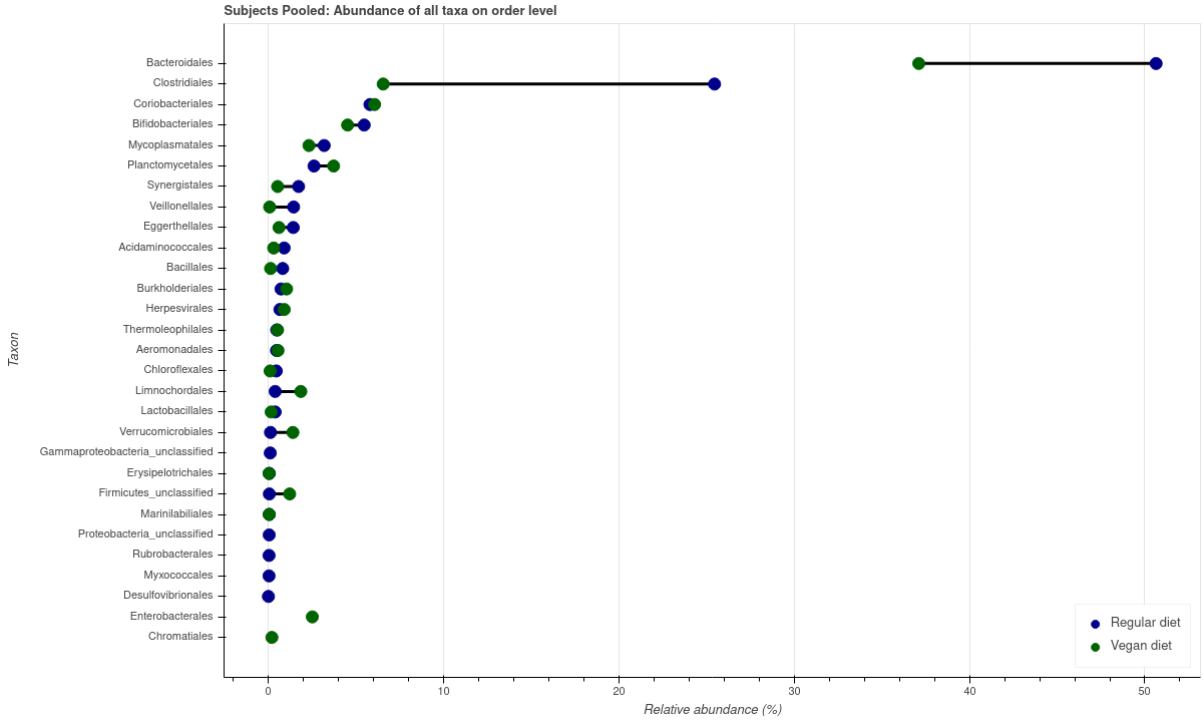

Figure 25. The abundance of all found taxa of pooled subjects on the taxonomic order level. *A line is seen as a difference between taxa (only shown for taxa found in both regular and vegan diet samples). In blue and green the mean regular diet and vegan diet samples of pooled samples are shown respectively.*

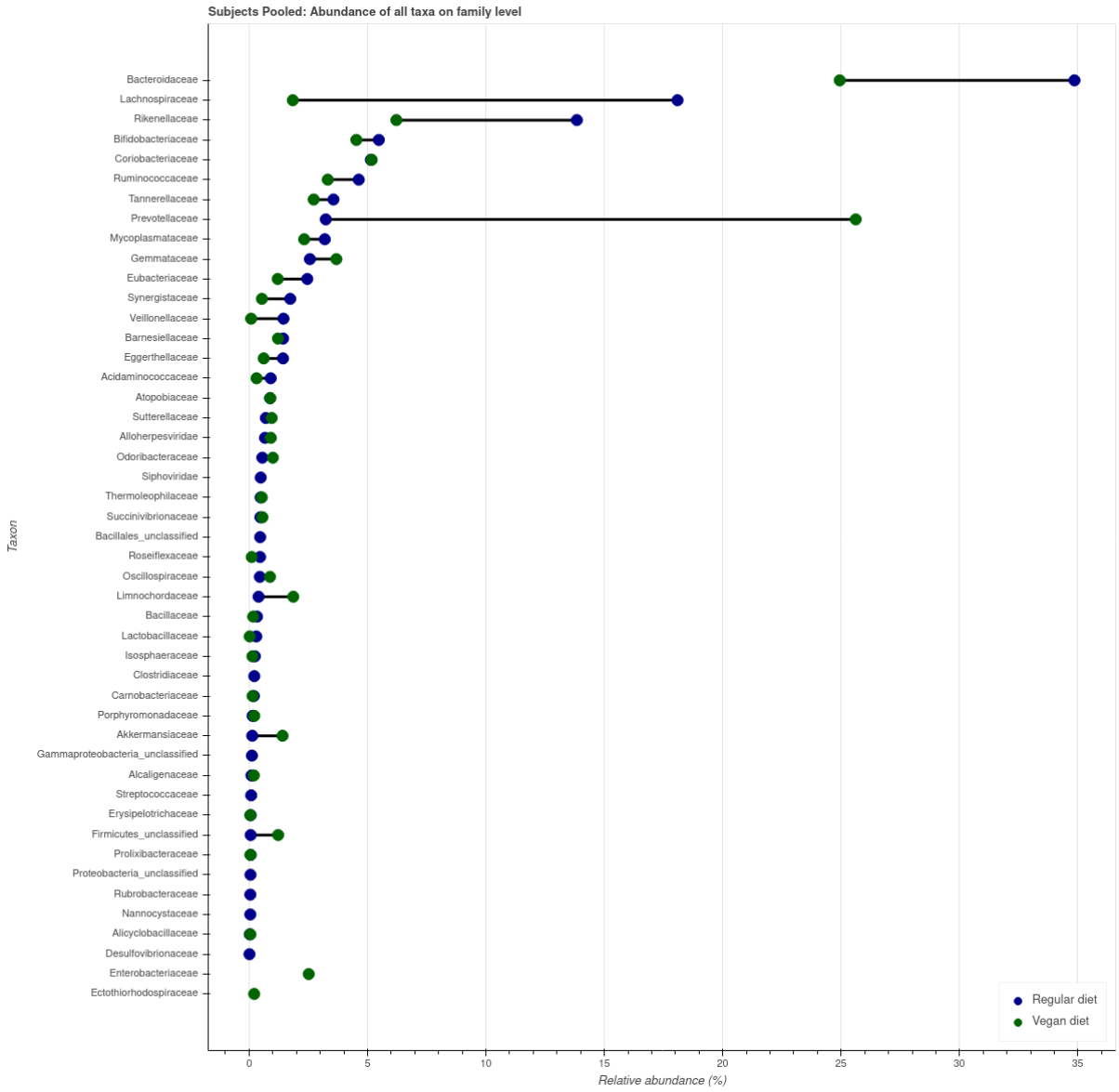

Figure 26. The abundance of all found taxa of pooled subjects on the taxonomic family level. *A line is seen as a difference between taxa (only shown for taxa found in both regular and vegan diet samples). In blue and green the mean regular diet and vegan diet samples of pooled samples are shown respectively.*

Taxanomic analysis with BLASTN

A second taxonomic analysis was performed with BLASTN to get an insight in the taxonomic profile of each subject before and after the vegan diet. FASTA files were aligned to a database, in contrast to MetaPhlAn reads were not only mapped to a database containing bacterial and viral sequences. This gave us a perception about the quality of the extracted DNA. The files generated by blast were moulded into a data frame holding the abundance of all the detected taxa on each of the hierarchical level. Abundances in bacteria are clearly noticeable before and after the intervention. These abundances have been visualized on superkingdom, genus and species level in **Figure 27** and **28**.

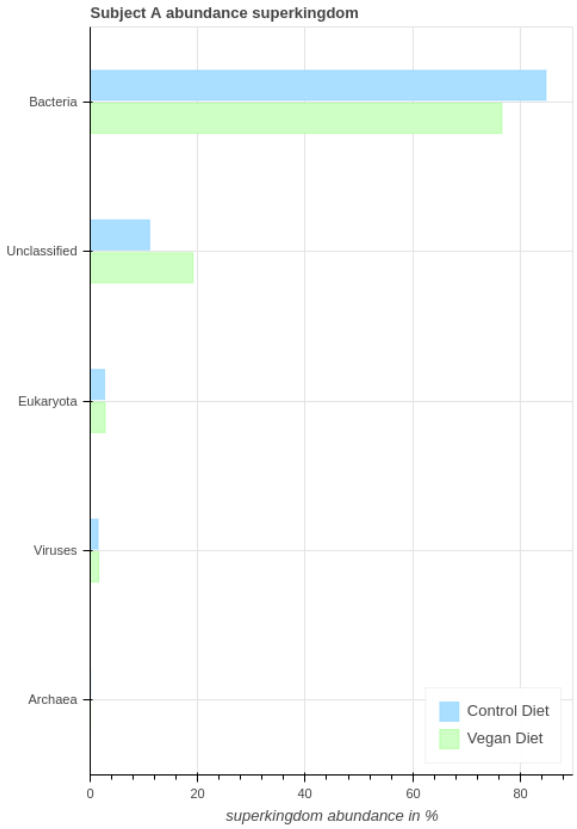

Figure 27. The abundance of taxa found of subject A on taxonomic superkingdom level. *Horizontal bars are shown representing relative abundance. Blue bars and green bars represent the control and the vegan condition.*

Relative abundance on genus(A) and species(B) level from subject A is displayed to gain a perception on possible shifts in microbiome. *Prevotella* genus shows a noticeable increase in relative abundance after the intervention in **Figure 28A**, this corresponds on species level where *Prevotella copri* increased after the intervention in **Figure 28B**. In contrast to MetaPhlAn, BLASTN detected *Escherichia coli* shown in **Figure 28B**. Interestingly, on genus level *Callithrix* is found in **Figure 28A**.

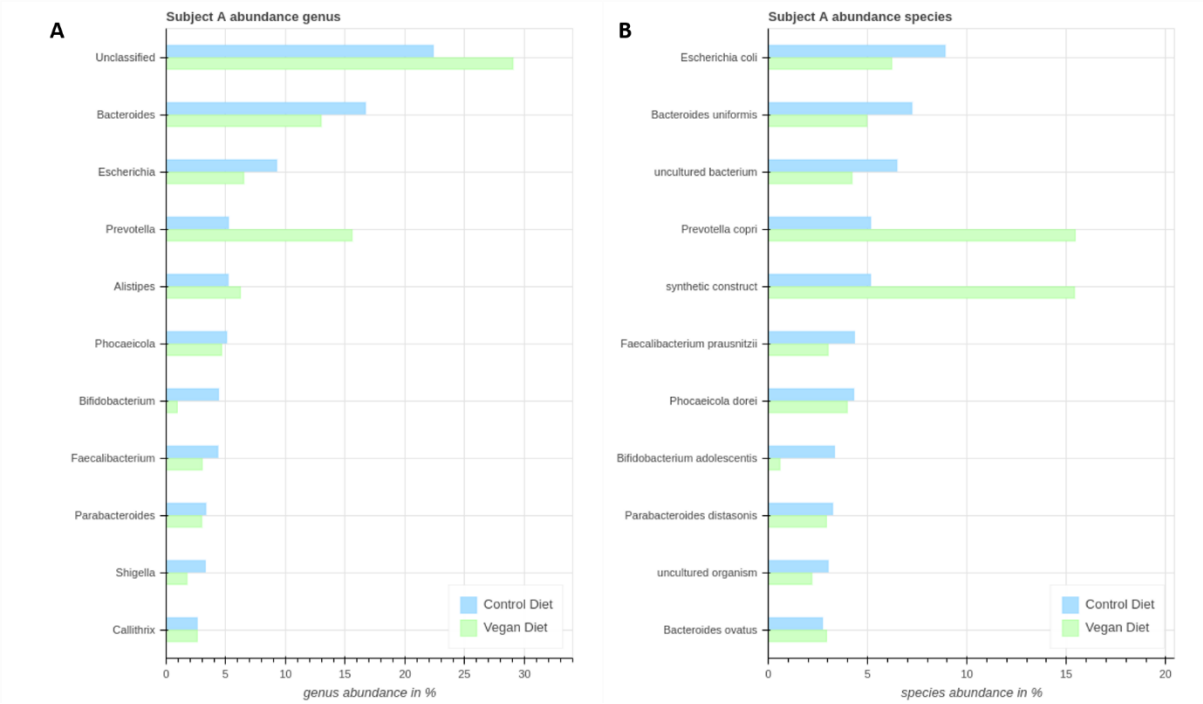

Figure 28. The abundance of taxa found of subject A on taxonomic genus(A) and species(B) level. *Horizontal bars are shown representing the relative abundance. Blue and green bars represent the control and the vegan condition.*

3.5 Interactive dashboard application

All visualizations can also be found in the created interactive dashboard. On the dashboard, the different research outcomes can be viewed per subject. Every measurement category has its own functionalities such as selecting a different plot type or viewing the detailed data frame. Instructions on how to run and use the dashboard can be found in the (Appendix C. GitHub repository).

4. Conclusion and Discussion

In the overall caloric intake of every subject were no significant changes observed during the vegan diet compared to the baseline diet. The goal of tracking food intake in this study was to maintain the same amount of intake nutrients during the vegan diet as during the baseline diet. This indicates that every subject did succeed in maintaining an overall stable caloric intake.
Looking at the food intake visualizations and comparisons, subject C consumed fewer carbohydrates and more fats during the vegan period. This can be due to consuming more nuts, nut butters, oils, or other products with a higher fat content instead of products with a higher carbohydrate content (Bushman, 1998). Subject B and D consumed a higher amount of carbohydrates during the vegan period. This can be due to the same reason as for subject C, instead that in this case it is the other way around. Many vegan food sources frequently contain relatively more carbohydrates, compared to non-vegan food sources (Kahleova et al., 2018). The protein intake of every subject decreased in the vegan period. Not consuming any meat and dairy can explain this occurrence since those products mostly contain a higher amount of protein (MacRae et al., 2005).

The carbohydrate contents did show interesting differences in fibre intake, every subject consumed more fibre during the vegan diet compared to the baseline diet. Increases in fibre intake can be due to consuming more vegetables, grains or other products high in fibre (Key et al., 2006). Consuming vegan carbohydrate sources will commonly result in a higher fibre intake compared to consuming non-vegan carbohydrate sources, because of consuming less processed foods and more whole foods (Kristensen et al., 2015). Only subject D showed a noticeable increase in sugar intake during the vegan diet. This can be caused by consuming whole foods that contain a higher sugar content, such as certain fruits and vegetables (Bazzano et al, 2008).

Based on visual inspection of body composition plots, there are no noticeable fluctuations in records of individuals. In other words, we cannot see a meaningful trend in weight, BMI, body fat percentage, skeletal muscle percentage. So, it cannot be concluded that a vegan diet does affect body composition in the short time period that we did our experiment.

Looking at subject B, there was a slight decrease in weight during the vegan diet. The average caloric intake of subject B during the vegan diet hardly differed from the baseline average caloric intake. This phenomenon was also observed in subject C and D. Losing weight while maintaining the same number of calories can be explained by burning more calories than consuming calories, which results in weight loss. Also, during the experiment the food products and liquids were not strictly measured. It could be that a subject loosely tracked some meals and estimated the amount of food incorrectly. This could have resulted in inaccuracy in the food intake measurements. The food intake measurements should, in this case, not be considered as a reliable explanation of body composition measurement fluctuations.

Fluctuations in body composition measurements can also be declared by the measurement errors from the Omron BF511 device. Omron Healthcare states that the device is clinically validated (OMRON Healthcare, 2020; Bosy-Westphal et al., 2008), which means that the measurement errors fall within the allowed limit. However, this is not an exemption of the device not having any inaccuracy that causes body composition measurement errors. The variables weight, body fat percentage, skeletal muscle percentage and visceral fat level have a standard error of estimate (S.E.E.), which are summarized in **Figure 23**. Unless the fact that these measurement errors fall within the permitted thresholds, they can cause any fluctuations in the variables.

Weight Accuracy	0.0 kg to 40.0 kg: ± 0.4 kg (0.0 lb to 88.2 lb: ± 0.88 lb) 40.0 kg to 150.0 kg: ± 1% (88.2 lb to 330.0 lb: ± 1%)	
Accuracy (S.E.E.)	**Body Fat percentage:**	3.5%
	Skeletal Muscle percentage:	3.5%
	Visceral Fat Level:	3 levels

***Figure 29**. Omron BF511 variable accuracy (OMRON Healthcare, 2020).*

Another explanation for body composition measurement fluctuations is the hydration of the human body. Changes in hydration of the human body can already influence the sensitivity of body composition measurement devices incredibly (Saunders et al., 1998). Consequently, consuming beverages prior to using the device influences the hydration of the human body, and therefore the body composition measurement results (Dixon et al., 2009).

The noticeable changes in mental state are mainly occurring for the negative type of emotions and occur mostly around the exam period, which was planned in the first few weeks of the vegan diet. E.g., the decrease in the response 'rather doing something else' for subject C, 'anxiety' for subject C, D and E and 'satisfied' for subject A.

The one mood that has surprisingly been increased for the better is the level of energy the participants feel they have. Three out of two subjects seem to have a higher energy level over the vegan period against the baseline. The macronutrients carbohydrates and proteins a known energy source (USDA, 2015). Subject C, D and E perceive a higher energy level during the vegan period than on the baseline period. Tracing this back to the food intake, subject C showed an increase in carbohydrates (fibre and sugar) but no increase in proteins. Subject D showed an increase in fibre (carbohydrates) but not in sugar and a noticeable drop in protein intake. As for subject E, there is both a decrease in fibre and sugar (carbohydrates), but an increase in protein. This showed that for the different subjects, there would be different sources of nutrients in the vegan diet which could have resulted in a higher energy level.

Noticeable is that only one subject felt less hungry during the vegan period. An interesting point of discussion, also during the experiment itself, was not specifically feeling hungry but more the feeling of not being satisfied after eating food from the vegan diet. Which can possibly also be the reasoning why the satisfied mood has decreased in score for most of the subjects. Two subjects are more hungry and less satisfied, and one subject is less hungry but more satisfied. Comparing the satisfied mood scores with the fibre intake (Slavin & Green, 2007, p. 36), subject B and E showed a decrease in intake which were not significantly enough to state that the fibre intake had anything to do with being satisfied. Note: the 'I am satisfied' question was not specified to focus on the feeling of hunger but can be traced back to the overall feeling of being satisfied.

Besides the energy level, tiredness is also something that is mentioned often when talking about the effects of veganism or even vegetarian diets (Aavik, 2019). For three of the six subjects, the tiredness decreased. For subject B there was a noticeable drop occurring over the whole period of veganism. Taking this to the nutrients that these subjects took, subject B and E's, protein, fibre and fats intake all decrease, meaning the vegan diet cannot be put out there as reasoning the subject's tiredness increased. For subject D it was the opposite: their fats, carbs and fibre increased during the vegan period, meaning for this subject the diet might had an influence.

Reasoning for the various results among the participants could be a result of the testing method. The questionnaire used PsyMate™ questions, which are based on the ESM principle, which could arguable be interpretable. Every participant seemed to interpretate several questions in different ways, such as the question 'are you satisfied?'. Which, in the end, seemed too general as one subject answered what they felt over the entire day and another subject answered for that specific moment.

In addition, the nature of the question might not have been correct. The ESM principle focusses on general health (Hektner et al., 2006), while this study also focused on general health improvements or deteriorate. Looking back, the nature of the questions could be more specified to the hypotheses and more focussed on specific moods which can be affected by someone's dietary restrictions. E.g., 'would you rather be doing something else?' might have given a better view on what impacted someone's mood, it has nothing to do with the general mental state.

It was found that the total number of reads and base pairs differed greatly per barcode, although it was expected that the distributions of total reads would be somewhat evenly distributed over the ten different barcodes (**Table 2**). The distribution of read length in base called reads matches with a gamma distribution with a long end tail as expected with MinION sequencing (Drs. M. Herber, personal communications, 2020). The median of the quality of the reads in Phred score is 11.28, which would result in a probability of the base called being called incorrectly of P = 0.0745. This probability of error may seem low and acceptable, however Laver et al., 2015 argues that MinION quality scores do not follow the Phred scale accurately and a Phred score of 11.28 does not equate the same error for MinION sequencing data compared with other Next-Generation Sequencing.

Calculating the most common taxa over a pooled subset of metagenomic samples is not a trivial task. Metagenomic samples can differ wildly resulting in taxa not being found in all samples. Although taking the total relative abundance seems better than taking the mean relative abundance of taxa over multiple metagenomic samples, there is still a bias in how many sequences the metagenomic samples contain. An absolute comparison in CFU or number of reads might be of higher interest, but as of the time of writing MetaPhlAn does not support this.

Running MetaPhlAn with the *stat_q* and *min_mapq_val* flags set to 0.1 and -1 resulted in a higher number of taxa identified, but it should be taken into consideration that this might introduce more false positives. These parameters lower the threshold of accepted read map qualities. By setting these flags to 0.1 and -1 the underlying BowTie2 accepts all mapped reads of sequences of at least 80 base pairs, the chosen allowed word length. Even with these considerations, the diversity (the number of different taxa) in samples was somewhat disappointing. With ChocoPhlAn containing more than one hundred thousand bacterial reference genomes, it is suspected that this is not a problem related to the references. The vegan samples for subject B, C, D and the control sample for subject C have the highest levels of unknown taxa and the lowest total base pairs. It is suspected that there has been an error in the wet lab work for these samples. Although the quality of the wet lab work was suspected at first to be the issue for all samples, the sequence quality seems adequate compared to literature for most samples (**table 2**) (Moss et al., 2020). The use of alternative taxonomic classifiers such as BLAST, DIAMOND or Kraken2 could be interesting to explore in the future (Altschul et al., 1990; Buchfink et al., 2015; Wood et al., 2019).

The most common taxa found in the pooled samples analysed by MetaPhlAn were cross-referenced to the Gut Feeling Knowledge Base v4 (gfkb) and to the NCBI taxonomy browser by their NCBI taxonomy ID to understand their characteristics and effect/function (King et al., 2019; Schoch et al., 2020). From these 5 different taxa, the Gut Feeling Knowledge Base only contained the NCBI taxonomy id of *Bacteroides Vulgate*. Identifying it as a mouse faecal isolate. Wu et al., 2011 shows

that for long-term diets *Bacteroides* are more common for those who eat a plethora of proteins and fats, with *Prevotella* being more common in carbohydrates rich diets. Looking at the most common taxa taken by the sum Bacteroides show up in the top three twice and *Prevotella* is found in the top three for the vegan diet. Further review of literature is needed for these specific species and their role in the microbiome.

To compare the MetaPhlAn results of the taxonomic analysis of the gut microbiota certain taxa were chosen to visualize separately that have been studied by Zimmer et al. 2012 and David et al. 2013 (**Figure 22**, **Figure 23**, **Figure 30**). These previous studies did not see a significant decline in Clostridia, while *Clostridia* showed a decrease in relative abundance outside of the S.E.M. in this study. However, this pooled comparison of relative taxa abundance cannot be interpreted as significant because of the low overall sample size of our data (n = 5 subjects, n = 7 samples containing *Clostridia*). The relative abundance of the other studied taxa were found to only change within one S.E.M. and had the problem of a sample size too low to be significant.

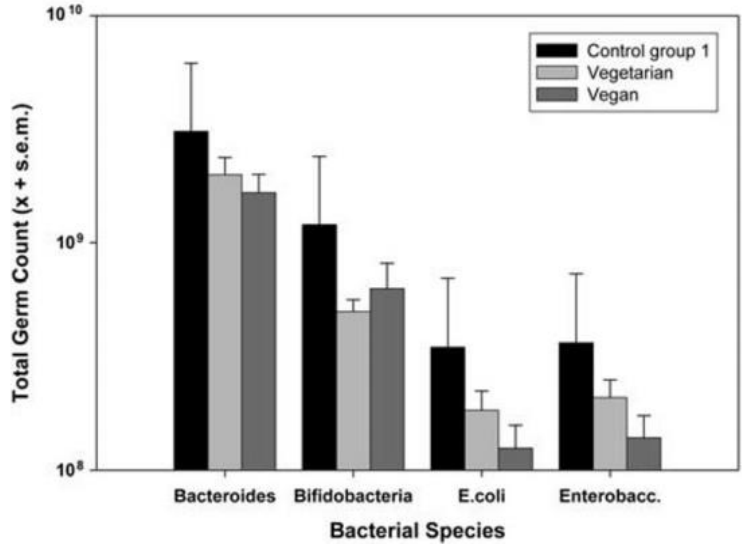

Figure 30. The abundance of taxa that significantly changed in the study done by Zimmer et al. 2012. Total Germ Count is expressed in CFU (Colony-Forming Units). Sample sizes of n = 249, n = 144 and n = 105 for the control group, vegetarian and vegan samples respectively. Adapted from Zimmer et al. 2012.

Plotting the difference between two metagenomic samples is not a trivial task. The properties of metagenomic samples being hierarchical, and possible large set difference makes plotting often either chaotic or lacking. An abundance heatmap clustered based on taxonomy would show all the taxonomic levels, but when there is a large set difference, the heatmap is sparsely filled with information. A higher diversity will also result in larger heatmaps possibly resulting in a very chaotic overview. Dumbbell plots can be a useful way of visualizing the change between two points in time or two conditions, which applies to the change in metagenomic samples (in paired datasets). The disadvantage of dumbbell plots for metagenomic comparisons arises with the lack of hierarchy, being able to only show one taxonomic level at a time.

BLASTN was used for a second taxonomic analysis to gain more insight on the possible shift in the gut microbiome and host to microbiome ratio. In all samples on the taxonomic superkingdom level there were substantially more bacteria compared to eukaryota. This means that the extraction method used was sufficient to yield an acceptable ratio between host and microbiome for this research purpose. The relative abundance on genus level resulted in a noticeable shift in *Prevotella,* this shift was also detected by MetaPhlAn in subject A. The *Prevotella* genus is strongly associated with a plant-based diet. A study by Filippo et al., 2010 noticed the difference in abundance in animal diets and plant-based diets, where *Prevotella* was highly present in subjects with a plant-based diet. While other studies observe these findings concerning *Prevotella* as well, in this study this finding cannot be validated due to the lack of statistical significance (Ruengsomwong et al., 2016; Matijašić et al., 2014). In contrast to MetaPhlAn, BLASTN did detect *Escherichia coli* in both samples for subject A, this could be due to the limited knowledge about the compatibility of the MinION, which uses long-read sequencing(LRS), in combination with MetaPhlAn. Hence it is important for future purposes to use Illumina and MinION in combination with MetaPhlAn or other LRS sequencing methods, e.g. PacBio's platform. PacBio's errors occur more randomly which are easier to correct in contrast to the frequent homopolymer errors of the ONT platform (Maghini et al., 2020), this could possibly lead to more hits using MetaPhlAn.

In conclusion, no significant change was found for the food intake, body composition and mental health as we expected. The low sample size and nonsufficient quality of the taxonomic analysis did not result in any findings of significance for the microbiome composition. Despite there being some tentative evidence for a lower occurrence of *Clostridia* in the vegan samples, more research is needed, preferably with alternative wet- and dry lab methods.

5. References

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Appendix

A. Measurement protocols

Measurement protocols

Ve-gang

October 12th – November 22nd

Definition of the vegan diet

In order to clarify the exact definition of a “vegan” diet in this experiment, there are some important points. Since definitions of terms are not widely accepted, there needed to be a clear definition for every term for this experiment.

1. Part time/full time:
   Some vegans follow their respective diets for weeks or months, but after that period they start to include (mostly little) animal products in their diet again, and eventually switch back to their normal old regime. This cycle continues.
2. Semi-diet:
   They are the ones who decide to eat less meat or animal products for sake of the planet or their health. This option often is taken as a first step to a vegan life. They try to use less meat, but from time to time they take their beloved dishes of food which are out of their diet.
3. Types of Veganism:
   There are different kinds of protocols among veganism, they are oriented to avoid animal products with a specified degree of commitment[1]. Here are three important categories:
   a. Vegan:
      Vegans do not eat any animal products. This means no meat, fish, dairy, eggs, honey or any animal derived products. Some vegans also refrain from buying leather, silk or wool, as well as visiting events in which animals are used for entertainment purposes.
      For this experiment the “simple vegan” term is defined for clarifying the diet and ethics. Simple vegan is a diet without meat, fish, diary, eggs, honey or any animal derived products. All other foods can be consumed. Subjects are not obliged to obey other ethical policies and this diet is only about the food.
   b. Plant-based:
      A plant-based diet is a diet based on whole grains, legumes, vegetables and fruits with small amounts of nuts and seeds[2]. No oil is used, not even olive oil or coconut oil. The diet includes both cooked food and raw food. Added sugars and salt should be kept to an absolute minimum.
   c. Raw vegan:
      Raw vegans only eat raw, plant-based foods which are not heated above 45 degrees Celsius.

Based on the above information, the diet in this experiment is full time and simple vegan.

[1] https://brendadegroot.com/en/types-vegans/
[2] https://www.wholefoodsplantbasedhealth.com.au/going-plant-strong/what-is-wfpb-diet/

1. Food intake measurement protocol

In order to have more reliable results out of the experiment, some rules and guidelines for entering the food intake are formulated as follows:

1. Subjects should import all data related to their intakes into the FatSecret application every day.
2. Subjects should do their best for entering data as accurate as possible.
3. In case of doubt if a product has animal derived ingredients or not, subjects are not allowed to consume that product.
4. In case the subject consumes a product that is out of the diet, the subject should continue the diet and add all information of that product to the data sheet (e.g. date, amount and type of animal derived products that subject took).
5. If any subject feels that the diet is dangerous for her/his health, he or she must immediately stop the diet and inform other subjects and supervisors about it.
6. Subjects should export their data once a week (preferably on Sunday after the last consumed product) and upload it to the shared "Food intake" folder (with proper naming). So, if their device/application is lost or broken, all needed information is still accessible in the shared folder.
7. If the subject forgets to enter their intakes into FatSecret, he or she should continue the diet and try to import the information as much as he/she remembers.

The format of files which will be exported from the FatSecret application should obey the following rules:

1. Report of intake calories should be generated every week and be uploaded to the shared folder:
   a. In the FatSecret application, under the "Diet Calendar" menu, there is a button at the end to export the intake information of the subjects (Figure 1).

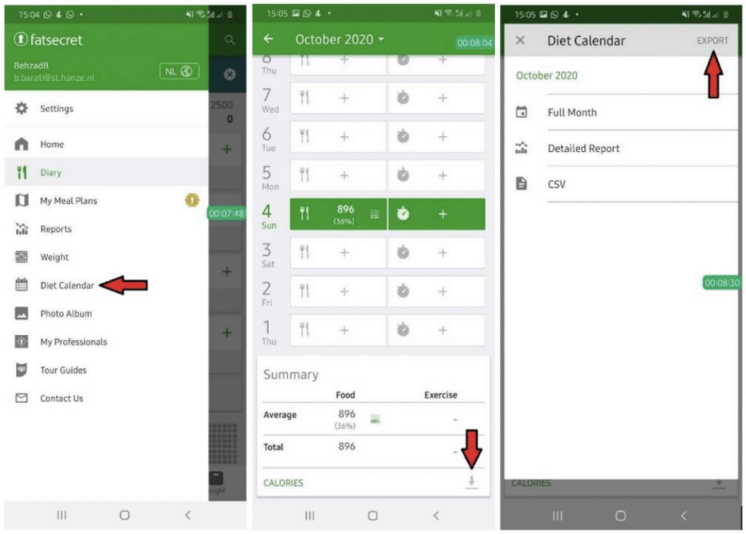

Figure 1. *FatSecret food diary exporting steps.*

3

2. The name of file must be in the following format:
   Week(number of experiment week)_(date of generated file)_subject_(first letter of subject).csv
   Replace the brackets with representing information.

2. Mental state measurement protocol

The mental state of all subjects is being monitored throughout this project with the use of a pre-selected mental health questionnaire. The possible changes and/or fluctuations in the mental state is being monitored in two categories: depression and stress.

How

- Daily ESM beep questionnaire (24 questions)
- Retrieved from *PsyMate 2*
- Using a OneDrive form

When (frequency)

- Once a day for six weeks (starting at 12 October, ending at 22 November)
- At the end of the day (e.g. every day at 19:00/20:00)

What (focus)

- Depression
- Anxiety

The PsyMate 2 application

The PsyMate 2 application's BEEP tests are mainly used to track a person's mood during the day. The BEEP tests consist of 24 questions which are the same every day and can be found in Appendix 1. 22 of the 24 questions have answer possibilities on the scale from 1-7 (going from completely disagree to completely agree). The remaining 3 questions are about your surroundings and activities.

Usage

The BEEP test will be used as a mental health survey once a day. These are the protocol rules that must be followed while working on this project and answering the questions within the application:

1. Answer the BEEP test once a day, in the evening.
    a. Answer the questions preferably a couple of hours before you go to bed
2. After finishing the survey:
    a. You can finish and be done!
    b. Add notes
        i. If you experienced something 'non-normal' that day, you could specify this in the notes so that can be taken into account when analyzing the results.
        ii. If something occurred while you were taking the test, which may influence the results; specify this in the notes.

Note: Adding notes is not mandatory. Only do so if you feel comfortable sharing certain information with the rest of the group.

File format

- All answers from the questionnaire will result in a csv file per subject:
  "Subject_(first letter of subject)_mental_health_week_(Number of experiment week).csv"
  Replace the brackets with the representing information.
- Weekly export on Sunday. The csv files should be uploaded to the shared "Mental state measurement files" folder.

3. Body composition measurement protocol

What

- Height (cm)
- Bodyweight (kg)
- Body Mass Index (score + classification)
- Bodyfat percentage (%)
- Skeletal muscle percentage (%)
- Resting metabolism (kcal)
- Visceral fat (level + classification)

When

Every day, starting on October 12th and doing the last measurement on November 23rd.

How

With the Omron BF511. Every subject takes one device home and uses it three times a week:

- After waking up in the morning
- After using the toilet
- Before consuming any beverage; including water (sober)

Steps:

1. Select your personal profile.
   Note: for the first measurement point (October 12th), this profile needs to be created first (Chapter 3 of the manual). Make sure that the right measurement units are selected.
2. Take a new measurement in your profile (Chapter 4 of the manual).
3. Check the measurement results (Chapter 4, step 5 of the manual).
4. Collect the measurement values in the corresponding unit (see "body_composition_measurements_template.xlsx"). The "Height" parameter only needs to be filled in once, for example on the first measurement point.
5. Clean the device.

Note: after taking last measurement (November 23rd), delete your profile from the device (Chapter 3 of the manual).

File format

All subjects collect their data in their own excel sheet and upload it to the shared "Body composition measurement files" folder. Every subject uses one file and updates it every time he/she takes a measurement. At the end of the experiment the data files of all subjects will be concatenated.
The name of the file must be in the following format:

body_composition_measurements_subject_(first letter of subject).xlsx
Replace the brackets with the representing information.

4. Microbiota measurement protocol

Faecal sample collection

What
Collection of faecal material.

When
Monday October 26th and Monday November 23rd.

Why
To collect the material for studying the microbiome per individual.

How

1. Label the provided collection container with your subject label.
2. Place something in the toilet, think of a clean empty plastic food container or a plastic bag. This will prevent contact between the inside of the toilet.
3. Collect three scoops of faecal matter. If it is sticky, use another piece of clean plastic or other clean material to get it of the "spoon" to place it in the container. Make sure that there are three clumps of ~2 grams in the container. Place it in the collection container and close it.
4. Place the collection container in a sealable plastic bag.
5. Wash your hands thoroughly.
6. Bring the sample to the Hanze University for storage in the -80°C freezer.

Important remarks:

- Faeces cannot touch the inside of the toilet.
- Avoid contamination with urine.
- Stool sample needs to be fresh.
- Stool samples will be collected in the morning and processed or properly stored the same morning in the lab.

Lab extraction

This protocol is retrieved from the QIAamp PowerFecal DNA Kit Handbook

1. Add 0.25 g of stool or biosolid to the Dry Bead Tube provided.
Note: For fecal samples that are especially high in lipids, polysaccharides and protein (e.g. meconium or some bird feces), smaller amounts of starting material (~0.10 g) may improve DNA yield and purity.
2. Add 750 µl of PowerBead Solution to the Dry Bead Tube.
3. Add 60 µl of Solution C1 and invert several times or vortex briefly.
4. Heat the tubes at 65°C for 10 min.
5. Secure tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000–V1–24). Vortex at maximum speed for 10 min.
6. Centrifuge the tubes at 13,000 x g for 1 min.
7. Transfer the supernatant to a clean 2 ml collection tube (provided). Expect between 400 to 500 µl of supernatant.
8. Add 250 µl of Solution C2 and vortex briefly to mix. Incubate at 2–8°C for 5 min.
Note: You can skip the 5 min incubation. However, if you have already validated the PowerFecal extractions with the incubation we recommend you retain the step.
9. Centrifuge the tubes at 13,000 x g for 1 min.
10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
Note: You can skip the 5 min incubation. However, if you have already validated the PowerFecal extractions with the incubation we recommend you retain the step.
12. Centrifuge the tubes at 13,000 x g for 1 min.
13. Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube (provided). Do not transfer more than 750 µl at this step.
14. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
15. Load 650 µl of supernatant onto a MB Spin Column and centrifuge at 13,000 x g for 1 min. Discard the flow through and repeat until all the supernatant has been processed.
Note: Each sample processed will require a total of three loads.
16. Add 500 µl of Solution C5 and centrifuge for 1 min at 13,000 x g.
17. Discard the flow through and centrifuge again for 1 min at 13,000 x g.
18. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided).
Note: Avoid splashing any of Solution C5 onto the MB Spin Column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile, DNA-free, PCR-grade water or TE buffer (cat. no. 17000-10).
Note: Eluting with 100 µl of Solution C6 will maximize DNA yield. For more concentrated DNA, a minimum of 50 µl of Solution C6 can be used.
20. Centrifuge at 13,000 x g for 1 min and discard the Spin Filter basket. The DNA in the tube is now ready for any downstream application.
Note: We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Hints & Troubleshooting Guide.

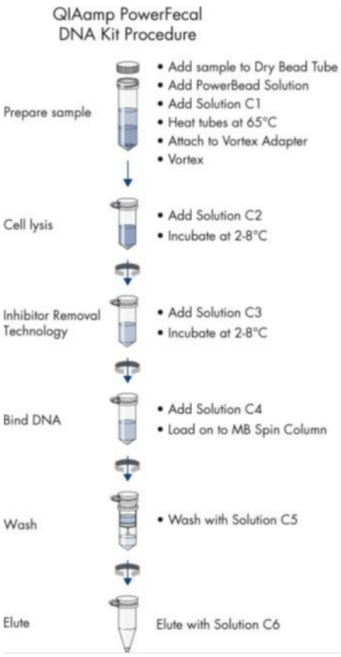

Figure 2. *Procedure QIAamp PowerFecal DNA kit*

Lab Sequencing

Materials

- ~400 ng high molecular weight genomic DNA
- Rapid Barcoding Sequencing Kit (SQK-RBK004)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads (optional)
- Freshly prepared 70% ethanol in nuclease-free water (optional)
- 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (optional)

Equipment

- Ice bucket with ice
- Microfuge
- Timer
- Thermal cycler or heat block at 30°C and 80°C
- P1000 pipette and tips

- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P2 pipette and tips

Optional Equipment

- Standard gel electrophoresis equipment
- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)
- Magnetic rack
- Hula mixer (gentle rotator mixer)

Library Preparation

1. Thaw kit components at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix RB01-12	Not frozen	✓	✓
Rapid Adapter (RAP)	Not frozen	✓	✓
Sequencing Buffer (SQB)	✓	✓	✓*
Loading Beads (LB)	✓	✓	Mix by pipetting or vortexing immediately before use
Flush Buffer (FLB) - 1 tube	✓	✓	✓*
Flush Tether (FLT)	✓	✓	✓

*Vortexing, followed by a brief spin in a microfuge, is recommended for Sequencing Buffer (SQB) and Flush Buffer (FLB).

2. Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.
   Prepare the DNA in nuclease-free water:
   - Transfer ~400 ng genomic DNA into a DNA LoBind tube
   - Adjust the volume to 7.5 µl with nuclease-free water
   - Mix by flicking the tube to avoid unwanted shearing
   - Spin down briefly in a microfuge
3. In a 0.2 ml thin-walled PCR tube, mix the following:

Reagent	Volume
400 ng template DNA	7.5 µl
Fragmentation Mix RB01-12 (one for each sample)	2.5 µl
Total	10 µl

4. Mix gently by flicking the tube and spin down
5. Incubate the tube at 30° C for 1 minute and then at 80° C for 1 minute. Briefly put the tube on ice to cool it down.
6. Pool all barcoded samples in your desired ratio, noting the total volume.
7. Resuspend the AMPure XP beads by vortexing.
8. To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube.
9. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
10. Prepare 500 µl of fresh 70% ethanol in nuclease-free water.
11. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
12. Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
13. Repeat the previous step.
14. Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.
15. Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at room temperature.
16. Pellet the beads on a magnet until the eluate is clear and colorless.
17. Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube and dispose of the pelleted beads
18. Add 1 µl of RAP to 10 µl of barcoded DNA.
19. Mix gently by flicking the tube and spin down.
20. Incubate the reaction for 5 minutes at room temperature.

For priming and loading the SpotON FlowCell please take a look here:
https://community.nanoporetech.com/protocols/rapid-barcoding-sequencing-sqk-rbk004/v/rbk_9054_v2_revn_14aug2019/priming-and-loading-the-sp?devices=minion

Appendix 1

I feel cheerful	1 = not at all, 4 = moderate, 7 = very
I feel insecure	1 = not at all, 4 = moderate, 7 = very
I feel relaxed	1 = not at all, 4 = moderate, 7 = very
I feel irritated	1 = not at all, 4 = moderate, 7 = very
I feel satisfied	1 = not at all, 4 = moderate, 7 = very
I feel down	1 = not at all, 4 = moderate, 7 = very
I feel energetic	1 = not at all, 4 = moderate, 7 = very
I feel anxious	1 = not at all, 4 = moderate, 7 = very
I feel happy	1 = not at all, 4 = moderate, 7 = very
I feel lonely	1 = not at all, 4 = moderate, 7 = very
I am concentrated	1 = not at all, 4 = moderate, 7 = very
I worry	1 = not at all, 4 = moderate, 7 = very
Depression	1 = not at all, 4 = moderate, 7 = very
Who am I with?	Select: partner, family resident, family non-resident, friends, colleagues, acquaintances, strangers or others, nobody
Do I feel comfortable in this situation?	1 = not at all, 4 = moderate, 7 = very
What am I doing?	Select: resting, work or study, household, hygiene, eating/drinking, leisure, other, nothing
I would rather be doing something else	1 = not at all, 4 = moderate, 7 = very
I am active	1 = not at all, 4 = moderate, 7 = very
Where am I?	Select: at home, at family or friend's place, at work or school, public place, transport, somewhere else
I am hungry	1 = not at all, 4 = moderate, 7 = very
I am tired	1 = not at all, 4 = moderate, 7 = very
I am in pain	1 = not at all, 4 = moderate, 7 = very
I generally feel well	1 = not at all, 4 = moderate, 7 = very
Since the last beep I used	Select: alcohol, medication, coffee, caffeine, smoking, nicotine, cannabis, energy drink, other substances, nothing
This beep disturbed me	1 = not at all, 4 = moderate, 7 = very

B. Shared files location

The shared and stored files created during this research can be found at https://hanzenl-my.sharepoint.com/:f:/r/personal/t_van_lieshout_st_hanze_nl/Documents/Project_Ve-Gang?csf=1&web=1&e=yX2Vg5.

C. GitHub repository

The repository containing all the code for processing and visualising the measurement data can be found at https://github.com/kyliekeijzer/project-vegang.