Cecum microbiota in mice affected by selenium level in diet

Anne Kristin Kjernlie
Preface and Acknowledgements

The work with the thesis finishing my master degree at the Norwegian University if Life Science (NMBU) has been performed at the Laboratory of Microbial Gene Technology (LMG), from December 2013 to May 2014.

Cecum samples and mice data for the experiment has been provided by Anne Graupner and Norwegian Institute of Public Health, Oslo via Dag Anders Brede.

I want to thank my supervisors Dzung Bao Diep and Dag Anders Brede for answering all my questions, discussing with me and learning me a lot.

Further, and most of all, I want to thank Özgün Candan Onarman Umu, without whom this thesis would not be the same. Thank you, for being my supervisor, working partner and friend.

I also want to thank Live Heldal Hagen, Anbjørg Kalland Haraldstad and Cyril Frantzen for helping with sample preparation and running of the Illumina MiSeq Sequencer.

Thanks to everyone at LMG for always being smiling and helpful.

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May 2014
Abstract

Selenium (Se) level in diet has been seen to affect gastrointestinal microbiota in mice, and microbiota has been seen to affect the uptake and expression of Se and selenoproteins. In this study, cecum from mice fed with low Se diet and normal Se diet has been examined, and their microbiota community composition compared. DNA from 16 cecum samples from each diet was extracted and 16S Metagenomic Sequencing was done with the Illumina MiSeq Sequencer. Output was processed with Qiime pipeline for alpha and beta diversity analyses. Significant difference in abundance was found in two genera, *Akkermansia* and *Oscillospira*. 
Sammendrag

Selen (Se) før påvirker gastrointestinal mikrobiota i mus, og motsatt påvirker mikrobiota opptak og uttrykk av Se og selenoprotein. I dette studiet har cecum fra mus føret med lavt Se før og normalt Se før blitt undersøkt, og sammensetning av mikrobiota har blitt sammenlignet. DNA fra 16 cecumprøver fra hver type føringstilfelle ble ekstrahert og 16S Metagenomisk sekvensering ble utført med Illumina MiSeq Sequencer. Resultatene ble bearbeidet med Qiime pipeline og alfa- og betadiversitet ble undersøkt. I to genera ble det funnet signifikante forskjeller i mengde bakterier, genera *Akkermansia* og *Oscillospira*. 
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1. Introduction

1.1 Selenium
The 34th chemical element, selenium (Se), is a trace mineral that is essential to animals, including humans, and is important in the matter of immunology and fertility (Hefnawy & Tortora-Perez 2010; Rayman 2000). Too low levels of Se can lead to deficiency diseases like Keshan disease (Chen 2012), while too high levels are toxic (Lee & Jeong 2012). For humans in Nordic countries the recommended level of Se intake is 50 µg/day and 60 µg/day for women and men, respectively (Nordic nutrition recommendations 2012: integrating nutrition and physical activity 2014). In its natural state Se is found in soil as selenate and selenite and is absorbed by plants which use these to biosynthesize mainly selenomethionine (SeMe) and selenocysteine (SeCys). These can further be absorbed by animals, or animals synthesize SeCys on their own (Rayman et al. 2008). The main source for human dietary Se is meat. The Se level in Nordic soil is low; hence the level of Se in Nordic people is low. Se in form of selenate or selenite is recommended as dietary supplement.

1.2 Selenoprotein
The essentiality of Se to mammals is due to its participation in selenoproteins. Se is incorporated into the 21th amino acid, selenocysteine, which further is incorporated into selenoproteins: glutathione peroxidases (GPx), thioredoxin reductases (Txnrd), deiodinases, selenophosphate-synthetase 2 and several other unrelated selenoprotein sequences (Reeves & Hoffmann 2009). These selenoproteins have been found to be important when it comes to mitigating oxidative stress, slow down tumor-cell growth, producing thyroid hormone and providing sperm motility and viability among other things (Rayman 2012).

In prokaryotes, selenoproteins similar to the mammalian ones are found, e.g. formate dehydrogenase, selenophosphate synthetase, glycine reductase and selenium-
dependent peroxiredoxin, in addition to a group of proteins where Se is bound to molybdenum, nickel or tungsten (Hrdina et al. 2009). Not all prokaryotes have selenoproteins, but they are found in 20% of known bacteria (Kryukov & Gladyshev 2004).

1.3 Cecum and bacterial composition
Cecum is a part of the intestinal system and is in mice unlike in human fully functional. Here the host gets help from the microbiota to digest food and get access to different minerals and metabolites (e.g. short chain fatty acids). Cecum is a part of the large intestine and is located between the ileum and colon (Snipes 1981). Gastrointestinal microbiota is present in abundance up to $10^{11}$ in the host (Xu & Gordon 2003). Microbiota community in mice intestine is a stable after 3 weeks, and is a result of factors like feeding, environment and heritage.

1.4 Metagenomics and Qiime
High throughput sequencing demands suitable software for further metagenomics analyzes of the output. Quantitative Insights Into Microbial Ecology (Qiime) is a pipeline that is used to compare and analyze the microbial communities using high-throughput amplicon sequencing data. It deals with raw data output from sequencer and processes it further to demultiplex and denoise the reads, pick operational taxonomic units (OTU) and assign taxanomies. It also construct phylogenetic trees from OTUs and do downstream statistical analysis (e.g. alpha diversity analysis, beta diversity analysis), generating plots and graphs.

1.5 Aim
Microbiota composition affects the Se uptake in mice intestine, and it also affects the selenoprotein expression in the host. This indicates a competition between the host and microbiota for the Se (Hrdina et al. 2009). Microbiota diversity is increasing with increasing Se level in diet (Kasaikina et al. 2011). In this study, microbiota compositions in cecum of mice fed with different diets were examined. Cecum samples of 32 mice,
16 of which were fed with low selenium diet and 16 fed a normal selenium diet, were used.

2. Materials and methods

2.1 Feed and living conditions
Sample intestines received for the experiment were from mice that were bred and fed at Norwegian Institute of Public Health, Oslo. Mice had been kept together in cages of two, one wild type individual and one knock out individual for a cell repair gene. Some cages had contained siblings (Table A1, Appendix). Low Se diet, containing 0.0198 mg Se/kg diet had been given to mice in 8 of the cages, while mice in the remaining 8 cages had been given the normal Se diet, containing 0.234 mg Se/kg diet. Mice was killed at age 11-20 weeks.

2.2 Cell dissociation
Cecum was cut out of the rest of the intestine, cut open and rehydrated by adding 200 \( \mu l \) of sterile water. Cecum content was transferred to a 15 ml cell star tube on ice. Cecum was washed with 2.5 ml + 1 ml dissociation buffer, and the solution was added to the cell star tube. Tubes were centrifuged at 4 °C at 1500 rpm for 5 minutes, and the supernatant transferred to a new cell star tube. This step was done twice. Bacterial cell pellet was collected by centrifuging at 4 °C at 10 000 rpm for 10 minutes, and discarding the supernatant. Pellet was resuspended in 1ml cell wash buffer, centrifuged at 1170 rpm for 30 seconds, and the supernatant transferred to 2 ml fresh tube. Cell pellet was collected by centrifuging at 14 000 rpm for 5 minutes, and the supernatant discarded.

2.3 DNA extraction
Bacterial cell pellet was resuspended in 1 ml RBB+C buffer. For each ml solution in sample, 30 \( \mu l \) lysozyme (40 mg/ml) and 25 \( \mu l \) mutanolysin (5000 U/ml) was added and the tubes incubated at 37 °C for 30 minutes. 20 % SDS was added to reach 4 % and the
tubes incubated at 70 °C for 20 minutes, while mixing them every 5 minutes. For each sample the solution was split into two 2 ml tubes, and 5 M NaCl was added to reach 0.7 M. Preheated (55 °C) CTAB buffer was added, 1/10 of the solution volume, and incubated at 70 °C for 10 minutes. In fume hood one volume chloroform was added, mixed with the sample by inversion, and the tube centrifuged at 14 000 rpm for 10 minutes. Upper phase of the solution was transferred to new 2 ml tube, the rest discarded. CTAB and chloroform steps were repeated until the upper phase became clear. One volume phenol:chloroform:isoamylalcohol (25:24:1) was added, mixed with sample and centrifuged at 14 000 rpm for 10 minutes. Upper phase was transferred to new 2 ml tube, the rest discarded. The step was repeated, but upper phase transferred to 1.5 ml eppendorf tube. One volume cold isopropanol was added and mixed with sample by several thoroughly inversions of tube. DNA pellet was collected by centrifuging at 14 000 rpm for 20 minutes. All isopropanol was discarded and pellet washed by adding 500 µl 70 % ethanol and centrifuging at 14 000 rpm for 10 minutes. All ethanol was removed and the tube left open for drying for 30 minutes. One pellet from each sample was resuspended in 50 µl MQ-water, and solution transferred to second sample tube. DNA concentration and quality was measured at nanodrop. Samples were kept at -20 °C. DNA quality was additionally checked by running samples on 0.7 % agarose gel. 4 µl of sample was dyed with 1 µl of Gel Red, loaded with 1 µl loading buffer (6X) and run on gel at 70 V for 30 minutes. Sample bands were visualized by UV light. DNA samples remaining in sample tube were cleaned up by adding 1 µl RNase (10 µl/ml) to each tube and incubating at 37 °C for 10 minutes. This step was done twice.

2.4 Amplicon preparation

2.4.1 16S Amplicon PCR and clean up
Samples were quantified with Qubit Broad Range, diluted to reach 5-10 ng/µl and quantified with Qubit High Spesificity. 2.5 µl of each sample was transferred to a 96-
well 0.2 ml PCR plate, 5 µl Amplicon PCR Forward Primer (1 µM), 5 µl Amplicon PCR Reverse Primer (1 µM) and 12.5 µl iProof buffer was added to every sample. Plate was sealed with microseal film and 16S Amplicon PCR preformed with following program:

- 98 °C for 3 minutes
- 25 cycles of
  - 98 °C for 30 second
  - 55 °C for 30 seconds
  - 72 °C for 30 seconds
- 72 °C for 5 minutes
- 4 °C until PCR clean up

PCR plate was centrifuged at 1000 x g at 20 °C for 1 minute, and seal removed. Cold AMPure XP beads were made evenly dispersed by vortexing for 30 seconds, and 1 ml of beads put in eppendorf tube to reach room temperature. 20 µl of AMPure XP beads were added to each sample and mixed by pipetting up and down 10 times. Samples were incubated for 5 minutes in room temperature before PCR plate was put on magnetic stand for a few minutes until the supernatant became clear. With PCR plate still on magnetic stand supernatant was discarded and samples washed with 200 µl freshly prepared 80% ethanol that was added, left to incubate for 30 seconds and discarded. Washing step was done twice. Beads were left to air dry for 10 minutes. PCR plate was removed from stand, 52.5 µl 10 mM Tris pH 8.5 was added to each sample and mixed by pipetting up and down 10 times. Samples were incubated in room temperature for 2 minutes and placed on magnetic stand for a few minutes until the supernatant became clear. With PCR plate still on magnetic stand 50 µl of supernatant was transferred to new 96-well PCR plate. PCR product quality was examined by running samples on gel with same procedure as for genomic DNA (2.3), but with 100 bp ladder.

2.4.2 Index PCR and clean up
5 µl of the cleaned 16S Amplicon PCR product of each sample was transferred to a new 96-well PCR plate and the plate arranged in TruSeq index Plate Fixture together with
Nextera XT Index 1 primers horizontally and Nextera XT Index 2 primers vertically in the rack (Figure 2-1). 5 µl each of Index 1 and Index 2 primers according to the Index Primer Sheet (Table A, Appendix), 10 µl MQ-water and 25 µl iProof buffer was added to each sample and mixed by pipetting up and down 10 times. Plate was covered with microseal and centrifuged at 1000 x g at 20 °C for 1 minute. Index PCR was preformed with following program:

- 98 °C for 3 minutes
- 8 cycles of
  - 98 °C for 30 seconds
  - 55 °C for 30 seconds
  - 72 °C for 30 seconds
- 72 °C for 5 minutes
- 4 °C until PCR clean up

![Figure 2-1 TruSeq Index Plate Fixture](Illumina MiSeq 16S Metagenomic Sequencing Library Preparation Protocol).

A: Nextera Index 2 Primers, B: Nextera Index 1 Primers, C: PCR Sample Plate.

PCR plate was centrifuged at 280 x g at 20 °C for 1 minute and seal removed. Cold AMPure XP beads we made evenly dispersed by vortexing for 30 seconds, and 2 ml of beads put in eppendorf to reach room temperature. 56 µl AMPure XP beads was added to each sample and mixed by pipetting up and down 10 times. Samples was
incubated, supernatant discarded, beads washed with ethanol and air dried as in PCR Clean Up 1(2.4.1). PCR plate was removed from magnetic stand, 27.5 µl 10 mM Tris pH 8.5 added to each sample and mixed by pipetting up and down 10 times. Samples were incubated in room temperature for 2 minutes and placed on magnetic stand for a few minutes until the supernatant became clear. With PCR plate still on magnetic stand 25 µl of supernatant was transferred to new 96-well PCR plate. PCR product quality was examined by running samples on gel with same procedure as after PCR Clean Up 1(2.4.1).

2.4.3 Library pooling
DNA concentration in ng/µl was measured with Qubit High Specificity and DNA concentration in nM was calculated using formula (1) in Illumina MiSeq 16S Metagenomic Sequencing Library Preparation Protocol where 630 bp is the average library size.

\[
\text{Concentration in nM} = \frac{\text{concentration in ng/µl}}{660 \times \frac{9}{\text{mol}}} \times 10^6
\]

Samples were diluted with MQ-water to reach a concentration of 4 nM each and 5 µl from every sample were pooled together in 1.5 ml eppendorf tube and mixed by pipetting up and down.

2.4.4 Library denaturing and MiSeq loading
Library denaturing and MiSeq sample loading was performed using MiSeq v3 reagent kit. 5 µl from pooled library was transferred to 1.5 ml eppendorf tube and 5 µl freshly diluted 0.2 N NaOH was added. In another 1.5 ml eppendorf tube control 4 nM PhiX library was prepared by adding 2 ul 10 nM PhiX library together with 3 µl Resuspension Buffer. Further, 5 µl freshly diluted 0.2 N NaOH was added to the tube. Both the
pooled library tube and the PhiX library tube were briefly vortexed and incubated for 5 minutes at room temperature. 990 µl of cold hybridization buffer, HT1, was added to each tube to reach concentration 20 pM for both libraries. In this sequencing, 8pM loading concentration was used, to get this concentration 240 µl of each library was transferred to new tubes and 360 µl cold HT1 was added to both tubes. 30 µl 8 pM PhiX library was transferred to new 1.5 eppendorf tube and 570 µl 8 pM amplicon library was added. Immediately before loading onto MiSeq v3 reagent cartridge tube was incubated at 96 °C for 2 minutes, mixed by inverting 2 times and placed in ice-water bath for 5 minutes. 16S amplicon sequencing was performed by Illumina MiSeq Sequencer.

2.5  Statistics
Illumina MiSeq output in fastq format was processed by Qiime online software. Qiime was further used for several statistical analyses; alpha diversity was examined by drawing rarefaction curves; Microbial community compositions of samples were summarized by plotting bar graphs of taxonomies from phylum to genus level; Beta diversity was examined by running Principal Coordinate Analyses (PCoAs). Tests were applied with concern to the Se diet parameter, but also cage, siblings, genotype, age and weight. In addition, OTU table obtained from Qiime was modified in format to be used in Calypso v3 for ANOVA test from phylum to genus level.

3. Results

3.1  Alpha diversity
Two of the samples, 2 and 38, had reads that ended around 100 000 sequences per sample in the rarefaction curve (Figure 3-1), while the rest of the samples had a rarefaction curve that ended around 200 000 sequences. Most of the samples had enough observed species to be used for further analyzes. Comparing the curves at a
given number of sequences showed some variation in observed species between samples, but this was not different between the different diets.

**Figure 3-1 Rarefaction analyses of cecum samples.** The curves show observed species as function of sequences per sample.

Further looking at the Taxa Summary for all samples from phylum to genus level (genus, Figure 3-2), the bar charts showed the bacterial composition in each sample. In general the same taxa were predominant in most samples at every level. At phylum level the predominant groups were Firmicutes and Bacteriodetes, while at class level Clostridia and Bacteriodia were most represented. Further, at order level, the Clostridales and Bacteriodales were the predominant groups, and at family level it was S24-7, Lachnospiraceae, Ruminicoccaceae and unclassified families from order Clostridales that were predominant. Finally, at genera level the far more predominant
Figure 3-2 Summary of taxonomy for cecum samples. a) Bar chart of all samples at genus level. b) List of bacteria in bar chart a).
groups were the same as at family level, without any specificity to genera. Individual samples showed variation compared to most of the other samples in the bar chart.

3.2 Beta diversity
Samples compared according to diet did not show clear grouping in the PCoA plot at genus level (Figure 3-3). When comparing according to the other parameters in the experiment it showed that most of the samples from the mice that lived in same cage grouped together in the PCoA plot, having more similar microbiota composition compared to others, but there was not a clear grouping for genotype or weight factors (Figure 3-4, 3-5, 3-6). Mice living in same cage were of same age, so they grouped in the same way as seen for cage. Sibling mice in a cage did not group more together than non sibling mice in a cage.

![Figure 3-3 Principle Coordinate Analyses, diet plot. Blue circle: Normal Se diet sample. Red square: Low Se diet sample.](image-url)
Figure 3-5 Principle Coordinate Analyses, genotype plot. Orange triangle: Normal Se, wild type mice. Green triangle: Normal Se, knock out mice. Red square: Low Se, wild type mice. Blue circle: Low Se, knock out mice.
3.3 Significant differences in community
Taxa summaries grouped in order of diet did not show clear differences between the groups in any level. Yet ANOVA showed significant difference for two genera, *Akkermansia* and *Oscillospira* (Figure 3-7). The abundance of *Akkermansia* was increased with the low Se diet, whereas the abundance of *Oscillospira* was decreased. For the other levels, ANOVA showed significant difference for Verrucomicroba, Verrucomicrobiae, Verrucobicrobiales and Verrucomicrobiaceae for phylum, class, order and family, respectively. The abundance of these increased in the low Se diet.
There were more genera that changed significantly in abundance when grouping the samples according to cage (Figure 3-8). Akkermansia was affected in this grouping as well, in addition to Desulfovibrio, Allobaculum and Dorea. A significant difference was also seen in family Lachnospiraces, orders Clostridiales and Bacteriodales, phylum Firmicutes and unclassified bacteria, neither of these with classified genera.
4. Discussion
Se level in diet affects the microbiota composition, increasing the diversity with increasing levels of Se (Kasaikina et al. 2011). Hence the expectations to see difference in the microbiota composition with low and normal Se diets in this thesis.

Principal coordinate analyses did not show a general grouping of samples according to different diets (Figure 3-3), and this factor cannot be said to affect the general composition of microbiota. Still, the ANOVA test showed significant difference for the two genera Akkermansia and Oscillospira. Why these two show significant difference, but no other genera does can have several explanations. One could be that the microbiota wins the competition against the host about the Se, and at the Se level examined in this experiment the genera Akkermansia and Oscillospira are the only ones that are sensitive to changes. The type of Se in the diets may also affect the results. Se in experimental diets was mainly SeMet (Appendix), and bacteria might easier absorb and incorporate SeCys or other forms of Se.
Another important point from the results is that they show large individual variety of the bacterial composition in the taxonomical summaries (Figure 3-2a). Every individual has an initial microbiota composition based on several factors. This is quite stable, and will at some extent resist outer strain and changes (Rasmussen et al. 2009). Having this in mind a sample set of 32 might not be large enough to reveal any differences between the groups.

When looking at the other factors in the experiment, neither genotype, nor weight or pair of siblings seemed to effect the bacterial composition, but the cage factor did. Figure 3-4 showed that mice living in the same cage mostly grouped together in the plot, indicating similarity in bacteria community. Mice that are living in the same cage will eat each other’s feces, and this might affect their microbiota composition to becoming more similar.

Additionally, even if the rarefaction curve gave enough species output to represent the samples in a good way, the light steepness of the curve indicates that there could be more species to investigate in the samples. The examined diet may have affected low abundance species that were not seen here.

5. Conclusions
Mice fed with low Se diet and normal Se diet showed significantly differences in abundance for the two genera, Akkermansia and Oscillospira. The rest of the microbiota community composition seemed to be unchanged.
References


Appendix

Mice diet

70% wheat
0.2% DL-Methionine
13.5% Torula Yeast
5% corn oil, 3.5% mineral mix
1.1% CaCO3, 1% Vitamin Mix
5.7% Sucrose with

0.234 mg Se/kg diet for sufficient Se diet and
0.0198 mg Se/kg for insufficient diet

Selenium content is mainly SeMet in the wheat, measured med ICP-MS at NMBU
## Table A1 Mice Sample Sheet

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<th>Mice name</th>
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<th>Genotype</th>
<th>Diet</th>
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</table>
0.7 % Agarose gel
2.8g ultra pure agarose in 400ml TAE-buffer
Microwave 10-15 minutes to dissolve
Keep on 55 °C water bath

Buffers

1M Tris(crystallized free base)
Tris(hydroxymethyl) aminomethane
FW = 121.4g/mol
6.057g dissolved in 50 ml MQ-water.
pH adjusted to 8 by 37 % HCl
Autoclaved

0.5M EDTA
Diaminoethane tetraacetic acid
FW = 372.2g/mol
9.3g in 50ml MQ-water
pH adjusted to 8.0 using NaOH pellets under vigorous stirring (EDTA not dissolved until pH 8.0)
Autoclaved

5M NaCl solution
FW = 58.44g/mol
29.22g dissolved in 100 ml MQ-water
Autoclaved

20% SDS
Sterilized by filtering

Dissociation buffer
1.5 ml methanol
150 µl Tween 80
1.5 ml 2-methyl-2-propanol
MQ-water added to 150 ml
pH adjusted to 2 with 37 % HCl
Sterilized by filtering

Cell wash buffer
1 ml 1M TrisHCl
20 ml 5M NaCl
MQ-water added to 100 ml

RBB+C lysis buffer
10 ml 5M NaCl
5 ml 1M TrisHCl
10 ml 0.5M EDTA
MQ-water added to 100 ml

CTAB buffer (10 % w/v CTAB, 0.7M NaCl)
3.5 ml 5M NaCl
2.5g CTAB
MQ-water added to 25 ml
Heated to 65 °C to dissolve CTAB
Sterilized by filtering