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| Chart, scatter chart  Description automatically generated **Fig. 2: Azo-casein digestion.** *Quantification of freed amino acids over time and after 70h for different casein concentrations.* |

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| **A picture containing diagram  Description automatically generated Fig. 1: Quantitative assay of H2S production** |

**Bio-X Seed Grant: Dynamics of hydrogen sulfide production by gut bacteria**Jonas Cremer and Alfred Spormann. Progress report, 25th July 2022.

**Scientific progress**Realizing that utilization of sulfur containing amino acids is likely the biggest source of bacterial H2S production, we focused over the last year particularly on the characterization of bacterial peptide digestion and cysteine utilization. Our experimental results revealed a strong variation among bacterial species. We thus also incorporated a bioinformatics approach to better understand this variation and predict how H2S production depends on the composition of the gut microbiota. The most important findings are summarized in the following.   
**Experimentally assessing H2S production from cysteine, methionine, and peptide digestion:** To qualitatively assess the capability of different gut strain to produce, we ran an improved colorimetric assay (see report from previous year) which provides a visual readout of black precipitate that forms as H2S binds to ferric ammonium citrate. We probed a selection of gut strains with cysteine, methionine, or casein provided as sole sulfur sources. The results show that (i) cysteine digestion is common but not universal among all gut strains; (ii) utilization of methionine, the only other sulfur-containing amino acid besides cysteine, does not lead to H2S production; (iii) only a fraction of the tested strains is capable of digesting casein (Fig. 1). We are currently running the assay to probe more strains and sulfur-containing proteins sources. We further observed that the accumulation of a black precipitate varied strongly among strains for protein digestion, between a few hours and a few days. We thus probed available assays to quantify the rate of protein digestion and determined that a calorimetric assay based on the release of azo-proteins provides the highest accuracy and most reliable digestion rates. Digestion activity of *B. fragilis* is shown in **Fig. 2** for digestion of azo-casein. We are currently running this assay on our collection of gut strains and will determine protein digestion rates for a range of different protein sources, including beef extract, albumin, and gelatin.  **Bioinformatics to better understand variation in cysteine utilization:** The strong variation in protein digestion is an important result that also highlights the challenges in determining the capability of gut microbiota, which contains hundreds of strains in varying abundances, to release H2S. We thus incorporated a thorough bioinformatics analysis into our investigation. The goal is to develop a pipeline to probe available metagenomics data for the abundance of genes and pathways involved in protein digestion and cysteine utilization. To develop this pipeline, we first analyzed the genomes of single strains, including those we analyzed experimentally. To probe for the genes involved in cysteine and protein digestion activities we used HMMER, a position-dependent and highly sensitive sequence homology algorithm. We started with the investigation of cysteine utilization and the metabolic pathways involved. **Fig. 3** shows an example of the homology quantification. We are currently probing the cysteine digestion capabilities of more strains to evaluate and determine the predictive powder of cutoff filters for the HMMER results. After this, we will run the analysis on metagenomics data and estimate the cysteine digestion capacities of different fecal samples. An undergraduate student is currently testing a similar approach to study extracellular proteases and the capability for protein digestion.

**The diversity of sulfate-reducing microbes within the human gut:** Sulfate and its reduction by sulfate-reducing microbes (SRM) is another potential source of H2S within the gut. To analyze the diversity of SRM we used HMMER to mine partially assembled human gut genomes for *dsrAB*, an SRM-specific gene encoding for dissimilatory sulfite reductase. Of the 4,228 subjects we analyzed, we identified *dsrAB* sequences in 31% of the subjects. Phylogenetic analysis of the *dsrAB* sequences revealed they were primarily from *Desulfovibrionaceae* and the Firmicutes group sensu lato. These findings together with a typically very low availability of sulfate in the large intestine support the idea that SRM do not majorly contribute to the overall H2S production of the microbiota. However, our analysis revealed important insights on the previously unacknowledged diversity of the SRM within the gut. We are currently finishing a manuscript on the OD.

**Request for no-cost extension and outlook**Due to the pandemic the start of the project got delayed. However, we are currently running the introduced assays and generate the data needed to finish our manuscripts and a major grant proposal. Specifically, we ask for an extension of nine months to (i) complete a first manuscript showing the strong variation of cysteine and peptide digestion capabilities of different gut bacteria, (ii) and apply for an NIH R01 grant to reveal how H2S production depends on diet and the composition of the microbiota. The extension will allow us to obtain the data critical to accomplish a highly competitive proposal. For the latter, we will specifically collect data to show the quantitative differences in peptide digestion among strains and proof our ability to quantify the peptide digestion capabilities of fecal samples.

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| **Chart, bar chart  Description automatically generated Fig. 3: Bioinformatics assay to probe for casein utilization.** Pipeline (A) and homology score distribution of 100 strains (B). ADD PIPELINE |

**Manuscripts and grant proposals (currently in preparation and submitted)**A. Salari, A. Spormann, J. Cremer. The dynamics of bacterial peptide digestion along the human large intestine.   
A. Salari, J. Cremer. The appendix promotes the stable composition of the human gut microbiota.  
R. Christensen, A. Mueller, J. Cremer, A. Spormann, J. Grembi, The human gut harbors a diverse selection of sulfate-reducing microbes.   
J. Cremer, NIH RO1. Original submission August 2021. Resubmission planned for February 2023.

**Presentations related to this work (past and upcoming)**R. Christensen, Exploring diverse metabolic characteristics of gut microbiota using sequence analysis in 3 different ways, Bio-X Stanford, 08/202   
R. Sharma, Quantifying the H2S release of different gut strains, Bio-X Stanford, 08/2022   
J. Cremer, Growth and impact of the human gut microbiota. MPI Plön, 09/2022   
J. Cremer, Grow with the flow – Bacterial Biomass Accumulation along the human intestine. Talks in English, Bio-X , Stanford, 02/2021   
J. Cremer, The appendix, an organ to control bacterial growth dynamics in the human large intestine? Biology Department, Stanford, 12/2020

**Individuals supported**   
Dr. Richa Sharma, a postdoctoral fellow, started Autumn last year. She developed and runs the experimental assays.   
Rebecca Christensen is a technician and performs the bioinformatics analysis of peptide digestion and cysteine utilization.   
Dr. Ali Salari was temporarily supported by the Bio-X and building on his mathematical strength modeled the diet dependent utilization of protein disgestion (see previous report). Unfortunately, due to visa issues, Dr. Salari could not join Stanford. But we are still collaboration to finish the manuscripts. His analysis is also an important part of the R01 grant proposal we are writing.