A peptide database from human feces to foster the discovery of novel bioactive peptides

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Abstract

Fecal microbiota transplantation (FMT) is known for its high efficacy in addressing gastrointestinal, metabolic, and neurological disorders, as well as various types of cancer. However, sterile stool filtrates (SSF) have also been proven effective in the treatment of a variety of diseases[1,2], posing a lower risk for bacterial infection than FMT treatment, particularly in immunocompromised patients[1]. SSF consists mainly of microbiota-derived polypeptides and gut metabolites, but the nature and identity of antimicrobial or immunomodulatory factors are largely unknown. We have previously shown that body fluid filtrates are a rich source of novel anticancer and antimicrobial peptides with diagnostic and therapeutic potential[4]. Therefore, in the present work, we focused on the generation and analysis of a peptide bank from human feces for the identification of novel antimicrobial, immunomodulatory, and anticancer peptides, either using *in silico* approaches or bioassay-guided isolation and characterization of specific peptide components. A liquid sample (2L) of human feces from healthy donors was filtered through a membrane with a cut-off of 30 kDa. The filtered sample was subjected to reversed-phase HPLC; 36 fractions were collected and stored at -80ºC until further use. A 0.1 mL aliquot of each fraction was used to form six small pools of adjacent fractions for sample preparation and mass spectrometry. All pools of fractions were processed for heme group removal by either cold acetone extraction or C18-SPE and then subjected to sequencing analysis by LC-MSMS and bioinformatics. Several hundred peptides were identified as bacterial or human protein fragments, as well as many *de novo*-sequenced peptides. An *in silico* approach was also used to predict antimicrobial peptides among them. We expect that our methodology, which includes the generation of the human feces peptide bank, sample processing, and bioinformatics analyses, will facilitate the discovery of novel human peptides of therapeutic interest.

[1]Ott SJ *et al.* Gastroenterology. 2017 Mar;152(4):799-811.
[2]Gedgaudas R *et al.* J Gastrointestin Liver Dis. 2023 Sep 28;32(3):332-338.
[3]Bosso M *et al.* Bioorg Med Chem. 2018 Jun 1;26(10):2719-2726.

General strategy

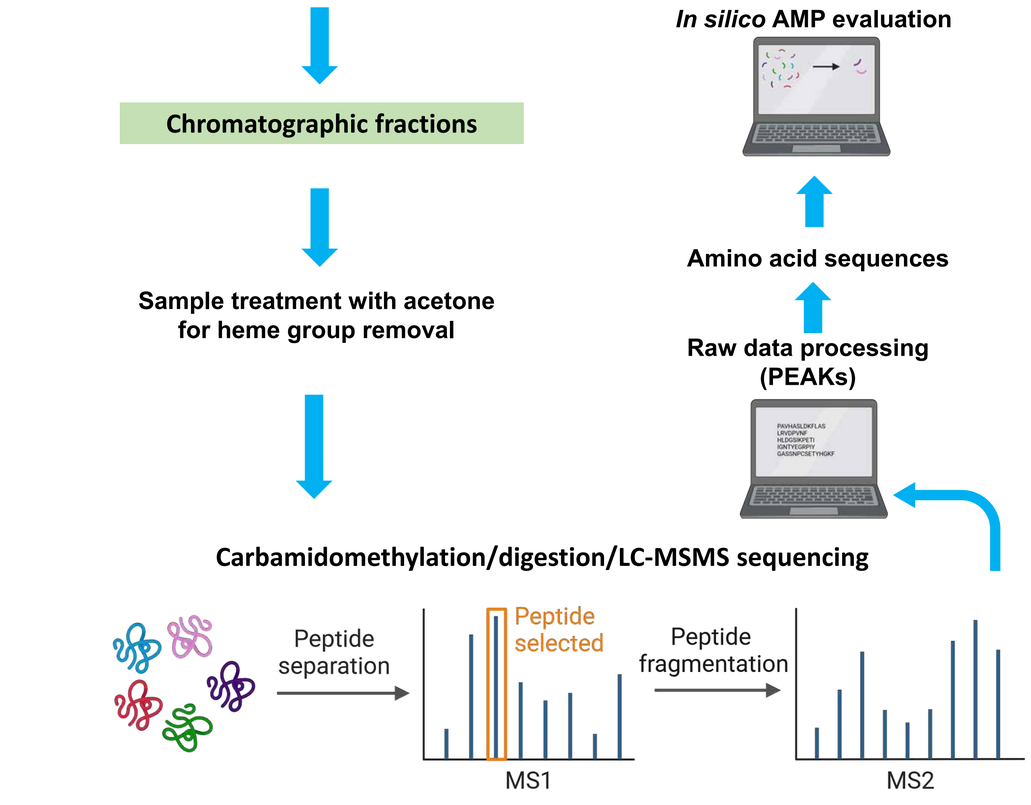

Figure 1. Chromatographic and MS workflow for the isolation and sequencing of human fecal peptides. A 2 L homogenate was subjected to ultrafiltration (cut-off 30 kDa). The ultrafiltered sample was applied to a preparative reversed-phase HPLC column (TFA/water/acetonitrile). The collected fractions were dried and then mixed with 10 volumes of acetone at -20ºC to remove the heme group. The samples were then treated with DTT (reduction) and iodoacetamide (carbamidomethylation) and analysed by nano-uPLC (reversed phase)/ESI-MS/MS Orbitrap Elite system. The raw data generated were processed by PEAKs X for peptide identification by searching human and bacterial protein databases or by *de novo* sequencing. Sequences obtained by database search and *de novo* sequencing (ALC>90%) were subjected to AMP prediction using CAMPR4, iAMPpred, AI4AMP, AMP scanner and DBAASP servers. All figures were created with Biorender.com

Results

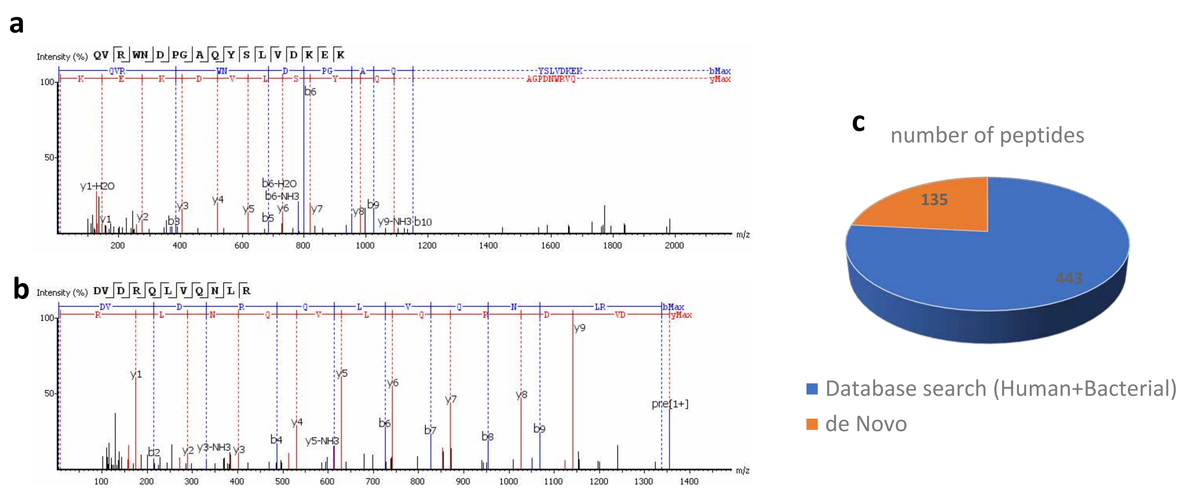

Figure 2. Mass spectrometry sequencing of fecal peptides. a) Fragmentation spectrum (MS2) of a peptide identified by database search as a fragment of cadherin-17 (Q12864|CAD17_HUMAN). b) Fragmentation spectrum (MS2) of a *de novo* peptide. Raw data were processed by PEAKs X, considering FDR=1%, Cys carbamidomethylation as fixed modification and Met oxidation as variable modification. The database search was performed against human proteins and gut (134) bacterial proteins from the UniProt database. c) Distribution of the number of peptides found in the present study: 23% *de novo* peptides vs. 77% database search peptides.

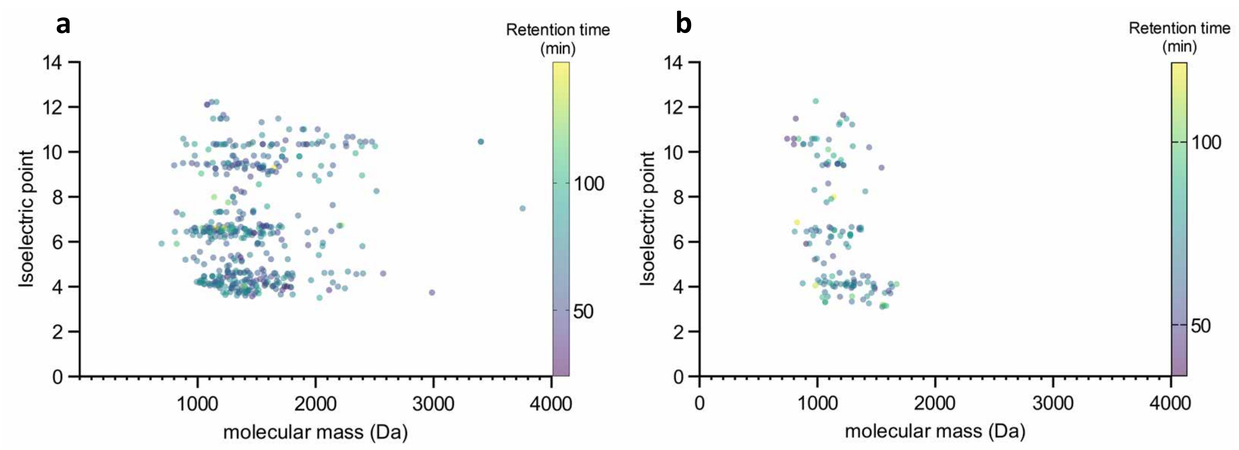

Figure 3. Isoelectric point/molecular mass/retention time (nanoLC-RP) distribution of human fecal peptides found in this study. a) Peptides found by database search. b) Peptides found by *de novo* sequencing. Graphs were generated using GraphPad Prism v10.2.3.

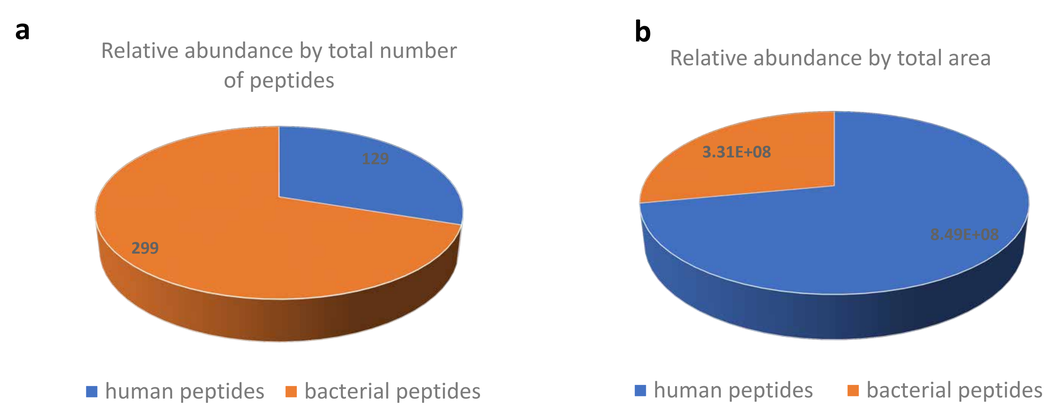

Figure 4. Distribution of total number of peptides and total area between peptides of human and bacterial origin. a) Relative abundance by total number: 70% bacterial peptides vs. 30% human peptides. b) Relative abundance by total area: 28% bacterial peptides vs. 72% human peptides.

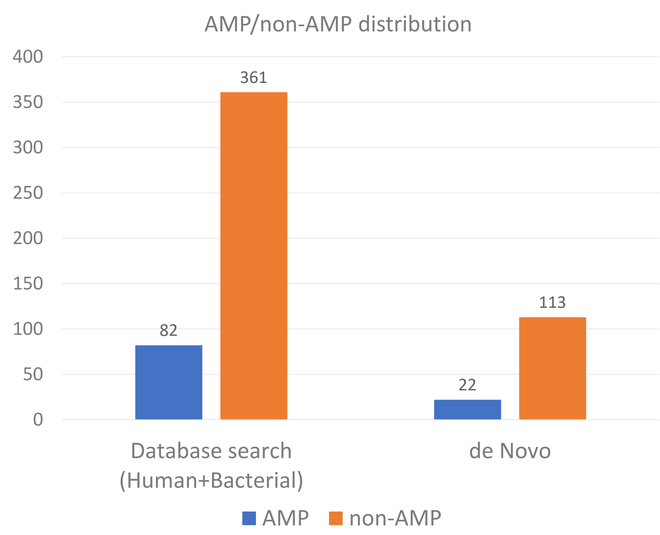

Figure 5. AMP prediction of fecal peptides using the AMP servers CAMPR4, iAMPpred, AI4AMP, AMP scanner, and DBAASP. A fasta file was created with all non-redundant sequences and subjected to *in-silico* evaluation. The results were similar for both groups of peptides, with AMP/non-AMP 18.5%/81.5% for database-found peptides and 19.4%/80.6% for *de Novo* peptides.

Conclusions

- Combining ultrafiltration, liquid chromatography, mass spectrometry and bioinformatics, we discovered a total of 578 peptides in human faeces (from database searches and de novo sequencing), most of which are fragments of gut bacterial proteins.
- These peptides are predominantly small, with molecular masses between 1-2 kDa and a wide range of isoelectric points and retention times in RPC (hydrophobicity).
- Approximately 20% of these peptides were predicted to be antimicrobial by the AMP servers.

What's next?

- Further *in silico* analysis of antimicrobial-predicted peptides and biological evaluation of the most interesting candidates on a variety of pathogens.

Acknowledgements

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