Global and temporal state of the human gut microbiome in health and disease

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# Abstract

The role of gut microbiota in humans is of great interest, and metagenomics provided the possibilities for extensively analysing bacterial diversity in health and disease. Here we explored the human gut microbiome samples across 19 countries, performing compositional, functional, and integrative analysis. To complement these data and analyse the stability of the microbiome, we followed 86 healthy Swedish individuals over one year, with four sampling times and extensive clinical phenotyping.

# Introduction

Metagenomic studies of the human microbiome enable the characterization of the microbial and functional diversity in health and disease[1]. Advances in metagenome assembly and various clustering methods enabled the generation of metagenome species[2]–[6]. Most of these studies focused on unveiling new uncultured genomes, while only a few focused on investigating the functional potentials and dynamic changes of the gut microbiome[7]–[9]. Understanding the functional and temporal behaviour of the microbiome may have great implications for the identification of its global signature in health and disease[8], [10], [11]. Additionally, short-term perturbations may trigger gut microbiota dysbiosis and changes at compositional and functional levels. Specifically, the negative selective microbe-microbe and host-microbe interactions, in the context of metabolism or antimicrobial machinery, could be the main mechanism underlying microbial dysbiosis[12]. Large-scale integration of microbiome functional changes and their associations with clinical data may provide novel information on temporal changes in the microbiome and host physiology[13].

Herein, we integrated publicly available data from many studies across different countries from healthy and diseased individuals. The analysis is presented in an open-access Human Gut Microbiome Atlas (www.microbiomeatlas.org), allowing researchers to explore for the first time an integrative analysis on composition, functional, richness, diseases, and region signatures for the gut microbiota across 19 geographical regions and 20 diseases.

# Human Gut Microbiome Atlas; Pan-metagenomics study on compositional and functional changes of the human gut microbiome

We performed a large-scale integrative analysis of 5,883 publicly available shotgun metagenomics stool samples, with at least 10 million high-quality sequencing reads from healthy and diseased cohorts from 19 different countries across five continents (Fig. 1a-b and Supplementary Table S1). We rarefied all metagenomic samples into 10 million reads per sample, which enables comparative analysis across different cohorts. We created the Human Gut Microbiome Atlas (HGMA) using quantitative analysis of shotgun metagenomics based on microbial genomes assembled using Metagenomic Species Pan-genomes (MSPs) (Fig. 1c). The MSP number was increased from 1,661 (previous release[5]) to 1,989 (average number of genes 1,894 ± 1,616) (Methods), and their taxonomy was updated. We generated gene counts and MSP abundances for all the samples using the 10.4 million gene catalogue[14]. We also characterized the functions and phenotype of the MSPs in 7 different categories (KO, PFAM, CAZyme, Mustard, JGI-GOLD phenotype, PATRIC virulence factor, and antiSMASH biosynthetic gene clusters) and identified co-conserved functional clusters across species (7,763 clusters) (Methods). This information was completed with 344 newly sequenced longitudinal samples from 86 Swedish individuals, described in detail in a subsequent section(Fig. 1d). All the data are freely available in the HGMA, without restrictions, in the public open access database (www.microbiomeatlas.org) that is part of the Human Protein Atlas program (<https://www.proteinatlas.org>). All MSPs and functions are highlighted together with the 5,224 samples across 19 countries with disease and healthy cohorts.

Using the 3,039 samples obtained from healthy individuals across 18 countries, including westernized and non-westernized regions, we uncovered the geographical distribution of the healthy gut microbiome. To this end, we applied the unsupervised clustering method, *monocle*, to MSP abundance profiles of the 3,039 samples (Methods)[15], [16]. We observed that there were two distinct ordinations of non-westernized and European samples of healthy subjects (Fig. 1e and Supplementary Fig. 1). Based on comparative analysis across different regions, we also identified 783 MSPs specifically enriched in certain countries (See Methods, Extended Fig 1a-d, Supplementary Fig. 2, and Supplementary Table S2). Functional annotation-based analysis across geographical clusters revealed enrichment of CAZymes for degrading host mucins and storage carbohydrates in westernized populations, where antimicrobial resistance (AMR) and virulence factors were also more prevalent (Fig. 1f). Comparison of functions of region-enriched MSPs in European countries and US/China/Japan revealed that fosfomycin and aminoglycoside resistance were the significant AMR, as deduced from the explained variances of ANOVA (Methods). Among the biosynthetic genes encoding the production of secondary metabolites, resorcinol, lanthipeptide, bacteriocin, and homoserine lactone were enriched in the European and US/China/Japan populations. These secondary metabolites, together with AMR, appeared to be the key feature in the westernized countries.

# Key enriched metagenome species share enrichment between countries and diseases

To distinguish diseased and healthy microbiomes from multiple cohorts, we performed a pan-metagenomics association study (Pan-MGAS) of multiple disease cohorts (23 diseases across 43 cohorts from 14 countries). We determined the enriched and depleted species within disease cohorts compared to healthy samples from the same country, showing an effect size greater than 0.3 (Fig. 1g and Supplementary Table S3).

In some cohorts a clear unbalance in the flora accompanies the disease, some cohorts show an intensive decrease of multiple species (i.e.  NSCLC:FRA,  RCC:FR, adenoma ITA figure a). At the same time, in some diseases several species increase their relative abundance, as we can see for most CRC cohorts. We also notice some cohorts with not very pronounced changes in the microbiome composition, for example, adenoma cohorts.

Some species were either enriched or depleted across multiple cohorts, regardless of geographical differences. For example, *Anaerostipes hadrus,* *Coprococcus comes,* and *Blautia obeum* areamong the most commonly depleted species,all of them found depleted in at least 6 different cohorts (figure c, \*add supplementary figure)*.* The firsttwo of them have been described as butyrate producers and dominant species isolated from the healthy human colon[17]–[19], and the third one has been associated with the gut microbiota recovery after cholera infection and with the normal maturation of the infant gut microbiota[20]

Between the species found enriched in at least 6 different cohorts we find *Fusobacterium nucleatum, Clostridium bolteae, clostridium clostridioforme, Clostriduium symbiosum,  Peptostreptococcus stomatis, Flavonifractor plautii,  Parvimonas micra*, among others (figure c). Several of them also have been isolated from oral samples (*Fusobacterium nucleatum*[21], *Peptostreptococcus stomatis*[22], *Parvimonas micra*[23]) and some of them have been identified infections including bacteremia (*Clostridium bolteae*[24], *Clostridium clostridioforme*[25], *Parvimonas micra*[26]).

Besides *Fusobacterium nucleatum* and *Clostridium symbiosum,* whichhas been previously linked with CRC[27]–[29], we also identify *Parvimonas micra* enriched several times in CRC and *Peptostreptococcus stomatis* enriched several times only in cancer (tumour) studies (Updated Supplementary table S4 (all diseases), supplementary figure).

Three different species belonging to the Streptococcus genus  (*Streptococcus anginosus*-msp\_1127, *Streptococcus parasanguinis*-msp\_0742, *Streptococcus vestibularis*-msp\_1453) and three more species from the *Veillonella* genus (*Veillonella atypica*-msp\_0884, *Veillonella dispar*-msp\_0148c, *Veillonella parvula*-msp\_0313)) were found enriched in two different liver disease cohorts, all of them commonly inhabit the oral cavity (Updated Supplementary table S4 (liver diseases)).

# Discussion

We have performed a comprehensive integrative analysis of global and temporal gut microbiomes, and we provide an open access HMGA portal (http://microbiomeatlas.org). Confirming previous observations[6], we have described the gut microbiome regional specificity, which needs to be considered before using the gut microbiome for the stratification of patients or for designing intervention studies. Beyond previous observations, our function-based analysis indicates that the western-enriched bacteria might dominate the gut microbial community with the production of antimicrobial peptides and homoserine lactone, which may inhibit their competitors.

Finally, the integration of metagenomics data from many studies spanning five continents provides valuable knowledge for researchers interested in the impact of the microbiome on individual health parameters. The open-access atlas will be updated routinely with the new publicly available gut metagenomics data, including the recently announced one million microbiome project aimed at providing comprehensive open-access metagenomics data from multiple research centres. In this manner, in-depth analysis of the impact of the gut microbiome on health and disease will be used to facilitate future studies to reveal the key role of the gut microbiome in human maintaining health.

JOSE:

## The causes for the observed dysbiosis and Dysbiosis effect in the disease’s progression

The physiological changes caused by the disease might partly explain why some diseases have a pronounced imbalance while some others don't, diseases affecting the bowel and CRC show a high species enrichment while some diseases affecting other body parts sometimes produce smaller imbalances. Some other factors might also be involved in the magnitude of the imbalance, for example the changes in diet[30], [31] or the use of drugs for treating the disease [32]–[34]).

## Effect of depleted/enriched species on disease development

The loss of the species actively contributing to keep a healthy environment could increase the host’s vulnerability to further health complications. For example, we observed some of the frequently depleted species have been described as butyrate producers. Butyrate has been associated with beneficial effects in the colon such as inhibition of inflammation, reinforcing the epithelial barrier and decreasing oxidative stress [35].

On the other hand, some of the enriched species might worsen the health status, by leading to new infections, potentiating the disease symptoms and even weakening the immune response. Some reports suggest *Fusobacterium nucleatum* promotes CRC development and metastasis[36], [37]. A previous report found *Flavonifractor plautii*, a species we found enriched in 6 cohorts, suppresses the Th2 immune responses in mice[38] which makes us speculate it could exert a similar effect in the humans.

The meta-pan genome association analysis we present shows a clear bias toward CRC studies, due to the increased availability of these studies, we expect new studies released in the future including more countries and diseases would help to update our analysis and balance this bias.

# Data availability

The datasets used in this study, including Swedish wellness cohort, are available in http://www.microbiomeatlas.org, with relevant project accession codes of raw data provided in Supplementary Table S1. Other data access and research questions related to the Swedish wellness cohort can be made available by contacting the corresponding author, Mathias Uhlen (mathias.uhlen@scilifelab.se).

# Code availability

The functional cluster analysis can be applied on gene counts and species abundances. The other pipeline scripts for analysis are also publicly shareable and available upon reasonable request from the corresponding authors.

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# Author contributions

S.S., S.D.E., and M.U. conceived the project. S.L. and S.S. led the design and analysis of the data. S.L. developed the temporal pipeline, analysis, and made the figures. L.E. and M.U. provided the wellness gut metagenomics samples. M.A., F.P., E.L., and S.D.E. generated the MSPs, performed quality check and taxonomy update. N.P. annotated the updated gut gene catalog. L.E.M and S.B.D performed the bioreactor fermentation experiment on healthy human stool samples. M.A. performed the GRiD analysis on bioreactor. G.B. applied metabolic models and performed simulations. M.A., V.M. and F.P. performed the analysis on the Italian and American cohorts for validation. N.B., C.P., S.V., D. R. and A.H. analyzed part of the data and prepared the materials for the HGMA. K.F. and F.J. developed the HGMA website. V.L. and B.H. annotated the gut catalog with new CAZymes. J.P. and D.L. annotated the secondary metabolites of the gene catalog. M.A. and G.B. contributed to testing the pipeline, statistical and functional analysis. S.S., S.L., M.U. and S.D.E wrote and drafted the manuscript. L.A.E, D.L.S, A.M., G.P. J.N. provided critical feedback on the data and manuscript. All authors read, edited and reviewed the manuscript.

# Competing interests

The authors declare no competing financial interests.

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# Methods

## Metagenomics species pan-genome (MSP) creation

1601 metagenomic samples used to build the Integrated Gene Catalog of the human gut microbiome (IGC2) were downloaded from the European Nucleotide Archive. Using the Meteor software suite[39], reads from each sample were mapped against the IGC2 catalog and a raw gene abundance table was generated. This table was submitted to MSPminer[5] that reconstituted 1,989 clusters of co-abundant genes named Metagenomic-Species Pangenomes (MSPs). Quality control of each MSP was manually performed by visualizing heatmaps representative of the normalized gene abundance profiles. In addition, MSPs completeness and contamination were assessed by searching for 40 universal single copy marker genes[40] and by checking taxonomic homogeneity.

MSP taxonomic annotation with phylogenetic tree.

MSPs taxonomic annotation was performed by aligning all core and accessory genes against *nt* and NCBI WGS (version of September 2018 restricted to the taxa Bacteria, Archaea, Fungi, Viruses and Blastocystis) using *blastn* (version 2.7.1, task = megablast, word\_size = 16)[41]. The 20 best hits for each gene were kept. A species-level assignment was given if more than 50% of the genes matched the RefSeq reference genome of a given species, with a mean identity ≥ 95% and mean gene length coverage ≥ 90%. The remaining MSPs were assigned to a higher taxonomic level (genus to superkingdom) if more than 50% of their genes had the same annotation.

40 universal phylogenetic markers genes were extracted from the MSPs with MOCAT[42]. MSPs with less than 5 markers were discarded. Then, the markers were separately aligned with MUSCLE[43]. The 40 alignments were merged and trimmed with trimAl[44]. Finally, the phylogenetic tree was computed with FastTreeMP[45] and visualized with iTOL[46].

Phylogenetic placement was further used to improve and correct taxonomic annotation.

## Functional annotation of the gut gene catalog and MSP

IGC2 catalog was annotated for the Antibiotic Resistant Determinants (ARD) described in Mustard database (v1.0) (http://www.mgps.eu/Mustard/)[47]. Protein sequences were aligned against 9,462 ARD sequences using *blastp* 2.7.1+ (option -evalue = 10-5). Best-hit alignments were filtered for identity ≥ 95% and bidirectional alignment coverage ≥ 90% (at query and subject level), giving a list of ARD candidates belonging to 30 families. Annotation of the carbohydrate-active enzymes (CAZymes) of the IGC2 catalog was performed by comparing the predicted protein sequences to those in the CAZy database and to Hidden Markov Models (HMMs) built from each CAZy family[48], following a procedure previously described for other metagenomics analysis[49]. Proteins of IGC2 catalog were also annotated to KEGG orthologous using Diamond (version 0.9.22.123)[50] against KEGG database (version 82). Best-hit alignments with e-value ≤ 10-5 and bit score ≥ 60 were considered. Proteins involved in virulence factors of PATRIC[51], [52] were matched against IGC2[14] by BLASTP (best identity > 50%, e-value < 10-10). Phenotype of MSP were manually checked and annotated based on JGI-GOLD phenotype (organism metadata)[53]. We identified biosynthetic genes of MSP with the use of standalone anti-SMASH program with minimal run option, focused on core detection modules (version 5)[54]. Loading antiSMASH into Amazon cloud computing (AWS) as docker image, we executed its mining process per MSP in a massive parallel setting.

## Quality control/normalization of gene counts and species abundance profiling

We filtered out human reads and then mapped metagenomic data (Supplementary Table S1) on IGC2 catalogue of human gut metagenome by METEOR[55] and based on the aligned reads, we estimated the abundance of each reference gene of the catalogue, normalizing multiple mapped reads by their numbers and summing up normalized counts for a given gene. Reducing the variability by sequencing depths, gene count values were downsized into 10 million reads per sample; and any samples less than 10 million mapped reads were excluded from our dataset. Normalized gene counts were used for the quantification of MSP abundance by R *momr* (*MetaOMineR*) package[56]. MSP abundances were estimated by the median abundance of the 25 marker genes representing the robust centroid of gene clusters of MSP. Sample metadata of all metagenomics data such as sequencing platform, geography, age, body-mass index, gender and the data source were provided under HGMA (http://microbiomeatlas.org).

## Tracing the diversifications of healthy metagenomic samples of different geography

After the quantification and per-million scaling of MSP abundance profiles, we employed trajectory analysis in R *monocle* ver.2 package to identify how samples were clustered[16]. In short, we selected the species profiles of all normal samples from different geographical origins and reduced the sample profiles into two dimensions by advanced nonlinear reconstruction algorithm, *DDRTree*. Based on the reduced two-dimensional components, we presented how samples were closely clustered as branches in scatter plots. Based on reduced profiles, we also calculated centroids and standard deviations of samples of given countries, except Finland population in toddlers (2 years).

## Identification of region-enriched species from geographically distinct cohorts

We selected healthy samples of 17 countries after excluding matched controls of two-year old subjects of Finland T1D cohort and redundant samples of subjects with multiple measurements (i.e. multiple visits). Among 17 countries, we estimated effect sizes for Wilcoxon signed rank (one-sided) tests[57] of different MSP abundances of two different countries. As one-sided tests were used, we set the lower bound of effect sizes as zero and the upper bound of effect sizes as one, avoiding negative and infinite values. Based on estimated effect sizes, we identified significantly enriched species having medium effect sizes of a specific country (effect size ≥ 0.3), compared to six or more countries, and defined those species as “region-enriched” species.

Next, we categories species if enriched in 1) European countries, 2) non-westernized countries, and 3) China/Japan/US and identified contrasted functions among those three clusters of countries by multivariate regressions as follows:

Yi = Ei βEi + Ni βNi + Ci βCi + ϵ

where *Yi* indicates a function with regards to species *i* like CAZyme, antibiotics resistance, anti-SMASH, and virulence factor (if a given function exists in species *i*, *Yi* =1, otherwise *Yi* = 0), *ϵ* indicates an intercept, *βEi*2*, βNi*2*, and βCi*2 are regression coefficients for *Ei*, *Ni*, and *Ci*, respectively and *Ei*, *Ni*, and *Ci* are categorial variables that indicate the region-enrichment of species *i*:

*Ei* = 1 if *i* ∊ species enriched in any of European countries, otherwise *Ei* = 0

*Ni* = 1 if *i* ∊ species enriched in any of non-westernized countries, otherwise *Ni* = 0

*Ci* = 1 if *i* ∊ species enriched in China, Japan, or US, otherwise, *Ci* = 0)

Functions significantly associated with enrichment of any of three geographical clusters were identified based on *F*-tests of regressions (p-value < 0.01; *βEi*, *βNi*, *βCi* > 0) and quaternary plots were shown based on squared regression coefficients (*βEi*2*, βNi*2*, βCi*2) normalized by their total sum.

## Gene richness and species associated with high and low gene richness

Gene counts (i.e. mapped read counts) of all dataset samples were downsized into 10 million reads by R *momr* (*MetaOMineR*) package. Based on detected genes from downsized gene count profiles, we identified gene richness of given samples. We first examined top-25% and bottom-25% gene richness of all HGMA samples and by Wilcoxon two-sided tests we compared species abundance between two groups of healthy samples: high gene richness (HGR) group for samples < first quartile of richness (Q1); and low gene richness group (LGR) for samples > third quartile of richness (Q3).

## Pan-metagenomics association studies (Pan-MGAS)

First, we selected of healthy and disease samples without interventions and redundant measurement (i.e. multiple visits) and performed comparative analyses of chosen samples (number of selected samples were shown in Supplementary Table S1). We estimated the effect sizes of Wilcoxon signed rank (one-sided) tests for MSP enrichment and depletion in diseases, compared to healthy controls of given country[57] and identified we identified significantly enriched or depleted species having medium effect sizes (effect size ≥ 0.3). Manhattan plots of pan-MGAS based on effect sizes were plotted with R *qqman* package[58].

## Unsupervised clustering of co-conserved functions of gut microbiota

We calculated Jaccard index among functional annotations to check how many species were sharing given a pair of functions together. We selected highly shared pairs of functions (Jaccard index >= 0.75) and merged into functional co-occurrence network using R *igraph* package[59]. Functional clusters within the network were identified by unsupervised community detection, short random work algorithm (*cluster\_walktrap* function)[60], [61] and identified singleton functions within the network as well. Among non-singleton functional clusters, we selected representative functional clusters if functions of given functional clusters were found more than three species, thereby excluding functional clusters sparsely annotated over MSPs. Associated MSPs to functional clusters were chosen if given MSP covered more than 75% functions of given functional cluster.

# Figure legends

**Fig. 1. Characterization of the global gut microbiome in health and disease.** Pan-metagenomics studies of health and disease. Corresponding datasets were publicly shared as a resource: human gut microbiome atlas (HGMA). **a,** geographical distribution of the datasets used in this study (the number of the samples is shown in parentheses). **b,** types of disease datasets of shotgun metagenomics used in this study. **c,** the workflow of the metagenomic species pan-genome (MSP) quantification together with functional characterization. In total, 5,224 shotgun metagenome samples, including 344 Swedish longitudinal samples, were aligned against the gene catalogue of the human gut microbiome and quantified at the level of MSP. All healthy samples (3,039) were used for the analysis of the global gut microbiome of healthy individuals, and all disease samples (2,185) were used for pan-disease analysis. For the functional characterization of human gut MSPs, we annotated respective genes with 19,540 features of microbial function/phenotype databases and identified 7,763 functional clusters better representing microbial functions. **d,** characterization of temporal changes of 86 Swedish healthy individuals (Swedish wellness cohort, S3WP) during a year (total 344 samples). **e,** *monocle* ordination of the gut microbiome in healthy samples. Individual samples from non-westernized countries, European countries, and US/China/Japan were coloured yellow, purple, and grey, respectively. **f,** contrasted functions among region-enriched species originated from three different geographical clusters, that is, non-westernized countries, European countries, and US/China/Japan. Based on functional annotations of CAZyme, antimicrobial resistance (AMR), secondary metabolism (antiSMASH), and virulence factors (PATRIC database), we checked the enrichment of functions in a given geographical cluster. Enrichment of species with respective functions was checked by ANOVA of multiple regressions, considering association of geographical clusters as dependent variables of regressions. **g,** pan-metagenomics association studies (Pan-MGAS) of 28 cohorts from 18 different diseases and 11 countries (n=2,185). We identified significantly enriched/depleted species of cohorts based on effect sizes (ESs) of Wilcoxon one-sided tests (ES ≥ 0.3). We found species enriched with diseases in different countries such as *A. histaminiformans* (NAFLD), and species depleted, such as *G. urolithinfaciens* (NAFLD). **h,** Jitter plots of frequency of the significantly enriched/depleted cohorts of all MSPs were calculated: total frequency of enriched/depleted cohorts (|number of enriched cohorts| + |number of depleted cohorts|) and subtracted frequency between enriched cohorts and depleted cohorts (|number of enriched cohorts| - |number of depleted cohorts|). Point colours changed from red (left) to blue (right) according to x-axis values. Common enriched/depleted species among cohorts were identified when total frequency ≥ 3 and absolute subtracted frequency ≥ 2.

**Fig. 2. Changes in inflow and outflow species populations linked to gene richness and host physiology. a,** modelling the temporal changes of species by Markov chain models (MCMs) estimating the state transition probabilities between absence and presence under detection limit (left top), inflow and outflow; dotted lines with a cross and gold arrows represent a failure and success to detect a species, respectively (left bottom). Inflow vs outflow score plot (right panel) identifies inflow species (IFS) (e.g. *A. butyriciproducens* and *B. obeum*), outflow species (OFS) (e.g. *V. parvula* and *H. effluvia*) or species that often change state between visits (*E. coli*, *S. salivarius*). **b,** we determined abundance changes between visits (*t* and *t+1*) and compared the total changes of IFS and OFS (ΔIFS and ΔOFS, respectively). In short, ΔIFS and ΔOFS were determined by differences of scaled total abundance between visits (i.e. Δ = Zt+1 - Zt) (See Methods). We observed negative correlation of abundance changes by visit between IFS species (ΔIFS) and OFS species populations (ΔOFS) (Spearman’s *ρ* = -0.334, p-value = 4.6×10-8). **c,** we compared the mean abundance of OFS species in visits (μOFS) and abundance changes between visits (∆OFS). The mean abundance of OFS species in visits was determined by the mean of scaled total abundance of OFS species in two sequential visits (i.e. μOFS = ½ × (Zt + Zt+1)). The higher the mean OFS species abundance (μOFS ≥ 2), the more its abundance changes between visits either increasing (∆OFS > 2) or decreasing (∆OFS < -2). **d,** Individuals enriched with OFS species at certain visits (μOFS ≥ 2) had significantly lower intra-individual similarity (Wilcoxon one-sided test *P* = 0.0034). **e,** we observed that individuals with an increase in IFS species abundance were significantly positively correlated with an increase in richness (Spearman’s *ρ* = 0.206, p-value = 9.0×10-4). We estimated physiological properties of IFS and OFS species by growth rate estimations by GRiD scores (**f-i**) and genome-scale metabolic modelling (j-k). We estimated GRiD scores of IFS and OFS species from (**f**) individuals highly enriched with IFS species and (**g**) individuals highly enriched with OFS species and observed higher GRiD scores of IFS species. In additional experiments of bioreactor fermentation of human faeces, we observed higher GRiD scores of IFS species after 24 hours, compared to original feces samples, whereas OFS species remained to be lower in GRiD scores at 24 hours. We evaluated our findings inflow/outflow scores estimated from Swedish cohort of four visits with those from (**l-m**) additional two more visits, (**n-o**) American HPFS cohort and (**p-q**) Italian DINAMIC cohort. We compared inflow scores (**l, n, p**) and outflow scores (**m, o, q**) between different datasets.

**Fig. 3. Inflow and outflow were associated with signatures of gene richness, and clinical parameters among healthy individuals**. a-b, comparison of inflow (a) and outflow (b) probability between high gene richness (HGR)- and low gene richness (LGR)-enriched MSPs; LGR-enriched species have a higher probability of outflow (Wilcoxon one-sided test *P* = 1.1×10-6). c, IFS/OFS species abundances were significantly associated with clinical parameters by linear mixed effect models (p-value < 0.05). Significant positive (+) and negative associations (-) are marked on a heatmap (size proportional to significance). Increase of IFS species abundance was associated with increase of muscle mass, whereas increase of OFS species was associated with increase of BNP, a heart failure marker.

**Fig. 4. Inflow and outflow were associated with disease signatures from pan-disease analysis, together with biodegradation of xenobiotics and host nutrient hijacking.**

**a-b,** we observed significant differences in inflow (**a**) and outflow (**b**) probability between common enriched and depleted MSPs in diseases (from Fig. 1h) (Wilcoxon one-sided tests p-values < 0.05). we observed that common depleted species were higher in inflow, whereas common enriched species were higher in outflow. **c-d,** radar plots showing the fraction of functional classes enriched in either (**c**) inflow or (**d**) outflow, tested by linear mixed effect models (adjusted p-value < 10-3). The inflows were enriched in core metabolism and outflow in accessory metabolism (e.g., BTEX contaminants). **e,** we identified functional clusters co-conserved across different species and distinguished functional clusters found in many species or few species (x-axis). Y-axis indicates the size of functional clusters. Here we identified two largest clusters enriched in pathobionts (CL-10, *patho-cluster*) and commensal bacteria (CL-12, *comm-cluster),* respectively. **f,** frequency of functional clusters associated with enriched/depleted MSPs in diseases: total frequency of enriched cohorts and depleted cohorts per functional cluster (|number of enriched cohorts| + |number of depleted cohorts|, y-axis) and subtracted frequency between enriched cohorts and depleted cohorts per functional cluster (|number of enriched cohorts| - |number of depleted cohorts|, x-axis). Functional clusters significantly associated with outflow species (hypergeometric tests *P* < 0.01) were mostly associated with depleted MSPs in diseases (points coloured purple). Points were plotted in a jittered setting to avoid overlaid points.

# Extended figure legends

**Extended Fig. 1. Regional differences in healthy gut microbiome composition and function. a,** fraction of region-enriched species. Among the 1,977 species identified from the datasets used in the present study, we identified 39.6% that were regionally enriched (783 species). We checked enrichment by comparing the abundance of a given MSP between two different countries with the effect size of Wilcoxon one-sided tests and identified them if the MSP abundance of a given country was higher than that of more than six countries, i.e., one-third of other countries among all comparisons (based on effect size >= 0.3). **b,** number of region-enriched species per country. We found that non-westernized countries had a higher number of region-enriched species, whereas non-European westernized countries such as Japan, the US, and China had a smaller number of region-enriched species. **c,** overlaps of region-enriched species among three different geographical clusters. Comparing three distinct geographical clusters, non-westernized countries, European countries, and the US/China/Japan, we found few overlaps of region-enriched species. However, regarding region-enriched species in the US/China/Japan, half of those species were shared with those in European countries. **d,** region-enriched species were clustered into two distinct clusters, westernized and non-westernized countries. Based on the scaled abundance of broadly region-enriched species (i.e., enriched in more than five countries), we performed hierarchical clustering. Here, we found two clear clusters of region-enriched species, which belong to either westernized countries or non-westernized countries. **e,** the number of total depleted/enriched species of all disease cohorts when different types of healthy samples were used as controls: healthy samples from the same country or same geographical cluster (e.g., European and China/Japan/US clusters). The total number of depleted species from healthy samples of the same country were much greater in number compared to those of the same geographical cluster (top), so depleted species can be missed many if controls were not taken from same country and same geographical cluster (bottom). **f,** disease network based on shared enriched/depleted species.More enriched than depleted species were shared among diseases. For the disease cohort from multiple countries, we averaged effect sizes to have representative effect sizes (assuming random-effects model rather than fixed-effects model; see Methods). Metabolic diseases are coloured red, and inflammatory/autoimmune diseases are coloured blue. **g,** the number of disease cohorts that can be compared with associated matched controls, country controls, or geographical cluster controls. Enriched/depleted species were identified using different types of controls. Depleted species varied more than the enriched ones with different types of controls. **h,** Venn diagrams of total depleted and enriched species detected from different types of controls. **i,** significance of depletion of region-enriched species of disease cohorts of given countries. We compared the effect sizes of country-enriched species by the Kolmogorov-Smirnov test (p-values < 0.01) and found that many disease cohorts were significantly depleted of country-enriched species (p-values on the y-axis).

**Extended Fig. 2. Temporal dynamics of individual gut microbiome composition. a,** the frequency of identified MSPs per individual. The median number of MSPs identified per individual for 86 individuals across quarterly visits had was 293. **b,** fraction of retained species between sequential visits. **c,** overlap of species identified by visits. Most species (1,159 MSPs) were shared among visits. **d,** gene richness of 86 individuals by visit. We examined the microbial diversity of 86 individuals by gene richness, counting the detected microbial genes of the reference catalogue per sample after downsizing mapped reads to 10 million reads. Based on *t*-tests, we found that there were no significant differences in gene richness among different visits of 86 individuals. **e,** species appearing or disappearing between visits. A median of 106.5 species appeared or disappeared per individual at the next visit. **f,** resilience of individual microbiomes observed in different cohorts. We checked how much species were retained by time based on Jaccard coefficients of shared species among different visits. First, we computed Jaccard coefficients for different individuals based on all healthy samples of our study (n=2,018) and found low Jaccard coefficients (≤0.3). In contrast, in healthy populations of Luxembourg (n=26) and Spain (n=47), we found high Jaccard coefficients for the same individuals with multiple visits (≥0.6). Similarly, high Jaccard coefficients were observed for the same individuals with multiple visits in the T2D and T1D disease cohorts. Except for antibiotics, Interventions such as metformin and immunotherapy, but not antibiotics, did not affect the similarity, as the Jaccard coefficients for the same individuals before/after treatment were still elevated. LU: Luxembourg, ES: Spain, DK: Denmark, FR: France. **g**, abundance changes of inflow species (IFS) and outflow species (OFS) between visits (∆IFS and ∆OFS, respectively) according to mean values of visits (μIFS and μOFS, respectively). Unlike OFS species, the abundance of IFS species rarely changed between visits (|∆IFS| < 2) according to the increase in mean values (μIFS ). **h,** intra-individual similarity of IFS-enriched individuals (right panel) and OFS-enriched individuals (left panel). We identified OFS-enriched individuals among 86 healthy individuals based on scaled total abundance of OFS species by Z-score (Zt and Zt+1) and its mean between visits (µOFS = ½ × (Zt + Zt+1) > 2). OFS-enriched individuals were less similar between visits than OFS-depleted individuals (t-test p-value < 0.01), whereas IFS-enriched individuals were similar between visits, similar to IFS-depleted individuals. **i,** correlations of changes in gene richness to OFS species, and IFS species (left, middle, and right, respectively). Changes in IFS species abundance (∆IFS) were significantly correlated with richness changes (p-value < 0.05), whereas changes in BMI and OFS species (∆BMI and ∆IFS, respectively) were not significant.

**Extended Fig. 3 Estimation of inflow, outflow probability at three depth levels of 15, 10 & 5 million reads.** We checked the probability of **a**, inflow, and **b**, outflow changes for different downsizing depths, 5M, 10M, and 15M. Inflow and outflow probability were highly correlated across different sequencing depths (Pearson’s correlation coefficients > 0.82, p-values < 10-15). **c,** in the scatter plots a and b, the undetected species at 5M reads and 10M reads downsizing were excluded. Venn diagram showed that there were 62 species missing at 5M reads downsizing compared to 10M reads, and 1 species missing at 15M reads compared to 10M reads.

**Extended Fig. 4. Estimation of species-retaining probability from Swedish longitudinal cohorts. a,** species-retaining probability estimated by Kaplan-Meier statistics. Based on the presence/absence events of a given species among the four visits, we estimated the retention probability from Kaplan-Meier estimators. As example, we show Kaplan-Meier plots of four species with different retaining probabilities. Higher outflow species, such as *S. parasanguinis* and *V. infantium*, had lower retaining probabilities, whereas lower outflow species, such as *B. vulgatus* and *P. copri*, had higher retaining probabilities. **b,** groups of species with different mean abundances (mean abundance bins) and corresponding species-retaining probabilities. We checked species-retaining probabilities according to mean abundance changes and found increasing trends, but with high variations. **c,** Cox regressions of MSP abundances to species-retention. No MSPs were significantly associated (all p-values > 0.05).

**Extended Fig. 5. Characteristics of populations with high and low gene richness. a,** Distribution of gene richness of all metagenomes sampled used in this study. We used the top 25% and bottom 25% over all samples as cut-offs to define high gene richness (HGR) and low gene richness (LGR) populations, respectively. **b,** significantly enriched/depleted species in populations with high gene richness (HGR) compared to those with low gene richness (LGR) (|Log fold change| > 2, Wilcoxon two-sided tests, adj. *P* < 10-3). For example, in the HGR population, *Faecalibacterium prausnitzii*, *Dorea longicatena*, *Blautia obeum*, and *Anaerobutyricum hallii* were enriched, whereas in the LGR population, *Ruminococcus gnavus*, *Flavonifractor plautii*, *Clostridium bolteae*, *Fusobacterium* spp., and *Clostridioides difficile* were enriched. **c,** Projected enrichment scores of HGR/LGR species (ZHGR – ZLGR) over all *monocle* ordinations in healthy populations (higher enrichment of HGR and LGR for red and blue, respectively). **d,** based on available clinical metadata of multiple cohorts, we associated clinical metadata with their HGR or LGR populations. We found that LGR populations had higher proportion of individuals displaying abnormal values of clinical variables such as CRP, ALT, TG, and HDL.

**Extended Fig. 6. Clinical and functional association with microbiome changes. a-b**, inflow/outflow score-associated functions. Based on univariate linear regressions, we found functions significantly enriched in MSPs with high inflow/outflow scores (adjusted p-value < 0.01) and showed some of enriched genera and functions as examples in the heatmap. By decreasing inflow/outflow scores (leftmost column), we showed the density of species of given taxonomy or functions within sliding windows of 100 MSPs along MSPs sorted with given inflow/outflow scores (right columns). We found *Dorea* and *Faecalibacterium* species were significantly associated with increased inflow scores (adjusted p-values 5.0×10-4 and 1.2×10-3, respectively), whereas *Faecalicatena* and *Clostridium* species significantly associated with increased outflow scores (adjusted p-values = 0.0069 for both genera). In addition, integrase (transposon) and MobC (plasmid mobilization relaxosome) were significantly associated with increased inflow scores (adjusted p-values 3.2×10-27 and 6.5×10-15), whereas facultative phenotype, microaerophilic phenotype, trigger factor (virulence) and ComGF (competence) associated with increased outflow scores (adjusted p-values 4.0×10-8, 3.3×10-3, 1.2×10-9, and 1.4×10-5, respectively). **c-d**, association of microbiota changes with clinical parameters. We associated microbiota changes with clinical parameter changes based on linear mixed effect models, considering the random effects of individuals. Based on the explained variance of MSP to clinical parameters, we found significant associations between them (explained variance > 10%) and showed their associations on the Chord diagram, presented with phyla **(c)** and genera **(d)** of respective MSPs. **e,** 10 clinical parameters significantly associated with MSPs by linear mixed effect models based on explained variance (> 10%; See Supplementary Table S10). **f-g,** *Bacteroides fragilis* (msp\_0033) was significantly associated with (**f**) CRP and (**g**) troponin T levels (explained variances, 62.5% and 36%,respectively).

**Extended Fig. 7. Analysis of functional clusters. a,** Overview of the identification of functional clusters. **b,** community network detected from the co-occurrence network of functional annotations. All functional clusters were shown as nodes and significant co-occurrences of functional clusters across species were shown as edges in the network. “Comm-cluster” and “patho-cluster” were coloured blue and red, respectively. **c,** Phyla associated with the functional clusters of known microbial functions. For example, we identified functional clusters enriched with enzymatic reactions producing butyrate and propionate (respective reactions of the cluster on left side) and reactions related to choline degradation, trimethylamine N-oxide (TMAO) production, acetogenesis (Wood-Ljungdahl pathway), and mucin degradation (sialidase). Phyla of MSPs associated with those clusters (hypergeometric test p-value < 10-3) are shown in the Chord diagram. **d,** number of functional clusters mapped with microbial function/phenotype databases and their singleton cluster fractions (singleton and non-singleton coloured green and red, respectively).

**Extended Fig. 8.** **a,** identification of functional clusters based on co-conserved genes across species. Unlike the manually curated module database, we identified functional clusters based on high co-conservation across species using the unsupervised clustering method. **b,** among different sources of microbial functional annotations (e.g. KEGG module and pathway), we found that co-conservation of genes across different species was substantially low (Jaccard index < 0.5).

# Supplementary Figures Legends

**Supplementary Fig. 1.** Preserved distinct geographical clusters through ordination analysis of *monocle*. We checked how clusters are changing after excluding (**a**) European countries, (**b**) US/Japan/China, and (**c**) Non-westernized countries. We still found that European samples were clustered together, and non-westernized countries clustered together, and US/Japan/China clustered together. In each subpanel (**a-c**), we showed monocle plots with clusters identified *monocle* (ordination plots) and fraction of samples per given clusters (bar plots).

**Supplementary Fig. 2.** Mapping rates of metagenomics reads of healthy samples to integrated gene catalogue. We showed the mapping rate per country and found majority of countries are above 70%; non-westernized countries tend to be lower in mapping rates (e.g. *Fiji*).

**Supplementary Fig. 3.** Essentiality analysis. The heatmap of metabolites leading to the decrease of growth rate or lethality in the IFS and OFS species showed that essentiality is significantly different between the group (fisher exact test, p-value = 0.05).

**Supplementary Fig. 4.** Constraint-based modelling of common IFS and OFS detected in three different cohorts, WELLNESS, DINAMIC, and HPFS cohorts. **a,** boxplot of growth rates of common IFS and OFS species, predicted in the four different diets **b,** boxplot of variance of growth rate changes for four different diets. To compare the variance among the MSPs the growth rates were normalized. **c,** the heatmap of metabolites leading to decrease of growth rate or lethality in the common IFS and OFS species by essentiality analysis showed that essentiality is significantly different between the group (fisher exact test, p-value = 0.05).

**Supplementary Fig. 5.** Examination of different enrichment functional terms between common enriched and depleted species (Chi-square tests p-values < 0.05, log odd ratio (LOD) > 3). We checked different enrichment of functional terms of IFS and OFS signatures, including vitamin synthesis, amino acid metabolism, nutrient transport, BTEX metabolism, phosphotransferase system (PTS), ABC transporters, drug efflux metabolism (KEGG), storage carbohydrate (CAZyme), mobile genetic elements (PFAM), virulence factors (PATRIC). Y-axis indicates -log 10 p-values of Chi-square tests and X-axis indicates log2 odd ratio. Among common enriched species, ABC transporters, PTS system were enriched, whereas among common depleted species, storage CAZyme, amino acids, vitamin, and mobile genetic elements were enriched.

## Supplementary Tables Legends

**Supplementary Table S1.** Description of Human Gut Microbiome Atlas (HGMA) datasets used in this study. We showed the overall statistics of HGMA datasets, including total number of samples, number of samples without intervention and multiple visits, number of matched healthy controls, sequencing platform, geography, reference, and raw data link.

**Supplementary Table S2.** List of region-enriched MSPs. Based on effect sizes (>0.3 in more than six countries of comparison), we identified total 782 regionally enriched MSPs among healthy samples of 17 countries.

**Supplementary Table S3.** Statistics of pan-metagenomics association study (pan-MGAS) results of 28 geographically distinct disease cohorts. We performed pan-MGAS analysis (shown in Fig. 1g) and identified enriched/depleted species in each cohort with effect sizes by each column (positive and negative values were assigned with effect sizes to indicate species enriched and depleted in given cohort, respectively). We identified total 441 MSPs significantly enriched/depleted species among cohorts. (effect sizes > 0.3).

**Supplementary Table S4.** Statistics of MSP common enriched/depleted in diseases. We examined the total and subtracted frequency of significantly enriched and depleted species in diseases (total frequency ≥ 3 and absolute subtracted frequency ≥ 2). Total 24 species were frequently enriched in diseases, whereas 12 species were frequently depleted in diseases. For more detailed understanding of those 36 species, we presented their IFS/OFS scores, log2 fold changes between HGR and LGR populations, and region-enrichment.

**Supplementary Table S5.** Summary of Markov Chain Model (MCM) and species-retaining probability. We showed estimated IFS and OFS probability with upper and lower bounds and standard errors from MCM analysis. We also presented prevalence and average abundance of given MSP of Swedish wellness cohort and species-retaining probability at 6 months by Kaplan-Meier analysis. IFS and OFS probability were estimated at 10 million reads and in parallel we also showed IFS and OFS probability estimated at 5 and 15 million reads. We also showed species present at 10 million reads but absent at 5 and 15 million reads. For better understanding, we also added columns describing common enriched/depleted species among 28 geographically distinct disease cohorts (defined in Fig. 1h), the number of significant cohorts identified enriched or depleted for common species, and microorganisms detected in bloodstream infection analysis (Ecker et al., Expert Review of Molecular Diagnostics, 2014).

**Supplementary Table S6.** Summary of the simulated growth rates for 34 OFS and 30 IFS GEMs based on four different dietary inputs.

**Supplementary Table S7.** Statistics of high-gene-richness (HGR)/low-gene-richness (LGR) enriched species. Comparing HGR and LGR populations (top-25% and bottom-25% in gene richness, respectively), we identified species significantly enriched in HGR or LGR (Wilcoxon two-sided tests adjusted p-value < 10-3 and |log 2 fold change| > 2) and presented relevant statistics of significant species, such as log 2 fold change, p-value (Wilcoxon two-sided test), adjusted p-value, relative abundance at HGR and LGR.

**Supplementary Table S8.** Statistics of associations between IFS/OFS species populations and clinical parameters. We identified six clinical parameters significantly associated with IFS/OFS species populations by linear mixed effect models. We showed corresponding t-statistics, and p-values of significantly associated clinical parameters.

**Supplementary Table S9.** Summary of clinical data of Swedish wellness cohorts of 86 individuals. Total 40 clinical parameters were presented with respective average score, standard deviation and the number of available samples.

**Supplementary Table S10.** Statistics of associations between species abundance and clinical parameters. based on linear mixed effect models, we identified significant associations of MSP abundance and clinical parameters. We showed corresponding t-statistics, p-value, adjusted p-value and explained variance (%).

**Supplementary Table S11.** Statistics of associations between IFS/OFS scores and microbial functions. Among 19,540 features from seven databases, we identified 4,464 microbial functions significantly associated with IFS/OFS scores among present species by linear regressions (adjusted p-value < 10-3). We showed t-statistics, p-value, and adjusted p-value of regression tests of all significantly associated functions. Source of given functional annotation was shown at the Class column.

**Supplementary Table S12.** Functional classes associated with IFS and OFS. Among 4,464 significantly associated microbial functions to IFS and OFS (adjusted p-values < 10-3), we found some functional classes were enriched with the associated functional terms. We showed functional classes and enriched fractions (%) in the table.

**Supplementary Table S13.** Characteristics of 7,763 functional clusters. For more detailed understanding, per functional cluster we presented the size of cluster, number of enriched species, enriched MSP identifiers, enriched species names, enriched KEGG modules (hypergeometric tests p-value < 0.01), subsystems of enriched KEGG modules, and KEGG orthology terms, PFAM terms, virulence term, CAZyme terms, anti-microbial resistance (Mustard) terms, secondary metabolism (anti-SMASH) terms, phenotype (JGI-GOLD) terms, product names of virulence terms, and all functional terms of given cluster.