Universitat Autònoma de Barcelona

GUT MICROBIOME IN HIV-1 INFECTION

MUNTSA ROCAFORT JUNCÀ

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DOCTORAL THESIS

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fan constar que

la realització experimental i la redacció d'aquesta tesis doctoral titulada **Gut Microbiome in HIV-1 Infection** han estat realitzades per l'estudiant **Muntsa Rocafort Juncà** sota la seva direcció

i consideren que

la memòria resultant és apta per optar al títol de **Doctor en Microbiologia** amb **menció internacional** per la Universitat Autònoma de Barcelona.

I per a deixar constància, firmen el següent document a Badalona, a 19 d'octubre de 2018.

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fa constar que

la realització experimental i la redacció d'aquesta tesis doctoral titulada **Gut Microbiome in HIV-1 Infection** han estat realitzades per l'estudiant **Muntsa Rocafort Juncà** sota la seva tutoria

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Dr. Pere Coll Figa

Per la mare, que ho és tot per mi.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein 1
ART	Antiretroviral treatment
ATB	Antibiotic
AUC	Area under the curve
BAFF	B-cell activating factor
BH	Benjamini-Hochberg
BMA	Barcelona metropolitan area
bp	Base pairs
CAG	Co-abundance genes
CCR5	C-C chemokine receptor type 5
CD14 / 163 / 27	Cluster of differentiation 14 / 163 / 27
CHI_ART	Chronically HIV-1 infected subjects on ART
CHI_noART	Chronically HIV-1 infected subjects ART-naive
cDNA	Complementary deoxyribonucleic acid
CRAI	Condomless receptive anal intercourse
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
FDR	False discovery rate
GALT	Gut associated lymphoid tissue
HBV	Human Hepatitis B virus
HCV	Human Hepatitis C virus
HGC	High gene count

HIV-1	Human immunodeficiency virus type-1
HMP	Human microbiome project
HPV	Human Papillomavirus
HTS	Heterosexuals
IBD	Inflammatory bowel disease
IDO1	Indoleamine-pyrrole 2,3-dioxygenase enzyme 1
IFABP	Intestinal fatty-acid binding protein
Ig	Immunoglobulin
IGC	Integrated gene catalogue
IL 12 / 17 / 22 / 23	Interleukin 12 / 17 / 22 / 23
IP10	Interferon gamma-induced protein 10
IQR	Interquartile range
ITS	Internal transcribed spacer
KT ratio	Kynurenine to Tryptophan ratio
LBP	Lipopolysaccharide binding protein
LGC	Low gene count
LMM	Linear Mixed Model
LPS	Lipopolysaccharide
MD2	Lymphocyte antigen 96
MDH	Manhiça District Hospital
MGS	Metagenomic species
MSM	Men who have sex with men
NFkB	Nuclear factor kappa of activated B-cells
NEG	HIV-1 negative subjects
NGS	Next generation sequencing
nM	Nanomolar
NHPM	Non-human primate model

NMDS	Non-metric muldimensional scaling
OTU	Operational taxonomic unit
PAM	Partitioning around medioids
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDL2	PD ligand type 2
PMN elastase	Leukocyte elastase
PWID	People who inject drugs
RHI	Recently HIV-1 infected / infection
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SCFA	Short chain fatty acids
SIV	Simian immunodeficiency virus
SOP	Standard operating procedure
Th17 / Th22	T helper 17 / 22 cells
TLR4	Toll-like receptor 4
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TNFα	Tumor necrosis factor α
TNFR2	Tumor necrosis factor receptor type 2
WGS	Whole genome sequencing

SUMMARY

Soon after Human Immunodeficiency Virus type 1 (HIV-1) infection, a severe and rapid depletion of the gut-associated lymphoid tissue (GALT) occurs, including significant loss of both, immune and epithelial host cells. This is followed by an increased permeability of the intestinal cell lining which facilitates microbial translocation. Microbial cells and products that are usually contained and controlled within the intestinal lumen, can now circulate in the bloodstream and become a new source for immune activation and inflammation. Because of the significant immune imbalance in the GALT after HIV-1 infection, recent microbiome research has focused on understanding the changes occurring in the microbial communities inhabiting the human gut after HIV-1 perturbation and in response to antiretroviral treatment (ART).

Initial cross-sectional studies provided contradictory associations between gut microbial richness and diversity and HIV-1 infection and suggested shifts from *Bacteroides* to *Prevotella* predominance following viral infection. Nonetheless, these results have not been confirmed in animal models or in studies matched for HIV-1 transmission risk groups. For instance, in non-human primate models (NHPM), Simian Immunodeficiency Virus (SIV) infection is followed by expansion of enteric virome but has a limited impact on the gut bacteriome.

In two independent European cross-sectionals cohorts of chronically HIV-1-infected subjects and uninfected controls, in Barcelona (n=156) and Stockholm (n=84), men-who-have-sex-with-men (MSM*)* showed a *Prevotella*-enriched gut microbiota and higher microbial richness and diversity compared to non-MSM individuals who predominantly showed a *Bacteroides*-enriched microbiota, regardless of HIV-1 infection. After stratifying for sexual orientation (MSM vs. non-MSM), we described lower microbial richness and diversity in HIV-1 infected subjects, more so in subjects with an immune-discordant response to ART, but there was no solid evidence of an HIV-1-specific *dysbiosis*. In our Barcelona cohort, diet did not have a major impact on gut microbiota composition.

To understand the longitudinal effects of HIV-1 infection on the human gut microbiota, we prospectively followed 49 Mozambican subjects diagnosed with recent HIV-1 infection and 54 uninfected controls for 9-18 months and compared them with 98 chronically HIV-1-infected subjects ART-treated (n=27) or not (n=71). Recent HIV-1

infection was characterized by increased fecal Adenovirus shedding, which persisted during chronic HIV-1 infection and did not resolve with ART. Recent HIV-1 infection was also followed by transient non-HIV-1-specific changes in the gut bacterial richness and composition. Despite early resilience to change, an HIV-1-specific signature in the gut bacteriome could be eventually identified in chronically HIV-1-infected subjects. Such signature featured depletion of *Akkermansia*, *Anaerovibrio*, *Bifidobacterium*, and *Clostridium*, and has been previously associated with chronic inflammation, CD8+ T-cell anergy and metabolic disorders.

In conclusion: 1) Sexual practice is an important confounding factor in microbiome studies that needs to be considered, especially in HIV-1-gut microbiota studies; 2) Gut microbiota is initially resilient to change right after HIV-1 acquisition but a pro-inflammatory-bacterial signature eventually appears in chronic phases of the infection, and 3) Changes on the gut microbiota do not only impact bacterial communities, but, at least, also viral communities.

RESUM

Una vegada el Virus de la Immunodeficiència Humana tipus 1 (HIV-1 de l'anglès *Human Immunodeficiency Virus type 1*) infecta l'organisme hoste es produeix una ràpida i severa destrucció del teixit limfoide associat a tracte gastrointestinal que inclou, entre d'altres, significants pèrdues cel·lulars del sistema immunitari i epitelial de l'hoste. Seguidament es produeix un increment de la permeabilitat de la paret intestinal que permet la translocació microbiana. Així i doncs, productes i cèl·lules microbianes que normalment queden confinats i controlats dins la llum intestinal ara poden travessar l'epiteli intestinal i entrar en circulació sanguínia convertint-se en un nou focus d'activació immunitària i inflamació. Per la seva significant contribució en la patogènesis del HIV-1, gran part de la recerca en microbioma dels últims anys s'ha centrat en entendre els canvis que tenen lloc en les comunitats microbianes que habiten dins l'intestí humà després de la infecció per HIV-1 i de l'inici de tractament antiretroviral (ART de l'anglès *Antiretroviral treatment*).

Els primers estudis transversals van descriure relacions contradictòries entre la riquesa i diversitat microbiana a l'intestí i la infecció per HIV-1 i van suggerir una substitució de *Bacteroides* per *Prevotella* com a conseqüència de l'adquisició viral. Aquests resultats però, no s'han confirmat ni en models animals ni en estudis balancejats per grups de risc de transmissió de VIH-1. En estudis en models de primats no humans (NHPM de l'anglès *non-human primate models*), la infecció per SIV (de l'anglès *Simian Immunodeficiency Virus*) s'ha associat a una expansió de les comunitats víriques intestinals però no s'ha descrit un canvi consistent en les comunitats bacterianes.

En dues cohorts europees independents de persones amb infecció crònica per HIV-1 i persones no infectades de Barcelona (n=156) i Estocolm (n=84), els individus homosexuals (MSM de l'anglès *Men-who-have-Sex-with-Men*), presentaven una microbiota intestinal més rica i diversa i una major abundància relativa de *Prevotella*, en comparació amb individus heterosexuals (no-MSM) que estaven enriquits per *Bacteroides*, independentment de la infecció per HIV-1. Estratificant els grups d'estudi en funció de la seva preferència sexual (MSM vs non-MSM), individus infectats amb HIV-1 presentaven valors inferiors de riquesa i diversitat bacteriana, amb els valors

més baixos en el grup de individus amb una resposta discordant a ART. Tot i així no hi havia canvis evidents respecte una *disbiosi* específica de infecció per VIH-1. En la nostra cohort d'estudi de Barcelona, la dieta tenia un efecte limitat sobre la composició de la microbiota intestinal.

Per estudiar els efectes longitudinals de la infecció per HIV-1 en la microbiota intestinal, vam fer un seguiment de 9-18 mesos a 49 persones de Moçambic diagnosticades amb infecció recent per HIV-1 i 54 persones no infectades, i les vam comparar amb dos grups de persones amb infecció crònica per HIV-1, tan si estaven amb ART (n=27) o no (n=71). En aquest estudi, seguint la infecció recent per HIV-1, es produïa una major secreció d'Adenovirus en femta, la qual persistia en infecció crònica i no desapareixia amb l'administració de ART. Paral·lelament també vam descriure canvis transitoris no específics en la riquesa i la composició bacteriana després de la infecció recent per HIV-1. Malgrat certa resiliència inicial al canvi, sí que s'evidenciava una signatura en quan a composició bacteriana en individus amb infecció crònica per HIV-1. Aquesta signatura, que incloïa pèrdua de *Akkermansia*, *Anaerovibrio*, *Bifidobacterium* i *Clostridium*, ja ha estat prèviament associada a inflamació crònica, anergia de limfòcits T $CD8^+$ i desordres metabòlics.

En conclusió: 1) La preferència sexual és un factor de confusió important que s'hauria de considerar d'ara endavant, especialment en estudis de microbioma intestinal i HIV-1; 2) La microbiota intestinal sembla ser inicialment resilient a canviar després la infecció per HIV-1 però amb el pas del temps i progressió a fase crònica sí que apareix una signatura bacteriana amb perfil pro-inflamatori; i 3) Els canvis en la microbiota intestinal no només afecten les comunitats bacterianes sinó també, com a mínim, les comunitats víriques.

RESUMEN

Una vez el Virus de la Inmunodeficiencia Humana tipo 1 (HIV-1 del inglés *Human Immunodeficiency Virus type 1*) entra en el organismo huésped, se produce una importante y severa destrucción del tejido linfoide asociado al tracto gastrointestinal que incluye, entre otros, significativas pérdidas celulares del sistema inmune y epitelial del huésped. Seguidamente se produce un incremento de la permeabilidad de la pared intestinal que permite la translocación microbiana. Entonces, productos y células microbianas que normalmente quedan confinadas y controladas en la luz intestinal, ahora pueden cruzar el epitelio intestinal y entrar en circulación sanguínea convirtiéndose en un nuevo foco de activación inmunitaria e inflamación. Debido a su significante contribución en la patogénesis del HIV-1, gran parte de la investigación reciente en microbioma se ha centrado en entender los cambios que tienen lugar en las comunidades microbianas que habitan dentro del intestino humano después de la infección por el HIV-1 y del inicio de tratamiento antirretroviral (ART del inglés *Antiretroviral treatment*).

Los primeros estudios transversales describieron relaciones contradictorias entre la riqueza y diversidad microbiana del intestino y la infección por HIV-1 y sugirieron una sustitución de *Bacteroides* por *Prevotella* como consecuencia de la adquisición viral. Estos resultados, pero, no se han confirmado ni en modelos animales ni en estudios balanceados por grupos de riesgo de transmisión de HIV-1. En estudios de modelos de primates no humanos (NHPM de l'anglès *non-human primate models*), la infección por SIV (del inglés *Simian Immunodeficiency Virus*) se ha asociado a una expansión de las comunidades víricas intestinales, pero no se ha descrito un cambio consistente en las comunidades de bacterias.

En dos cohortes europeas independientes de personas con infección crónica por HIV-1 y personas no infectadas de Barcelona (n=156) y Estocolmo (n=84), los individuos homosexuales (MSM del inglés *Men-who-have-Sex-with-Men*), presentaban una microbiota intestinal más rica y diversa, y mayor abundancia relativa de *Prevotella* en comparación con individuos heterosexuales (non-MSM) que estaban enriquecidos por *Bacteroides*, independientemente de la infección por HIV-1. Estratificando los grupos de estudio según su preferencia sexual (MSM vs non-MSM), las personas

infectadas por HIV-1 presentaban valores inferiores de riqueza y diversidad bacteriana, con los valores más bajos en el grupo de individuos con una respuesta discordante a ART. Aun así, no había cambios evidentes respecto a una *disbiosi* específica de infección por HIV-1. En nuestra cohorte de estudio de Barcelona, la dieta tenía un efecto limitado sobre la composición de la flora intestinal.

Para estudiar los efectos longitudinales de la infección por HIV-1 en la microbiota intestinal, también hicimos un seguimiento de 9-18 meses a 49 personas de Mozambique diagnosticadas con infección reciente por HIV-1 y a 54 personas no infectadas, y las comparamos con dos grupos de personas con infección crónica por HIV-1, estando con ART (n=27) o no (n=71). En este estudio, siguiendo la infección reciente por HIV-1, se producía una mayor secreción de Adenovirus en heces que persistía en infección crónica y no desaparecía con la administración de ART. Paralelamente también se describieron cambios transitorios y no específicos en la riqueza y la composición bacteriana después de la infección reciente por HIV-1. A pesar de cierta resiliencia inicial al cambio después de la infección por HIV-1, sí que se evidenciaba una signatura en cuanto a composición bacteriana en individuos con infección crónica por HIV-1. Dicha signatura incluía depleción de *Akkermansia*, *Anaerovibrio*, *Bifidobacterium* y *Clostridium*, y ya ha estado previamente asociada a inflamación crónica, anergia de linfocitos T $CD8^+$ y desórdenes metabólicos.

En conclusión: 1) La preferencia sexual es un factor de confusión importante que se debería tener en cuenta en el futuro, especialmente en estudios de microbioma intestinal y HIV-1; 2) La microbiota intestinal parece ser resiliente al cambio justo después de la adquisición del VIH-1 pero con el paso del tiempo y progresión a SIDA sí que aparece una signatura bacteriana con perfil pro-inflamatorio; y 3) Los cambios en la microbiota intestinal no solo afectan las comunidades bacterianas sino también, como mínimo, las comunidades víricas.

1. INTRODUCTION

1.1. MICROBES MATTER

Prokaryotes have been evolving for more than 3.8 billion years in almost every niche on planet Earth. It is estimated that about 4-6x10^{30} prokaryotic cells exist all over the biosphere, 2 to 3 orders of magnitude more than all plant and animal cells. Constituting up to 60% of overall biomass, their practically ubiquitous presence makes prokaryotes essential components of the Earth's biota and represents a huge and mostly unexplored source of genetic information and diversity (Whitman et al., 1998).

Microorganisms participate in many activities in a wide range of environments and conditions: 1) modulation of biogeochemical cycles either through active metabolism or by recycling other organisms' dead remains (Falkowski et al., 2008), 2) applicability in industry thanks to their broad metabolic potential (e.g. food-industry or biotechnical industry) (reviewed in (Taylor and Richardson, 1979)), 3) applicability in energy production, transformation of unusable fuels and water treatments (Min and Logan, 2004), 4) interaction with higher order multicellular organisms (Casadevall and Pirofski, 2000).

Interestingly, microorganisms not only interact with superior organisms in a parasitic manner, but some microbes are known to be commensals or mutualists to their hosts. For instance, the microbial biota residing in the human's gut (the gut microbiota) helps the host in gut immunity development, synthesis of vitamins such as biotin or folic acid, and in digestion (reviewed in (Clemente et al., 2012)). Similarly, microorganisms inhabiting the human skin help the human host to protect against invasion by other pathogenic or harmful microorganisms by directly interacting with closely-located host immune cells (Christensen and Brüggemann, 2014). Other niches in the human body such as the vaginal tract, respiratory tract and the oral cavity, are also known to have been colonized by microbial communities through evolution (reviewed in (Meisel and Grice, 2017)).

1.1.1. CLASSIC MICROBIOLOGY

When discovered in the 17th century, microorganisms were classified relying uniquely on their phenotypic characteristics. By the early 20th century, researchers agreed that microorganisms with different biochemical, physiological and metabolic

characteristics could share observable phenotypic traits and that the former should also be used for classification. However, major caveats were still present: 1) the number of biochemical and phenotypic properties to test was limited, 2) the observable characteristics were restricted to those bacteria that could be isolated and cultured in the lab (*The Great Plate Count Anomaly* coined by Staley and Konopka in 1985 estimated this microbial cultivable-fraction to be that less than 1% of the observable bacteria in oligo/meso-trophic aquatic habitats), and 3) microorganisms could have different behavior in pure culture compared to their natural niche where they might be involved in significant co-operation networks with other members of the microbial community (Wintermute and Silver, 2010). Thus, the research community was already aware of the important biases and limitations linked to the characterization of microorganisms based on pure phenotypic traits.

1.1.2. MOLECULAR APPROACH

Molecular biology started to gain weight around 1960 and provided microbiologists with a new perspective for characterizing bacteria using genetic information. The main advantages of the genotypic characterization included: 1) genotypic variation is continually producing multiple changes that do not necessarily imply phenotypic changes, 2) the way information is exposed at the genotypic level is much more constrained with 4 nucleotides and 20 amino acids allowing more precise evaluation and quantification, and 3) many different genetic changes can produce similar phenotypes. Thus, evaluation of genotypes led to a more objective, reproducible and comprehensive concept of species, thus reducing errors in microbial taxonomic classification.

At first, properties derived from DNA composition such as GC content or DNA-DNA hybridization were used to taxonomically characterize microbes (Meier-Kolthoff et al., 2014). However, the highest revolutionary event in molecular taxonomy took place in the mid-1980s with the advent of full-sequence analysis of molecular chronometers such as the ribosomal RNA (rRNA) genes. Confirming prior suspicions, new phylogenetic branching based on molecular markers showed that characters traditionally used to cluster microorganisms had restricted phylogenetic linkage (reviewed in (Amit Roy, 2014)). By the end of 1980s, most molecular techniques relied on polymerase-chain-reaction (PCR) amplification and targeted Sanger sequencing

thus increasing the capabity to detect and properly identify a wider range of microorganisms in a wider range of ecological niches based on their genetic code. Nevertheless, the real explosion of microbial community analysis did not occur until the advent of next-generation sequencing (NGS).

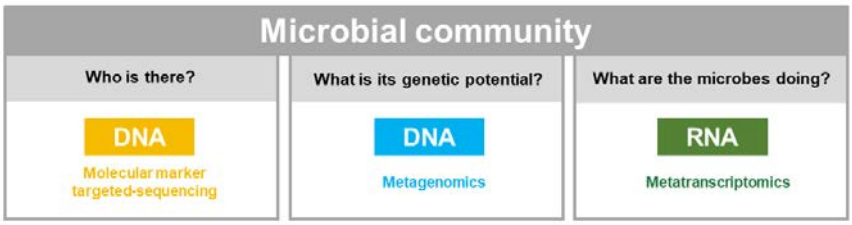
1.1.3. SEQUENCING APPROACHES

It was 2005 when first Roche's 454-sequencing technology was launched. This new sequencing technology, popularly named NGS, produced higher throughput at lower costs. At the molecular level, and compared to the previous Sanger sequencing, multiple short input DNA fragments were concurrently sequenced following an automated process of spatially clustered PCR of single DNA templates. The input DNA could either be genomic DNA fragmented into smaller fragments or a PCR-amplified targeted library. Hence, the higher throughput enhanced analysis on whole eukaryotic genomes and/or on DNA mixtures including multiple molecules from one or various sources.

Since the advent of NGS, multiple improvements have been made to reduce costs and increase relationship between coverage and read length. Increasing the coverage (i.e. number of sequencing reads mapping a given region of the input DNA), increased sensitivity in detecting minor changes in input genomic DNA, such as when discriminating two phylogenetically close taxa (reviewed in (Loman and Pallen, 2015)).

In microbiome research, NGS has become a highly useful culture-independent technique to characterize communities of microorganisms and predict their functional interactions within a given sample. High throughput sequencing is not restricted to taxonomical profiling of biological samples (phylogeny) but also might provide data on gene content (metagenomics) and gene expression profiling (metatranscriptomics) (Figure 1).

Figure 1. Main *–omics* sciences used in microbiome studies.



Main questions regarding microbial communities resolved using different input material and different sequencing approaches.

Nowadays, more recent third-generation sequencing platforms allow sequencing of a single molecule without having to create amplification libraries and still generate longer reads in shorter periods of time and at lower costs (reviewed in (Kchouk et al., 2018)). These new technologies broaden the scope and potential of the applications in microbial studies even more.

1.1.3.1. LAB PROCEDURES

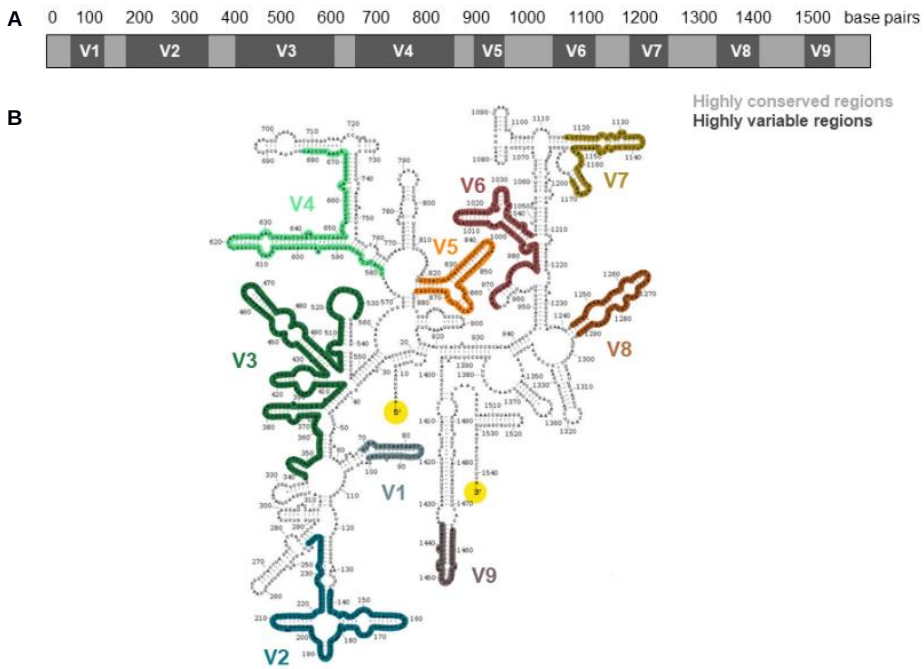
Most common sequencing approaches to taxonomically characterize microbial communities are: targeted-sequencing and whole genome sequencing (WGS) (reviewed in (Clooney et al., 2016)).

In a targeted-sequencing approach, PCR protocols are used to specifically amplify genetic molecular markers. The choice of this molecular marker is crucial since it determines the extent and precision of the derived taxonomic and phylogenetic information. In the 1970s Carl Woese described the usefulness of the ribosomic RNA genes as molecular chronometers in bacterial phylogeny (Fox et al., 1977). He defended that rRNA genes were a good reflection of the evolution of the entire genome (Woese et al., 1990).

The 16S rRNA gene is found in all prokaryotic genomes (bacteria and archaea) and codifies for a ribosomal RNA molecule which is part of the ribosome. This RNA molecule is in average 1500 ribonucleotides long and it is characterized by the alternation of highly conserved and highly variable regions within its primary structure (Figure 2). This primary structure is ideal to design universal primers to taxonomically

identify prokaryotes since within the variable regions there are several characteristic sequences known as *sign oligonucleotides*, that appear in most, if not all microorganisms within a certain phylogenetic group (ranging from domain to genus, or even to species level), and rather never in other groups of microorganisms.

Figure 2. Primary and secondary structure of the prokaryotic 16S rRNA gene.



***A**, Schematic representation of the 16S rRNA gene showing alternation of highly variable (colored in dark grey and labeled from V1 to V9) and highly conserved regions (colored in light grey). **B**, Primary and secondary structure of the Escherichia coli 16S rDNA gene with its 9 described hypervariable regions (named V1 to V9) through its complete length. Source of 16S rDNA gene: http://rna.ucsc.edu/rnacenter/xrna/xrna_gallery.html*

Concurrently to the 16S rRNA gene in prokaryotes, the 18S rRNA gene is usually used to classify eukaryotes and the internal transcribed spacers (ITS) within the eukaryotic ribosomal RNA genes operon for high resolution classification of fungi.

In the WGS approach we sequence all raw DNA content directly extracted from the sample rather than focusing on a single target. Following this approach, we get

massive amounts of sequencing data that go beyond taxonomic profiling. For instance, it also provides insights into communities' internal metabolic and gene function networks. Moreover, WGS also allows profiling other microbial populations beyond bacteria (e.g. virus, minor eukaryotes, fungi, etc.). Of note, taxonomic characterization using WGS not only reaches higher resolution (usually species level compared to the genus level with 16S rRNA sequencing), but also provides less-biased data since no PCR of molecular markers is necessary.

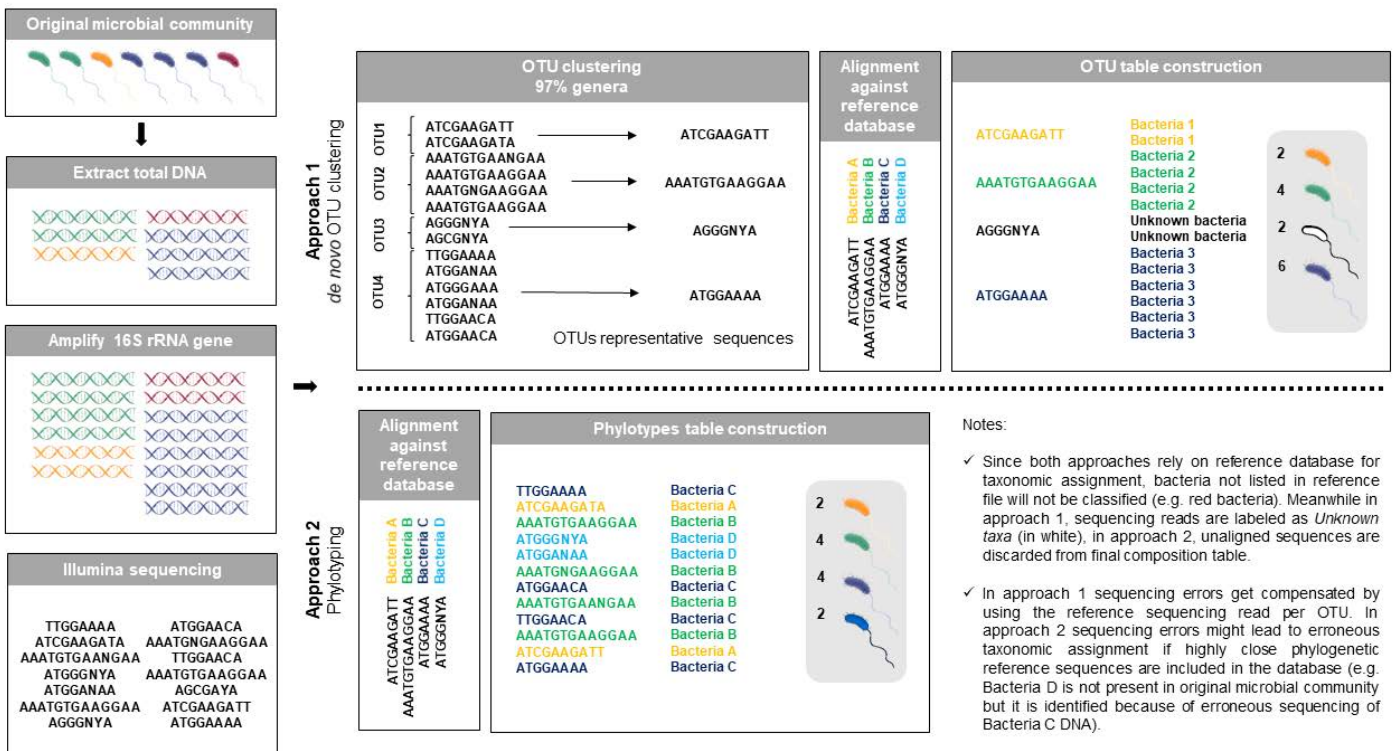
1.1.3.2. BIOINFORMATIC ANALYSIS: SOFTWARE AND DATABASES

Either by using a targeted-sequencing or a WGS approach, the use of bioinformatics tools is required to analyze the data generated. Targeted-sequencing is commonly used to taxonomically characterize microbial communities. Depending on the molecular marker chosen, it is possible to characterize bacteria and archaea, fungi or eukaryotes. Once the amplicons are generated and sequence data is obtained, resolution of taxonomic profiling essentially depends on the algorithm used to process and annotate query sequences and the taxonomic reference database chosen.

Targeted sequencing data can basically follow two analysis routes: *phylotyping*, where query sequences are individually aligned against the taxonomic reference, or *de novo* OTU clustering, where sequences are clustered into *operational taxonomic units* (OTU) uniquely based on overall sequence similarity prior to alignment. Grouping sequencing reads into OTUs has three major advantages (Figure 3): 1) reduces the impact of sequencing errors in individual sequencing reads, 2) sequences not aligned against the taxonomic reference are kept and labeled as *unknown taxa* and not discarded as with *phylotyping*, and 3) a given taxa can be represented by more than one representative sequence (i.e. two or more OTUs with same taxonomic assignment representing distinct subspecies or strains). Minimum percentage of sequence similarity among sequences clustered within the same OTUs are usually 97% and 99% for genus and species identification, respectively.

Regardless of the approach used, we eventually generate a matrix, known as OTU table, indicating how many sequencing read belong to each of the taxa identified in each sample, thus quantifying the taxonomical profile of each sample.

Figure 3. Differences and similarities between *phylotyping* and *de novo* OTU clustering.



Example dataset to show differences in microbial taxonomic characterization derived from 16S rRNA gene sequencing data using either phylotyping (approach 2) or de novo OTU clustering (approach 1). Main constraints using phylotyping are listed in the Notes section.

Although the number of data analysis pipelines has significantly increased over the past years, most common used bioinformatic pipelines to process targeted sequencing data so far are QIIME (Caporaso et al., 2010) and Mothur (Schloss et al., 2009). Concurrently, most widely used reference databases used for taxonomic profiling are GreenGenes (DeSantis et al., 2006) and SILVA (Quast et al., 2013) for bacteria, archaea and eukaryotes, and UNITE (Kõljalg et al., 2013) for fungi.

Although targeted-sequencing aims to perform the taxonomic profiling of the sequencing dataset, there are various bioinformatic tools designed to infer the functional capacity of the microbial communities relying only on taxonomic characterization such as PICRUSt (Langille et al., 2013), Tax4Fun (Aßhauer et al., 2015) and Piphillin (Iwai et al., 2016). Overall, these software use the OTU table with relative abundances as input and, considering both, the 16S rRNA copy number per bacteria and the gene copy number for each gene family, predict the contribution of each taxa to the overall gene content of the sample. Eventually, they sum all individual contributions to produce an estimate of the genes present in the sample.

For the WGS data analysis, MetaPhlan2 (Truong et al., 2015) uses approximately 1 million clade specific marker genes derived from almost 18000 reference genomes to eventually generate a bacterial species abundance table providing relative abundance of each of the identified taxa. WGS reads can also be directly mapped against other available reference databases. Although metagenomics does not provide information on *in vivo* enzymatic activity (contrary to metatranscriptomics), it provides information about the functional potential of microorganisms identified. For instance, by aligning WGS reads to the Integrated reference catalogue of the human gut microbiome (Li et al., 2014) we can calculate richness (i.e. how many distinct categories are detected) of enzymes, modules and metabolic pathways in each sample. Similarly, other databases commonly used to characterize microbial communities are those including references on antibiotic resistance genes (Liu and Pop, 2009) and/or virulence factors (Chen et al., 2016).

Analyses of WGS can also be made independently of any reference database. For instance, it is common to compute gene richness on microbial communities (i.e. total number of different genes identified in a given sample). To do so, MOCAT (Kultima et al., 2012) and Prokka (Seemann, 2014) are useful tools to quality-trim and filter

Illumina-derived sequencing reads to further assemble them into longer units, known as contigs, to enhance protein-coding gene prediction. Interestingly, in 2014 Nielsen et al. used a WGS approach to identify microbial species (labeled as metagenomic species, MGS) through grouping genes that reported similar abundances across the different samples (labeled co-abundance genes, CAG)(Nielsen et al., 2014).

With the data obtained from either approach (targeted- or whole genome-sequencing), downstream analyses take advantage of many of the theoretical concepts from classical ecology (Figure 4). Alpha diversity measures are intrinsic to each sample and independent from other samples. Both, parametric and non-parametric statistical tests are commonly used to compare these measures among groups of samples. Simplest example would be to compute richness (i.e number of different taxa detected in a given sample) and evenness (i.e. distribution of abundances within all taxa detected in a given sample) ecological indices and compare mean or median values between groups of samples. A common, but not globally accepted practice in alpha diversity analysis, is to overcome unequal sequencing coverage between samples through rarefying all samples to a given number of randomly selected reads (McMurdie and Holmes, 2014).

Beta diversity measures need to be defined between two or more samples and aim to determine their degree of similarity based on the composition data. Most common inter-sample test involves computing ecological pairwise distance matrices between various samples and then project such distance matrix in a low-dimension ordination plot. Many similarity metrics exist, but they can be classified into three groups: a) those that only consider presence/absence of taxa, b) those that also consider abundance of the taxa and c) those that also consider the phylogenetic relationship among the taxa identified. Although QIIME and MOTHUR have embedded scripts to perform basic alpha and beta diversity analysis, many R packages have also been published to do so: Vegan (Oksanen et al., 2013), Phyloseq (McMurdie and Holmes, 2013), and BiodiversityR (Kindt, 2016), among others.

Figure 4. Main alpha and beta diversity ecological parameters.

MEASUREMENT	DESCRIPTION	PROS	CONS
RICHNESS: Measures the total number of taxa in each sample.			
Observed richness (S)	Number of unique taxa identified.	Rapid to calculate.	Comparisons in richness can only be made among samples if they have sample size (i.e. number of sequencing reads). Highly dependent on sample size and strongly influenced by evenness.
Chao1	Estimation of real richness based on the number of taxa supported by either 1 or 2 sequencing reads.	Assume that datasets are open communities so that other taxa beyond those characterized might exist and not be represented in the sequencing data.	
ACE	Estimation of real richness based on the number of taxa supported by 10 reads or less reads.		
EVENNESS: Measures how evenly distributed the sequencing reads are among all taxa identified in each sample.			
Shannon (H)	Quantifies the uncertainty of the taxonomic identity of an unknown entity in a community based on number of different entities and their abundance.	Multiple values follow a normal distribution so parametric statistics can be used to compare multiple groups.	Also take richness in consideration, thus comparisons can only be made among samples if they have sample size (i.e. number of reads). — Highly weighted towards rare species compared to abundant ones.
Simpson (D)	Quantifies the probability that 2 randomly chosen sequencing reads belong to different taxa. It is usually represented as 1/D or 1-D to achieve a positive meaning.		Highly weighted towards the most abundant taxa.
ECOLOGICAL DISTANCE METRICS: Indices of dissimilarity as surrogates of ecological distances between samples: the more different two communities are in composition, the higher the index.			
Jaccard	Quantifies the compositional dissimilarity between samples based on presence/absence of the taxa.	The most simple and with less requirements.	It only relies on richness ignoring evenness between taxa.
Bray-Curtis	Quantifies the compositional dissimilarity between samples based on raw abundance data (not relative abundances).	Takes evenness of taxa into account without the need to fit compositional requirements (i.e. sum of relative abundances must always be 1).	Highly influenced by significant differences in abundances. Because uses raw input data, assumes that all samples have the same size.
UnWeighted UniFrac	Quantifies the compositional dissimilarity between samples based on presence/absence of taxa and their phylogenetic relatedness.	They incorporate phylogenetic relatedness information of taxa in samples.	They require phylogenetic information to be computed. It only relies on richness ignoring evenness between taxa.
Weighted UniFrac	Quantifies the compositional dissimilarity between samples based on relative abundance of taxa and their phylogenetic relatedness.		They require phylogenetic information to be computed. Working with relative abundances force sequencing data to be compositional (i.e. sum of relative abundances must always be 1).

Brief description and pros and cons of the main ecological parameters used for alpha and beta diversity assessment of sequencing data on microbiome studies.

1.2. HUMAN MICROBIOME

The *human microbiome* refers to the entire genomic content derived from all microorganisms inhabiting *in* and *on* the human body in association with the human host. Although these microbial communities, globally known as the *human microbiota,* are primarily dominated by bacteria (initially described to outnumber human cells by a factor of 10 and human genes by a factor of 1000 (ratios reviewed in (Sender et al., 2016))); archaea, minor eukaryotes and viruses are also present. Contrary to the *The Germ Theory* initially coined in the 14th century, most of these microorganisms were further seen to be harmless to the host. Some of them were even described as key members in maintaining human health and cell homeostasis (Clemente et al., 2012). This discovery led researchers to describe the human as a *superorganism* composed, not only of human cells, but also of large invisible communities of microorganisms that live in homeostasis with the host cells through mutualistic relationships (Caricilli, 2014)(Wang et al., 2017).

The microorganisms inhabiting in the human intestines, the *human gut microbiota*, can reach up to 10 trillion cells including hundreds of different microbial species (Bäckhed et al., 2005). Both, the human enteric cells and the microorganisms, establish a delicate mutualistic relationship in the gut. On one hand, the human host provides the microbial cells with a continuous food source and many diverse ecological niches where to grow and reproduce (Pereira and Berry, 2017). On the other hand, the microorganisms help the host in several ways: 1) produce vitamins like biotin, thiamine and folate (LeBlanc et al., 2013) and various short-chain fatty acids (SCFA) such as butyrate, propionate and acetate (Ramakrishna, 2013), 2) break down food components and extract nutrients to help digestion (Flint et al., 2012), 3) help develop a mature and fully competent immune system (Belizário and Napolitano, 2015)(Clemente et al., 2012), and 4) produce anti-inflammatory compounds that help the human defense system to fight off other potentially pathogenic invading microorganisms (Tanaka and Nakayama, 2017)(Francino, 2014).

If this delicate equilibrium between the human host and the microbial communities is perturbed, microbial *dysbiosis* might occur and some of these microbes may become harmful to the host. Even though *dysbiosis* might have different outcomes in different

conditions, it is usually described as an imbalance that disturbs the microbial functions which are essentials for maintaining health and introduces processes that eventually promote disease (i.e. adverse phenotype) (reviewed in (Kriss et al., 2018)). Hence, *dysbiosis* usually includes: 1) presence of pathogenic microorganisms related to inefficient natural microbial defense barriers, 2) low microbial richness and diversity, 3) presence of bacterial products in blood which cause inflammation, and 4) altered metabolic functions, among others. Specifically, in the human gut, the most common manifestation of *dysbiosis* is the replacement of complex communities of anaerobic microbes with a community of lower overall microbial richness and diversity with increased presence of facultative anaerobes.

1.2.1. HUMAN MICROBIOME PROJECT

The human microbiome project (HMP) started in 2008 in the United States aiming to identify and characterize the human microbiota both in health and disease. HMP researchers performed WGS of many diverse human samples (collected from oral cavity, skin, vagina, gut and nose/lung) to provide a deep genetic perspective of the human microbial communities.

Alongside with the development of standard operating procedures (SOP) for experimental and bioinformatics analysis in metagenomics studies, it also aimed to develop a reference set of microbial genome sequences which would serve as a resource repository for further studies on the human microbiome (NIH HMP Working Group et al., 2009)(Gevers et al., 2012).

1.2.2. META-HIT

The META-HIT project was also launched in 2008 aiming to specifically characterize the human gut microbiota in human health and disease such as in obesity and in inflammatory bowel disease (IBD).

Authors initially created a reference catalogue of microbial genes present in the human gut through sequencing stool samples from more than one hundred Spanish and Danish individuals (Qin et al., 2010). They concluded that a healthy adult carries in his gut around 160 different bacterial species out from a 1000-pool of described bacteria and that this bacterial richness corresponds to more than 500000 bacterial

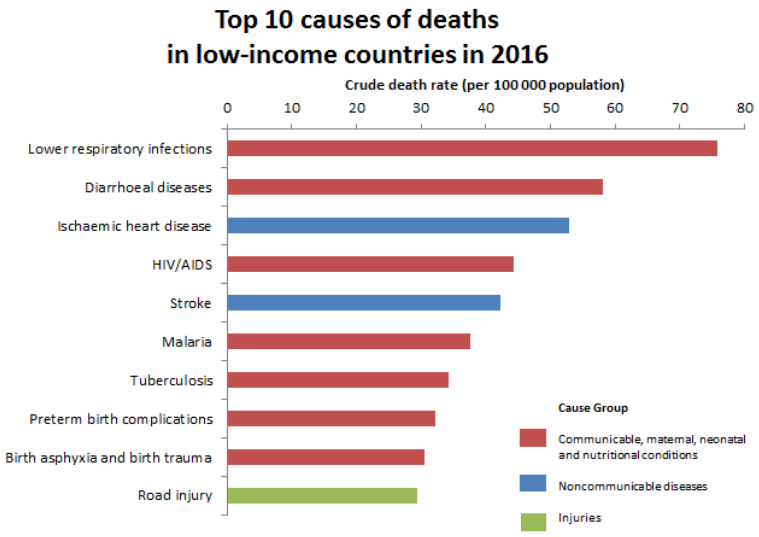
genes. They also stated that up to 60 bacterial species are present in everyone's gut as well as that approximately 40% of a given subject's bacterial genes are undoubtedly present in at least 50% of the rest of the population. Of note, they also reported for the first time the stratification of the human population into, at least, three main enterotypes, each characterized by a dominant bacterial genus: *Bacteroides*, *Prevotella* and *Ruminococcus* (Arumugam et al., 2011).

Of note, in 2014, they integrated the Meta-HIT derived sequencing data to the already existing microbial genomic sequencing data derived from the HMP. Authors thus defined a new Integrated Gene Catalogue (IGC) which comprised 9,750,788 non-redundant genes derived from 1267 gut metagenomes from individuals spread through 3 different continents (America, Europe and Asia) (Li et al., 2014). With this expanded catalog, they expected to enhance characterization of metagenomic and metatranscriptomic data from the gut microbiota to better understand its variation across populations in human health and disease.

1.3. ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

Since AIDS was first reported in the United States of America in 1981 (CDC, 1981) and the HIV was declared its etiologic agent in 1983 (Barre-Sinoussi, F., J. C. Chermann, 1983), the HIV pandemic has become one of the most devastating on human history ever recorded (Sharp and Hahn, 2011). Regardless of the improvements and efforts in both the clinic and the society on increasing consciousness about HIV/AIDS during the last two decades, AIDS remains among the top 10 leading causes of death especially in non-developed countries (Figure 5).

Figure 5. Top 10 causes of death in low-income economies in 2016



Ten leading causes of death in low-income countries worldwide in 2016. Image source: http://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

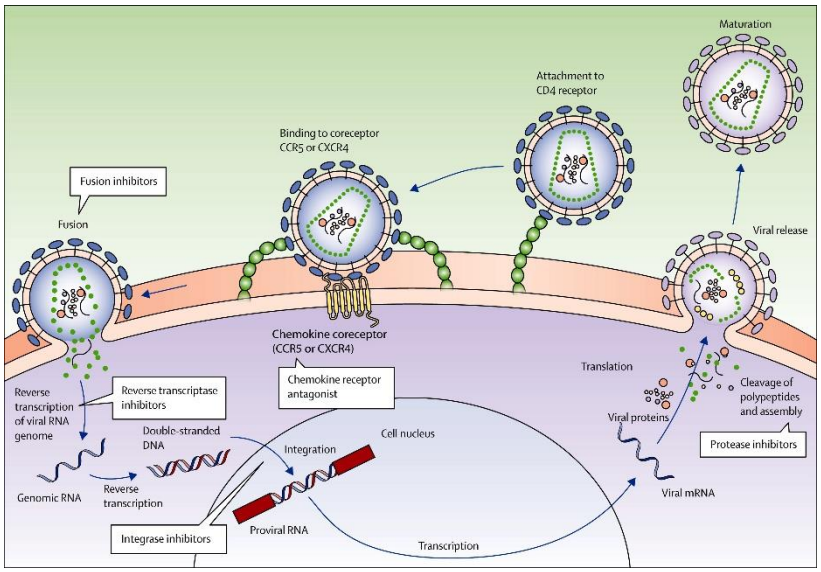
In 2017, 36.7 million individuals were living with HIV with 1.8 million new infections reported per year and 1 million AIDS-related illnesses reported as death causes worldwide. Up to 76.1 million people have become infected since the start of the epidemic and over half of them, 35 million, have already died because of AIDS-related illnesses (Joint United Nations Programme on HIV/AIDS (UNAIDS), 2017).

1.3.1. HIV - CHARACTERISTICS AND LIFE CYCLE

HIV-1 is a lentivirus within the *Retroviridae* family that has an enveloped capsid and a viral core with two single-stranded RNA molecules. The HIV-1 can infect host immune cells expressing the surface receptor CD4 as well as either the CCR5 or CXCR4 co-receptor. Lymphocytes, monocytes and dendritic cells, among others, are either directly destroyed by HIV-1 or, at least, their defensive function results impaired (Swanstrom and Coffin, 2012). Once inside the host cell, the virus generates a double-stranded cDNA copy by reverse transcribing the RNA template, and integrates its proviral DNA into the human genome using its viral integrase enzyme, thus establishing its own viral reservoir in the host (Schacker et al., 2000). If environmental

conditions are favorable for the virus, the proviral DNA is transcribed and translated using host's enzymatic machinery so that new progeny is eventually released to continue with the infection (Figure 6). ART can block one or more of the enzymatic steps in the replication process (Figure 6), but without ART, HIV-1 infection naturally progresses weakening the host's immune system and leading the host to a state of immunosuppression in 8 to 10 years where a number of opportunistic infections and AIDS-related illnesses can occur and eventually cause the host's death (Simon et al., 2006).

Figure 6. HIV-1 infection and replication cycle.



HIV-1 replication cycle. Potential ART targets listed in white boxes. Image source: https://codehealth.io/library/article-128/opportunistic-infections-and-clinical-monitoring-in-acquired-immune-deficiency-syndrome-aids/

1.3.2. HIV - MICROBIAL TRANSLOCATION, IMMUNE ACTIVATION AND CHRONIC INFLAMMATION

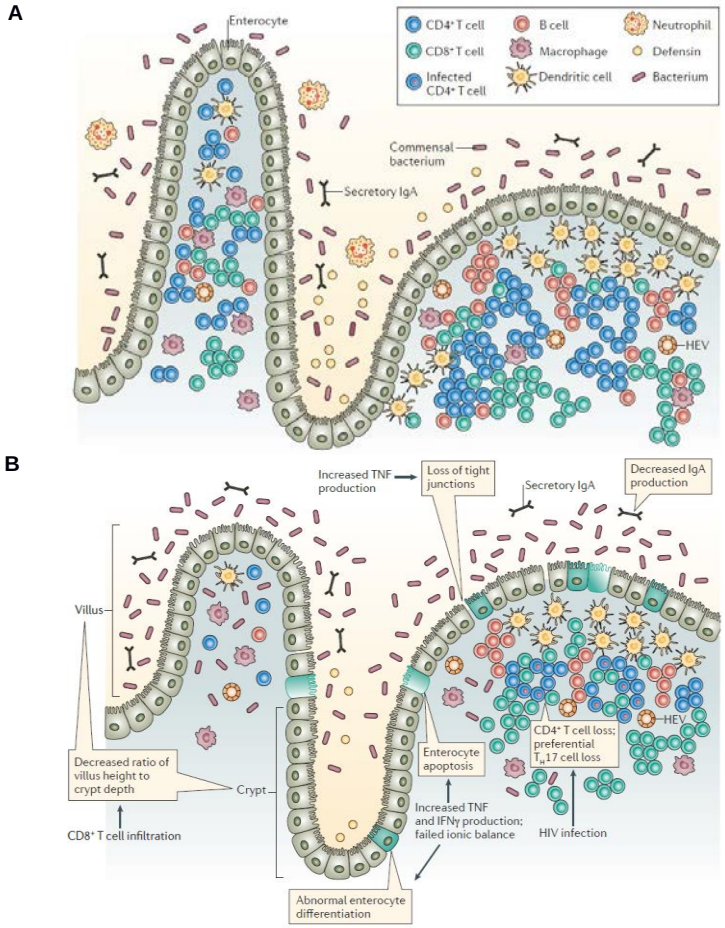
The GALT is the largest lymphoid tissue in the body harboring more than the half of the total lymphoid cells (Lefrançois and Puddington, 2006). Because the intestinal mucosa is constantly exposed to external antigen circulating derived from food ingestion, the GALT plays a crucial role for maintaining the immunological homeostasis through innate and acquired immunological responses (Cheroutre and

Madakamutil, 2004). Alongside with the diversity of immune cells (e.g. neutrophils, macrophages and monocytes) and immune products (e.g. secretory IgA and antimicrobial peptides) involved in controlling bacterial invasion and growth in the gut, the gut-blood physical barrier is composed of tightly connected enterocytes that strictly regulate the transfer of fluids, nutrients and electrolytes (Nagler-Anderson, 2001)(Kayama and Takeda, 2015)(Zhang and Luo, 2015) (Figure 7A).

The gut $CD4^+$ T-cell population is rapidly depleted in primary HIV-1 infection, more so than in the blood or in peripheral lymphoid sites (Mehandru et al., 2007)(Poles et al., 2001). This severe and early damage to the GALT immune cell population contributes to weaken the intestinal lining. Consequent increased permeability allows bacterial cells and products usually contained in the gut lumen to translocate into the blood circulation (Nazli et al., 2010) (Figure 7B). Once in the bloodstream, these microbial particles have the potential to enhance immune activation and inflammation which is a key feature of HIV-1 infection and disease-progression to AIDS (Brenchley et al., 2006)(Ancuta et al., 2008)(Dinh et al., 2015)(Hofer and Speck, 2009).

The exact mechanisms involved in this loss of intestinal barrier function are not completely understood. Nonetheless, direct epithelial damage including enterocyte apoptosis, high cell turnover, impaired phagocytic activity and physical destruction of tight junctions have been reported (Kotler et al., 1984)(Estes et al., 2010). It has also been highlighted a preferential loss of Th17 $CD4^+$ T-cells at mucosal sites in HIV-1 infection (Brenchley et al., 2008)(Prendergast et al., 2010)(Shaw et al., 2011)(Lu et al., 2016). These cells are known to be essential for the gut epithelial cells homeostasis, help recruit neutrophils when fighting opportunistic commensal bacteria, enhance proliferation of enterocytes, as well as produce significant levels of IL-17, IL-22/IL-23 and several defensins (Guglani and Khader, 2010)(Maloy and Kullberg, 2008). Scamurra et al. also observed decreased population of functional mucosal plasma cells as well as low intraluminal concentration of soluble IgA which has a significant role in the gut homeostasis by neutralizing microbial products hampering their attachment and translocation across the epithelial barrier (Scamurra et al., 2002).

Figure 7. Gut epithelium and mucosal tissue in health and in HIV-1 infection.



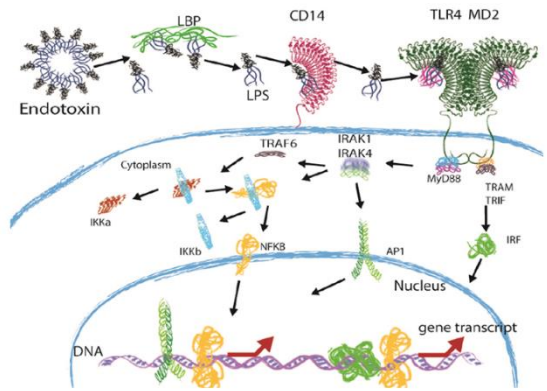
***A**, Gut mucosa and epithelial barrier in health. **B**, Gut mucosa and epithelial barrier in HIV-1 infected subjects showing enterocyte damage, loss of tight-junctions, decreased IgA production, etc. Image source: Microbial translocation in HIV infection: causes, consequences and treatment opportunities (Sandler and Douek, 2012)*

Microbial products reported to translocate through the weakened gut barrier and be potentially recognized by the host's immune system include: peptidoglycans which are present in the cell wall from gram-positive bacteria (Clarke et al., 2010), flagellin (Svärd et al., 2015), bacterial DNA (Jiang et al., 2009), viral RNA/DNA (Li et al., 2013),

and β-glucans which are present in cell walls from bacteria and fungi (Morris et al., 2012), among others.

For instance, lipopolysaccharide (LPS), a major component of the cell wall from gram-negative bacteria also known as endotoxin, has been suggested a major player in the generalized chronic immune activation commonly observed in the HIV-1 infected population (Fujigaki et al., 2001)(Figure 8). It contributes to the host immune activation by binding to the LPS-binding protein (LBP), the pattern recognition receptor CD14, MD-2 and the toll-like receptor 4 (TLR4) present in the cell membranes of monocytes and macrophages. Consequently, many intracellular signaling pathways get activated leading to increased levels of NF-kB nuclear transcription factor and activator protein AP-1. In turn, these increases result in higher production of inflammatory cytokines including several interleukins, TNFα and interferons (Rhee, 2014). The LPS commonly translocate from the gut lumen to the hepatic portal vein and travel towards the liver where Kupffer cells (i.e. specialized liver-resident macrophages) get activated by TLR4-CD14 interaction and clear the enteric-derived LPS (Su et al., 2000). HIV-1 infection has been associated with decreased abundance of functional Kupffer cells (Balagopal et al., 2009), thus reinforcing the role of translocated LPS in promoting immune activation because of impaired bacterial product clearance in blood (Balmer et al., 2014).

Figure 8. TLR4 signaling pathway in response to bacterial LPS.



The TLR4 signaling pathway in response to LPS activation. Image source: Recognition of LPS by TLR4: Potential for anti-inflammatory therapies (Nijland, Reindert Hofland, Tom Van Strijp, Jos A G, 2014).

The administration of ART has been proved to significantly reduce plasma viral loads hampering HIV-1 replication at various levels (extensively reviewed in (Arts and Hazuda, 2012)). Nevertheless, immune cell recovery after treatment initiation is more variable and not all subjects on ART recover pre-infection $CD4^+$ T-cell counts (Gianella et al., 2016). Actually, subjects with good immune recovery and viral suppression upon ART administration, also show symptoms of residual immune activation and chronic inflammation that cannot be pharmacologically fully suppressed (Guadalupe et al., 2003)(Almeida et al., 2002)(Stone et al., 2005). Residual replication in pre-established HIV-1 reservoirs has been proposed as a main actor in this chronic immune activation (Martinez-Picado and Deeks, 2016). But, it is still unclear the contribution of microbial translocation to this chronic immune activation even in ART-treated immune responders (Cassol et al., 2010).

1.4. HUMAN GUT MICROBIOTA AND HIV-1

1.4.1. CHRONIC HIV-1 INFECTION

Studies in NHPM reported that chronic SIV infection did not result in an universal and clearly defined *dysbiosis* of the gut microbiota (McKenna et al., 2008)(Klase et al., 2015)(Moeller et al., 2013)(Moeller et al., 2015). Otherwise, multiple groups reported significant changes on the human gut microbiota linked to either ART treated or non-treated chronic HIV-1 infection (results summarized in Table 1).

Enrichment of Proteobacteria and pro-inflammatory bacteria in HIV-1 infection

Most consistent change on the human gut microbiota in relation to HIV-1 infection is a significant increase in the relative abundance of bacteria from phylum Proteobacteria, especially, members of order Enterobacteriales and family *Enterobacteriaceae*. This increase has been reported, independently of ART-administration, in both, mucosa (Dillon et al., 2014)(Vujkovic-Cvijin et al., 2013)(Mutlu et al., 2014) and stool samples from HIV-1 infected individuals (Ellis et al., 2011)(Lozupone et al., 2013)(Dinh et al., 2015). More precisely, Yang et al. found enrichment in *Burkholderia fungorium* and *Bradyrhizobium pachyrhizi* in mucosal samples from HIV-1 infected individuals and hypothesized that a decrease of colonization resistance in the gut was occurring after HIV-1 infection so that non-commensal environmental species could easily appear in the gut (Yang et al., 2016).

Similarly, Dillon et al. found increases in *Acitenobacter junii* and *Schlegella thermodepolymerans* in colonic mucosal samples (Dillon et al., 2016a) and Lozupone et al. increases in *Desulfovibrio piger* in stool samples from HIV-1 infected subjects (Lozupone et al., 2013). Both, *in vitro* and *in vivo* observations confirmed that both, Enterobacteriales and *Enterobacteriaceae*, strongly correlate with gut $CD4^+$ and $CD8^+$ T-cell depletion, activation and death (Dillon et al., 2016a)(Vujkovic-Cvijin et al., 2013)(Ellis et al., 2011)(Steele et al., 2014), increased levels of sCD14 and other inflammation markers such as IFNγ and IL-1β (Dinh et al., 2015), and lower values of circulating $CD4^+$ T-cell counts (Yang et al., 2016).

Depletion of Firmicutes and Bacteroidetes in HIV-1 infection

Published data also suggested that the abovementioned colonization by Proteobacteria and other potentially pathogenic bacteria might occur alongside significant decreases in relative abundance of commensal members of the phyla Firmicutes and Bacteroidetes in both, treated and untreated HIV-1 subjects (Dillon et al., 2014)(McHardy et al., 2013)(Vázquez-Castellanos et al., 2014)(Vujkovic-Cvijin et al., 2013). Yang et al. and Perez-Santiago et al. both reported decreases in genus *Lactobacillus* following HIV-1 infection and the former linked such decreases to low $CD4^+$ T-cell counts and higher HIV-1 viremia and sCD14 levels (Yang et al., 2016)(Pérez-Santiago et al., 2013). Class Clostridia, including genera *Lachnospira* and *Oribacterium*, have also been reported to be impacted, not only by HIV-1 infection (Nowak et al., 2015), but also by other diseases affecting gut homeostasis (Sokol et al., 2009). Butyrate, as well as other SCFA, is a key element for the regulation of the intestinal homeostasis acting as an energy source for enterocytes and other epithelial cells, and a signaling molecule that modulates intestinal immune responses (Hamer et al., 2008). Not surprisingly, other taxa reported to be depleted in HIV-1 infected subjects contain numerous butyrate producing bacteria such as *Faecalibacterium*, *Roseburia*, *Coprococcus* and *Eubacterium*, among others (Dillon et al., 2014)(McHardy et al., 2013)(Nowak et al., 2015)(Vázquez-Castellanos et al., 2014). Additionally, Mutlu et al. found negative correlations between relative abundance of *Bacteroides* and *Faecalibacterium* and levels of IL-6 and soluble CD14 (Mutlu et al., 2014). Of note, family *Erysipelotrichaceae* within phylum Firmicutes, has been found increased in both, treated and untreated HIV-1 individuals (Dinh et al.,

2015)(Lozupone et al., 2013)(Vujkovic-Cvijin et al., 2013) and has also been linked to other inflammatory disorders (Kaakoush, 2015).

Prevotella to *Bacteroides* shift following HIV-1 infection

While several investigators also found a shift from a *Bacteroides*- to a *Prevotella*-dominated gut microbiota as the main association to HIV-1 infection (Dillon et al., 2014)(Lozupone et al., 2013)(Mutlu et al., 2014)(Vázquez-Castellanos et al., 2014)(Vujkovic-Cvijin et al., 2013), others found no significant changes (McHardy et al., 2013)(Dinh et al., 2015). Nowak et al. found higher relative abundance of *Prevotella* in subjects on ART compared to non-treated HIV-1 infected subjects (Nowak et al., 2015).

Regarding the role of *Bacteroides* and *Prevotella* in immune cell activation, Dillon et al. found a slight inverse correlation between abundance of *Bacteroides* and $CD4^+$ T-cell counts in HIV-1 infected non-treated subjects and that abundance of *Prevotella* was strongly positively associated to activation levels of T-lymphocytes (measured by CD38+ HLA-DR+) as well as to numbers of mucosal Th22/Th17 T-cells and mucosal dendritic cells (mDC) (Dillon et al., 2014)(Dillon et al., 2016b). They described *Prevotella* as a *pathobiont* after finding that this bacterial genus induced higher production and secretion of pro-inflammatory cytokines compared to other genera such as *Ruminococcus* in animal models. Vázquez-Castellanos et al. linked a *dysbiotic* gut microbiota with HIV-1-derived immune alteration by reporting positive correlation between relative abundance of *Prevotella* and *Succinivibrio*, with various markers of T-cell activation and the inflammation marker C-reactive protein (CRP) (Vázquez-Castellanos et al., 2014). Significantly, *Prevotella* has also been described as a pro-inflammatory bacteria in other studies beyond of HIV-1 infection (Lucke et al., 2006)(Kumar et al., 2003).

Reduced bacterial richness and diversity in HIV-1 infection

Following ecology basics, a healthy gut microbiota community (*eubiotic* community) is expected to be richer and more diverse compared to a *dysbiotic* community. While Lozupone et al. reported in 2013 higher richness and diversity in HIV-1 infected ART-naïve individuals than in the corresponding HIV-1 negative controls (Lozupone et al., 2013), other groups reported either no changes (Dillon et al., 2014)(Dinh et al.,

2015)(Vujkovic-Cvijin et al., 2013)(Yang et al., 2016) or reduced bacterial richness and diversity in the HIV-1 infected group (Mutlu et al., 2014)(McHardy et al., 2013)(Nowak et al., 2015). Low gut microbial diversity has been correlated with several markers of disease progression such as $CD4^+/CD8^+$ T-cell ratio, as well as to several markers of monocyte activation (soluble CD14 and soluble CD163) and microbial translocation (LPS and LBP)(Nowak et al., 2015).

Feces vs mucosal samples to characterize the gut microbiota

The use of fecal samples as a proxy of gut microbiota is still on debate since sample source has been underlined as a possible factor affecting gut microbiota composition. Significant differences in taxonomic composition have been observed between stool and gut mucosal samples from same individual independently of HIV-1 infection immune status (Nava et al., 2011)(Eckburg et al., 2005)(Zoetendal et al., 2002). Although mucosal samples might be more relevant because of its proximity to real bacterial niches within human gastrointestinal tract, its difficulty to collect, makes gut mucosa a very interesting but poorly explored environment. Of note, it has been highlighted that differences between HIV-1 positive and HIV-1 negative subjects are more evident in mucosal than in lumen-derived samples (i.e. stool)(Mutlu et al., 2014).

Whole genome sequencing and gut microbiota functional potential

WGS data have shed light on metabolic pathways that result altered in response to HIV-1 infection and have linked them to microbial translocation and immune activation (Vázquez-Castellanos et al., 2014)(Vujkovic-Cvijin et al., 2013). Indoleamine-pyrrole 2,3-dioxygenase enzyme 1 (IDO1) is a human enzyme known to participate in the tryptophan to kynurenine catabolism pathway and in the control of lymphocyte T-cell population functioning and tolerance (Munn and Mellor, 2013)(Katz et al., 2008). It is already well described that kynurenine to tryptophan ratio (KT) in blood is altered in HIV-1 infection and linked to progression to AIDS (Huengsberg et al., 1998). The hypothesis in relation to the gut microbiota is that the enrichment of bacteria capable to catabolize tryptophan to kynurenine using an IDO1 homolog gene favors the gut mucosa disruption hence contributing to microbial translocation and immune activation. While most bacteria are primary producers of tryptophan as well as other amino acids, very few bacteria are known to contain the homolog enzymatic

machinery to the human IDO1 enzyme. Interestingly, bacteria favoring higher levels of KT ratio belong to phyla Proteobacteria which is consistently reported to increase and translocate in HIV-1 infection (Vujkovic-Cvijin et al., 2013)(Mutlu et al., 2014). Particularly, Vujkovic-Cvijin et al. reported positive correlation between levels of KT ratio and relative abundance of *Pseudomonas, Xantomonas, Burkholderia, Stenotrophomonas, Shewanella* and *Bacillus* (Vujkovic-Cvijin et al., 2013). LPS biosynthesis is another metabolic pathway found enriched in the microbiome of HIV-1 infected subjects and positively correlated to reduced bacterial diversity and increased proportion of gram-negative bacteria (Vázquez-Castellanos et al., 2014)(Vujkovic-Cvijin et al., 2013). Genes involved in glutathione and seleno-compound metabolism, as well as in folate and siderophore biosynthesis have also been found enriched in HIV-1 infected individuals (McHardy et al., 2013). Conversely, McHardy et al. also reported lower levels of genes involved in amino acid production and metabolism, fructose/mannose metabolism, CoA biosynthesis and energy processing in HIV-1 infected individuals. Other metabolic candidates linked to HIV-1 gut *dysbiosis* are the choline-derived trimethylamine (TMA) and trimethylamine oxide (TMAO) compounds. Choline degradation is a major source of TMA formation in the human gut by symbiotic anaerobic bacteria. Interestingly, choline catabolism genes were not found to be evenly distributed between major bacterial phyla: while genes were commonly found in phyla Firmicutes and Proteobacteria, they were almost absent in Bacteroidetes (Craciun and Balskus, 2012). A recent study showed that HIV-1 positive individuals exhibited worsened calcified coronary plaque features compared to non-HIV-1 infected controls and that such features were correlated to TMA and LPS levels (Srinivasa et al., 2015). These results would support the pro-inflammatory-bacteria label on *Prevotella* since it is able to produce TMA and derived metabolites, as well as the anti-inflammatory-bacteria label on *Akkermansia* and other bacteria found depleted in HIV-1 infection that are known to be protective against metabolic disorders (Everard et al., 2013).

Effect of ART on the gut microbiota in HIV-1 infection

How the administration of ART modulates the gut microbiota and its contribution to AIDS progression is still unclear. Initial results suggested that ART could have a

negative impact on gut microbial diversity as unexpectedly these ecological values were lower after ART initiation (Nowak et al., 2015).

Table 1. Main findings on human gut microbiota in chronically HIV-1 infected subjects.

Study	Participants		Sample type	Approach	Main findings in HIV+			
	HIV+ (ART)	HIV-			Richness and diversity	Proteobacteria	Firmicutes - Bacteroidetes	Bacteroides to Prevotella
Ellis et al. 2011	10 (6)	5	Mucosa & Stool	16S rRNA	↑	↑		
Lozupone et al. 2013	28 (14)	13	Stool	16S rRNA	↓	↑ Desulfovibrio	↑ Erysipelotrichy	✓
McHardy et al. 2013	40 (20)	20	Mucosa	16S rRNA			↓	
Vujkovic-Cvijin et al 2013	32 (18)	9	Mucosa	16S rRNA	=	↑	↓ Clostridia Bacteroidia	✓
Perez-Santiago et al. 2013	13	0	Stool	16S rRNA			↓ Lactobacillus	
Lozupone et al. 2014	40 (28)	15	Stool	16S rRNA	↓			✓
Mutlu et al. 2014	21 (19)	22	Mucosa & Stool	16S rRNA	↓	↑	↓	✓
Dillon et al. 2014	18 (0)	14	Mucosa	16S rRNA	=	↑	↓	✓
Dinh et al. 2015	21 (12)	16	Stool	16S rRNA	=	↑	↑ Erysipelotrichy	
Vazquez-Castellanos et al. 2015	15 (15)	15	Stool	16S rRNA & Shotgun	↓	↑	↓	✓
Nowak et al. 2015	31 (19)	9	Stool	16S rRNA	↓		↓	
Yang et al. 2016	8	8	Mucosa	16S rRNA	=	↑	↓	✓
Dillon et al. 2016	17 (0)	14	Mucosa	16S rRNA		↑	↓	✓

Main results on gut microbiota analyses in relation to HIV-1 infection.

1.4.2. ACUTE HIV-1 INFECTION

Because of the very early effects of HIV-1 infection on the human gut homeostasis (Sankaran et al., 2008), collecting human's fecal samples soon enough to see the hyper-acute effects on the gut microbiota turns out to be logistically very complex. Hence SIV/NHPM have been used to investigate the HIV-1 pathogenesis during acute infection.

In 2008, McKenna et al. described that the macaque gut microbiota was, similarly to humans, primarily dominated by phyla Firmicutes and Bacteroidetes with low abundance of Proteobacteria and Actinobacteria, even though significant differences appeared at lower taxonomic ranks (McKenna et al., 2008). In 2016, Glavan et al. reported transient increases in relative abundance of potentially pathogenic bacterial taxa including *Pasteurellaceae*, *Aggregatibacter*, *Actinobacillus* and *Mycoplasmataceae* in acute SIV infection (Glavan et al., 2016). Increased levels of phylum Proteobacteria were found in macaque mesenteric lymph nodes supporting that these bacteria would be the ones translocating from gut lumen after SIV infection (Klase et al., 2015). Significant decreases in *Lactobacillus,* a bacterial genus known to be important for regulating and maintaining physiological gut immunity, have been also reported after SIV challenge (Vujkovic-Cvijin et al., 2015).

1.4.3. NON-BACTERIAL MICROBIOME

A longitudinal study in 2010 described the evolution of the fecal bacteriome and virome in healthy individuals and reported a significant temporal covariation between both microbial community types (Minot et al., 2011). Of note, they also reported that the major source of divergence in the virome composition over time was interpersonal variation and highlighted diet as being a key modulator on gut microbial communities. Similar results were published in 2015 when Lim et al. reported that viral richness was low early in life and increased with age, thus suggesting that the virome, similarly to bacteriome, was mainly established through environmental exposure (Lim et al., 2015).

Beyond health, De Vlaminck et al. observed significant changes on the plasma virome, including overall increased viral load and increased abundance of Anellovirus, in immunosuppressed individuals after organ transplant reception (De Vlaminck et al.,

2013). Same year, Li et al. reported similar increase in Anellovirus in plasma from subjects with AIDS both from Africa and United States of America, especially in subjects with low $CD4^+$ T-cell counts (Li et al., 2013). Focusing on HIV-1/SIV recent infection, Handley et al. reported in 2012 a significant expansion of the enteric virome in progressive, but not in non-progressive, SIV-infected monkeys after 24 weeks post-infection (Handley et al., 2012). Authors also highlighted the existence of a common core of enteropathogenic eukaryotic viruses expanding in most of their rhesus macaque models including *Adenoviridae*, *Calciviridae*, *Papillomaviridae* and *Parvoviridae*, after SIV-challenge. Norman et al. highlighted the role of enteric Caudovirales as a key contributors to intestinal inflammation and concurrent bacterial dysbiosis in IBD (Norman et al., 2015).

2. OBJECTIVES

1. Analyze the influence of the gut microbiota in the ability of HIV-1 infected subjects to reach a proper immune reconstitution, control HIV-1 replication and control the chronic inflammation associated to HIV-1 infection.

2. Characterize the coevolution of the gut microbiota and the immune system following acute HIV-1 infection.

3. MATERIAL AND METHODS

3.1. STUDY 1: META HIV-PHENO

3.1.1. STUDY COHORT

To assess the link between HIV-1 infection, gut microbiota and host's immune deterioration and restoration on ART, we set up one test cohort in Barcelona and then validated our 16S rRNA findings in another European independent cohort from Stockholm.

The original test cohort, named BCN0, was enrolled in Barcelona, Catalonia, Spain, between January and December 2014. HIV-1 infected individuals were recruited from the HIV Clinics at the University Hospitals Germans Trias i Pujol and Vall d'Hebrón. HIV-1-negative individuals were mainly recruited from an ongoing prospective cohort of HIV-negative homosexual men at risk of becoming infected by HIV-1 (Coll et al., 2017) who attend quarterly medical and counseling visits including HIV-1 testing at BCN Checkpoint, a community-based center for MSM in Barcelona (Meulbroek et al., 2013). Additional controls were HIV-1-negative partners from HIV-1 infected subjects attending the HIV clinics. The inclusion criteria were: age within 18 and 60 years and body mass index within 18.5 and 30. Exclusion criteria were: (a) any gross dietary deviation from a regular diet including vegetarian, low-carb, etc., (b) antibiotic use during the previous 3 months, (c) pregnancy or willingness to become pregnant, (d) current drug consumption or alcohol abuse, (e) any chronic digestive disease such as peptic ulcer, Crohn's disease, ulcerative colitis or coeliac disease, (f) any surgical resection of the intestines except for appendectomy, (g) any autoimmune disease; and (h) any symptomatic chronic liver disease or presence of hepatic insufficiency defined as a Child–Pugh C score.

HIV-1 infected subjects in the study were selected to represent a wide spectrum of different HIV-1 phenotypes according to their immune status, ART administration, and capacity to control viral replication (Table 2).

Table 2. HIV-1 phenotypes included in the BCN0 cohort.

HIV-1 Phenotype	Antiretroviral treatment	CD4+ T-cell counts (cells/mm^3)	Viral Load (copies/mL)
Late Presenters	No	<200	-
Concordants	Yes (>2 years)	>500	<50
Discordants	Yes (>2 years)	<300 (>2 years)	<50
Early Treated	Yes (started within the first 6 months after HIV-1 infection)	-	<50 for 3 months and no blips once reached under-detectability
ART-Naive	No	>500	>10000
Viremic Controllers	No	-	50-2000 (>2 years)
Elite Controllers	No	-	<50 (>2 years)

Main clinical parameters for the HIV-1 phenotypes included in the BCN0 cohort. Phenotypes were described based on their CD4+ T-cell counts, ART-administration and viral load.

All subjects went through a clinical evaluation following a standardized questionnaire including: a checklist for fulfillment of inclusion and exclusion criteria, anthropometric data, age at study entry, age at HIV-1 diagnosis, gender, ethnicity, city of residence, HIV-1 transmission risk group, history of allergies, antibiotic intake between 3 and 6 months before inclusion, frequency and consistency of feces, history of medical or surgical problems and/or interventions, present and previous ART, history of AIDS- and non-AIDS-related diseases, nadir and most recent $CD4^+$ T-cell counts, HIV-1 RNA levels, history of sexually transmitted diseases and infection by the human papillomavirus (HPV) and/or hepatitis B (HBV) or C (HCV) viruses. The HIV-1 transmission risk categories in our study were mutually excluding: male study participants who reported being homosexual or referred insertive or receptive anal intercourse with other men were included in the MSM category, even if they also reported intravenous drug use or sex with women. Females and non-MSM males reporting past intravenous drug use were classified as drug users (people-who-inject-drugs, PWID). Participants not included in the preceding categories were classified as heterosexual (HTS). All participants went through a proctology evaluation by a specialized HIV-1 physician/proctologist to assess perianal lesions, either HPV-related or not, and test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. No cases of anal cancer were detected.

An internal validation cohort, named BCN1, included 110 individuals from the BCN0 who provided a second fecal sample one month later.

Observations in Barcelona were also externally validated in an independent observational cohort, named STK, which was recruited at the HIV outpatient clinic in the Karolinska University Hospital, Stockholm, Sweden. All HIV-1-infected subjects in the STK cohort were at least 18 years old, had been diagnosed with HIV-1 between 1 and 25 years earlier and were ART-naïve at the time of fecal sampling. HIV-1 uninfected controls were healthy individuals matched by sex and age. Neither patients nor controls had been prescribed antibiotics or probiotics; or had had infectious diarrhea during the preceding 2 months.

3.1.2. LAB PROCEDURES

3.1.2.1. Sample collection

Fecal samples from Barcelona were collected using sterile fecal collection tubes the same day or the day before their clinical appointment and following instructions pre-specified on SOP. Samples were immediately extracted upon arrival to the laboratory. If required, samples were stored at 4 °C overnight until DNA extraction. Before discarding the samples, small amounts of feces were saved both, in dry conditions and suspended in RNA later, for preserving DNA and RNA molecules for further analysis.

Blood samples from Barcelona were collected at the HIV Clinics at University Hospitals Germans Trias I Pujol and Vall d'Hebrón and cryopreserved at -80ºC until immune markers quantification.

Fecal samples from Stockholm were cryopreserved at −80 °C within the first 36 hours after collection and shipped on dry ice in batch to IrsiCaixa AIDS Research Institute, where they were stored at -80ºC until further use.

3.1.2.2. DNA/RNA extraction

Fecal DNA was extracted from fecal samples using the PowerSoil DNA Extraction Kit following manufacturer's instructions. RNA was extracted from RNA later-cryopreserved fecal samples using the Stool total RNA Purification Kit following manufacturer's instructions.

3.1.2.3. 16S rRNA gene amplification

To amplify the variable region V3-V4 from the 16S rRNA gene (expected amplicon size ~460 bp), we used the primer pair described in the MiSeq rRNA Amplicon Sequencing protocol which already have the Illumina adapter overhang nucleotide sequences added to the 16S rRNA V3-V4 specific primers, i.e.: 16S_F 5'-(TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **CCT ACG GGN GGC WGC AG**)-3' and 16S_R 5'-(GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA **GGA CTA CHV GGG TAT CTA ATC C**)-3'.

DNA from BCN0, BCN1 and STK datasets, was amplified in triplicate 25 µL reactions under the PCR conditions listed in Table 3.

Table 3. PCR protocol for the 16S rRNA gene amplification in the MetaHIV-Pheno study.

<table>
<tr><th>AMPLIFICATION MIX</th><th colspan="2">PCR CYCLING CONDITIONS</th></tr>
<tr><td rowspan="5">2,5 µl of non-diluted DNA template
12,5 µl of KAPA HiFi HotStart Ready Mix
5 µl 16S_F (1 µM)
5 µl 16S_R (1 µM)</td><td colspan="2">3 minutes at 95ºC</td></tr>
<tr><td rowspan="3">30 cycles</td><td>30 seconds at 95ºC</td></tr>
<tr><td>30 seconds at 55ºC</td></tr>
<tr><td>30 seconds at 72ºC</td></tr>
<tr><td colspan="2">10 minutes at 72ºC</td></tr>
</table>

PCR conditions to amplify the 16S rRNA gene in fecal samples from Barcelona and Stockholm cohorts.

Once the desired amplicon was confirmed in 1% agarose gel electrophoresis, all three replicates were pooled and stored at -30ºC until sequencing library preparation.

3.1.2.4. Illumina MiSeq sequencing

16S rRNA amplicons were cleaned-up for non-DNA molecules and Illumina sequencing adapters, and dual indices were further attached using the Nextera XT Index Kit as described in the MiSeq 16S rRNA Amplicon Sequencing protocol. After a second round of clean-up, amplicons were quantified using the Quant-iT PicoGreen double strand DNA Assay Kit and Nanodrop 1000, and diluted in equimolar concentrations (4nM) for further pooling. Sequencing was performed on an Illumina MiSeq platform according to the manufacturer's specifications to generate paired-end

reads of 300 base lengths in each direction at the Institut Germans Trias I Pujol (IGTP), Badalona, Spain.

3.1.2.5. Immune measurements

Immune measurements were only performed in blood samples from the BCN0 cohort. Plasma levels markers of enterocyte damage (intestinal fatty acid binding protein (IFABP)), microbial translocation (soluble CD14 and LBP) and systemic inflammation (IL6, CRP and IP-10) were analyzed using commercial ELISA kits in accordance with manufacturer's instructions. If plasma levels were above the upper detection limit, its concentration was calculated as the maximum quantifiable value, i.e. the highest concentration included in the standard curve.

3.1.2.6. Nutritional assessment

The nutritional assessment was only performed in the BCN0 cohort. In a first visit, the HIV Unit dietitian in the University Hospital Germans Trias I Pujol, provided two different validated questionnaires to the study participants and explained them how to complete them.

The first questionnaire was a standardized prospective dietary nutrient survey consisting on recording, as precisely as possible, everything that the patient ate or drank during 3 to 5 consecutive days, including at least one weekend day. Particular emphasis was put on recording the amounts of dietary complements like sugar, salt, sauces, etc. and/or the amount of oil used for cooking or dressing, for example. The collected information was translated into numerical estimates of energy (kcal), and grams and percentage of the total energy intake contributed by proteins, total lipids, saturated, monounsaturated and polyunsaturated fat, total carbohydrates, sugars and digestive polysaccharides, as well as grams of total dietary fiber, vitamins and minerals. Such transformation was done using a non-commercial software (*Programa de Càlcul Nutricional - PCN Pro, version 1.0, compilation 32, authors: David Cantós López, Andreu Farran Codina and Imma Palma Linares)* developed by the University of Barcelona. This program implements the nutrient composition tables developed by the *Centre d'Ensenyament de Nutrició Humana i Dietètica, CESNID (Tablas de composición de alimentos del CESNID, ISBN 84-486-0590-X),* which are the

validated reference tables for nutritional composition used by all dietitians and nutritionists in Catalonia.

The second questionnaire was a recall of food portions taken per week, on average, over the last year. For example, how many glasses of milk the subject usually drinks per week: 1 per day, 1 every 2-3 days, etc. To a great extent, this questionnaire was equivalent to the one used by the Health Professionals Follow-up Study of the Harvard School of Public Health (Wu et al., 2011) but was adapted to include local dietary products and exclude items that are almost not consumed at all in our environment (e.g. peanut butter, etc.).

After the completion of both questionnaires, all study subjects reviewed them with the study dietitian. This allowed clarifying questions, identifying errors in completion and reaching a final agreement on diet consumption.

3.1.3. BIOINFORMATIC ANALYSES

3.1.3.1. OTU picking and taxonomic assessment

Raw amplicon-based MiSeq sequences were initially assessed using the FastQC software (Andrews, 2010) and trimmed with Trimmomatic (Bolger et al., 2014) using adjusted quality and length values: a quality cutoff value of Q30 for both ends, a minimum mean threshold of Q20 for a 30bp-sliding window across sequences and a minimum read length of 250bp. Mothur (Schloss et al., 2009) and the SILVA nr v.119 reference database (Quast et al., 2013) were used for the taxonomic classification of a subset of 10000 quality-filtered and randomly selected 16S rRNA sequencing reads per sample. Additional quality filtering of reads was performed by allowing no ambiguous base calls and discarding those reads out of the limits defined by the primer design when aligned against the SILVA database. Reference-based chimera filtering using the UCHIME algorithm (Edgar et al., 2011) and the SILVA Gold alignment were used to discard chimeric sequences, and the RDP classifier was used for taxonomic classification (Wang et al., 2007). Sequences taxonomically classified as having mitochondrial, eukaryotic, chloroplast or unknown origin were disregarded. OTUs were clustered *de novo* using UPARSE (Edgar, 2013) with a 97% similarity threshold using family level information to reduce the computational load. A

representative sequence for each of the OTUs was extracted and a phylogenetic tree was created using the FastTree2 software (Price et al., 2010) upon Mothur derived alignments in order to calculate phylogenetic UniFrac (weighted and unweighted) distances (Lozupone et al., 2011).

To provide a technical validation of this procedure and confirm the absence of user- or pipeline-dependent biases, similar analyses were repeated by Prof. Dr. Peer Bork's group in EMBL in Heidelberg using LotuS1.35 (Hildebrand et al., 2014) with a maximum accumulated error set to 0.75, a minimal average quality of 27 and truncating reads to 170 bp in short paired-end mode.

3.1.4. STATISTICAL ANALYSES

3.1.4.1. Ecological parameters

To characterize alpha diversity, species richness (Observed richness and the numeric richness estimators Chao1 and ACE) as well as diversity / evenness measurements (Shannon and Simpson indices) were calculated using Vegan (Oksanen et al., 2013) and BiodiversityR (Kindt, 2016) /R packages. For each sample, a subset of 4000 sequencing reads was randomly selected per sample using the *rrarefy* function from Vegan, as representative of the entire dataset. Samples below the corresponding threshold were not considered for the ecological analysis.

Cross-sectional comparisons between groups in each cohort were done using the Wilcoxon Rank-Sum Test or the Kruskal-Wallis and post-hoc pairwise tests corrected for multiple comparisons using Benjamini-Hochberg (BH) or False Discovery Rate (FDR), as corresponded.

3.1.4.2. Taxonomic composition

For the taxonomic characterization, all OTUs with less than 10 counts in 10% of the samples were excluded for each of the cohorts studied. To assess bacterial genera and phyla relative abundances, OTUs were collapsed to these taxonomic levels for each sample so that relative abundances were summed. Cross-sectional comparisons of specific taxa between groups in the different cohorts were done using

the Wilcoxon Rank-Sum Test or the Kruskal-Wallis and post-hoc pairwise tests corrected for multiple comparisons using BH or FDR, as corresponded.

The stringent criterion on the LEfSe algorithm (Segata et al., 2011) (i.e. significant taxa had to differ between every pair of class values) was used to describe which bacterial genera were significantly enriched between sexual orientation groups (MSM vs non-MSM) consistently across HIV-1 immune status (class: sexual orientation, subclass: HIV-1 immune status) and vice versa (class: HIV-1 immune status, subclass: sexual orientation) in the BCN0 and STK cohorts.

To explore if the datasets were structured in clusters, the Partitioning Around Medoids (PAM) algorithm (Reynolds et al., 2006) (Maechler et al., 2015), as implemented in the Cluster R/ package, was used considering Bray-Curtis distances between microbiota samples. Silhouette coefficients were used to assess the goodness-of-fit of the different possible partitions of each cohort sequencing dataset.

Ordination analyses were performed using non-metric multidimensional scaling (NMDS) in a two-dimensional space based on ecological distance matrices calculated by Bray-Curtis as well as UnWeighted and Weighted UniFrac dissimilarities (Lozupone et al., 2011) as implemented in Phyloseq R/ package (McMurdie and Holmes, 2013).

Permutational analysis of variance using Bray-Curtis distances was used to test the influence of different covariates on microbiota structure similarity measures as implemented in Adonis function from Vegan R/ package (Oksanen et al., 2013).

To evaluate the concordance between the NMDS coordinate matrices of the BCN0 and BCN1 datasets we performed a Procrustes analysis (Gower, 1975) and used a Procrustean randomization test (PROTEST) (Jackson, 1995) to test for statistical significance.

A statistical procedure named SIAMCAT and equivalent to one previously developed, tested and validated to classify risk of colorectal cancer based on fecal metagenomic

biomarkers based on LASSO regression classifier (Zeller et al., 2014), was used to characterize the strength of the association between sexual orientation (MSM vs. non-MSM), HIV-1 immune status and global fecal microbiota composition in the BCN0 cohort.

3.1.4.3. Nutrient assessment

To ensure that nutrient amounts were comparable between individuals with different total energy intake, individual nutritional data was standardized by the corresponding energy intake by fitting a linear model (mean= 0 and standard deviation=1) and taking residuals as new nutritional values (Willett et al., 1997). To identify food portion/nutrients-microbiota associations, we used the Dirichlet-Multinomial distribution to implement a penalized logistic regression model (Chen and Li, 2013). Hence, we identified the strongest relationships between nutritional intake and genera abundance and variance in the BCN0 dataset by applying strong penalties to weak associations using a penalized likelihood approach.

Spearman correlations were used to assess the global relationship between nutrients / food portions and the overall gut microbiota composition. Strength of associations was described using R-square and BH-adjusted p-values.

A Permanova test was used to assess overall differences in nutrient consumption between clusters in the BCN0 dataset. The relative consumption of nutrients and food portions by sexual orientation group and microbiota clusters (*Prevotella* vs. *Bacteroides*) was assessed in the BCN0 cohort using bootstrapping to build 95% confidence intervals for the mean difference between groups.

3.1.4.4. Immune markers assessment

Cross-sectional differences between groups in the BCN0 dataset were tested using Wilcoxon-Rank-Sum test or the Kruskal-Wallis and post-hoc pairwise tests corrected for multiple comparisons as corresponded.

3.2. STUDY 2: META HIV-ACUTE

3.2.1. STUDY COHORT

To study the coevolution of the gut microbiota and the immune system after acute HIV-1 infection, we studied 4 groups of subjects recruited in Mozambique: individuals with recent HIV-1 infection (RHI) and HIV-1 negative controls (NEG), which were followed prospectively during 9-18 months, and chronic HIV-1-infected subjects either on ART (CHI_ART) or not (CHI_noART), who provided a single cross-sectional sample for comparison. The study population was enrolled between April 2013 and May 2014 at the Manhiça District Hospital (MDH) in Manhiça, Southern Mozambique. Written informed consent was obtained from all subjects before participation. Adults older than 18 years, who were residents of the established District Surveillance System study area, and who presented to the outpatient clinic of the MDH for non-specific febrile symptoms or voluntary HIV counseling and testing were invited to participate in the study. Chronically HIV-1-infected adults were selected from the outpatient HIV-1 clinic at the MDH. Pregnant women were not allowed to enter the study. Blood was collected by finger prick for HIV-1 rapid antibody testing with Determine HIV ½. Positive results were then confirmed with a more specific Uni-Gold rapid test. Individuals with a positive HIV-1 serology in both rapid tests were referred for clinical management and were not eligible for enrollment into the study. Recent HIV-1 infection was diagnosed in patients with a negative or indeterminate rapid test serology (first test negative or first test positive and second test negative) and positive HIV-1 viremia by reverse transcriptase–polymerase chain reaction testing on frozen plasma using Abbott Real-Time HIV-1 assay according to the manufacturer's instructions with a sensitivity of detection of 150 copies/mL. HIV-1 RNA was quantified by applying a multilevel pooling scheme of 10 samples/pool as described and validated in previous works (Serna-Bolea et al., 2010). The group of HIV-1 negative time-matched controls was selected by computer randomization. RHI and NEG individuals were invited to attend a first study visit 1 month after the screening date to start the follow-up in the context of the GAMA cohort (Pastor et al., 2017). RHI individuals were followed during months 1, 2, 3, 4, 6, 9, 12, 15 and 18 after study enrollment, whereas NEG controls were seen at months 1, 4 and 9 after study enrollment, which was considered as the only realistic feasible follow-up in otherwise healthy subjects in the Mozambican setting. Medical consultation and HIV counseling

were provided at each medical visit. Study participants received antibiotic treatment when required according to their clinical status. They also began ART when needed according to the Mozambican ART guidelines applicable at the time of the study; i.e., when $CD4^+$ T-cell counts were lower than 250 cells/mm^3 or if clinical complications requiring ART initiation developed. Follow-up was terminated if subjects started antibiotic or ART or if they became pregnant. Chronically HIV-1-infected adults (CHI_ART and CHI_noART) provided only one cross-sectional clinical and analytical assessment. In this pilot exploratory study no formal sample size calculation was performed.

Demographic and clinical data were collected in a specific questionnaire which included the study group to which the subject belonged (RHI, NEG, CHI_ART and CHI_noART), the time point of follow-up in months, the participant's gender, age, weight and height, $CD4^+$ and $CD8^+$ T-cell counts, $CD4^+/CD8^+$ ratio, HIV-1 RNA levels, hemogram, body temperature, whether fever was reported or not within the 24h prior to sample collection, whether the patient reported or not diarrhea the week before sample collection and its severity, as well as information on pregnancy, ART and antibiotic use. The dataset also included information on a malaria optic microscopic determination, whether the participant reported malaria within the month before sample collection, the presence of occult blood in feces, the result of several microbiological tests in feces including *Giardia* spp., *Cryptosporidium* spp., *Entamoeba* spp., *Clostridium difficile* toxin, and the result of serologies for HBV, syphilis and *Strongyloides stercoralis*.

3.2.2. LAB PROCEDURE

3.2.2.1. Sample collection

Fecal samples were collected and stored in sterile tubes at -80ºC either in dry conditions and/or suspended in 1ml of RNAlater to preserve RNA molecules in Mozambique. Samples were properly labeled according to subject ID and number of follow-up visit and directly shipped in dry ice to the IrsiCaixa AIDS Research Institute. Once in the receiving lab, they were directly stored at -80ºC until further use.

In all cases, collection procedures were the same for cases and controls.

3.2.2.2. DNA/RNA extraction

Fecal DNA was extracted using the PowerSoil DNA Extraction Kit following manufacturer's instructions from dry frozen fecal samples. RNA was extracted from RNA later cryopreserved fecal samples using the Stool total RNA Purification Kit following manufacturer's instructions.

3.2.2.3. 16S rRNA gene amplification

To amplify the variable region V3-V4 from the 16S rRNA gene (expected amplicon size ~460 bp), we used the primer pair described in the MiSeq rRNA Amplicon Sequencing protocol which already have the Illumina adapter overhang nucleotide sequences added to the 16S rRNA V3-V4 specific primers, i.e.: 16S_F 5'-(TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **CCT ACG GGN GGC WGC AG**)-3' and 16S_R 5'-(GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA **GGA CTA CHV GGG TAT CTA ATC C**)-3'.

All samples from the Mozambican cohort were amplified once in a 75µl reaction under the PCR conditions listed in Table 4.

Table 4. PCR protocol for 16S rRNA gene amplification in the MetaHIV-Acute study.

AMPLIFICATION MIX	PCR CYCLING CONDITIONS	
7,5 µl of non-diluted DNA template 37,5 µl of KAPA HiFi HotStart Ready Mix 25,2 µl of DNA/RNA-free water 2,4 µl 16S_F (10 µM) 2,4 µl 16S_R (10 µM)	5 minutes at 95ºC	
	30 cycles	20 seconds at 98ºC
		15 seconds at 69ºC
		15 seconds at 72ºC
	10 minutes at 72ºC	

PCR conditions to amplify the 16S rRNA gene in fecal samples from the Mozambican cohort.

Once the desired amplicon was confirmed in 1% agarose gel electrophoresis, amplified solution was stored at -30ºC until further sequencing library preparation.

3.2.2.4. RT-PCR testing of eukaryotic viruses

Biomérieux RT-PCR Rgene kits were used to detect Adenovirus and Enterovirus in cryopreserved extracted stool DNA/RNA aliquots following the manufacturer's instructions, respectively. Quantitative results were adjusted using a sensitivity control at 1 copy/µl.

Specific probe/primer mixes for real time-PCR assays (RT-PCR) were used to quantitatively detect Cytomegalovirus, Human Herpesvirus 6A&6B and Human Herpesvirus 8 in cryopreserved DNA aliquots. Probe/primer mixes were combined with the TaqMan Gene Expression Master Mix at 1/20 dilution. Quantitative results were adjusted using the corresponding sensitivity control at 1 copy/µl.

3.2.2.5. Illumina MiSeq and Illumina HiSeq sequencing

16S rRNA amplicons were cleaned-up for non-DNA molecules and Illumina sequencing adaptersm and dual indices were attached using the Nextera XT Index Kit as described in the MiSeq 16S rRNA Amplicon Sequencing protocol. After a second round of clean-up, amplicons were quantified using the Quant-iT PicoGreen double strand DNA Assay Kit and Nanodrop 1000, and diluted in equimolar concentrations (4nM) for further pooling. Sequencing was performed on an Illumina MiSeq platform according to the manufacturer's specifications to generate paired-end reads of 300 base lengths in each direction at the Institut Germans Trias I Pujol (IGTP), Badalona, Spain.

For the WGS, total fecal DNA was fragmented using the Nextera-XT Illumina kit which uses modified transposons to randomly fragment DNA and incorporates nucleotide tags to identify samples (approximately 125 base pairs are added per paired-end reads). One library of approximately 300-basepair-clone insert size was constructed for each sample. Total fecal DNA was sequenced in an Illumina Hi-SeqTM platform at the Institut de Medicina Predictiva Del Cancer (IMPPC), Badalona, Spain.

3.2.2.6. Immune measurements

Plasma and fecal samples from Mozambique were collected and cryopreserved at -80ºC until quantification. A total number of 51 cytokines were quantified in plasma

including general markers of inflammation, cell death and growth factors, angiogenesis, monocyte function and mobility, T- and B- cell function, neutrophil and eosinophil activation, enterocyte damage, intestinal permeability and microbial translocation (Table 5).

Table 5. Immune measurements in blood and feces tested in the Mozambican samples.

BLOOD	IL-1A, MCP-1, GCSF, IFN γ, IL-12, IL-13, IL-7, VEGF, MIG, RANTES, Eotaxin, MIP1 β, IP-10, IL-2R, IFN α, IL-15, GMCSF, TNF α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, MIP1 α, IL-17, IL-8, EGF, CD40 ligand, IL-21, BAFF, CD27, TNFR2, sCD14, LBP, FABP 2, CRP, sCD163, CXCL16, sCD23, B7H1, PDL2, TRAIL, Fas ligand, TGFβ1, IgG and IgA ASCA, EndoCab IgG and EndoCab IgA, zonulin, general antibody types and subtypes
STOOL	Calprotectin, PMN-elastase, zonulin, EDN/EPX, HBD2, secretory IgA1, α1–antitrypsin, S100A12, lactoferrin, ASCA and pANCA and claudin.

List of all immune measurements tested in fecal and blood samples in the Mozambican cohort.

All immune quantifications were performed according to the manufacturer's instructions. Overflow and under limit of detection values were validated for every analyte as the double and the half of the detection limit, respectively.

3.2.3. BIOINFORMATIC ANALYSIS

3.2.3.1. OTU picking and taxonomic assessment

Raw MiSeq sequences were initially assessed using the FastQC software (Andrews, 2010) and trimmed with Trimmomatic (Bolger et al., 2014) using adjusted quality and length values: a quality cutoff value of Q20 in at least 50% of the bases and a minimum sequence average quality of Q20. QIIME 1.9.1 (Caporaso et al., 2010) was used for the taxonomic classification of the 16S rDNA sequences contained in each sample using GreenGenes 13.8 as the reference database (DeSantis et al., 2006). The first step was to join forward and reverse reads per sample using the *join_paired_ends.py* script. The maximum percent differences accepted in the overlapping region was set to 15. Reads were then filtered to contain a maximum of 2 ambiguous bases (N) using the *split_libraries_fastq.py* script. An open-reference OTU picking approach was used to cluster the sequencing reads at 97% sequence similarity and construct OTUs. Briefly, in this *pick_open_reference_otus.py* approach, reads were clustered against

a reference sequence collection and those reads that did not hit any reference sequence were subsequently clustered using UCLUST (Edgar, 2010) in a *de novo* clustering approach. We then used the ChimeraSlayer method (Haas et al., 2011) in the *parallel_identify_chimeric_seqs.py* script to identify chimeric sequences using a PyNast reference from the GreenGenes 13.8 database. Finally, the *make_otu_table.py*, *filter_alignment.py* and *make_phylogeny.py* scripts were used to create final *biom* (including OTU table and taxonomic information) and phylogenetic tree files.

3.2.3.2. Whole genome sequencing

Raw HiSeq sequences were initially filtered using Trimmomatic (Bolger et al., 2014). After removing Nextera adapters, reads were trimmed by their ends by a minimum quality of Q30, by a global minimum length of 100 base pairs and by using a sliding window set at a minimum quality of Q20 each 30 base pairs-long consecutive segments. Human contamination was minimized by mapping the filtered sequencing reads against the human genome and removing the mapped reads with an alignment quality above Q20. Paired filtered reads were then used for the taxonomic characterization of the microbial communities using the MetaPhlan2 software (Truong et al., 2015) with default parameters. Entire set of filtered reads was also aligned using Bowtie2 (Langmead et al., 2013) to the IGC (Li et al., 2014). Resulting alignments were filtered with SAMtools so that only reads with a mapping quality above Q20 were kept. Eventually a subset of 6 million aligned reads was created per sample alignment to ensure that results of gene, enzyme and metabolic pathway richness were comparable between samples.

3.2.4. STATISTICAL ANALYSIS

3.2.4.1. Ecological parameters

To characterize alpha diversity, species richness (Observed richness and the numeric richness estimators Chao1 and ACE) as well as diversity / evenness measurements (Shannon and Simpson indices) were calculated using Vegan (Oksanen et al., 2013) and BiodiversityR (Kindt, 2016) /R packages. For each sample, a subset of 5000 sequencing reads was randomly selected per sample using the *rrarefy* function from

Vegan, as representative of the entire dataset. Samples below the corresponding threshold were not considered for the ecological analyses.

Cross-sectional comparisons between groups were done using the Wilcoxon Rank-Sum Test or the Kruskal-Wallis and post-hoc pairwise tests corrected for multiple comparisons using BH or FDR, as corresponded. To avoid over-representation of groups with longitudinal follow-up compared to the chronically HIV-1 infected groups, we only considered first sample available per individual for the RHI and NEG groups. Moreover, we split RHI samples into two groups: RHI≤6 including first sample collected per individual within the first 6 months of follow-up, and RHI>6 including first sample per individual collected beyond.

Longitudinal changes on the abovementioned ecological parameters in the RHI and NEG groups were tested using Linear Mixed Models (LMM). These models were adjusted to test overall community dynamics and to detect statistically significant increases or decreases of alpha diversity estimators in the defined time ranges (i.e. 1 to 6 months for the RHI group and 1 to 4 months in the NEG group).

3.2.4.2. Taxonomic composition

For the taxonomic characterization, all OTUs with less than 10 counts in 10% of the samples were excluded. To assess genus and phylum relative abundances, OTUs were collapsed to these taxonomic levels for each sample and relative abundances were summed. Cross-sectional comparisons of individual taxa between groups in the different cohorts were done using the Wilcoxon Rank-Sum Test or the Kruskal-Wallis and post-hoc pairwise tests corrected for multiple comparisons using BH or FDR, as corresponded. We used same cross-sectional representation of RHI and NEG groups as cited in the section above.

Longitudinal changes on relative abundances in the RHI and NEG groups in the cohort, were tested using LMM providing same statistical information as cited above for the ecological parameters. All pairwise associations between bacterial taxa were assessed using Spearman's correlation statistic and BH-correction.

Ordination analyses were performed using NMDS in a two-dimensional space based on ecological distance matrices calculated by Bray-Curtis as well as UnWeighted and Weighted UniFrac dissimilarities (Lozupone et al., 2011) as implemented in R/ Phyloseq (McMurdie and Holmes, 2013) wrappers.

Permutational analysis of variance using Bray-Curtis distances as implemented in Adonis function from Vegan (Oksanen et al., 2013) package was used to test the influence of different covariates on microbiota structure similarity measures.

Clustering of bacterial genera into co-abundance groups was performed using the hclust algorithm in stats R/ package (R Core Team, 2014) with ward.D2 methodology on Euclidean distance matrix computed from a relative abundance table of bacterial genera across groups. For each genus and group, we calculated the mean value of all previously scaled individual abundance percentages (force mean=0 and standard deviation=1). A phylogenetic distance threshold of 0.8 allowed us to identify 7 different bacterial clusters on the resulting dendrogram.

3.2.4.3. Eukaryotic virus expansion

Results derived from RT-PCR testing were used as presence/absence of viral DNA/RNA in feces. Comparison of number of individuals with virus in feces among groups at study entry was performed using Fisher's exact statistical test. To resume results from tested samples from same individual at different time points to a single value per individual we used two different criteria: 1) if first sample available from that given individual was positive, that subject was positive, 2) if that given individual had at least 1 sample testing positive along its follow-up, that subject was positive.

4. RESULTS

4.1. STUDY 1: META-HIV PHENO

4.1.1. Baseline characteristics of the study cohort

The test cohort (BCN0) included a total number of 156 subjects from which 129 were HIV-1 positive (82.7%) and 27 HIV-1 negative (17.3%). The internal validation cohort (BCN1) included 110 individuals from which 87 (79.1%) were HIV-1 positive and 23 (20.9%) HIV-1 negative. The external validation cohort (STK) included 84 individuals from which 77 were HIV-1 infected (91.6%) and 7 HIV-1 negative (8.4%).

In Barcelona, the median age of the study participants was 43 years and their median body mass index was 23.8 kg/m^2. Eighty percent of subjects were men, mostly from Caucasian ethnicity. Regarding their transmission risk group, 64% of all subjects were MSM, 26% HTS and 10% PWID. There were 8 (5.1%) elite controllers, 11 (7.1%) viremic controllers, 15 (9.6%) ART-naïve, 13 (8.3%) early-treated, 53 (34.1%) immune concordant, 18 (11.5%) immune discordant, and 11 (7.1%) late presenters. HIV-1-infected subjects were slightly older and were more likely to be HBV and HCV positive than HIV-1 negative controls. HIV-1 positive and HIV-1 negative groups were well balanced in all other factors as shown in Table 6.

Table 6. Demographics of the BCN0 cohort.

		Full dataset	HIV-1 Positive	HIV-1 Negative	P-value (HIV-1 Pos. vs. Neg)	
N (number of subjects)		156	129	27	-	
Gender	Male	124 (79.5%)	101 (78.3%)	23 (85.2%)	0.076	0.600
	Female	31 (19.9%)	28 (21.7%)	3 (11.1%)		0.291
	Transgender	1 (0.6%)	0	1 (3.7%)		0.173
Ethnic Group	Asiatic	1 (0.6%)	1 (0.8%)	0	0.900	1
	Caucasian	124 (79.5%)	101 (78.3%)	23 (85.2%)		0.600
	Hispanic-Latino	28 (18%)	24 (18.6%)	4 (14.8%)		0.786
	Others	3 (1.9%)	3 (2.3%)	0		1
Risk Group	hts	41 (26.3%)	37 (28.7%)	4 (14.8%)	0.027	0.156
	msm	100 (64.1%)	77 (59.7%)	23 (85.2%)		0.014
	pwid	15 (9.6%)	15 (11.6%)	0		0.075
ATB3		2 (1.3%)	2 (1.6%)	0	1	
ATB6		36 (22.4%)	32 (24%)	4 (14.8%)	0.446	
Age (years, median(IQR))		43 (35, 51.3)	44 (36, 52)	37 (34, 44)	0.021	
Profile	Late presenters	11 (7.1%)	11 (8.5%)	0	-	-
	Discordant	18 (11.5%)	18 (14%)	0		-
	Concordant	53 (34%)	53 (41.1%)	0		-
	Early-treated	13 (8.3%)	13 (10.1%)	0		-
	Naive	15 (9.6%)	15 (11.6%)	0		-
	Viremic controllers	11 (7.1%)	11 (8.5%)	0		-
	Elite controllers	8 (5.1%)	8 (6.2%)	0		-
	HIV-1 negative	27 (17.3%)	0	27 (100%)		-
Residency	BCN	51 (32.6%)	36 (27.9%)	15 (55.6%)	0.058	0.007
	BMA	56 (35.9%)	50 (38.8%)	6 (22.2%)		0.125
	Rest	38 (24.4%)	33 (25.6%)	5 (18.5%)		0.622
	NA	11 (7.1%)	10 (7.7%)	1 (3.7%)		0.690
Allergy	No	122 (78.2%)	101 (78.3%)	21 (77.8%)	0.205	1
	Yes	30 (19.2%)	26 (20.2%)	4 (14.8%)		0.603
	NA	4 (2.6%)	2 (1.5%)	2 (7.4%)		0.138
Fecal consistency	Hard	56 (35.9%)	44 (34.1%)	12 (44.4%)	0.535	0.378
	Soft	91 (58.3%)	77 (59.7%)	14 (51.9%)		0.521
	Liquid	5 (3.2%)	5 (3.9%)	0		0.588
	NA	4 (2.6%)	3 (2.3%)	1 (3.7%)		0.536
Abdominal alterations	Yes	23 (14.7%)	22 (17.1%)	1 (3.7%)	0.089	0.134
	No	127 (81.4%)	103 (79.8%)	24 (88.9%)		0.414
	NA	6 (3.9%)	4 (3.1%)	2 (7.4%)		0.277
Deposition frequency	1	88 (56.4%)	70 (54.3%)	18 (66.7%)	0.669	0.288
	2	47 (30.1%)	40 (31%)	7 (25.9%)		0.653
	3	12 (7.7%)	11 (8.5%)	1 (3.7%)		0.692
	4	5 (3.2%)	5 (3.9%)	0		0.588
	NA	4 (2.6%)	3 (2.3%)	1 (3.7%)		0.536
BMI		23.8 (22, 26.1) (n=138)	23.8 (22.1, 26) (n=122)	24.9 (22, 26.9) (n=16)	0.469	
PCR Chlamydia	Positive	9 (5.8%)	9 (7%)	0	0.161	0.360
	Negative	115 (73.7%)	91 (70.54)	24 (88.9%)		0.055
	NA	32 (20.5%)	29 (22.5%)	3 (11.1%)		0.293
PCR Neisseria	Positive	0	0	0	0.109	
	Negative	125 (80.1%)	100 (77.5%)	25 (92.6%)		
	NA	31 (19.9%)	29 (22.5%)	2 (7.4%)		
PCR Human Papilloma Virus	Yes	72 (46.2%)	61 (47.3%)	11 (40.7%)	0.613	0.671
	No	83 (53.2%)	67 (51.9%)	16 (59.3%)		0.530
	NA	1 (0.6%)	1 (0.8%)	0		1
Anal cytology	ASCUS	22 (14.1%)	17 (13.2%)	5 (18.5%)	0.664	0.542
	HSIL	7 (4.5%)	7 (5.4%)	0		0.605
	LSIL	30 (19.2%)	26 (20.2%)	4 (14.8%)		0.603
	Normal	80 (51.3%)	64 (49.6%)	16 (59.3%)		0.402
	NA	17 (10.9%)	15 (11.6%)	2 (7.4%)		0.738
Syphilis serology	Positive	21 (13.5%)	20 (15.5%)	1 (3.7%)	0.262	0.128
	Negative	116 (74.3%)	93 (72.1%)	23 (85.2%)		0.225
	NA	19 (12.2%)	16 (12.4%)	3 (11.1%)		1
HBV co-infection	Positive	19 (12.2%)	19 (14.7%)	0	0.054	0.045
	Negative	112 (71.8%)	91 (70.6%)	21 (77.8%)		0.638
	NA	25 (16%)	19 (14.7%)	6 (22.2%)		0.386
HCV co-infection	Positive	24 (15.4%)	24 (18.6%)	0	0.013	0.015
	Negative	120 (76.9%)	94 (72.9%)	26 (96.3%)		0.005
	NA	12 (7.7%)	11 (8.5%)	1 (3.7%)		0.692
ALT (U/L, median(IQR))		23 (18, 33)	24 (19, 36)	20 (16.5, 24)	0.017	
AST (U/L, median(IQR))		24 (21, 31) (n=138)	25 (21, 31.5) (n=111)	23 (20.5, 27.5)	0.450	
Bilirubin (mg/dl, median(IQR))		0.43 (0.32, 0.58) (n=152)	0.42 (0.32, 0.58) (n=128)	0.43 (0.31, 0.61) (n=24)	0.831	
GGT (U/L, median(IQR))		24 (14, 36) (n=129)	25 (14.2, 39.2) (n=102)	20 (13.5, 30.5)	0.145	

FA (U/L, median(IQR))	70 (58, 82.5) (n=155)	71.5 (61.7, 84) (n=128)	57 (51, 66.50)	<0.01 * (1 outlier)
Cholesterol (mg/dl, median(IQR))	181 (155, 205.5) (n=155)	185 (154.5, 206) (n=128)	164 (157, 197)	0.322
HDL (mg/dl, median(IQR))	48 (41, 58) (n=154)	48 (39, 56.5) (n=128)	49 (43.7, 66.5) (n=26)	0.099
LDL (mg/dl, median(IQR))	106 (87, 128.5) (n=152)	108.5 (87, 130.2) (n=128)	93.5 (85.5, 121.8) (n=24)	0.232
Triglycerides (mg/dl, median(IQR))	104 (71.2, 139) (n=154)	108 (74.5, 142) (n=127)	80 (64.5, 102.5)	0.025
Creatinine (mg/dl, median(IQR))	0.84 (0.75, 0.94) (n=150)	0.84 (0.75, 0.94) (n=127)	0.88 (0.80, 0.99) (n=23)	0.226
Fibrinogen (mg/dl, median(IQR))	345 (302, 399) (n=143)	348.5 (312, 402.8) (n=118)	333 (283, 371) (n=25)	0.149 * (1 outlier)
INR	10.5 (10, 10.9) (n=142)	10.5 (10, 11) (n=117)	10.6 (9.8, 10.7) (n=25)	0.778
Potassium (mmol/L, median(IQR))	4.3 (4, 4.5) (n=152)	4.3 (4, 4.5) (n=127)	4.4 (4.1, 4.6) (n=25)	0.290
Sodium (mmol/L, median(IQR))	141 (139, 142) (n=154)	141 (139, 142) (n=128)	140 (139, 141.8) (n=26)	0.602
Urea (mg/dl, median(IQR))	31 (25, 38) (n=150)	31 (26, 39) (n=126)	28 (23.7, 35) (n=24)	0.063
Hematocrit (%, median(IQR))	42.8 (40.3, 45)	42.4 (40.1, 45)	43.6 (42.3, 45)	0.130
Erythrocytes (x10x12/L, median(IQR))	4.7 (4.3, 4.9)	4.6 (4.2, 4.9)	4.7 (4.5, 4.9)	0.357
Hemoglobin (gr/dl, median(IQR))	14.4 (13.4, 15.1)	14.30 (13.4, 15.1)	14.6 (14.1, 15.0)	0.379
Plaquetas (x10x9/L, median(IQR))	193.5 (167.8, 226.2)	190 (161, 226)	211 (186.5, 226)	0.071
CD4+ T-cell count (cells/mm3, median(IQR))	-	704.5 (468.8, 856.2) (n=128)	-	-
Nadir CD4+ T-cell count (cells/mm3, median(IQR))	-	337 (140, 528.5) (n=127)	-	-
Absolute CD4+ T-cell count (cells/mm3 , median(IQR))	-	700 (462, 860)	-	-
Relative CD4+ (%, median(IQR))	-	32.9 (24.2, 40.2) (n=128)	-	-
Absolute CD8+ T-cell count (cells/mm3 , median(IQR))	-	776.5 (576, 1012) (n=128)	-	-
Relative CD8+ (%, median(IQR))	-	39.3 (33.0, 48.9)	-	-
Lymphocytes (x10x9/L, median(IQR))	2 (1.7, 2.5) (n=146)	2 (1.6, 2.5) (n=122)	2.1 (1.8, 2.3) (n=24)	0.438
Leukocytes (x10x9/L, median(IQR))	5.8 (4.8, 7.2)	5.6 (4.8, 6.7)	7.1 (5.2, 8.4)	0.011

Median (IQR) and p-values for continuous and discrete variables were calculated with the Wilcoxon rank sum and Fisher's tests, respectively. ATB, antibiotic; BMA, Barcelona Metropolitan Area; NA, not available; IQR, interquartile range; BMI, Body Mass Index; U, units.

In Stockholm, 60% of all subjects were men; 23% were MSM, 66% HTS and 11% PWID. Only half were nationals from Scandinavian countries; 62% individuals were Caucasian and 33% were black (Table 7).

Table 7. Demographics of the STK cohort.

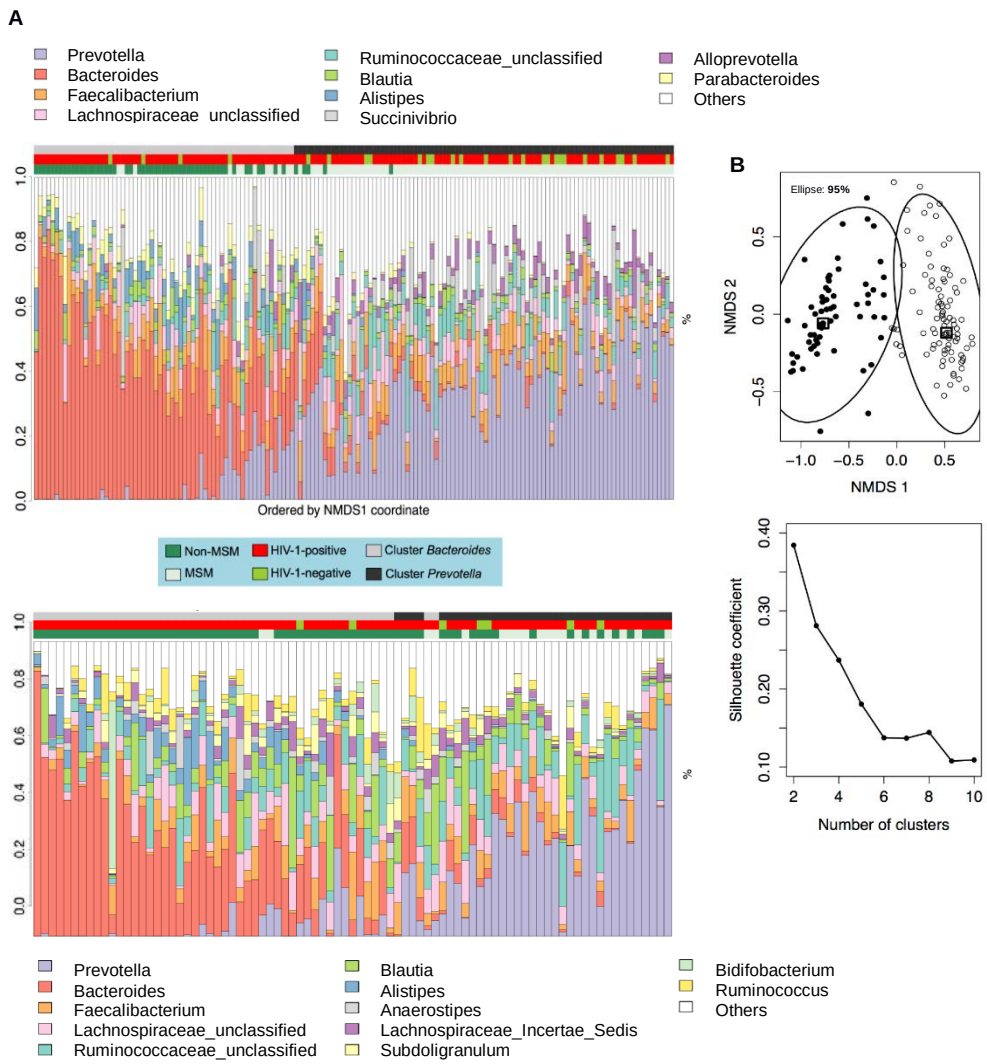
		Full dataset	HIV-1 Positive	HIV-1 Negative	P-value
N of subjects		84	77	7	-
Age (years)		40 (32, 48)	38 (32, 49)	44 (38, 47)	0.615
Gender	Male	51 (60.7%)	46 (59.7%)	5 (71.4%)	0.699
	Female	33 (39.3%)	31 (40.3%)	2 (28.6%)	
Risk Group	HTS	55 (66.5%)	48 (62.3%)	7 (100%)	0.214
	MSM	19 (22.6%)	19 (24.7%)	0	
	PWID	10 (11.9%)	10 (13.0%)	0	
Ethnicity	Asian	2 (2.4%)	2 (2.6%)	0	1
	Black	28 (33.3%)	26 (33.8%)	2 (28.6%)	
	Caucasian	52 (61.9%)	47 (61.0%)	5 (71.4%)	
	Hispanic-Latino	2 (2.4%)	2 (2.6%)	0	
Country of origin	Sweden	39 (46.4%)	34 (43.4%)	5 (71.4%)	0.069
	Kenya	5 (5.9%)	5 (6.5%)	0	
	Finland	4 (4.8%)	4 (5.2%)	0	
	Ethiopia	3 (3.6%)	1 (1.3%)	2 (28.6%)	
	Eritrea	3 (3.6%)	3 (3.9%)	0	
	Nigeria	3 (3.6%)	3 (3.9%)	0	
	Uganda	3 (3.6%)	3 (3.9%)	0	
	Other	24 (28.5%)	24 (31.2%)	0	
CD4+ T-cell count (cells/mm3)*		-	480 (380, 630)	-	-
CD4+ T-cell count (%)*		-	26 (20, 32)	-	-
CD8+ T-cell count (cells/mm3)*		-	970 (660, 1290)	-	-
CD8+ T-cell count (%)*		-	51 (44, 59)	-	-
HIV-1 RNA copies/mL*		-	19100 (1590, 69900)	-	-

Median (IQR) and p-values for continuous and discrete variables were calculated with the Wilcoxon rank sum and Fisher's tests, respectively.

4.1.2. European gay men have a distinct gut microbiota composition

Clustering of the fecal microbiota in BCN0 using a PAM algorithm suggested the presence of at least 2 clusters of bacterial communities (Figure 9A). Such clusters were primarily enriched either in genus *Bacteroides* or *Prevotella* and had a similar bacterial composition to the corresponding previously described human gut microbial enterotypes (Figure 9B)(Arumugam et al., 2011)(Koren et al., 2013).

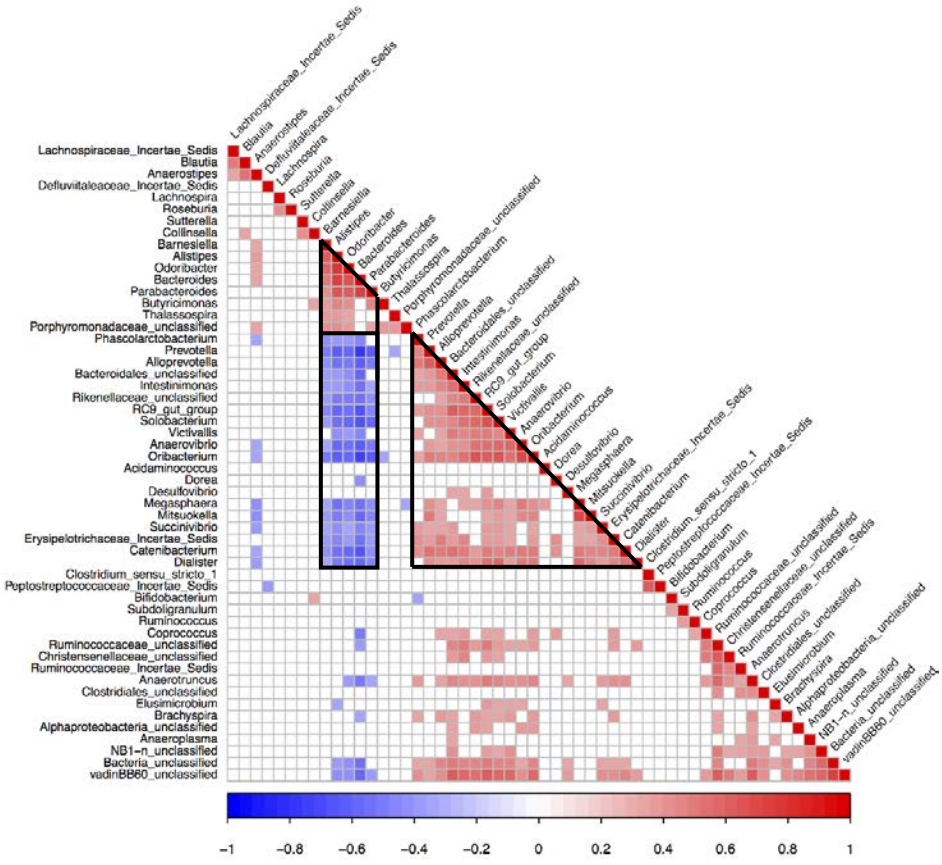
Figure 9. Bacterial composition of the fecal samples from BCN0 and STK.



A, *The bacterial genera composition of the fecal microbiota in the BCN0 cohort (top) was largely determined by HIV transmission group (third horizontal line on the top of the graph), with MSM (light green) being enriched in Prevotella (in purple in the barplot) and non-MSM individuals (dark green) in the Bacteroides (in red in the barplot). Genera with mean abundance of at least 2% across all samples are represented in colors; those with b2% abundance are grouped into the category "Others". Each column represents one individual. Below, same plot for the STK cohort.* ***B****, The PAM analysis of the BCN0 dataset shows that this population structure in this dataset is better explained by 2 rather than more clusters, with reasonable Silhouette support (0.38).*

There were strong positive correlations between the genus *Bacteroides* and *Parabacteroides*, *Barnesiella*, *Alistipes* and *Odoribacter*, as well as between *Prevotella* and *Alloprevotella*, *Catenibacterium*, *Mitsuokella* and *Intestinimonas*, among others. These results highlight that differences between the clustering groups extended beyond the *Bacteroides*/*Prevotella* ratio. The genera correlating with *Prevotella* were negatively correlated with *Bacteroides* and vice versa (Figure 10).

Figure 10. Correlations between *Bacteroides* and *Prevotella*-relative abundances and other bacterial genera.



Spearman correlation between bacterial genera's relative abundances. Only significant values (Holm's-corrected p-values<0.05) are shown. The plot confirms previous observations, i.e.: a) strong positive correlations between Bacteroides, Parabacteroides, Barnesiella, Alistipes and Odoribacter, b) strong positive correlations between Prevotella, Alloprevotella, Mitsuokella and Intestinimonas, among others, and c) strong inverse correlations between the groups including Prevotella and Bacteroides.

We then explored the metadata variables potentially explaining variation observed in the composition of the fecal microbiota according to a univariate ADONIS test of ecological distance. We found possible effects of HIV-1 transmission risk group, gender, feces consistency, place of residency, ethnicity, HIV-1 immune status and altered abdominal transit (Table 8). However, only the HIV-1 transmission risk group (MSM, HTS, PWID) retained statistical significance in a multivariate ADONIS analysis with terms added sequentially (R2: 0.373, p<0.001).

Table 8. ADONIS test on metadata variables collected for the BCN0 dataset.

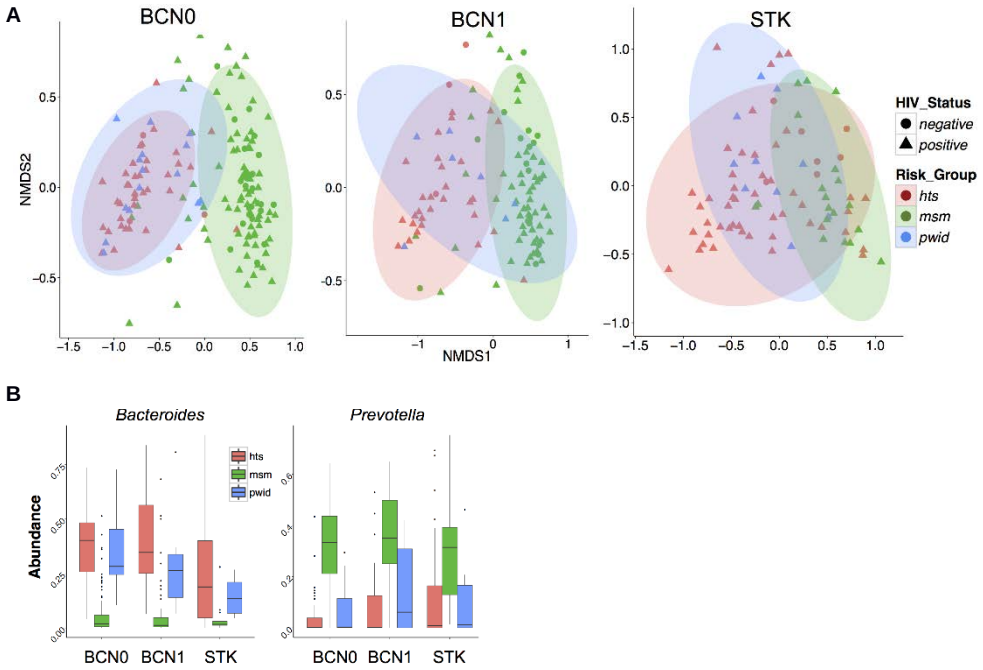
	Univariate		Multivariate *	
	R2	P-value	R2	P-value
HIV-1 Risk group	0,367	<0,001	0,373	<0,001
Gender	0,152	<0,001	0,008	0,407
Feces consistency	0,049	<0,001	0,009	0,335
Residency	0,044	0,014	0,008	0,421
Ethnicity	0,037	0,022	0,018	0,156
HIV-1 Serostatus	0,035	0,003	0,011	0,054
Altered abdominal transit	0,031	0,023	0,005	0,238

* terms added sequentially (first to last)

Based on an ADONIS statistical test, only those factors explaining distance variations among human microbiota profiles in the BCN0 dataset are shown.

As expected, fecal microbiota in the BCN0, BCN1 and STK datasets clustered by HIV-1 risk group rather than by HIV-1 immune status, either using Bray–Curtis or other ecological distances in two-dimensional ordination plots (Figure 11). MSM individuals, both in BCN0 and STK cohorts, belonged to the *Prevotella*-rich cluster, whereas non-MSM individuals (including PWID and HTS risk groups) mainly belonged to the *Bacteroides*-rich cluster.

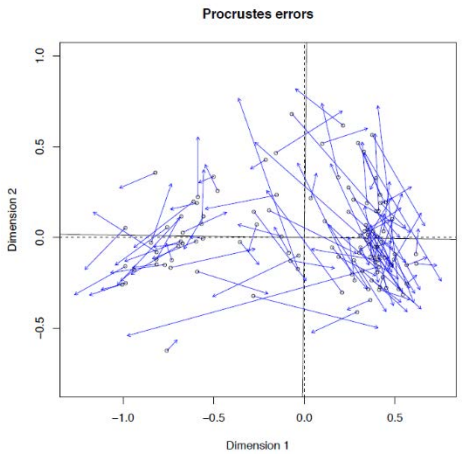
Figure 11. Bacterial composition clustering in the BCN0 and STK cohorts.



***A**, Two-dimensional ordination plots showing that cluster on the left included both, HTS and PWID, whereas cluster on the right, mainly included MSM individuals. **B,** Boxplots (median±IQR) on Bacteroides and Prevotella relative abundances. HTS and PWID were highly enriched in Bacteroides and showed almost no Prevotella, whereas MSM individuals had really high levels of Prevotella and almost no presence of Bacteroides.*

Although a few individuals showed marked differences in their gut bacterial composition between the two-time points in the Barcelona cohorts, fecal microbiota ordination was highly concordant between BCN0 and BCN1 datasets (Procrustes m2= 0.3475, PROTEST p = 0.001), indicating that differences in microbial ordination were not due to random variation but quite stable over time (Figure 12).

Figure 12. Procrustes analysis testing the stability of the gut microbiota composition between BCN0 and BCN1 datasets.



PROTEST Statistics:
Procrustes Sum of Squares (m2): 0.3475
Correlation in a symmetric Procrustes rotation: 0.8078
Significance: 0.001
Permutation: free
Number of permutations: 999

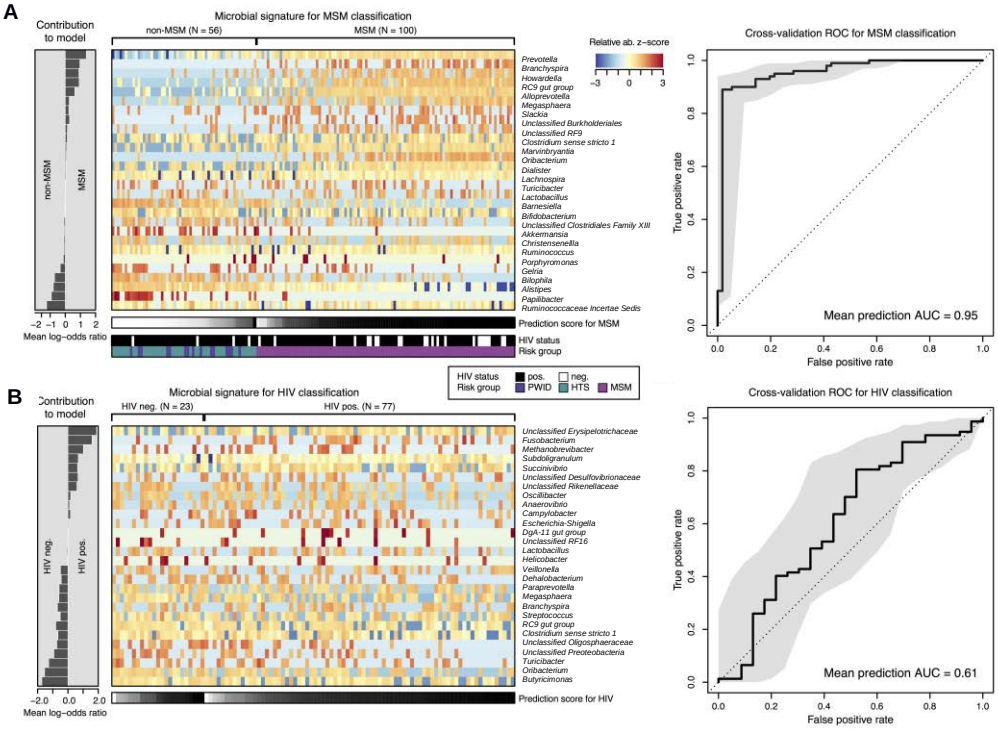
Procrustes analysis was performed with the 110 samples with the two-time points available (BCN0 and BCN1). The analysis shows high overall concordance between the NMDS matrices of the two datasets, even though a few individuals showed marked variations in microbiota ordination. The NMDS matrices were constructed using Bray-Curtis distance. Circles are samples at BCN01 and each circle is connected to the corresponding sample at BCN1 with a blue arrow.

In an analysis accounting for the potential interdependency of HIV-1 transmission risk group and HIV-1 immune status using LEfSe (Segata et al., 2011), we detected consistent differences in both, BCN0 and STK cohorts only by sexual preference group (MSM vs. non-MSM), with enrichment of *Prevotella*, *Alloprevotella*, *Succinvibrio*, *Dorea*, RC 9 gut group, *Desulfovibrio*, *Phascolarctobacterium* and unclassified *Bacteroidales* in MSM, and enrichment in *Bacteroides*, *Odoribacter* and *Barnesiella* in non-MSM individuals.

To quantify the strength of the association between sexual preference group, HIV-1 immune status and global fecal microbiota composition, we applied a previously validated global microbiota classification concept based on LASSO regression (Zeller et al., 2014) to the BCN0 dataset. Cross-validation accuracy was extraordinarily high for sexual preference group (mean area under the curve (AUC) = 95%), confirming a different fecal microbiota composition between MSM and non-MSM individuals (Figure 13A). When we considered only MSM individuals, HIV-1 immune status was

not associated with consistent changes in the global fecal microbiota composition at the genus level (mean AUC=61%) (Figure 13B).

Figure 13. LASSO regression model testing the predictability of HIV-1 infection status and sexual orientation relying on gut microbiota composition.



*Global microbiota classifier by sexual preference group (**A**) and HIV-1 status (**B**). Relative abundances of 28 gut microbial genera collectively associated with MSM and HIV-1 infection, respectively, are displayed as heatmap of log-abundance z-scores with the direction of association indicated to the left. To avoid confounding by sexual preference, the HIV-1 classifier (B) only includes MSM subjects. The mean contribution of each marker bacterial genus to the classification is shown to the left (bars correspond to log-odds ratio in logistic regression). Below each heatmap the classification score of the microbial signature from cross-validation is shown as gray scale. HIV-1 status and HIV-1 risk group are color-coded below the first heatmap (see color key). Cross-validation accuracy of the microbiota classifier is depicted as receiver–operator-characteristic (ROC) curve summarizing mean test predictions made in ten times resampled tenfold cross-validation with the area under the curve (AUC) indicated inside each plot.*

Relative to non-MSM subjects, MSM were younger, were more likely to live in Barcelona city, reported softer fecal consistency, and were less likely to be infected

with HBV and HCV (Table 9). However, none of these factors among others were likely to confound the previous LASSO models.

Table 9. Potential microbiota confounders in MSM vs. non MSM in BCN0 cohort.

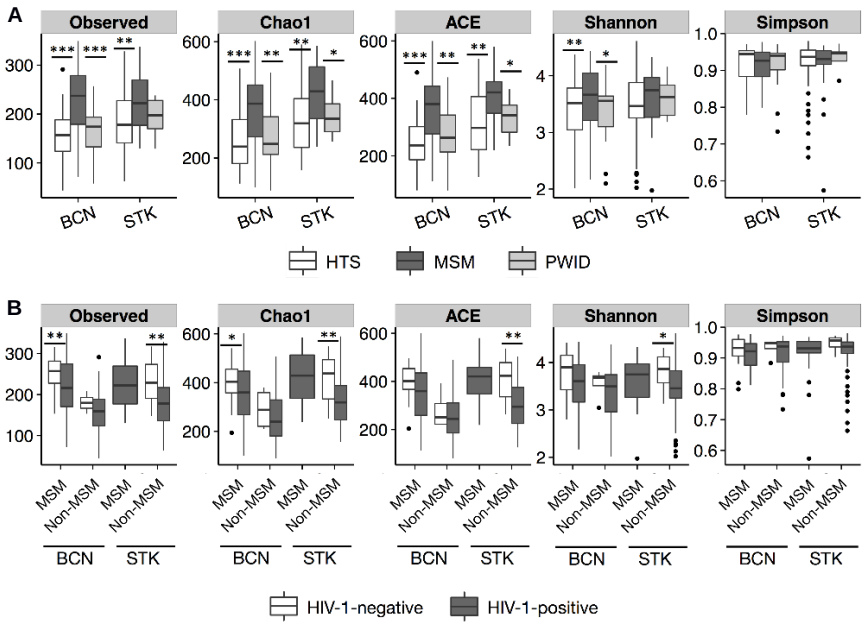
		All (n=156)	MSM (n=100)	Non-MSM (n=56)	P-value	
Age (years)*		43 (35, 51)	38 (34, 46.3)	50 (42, 54)	<0.01	
Gender	**Male**	124 (79.5%)	99 (99%)	25 (44.7%)	<0.01	<0.01
	Female	31 (19.9%)	0	31 (55.3%)		<0.01
	Transgender	1 (0.6%)	1 (1%)	0		1
Ethnicity	**Asiatic**	1 (0.6%)	0	1 (1.8%)	0.163	0.359
	Caucasian	124 (79.5%)	78 (78%)	46 (82.1%)		0.680
	Hispanic-Latino	28 (18%)	21 (21%)	7 (12.5%)		0.201
	Others	3 (1.9%)	1 (1%)	2 (3.6%)		0.293
Residency	**Barcelona**	51 (32.7%)	41 (41%)	10 (17.9%)	0.022	0.004
	BCN Met	56 (35.8%)	30 (30%)	26 (46.4%)		0.055
	Outside BCN Met	38 (24.4%)	22 (22%)	16 (28.6%)		0.437
	NA	11 (7.1%)	7 (7%)	4 (7.1%)		1
BMI (kg/m²)		23.8 (22, 26)	24.3 (22.3, 26.2)	23.5 (20.9, 25.17)	0.053	
Allergy	**No**	122 (78.2%)	79 (79%)	43 (76.8%)	0.876	0.840
	Yes	30 (19.2%)	18 (18%)	12 (21.4%)		0.673
	NA	4 (2.6%)	3 (3%)	1 (1.8%)		1
ATB during the previous 3-6 months		35 (22.4%)	20 (20%)	15 (26.8%)	0.4238	
Fecal consistency	**Hard**	56 (35.9%)	26 (26%)	30 (53.6%)	<0.01	<0.01
	Soft	91 (58.3%)	68 (68%)	23 (41.1%)		0.001
	Liquid	5 (3.2%)	2 (2%)	3 (5.3%)		0.363
	NA	4 (2.6%)	4 (4%)	0		0.297
Abdominal transit alterations	**Yes**	23 (14.7%)	14 (14%)	9 (16%)	0.191	0.815
	No	127 (81.4%)	80 (80%)	47 (84%)		0.669
	NA	6 (3.9%)	6 (6%)	0		0.088
Defecation frequency (per day)	**1**	88 (56.4%)	51 (51%)	37 (66.1%)	0.221	0.091
	2	47 (30.1%)	31 (31%)	16 (28.6%)		0.856
	3	12 (7.7%)	10 (10%)	2 (3.5%)		0.131
	4	5 (3.2%)	4 (4%)	1 (1.8%)		0.655
	NA	4 (2.6%)	4 (4%)	0		0.297
HBV co-infection	**Positive**	19 (12.2%)	2 (2%)	17 (30.4%)	<0.01	<0.01
	Negative	112 (71.8%)	79 (79%)	33 (58.9%)		0.009
	NA	25 (16%)	19 (19%)	6 (10.7%)		0.255
HCV co-infection	**Positive**	24 (15.4%)	6 (6%)	18 (32.1%)	<0.01	<0.01
	Negative	120 (76.9%)	88 (88%)	32 (57.1%)		<0.01
	NA	12 (7.7%)	6 (6%)	6 (10.8%)		0.352
Syphilis serology	**Positive**	21 (13.5%)	18 (18%)	3 (5.4%)	0.056	0.029
	Negative	116 (74.3%)	72 (72%)	44 (78.6%)		0.446
	NA	19 (12.2%)	10 (10%)	9 (16%)		0.311
PCR Chlamydia trachomatis	**Positive**	9 (5.8%)	8 (8%)	1 (1.8%)	0.216	0.158
	Negative	115 (73.7%)	74 (74%)	41 (73.2%)		1
	NA	32 (20.5%)	18 (18%)	14 (25%)		0.309
PCR Neisseria gonorrhoeae	**Positive**	0	0	0	0.296	-
	Negative	125 (80.1%)	83 (83%)	42 (75%)		-
	NA	31 (19.9%)	17 (17%)	14 (25%)		-
PCR Human Papilloma Virus	**Yes**	72 (46.2%)	49 (49%)	23 (41.1%)	0.229	0.403
	No	83 (53.2%)	51 (51%)	32 (57.1%)		0.506
	NA	1 (0.6%)	0	1 (1.8%)		0.359
Anal cytology	**ASCUS**	22 (14.1%)	15 (15%)	7 (12.5%)	0.715	0.812
	HSIL	7 (4.5%)	5 (5%)	2 (3.6%)		1
	LSIL	30 (19.2%)	22 (22%)	8 (14.3%)		0.293
	Normal	80 (51.3%)	48 (48%)	32 (57.1%)		0.318
	NA	17 (10.9%)	10 (10%)	7 (12.5%)		0.779

Median (IQR) and p-values for continuous and discrete variables were calculated with the Wilcoxon rank sum and Fisher's tests, respectively. ATB, antibiotic; BMA, Barcelona Metropolitan Area; NA, not available; IQR, interquartile range; BMI, Body Mass Index; U, units.

4.1.3. HIV-1 infection is independently associated with reduced bacterial richness.

The fecal microbiota was significantly richer and more diverse in MSM compared to non-MSM individuals both in BCN0 and STK, also after correcting for multiple comparisons (Figure 14A). Because of the significance of sexual orientation on overall gut microbiota composition, when we stratified for MSM vs. non-MSM, HIV-1 infection remained consistently associated with reduced bacterial richness in both cohorts (15% to 30% reduction in HIV-1 infected individuals compared to HIV-1 negative individuals) (Figure 14B). Since LASSO predictor did not found consistent changes in overall bacterial composition and HIV-1 infection status, these results suggest that this reduction in microbial richness observed in HIV-1 infected individuals is not bacterial genus-specific.

Figure 14. Bacterial richness and diversity in the cross-sectional European cohorts.

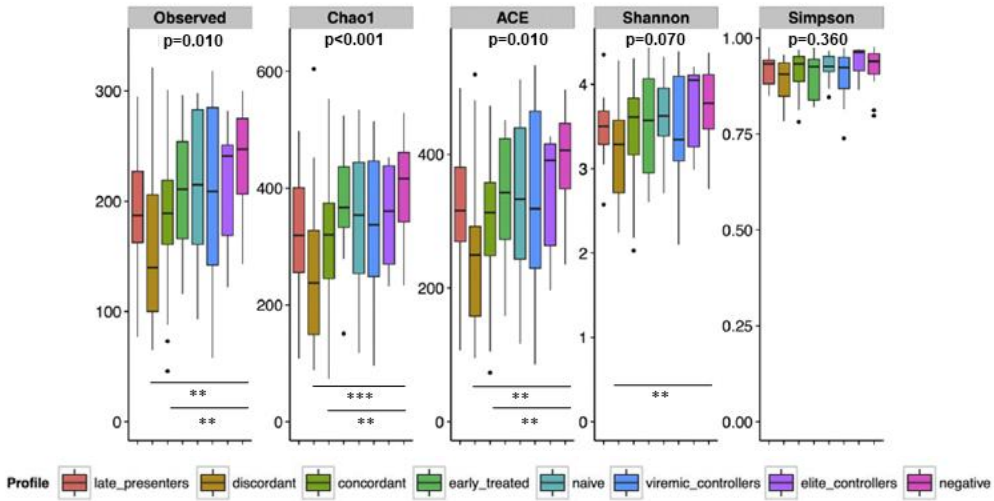


***A**, The highest richness and diversity values in the human fecal microbiota were observed in MSM in both, BCN0 and STK cohorts, compared to both non-MSM transmission risk categories. There were no differences between HTS and PWID. Kruskal–Wallis p-values were adjusted for multiple comparisons using the BH method. Note: "Simpson" refers to 1-Simpson index. The remaining ecological index names are self-explanatory. *p b 0.1, **p b 0.05, ***p b 0.001. **B**, When stratifying for sexual orientation, in both*

*European cohorts, HIV-1 infection was consistently associated with reduced values for bacterial richness and diversity irrespective of individuals being MSM or non-MSM. Comparisons were made with the Wilcoxon rank sum test with continuity correction. Note: "Simpson" refers to 1-Simpson index. The remaining ecological index names are self-explanatory. *p b 0.1, **p b 0.05, ***p b 0.001.*

In the BCN0 cohort, the lowest microbial richness and diversity values were observed among the HIV-1-infected individuals with an immune-virological discordant phenotype (Figure 15). Subjects with an immune-virological concordant phenotype had higher microbial richness than those immune-virological discordant individuals, but, still showed reduced bacterial richness compared to HIV-1 negative controls. This observation suggests that despite adequate immune recovery [median (IQR) $CD4^+$ T-cell counts: 761 (640, 932) cells/mm^3] at the time of testing, ART had not been able to fully normalize microbial richness in these subjects.

Figure 15. Bacterial richness and diversity among different HIV-1 phenotypes.



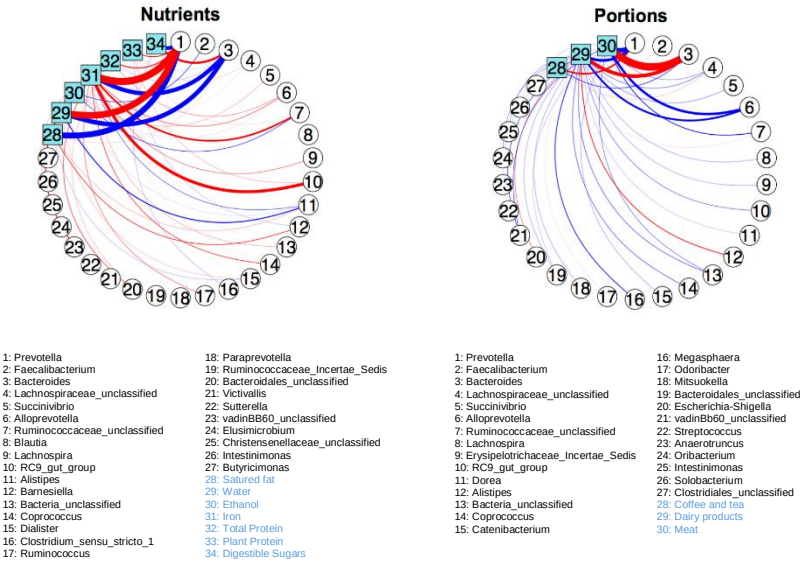
*HIV-1-infected subjects with an immune discordant phenotype (i.e. those which do not recover good $CD4^+$ T-cell counts despite at least 2 years of effective ART) had the lowest microbiota richness of all HIV-1 phenotypes. Individuals with an immune concordant phenotype (i.e. those achieving good $CD4^+$ T-cell counts reconstitution on ART) also had lower microbiome richness than HIV-1-negative individuals, but not as low as immune discordant subjects. "Simpson" refers to 1-Simpson index. The remaining ecological index names are self-explanatory. Comparisons were done using a Kruskal–Wallis test including post-hoc pairwise analyses. Benjamini–Hochberg-adjusted p-values are shown at the top of each index; for post-hoc pairwise comparisons: *p b 0.1, **p b 0.05, ***p b 0.001*

4.1.4. Diet had a minor impact on gut microbiota composition in the BCN0 cohort.

Although long-term dietary patterns have been previously linked to alternative enterotype states (Wu et al., 2011), the effect of diet on the gut microbiota composition was limited in our BCN0 cohort. None of the diet components tested was selected by a multivariate LASSO regression model as a consistent predictor of the microbiota clustering. Subjects belonging to the *Prevotella* cluster and MSM individuals had significantly higher total energy intake. Therefore, all subsequent nutritional analyses were normalized for this factor.

The Dirichlet statistical approach used to seek for links between bacterial genera and diet highlighted several categories: saturated-fat, water, ethanol, iron, total protein, plant protein and digestible sugars for nutrients; and coffee/tea, dairy products and meat for food portions (Figure 16).

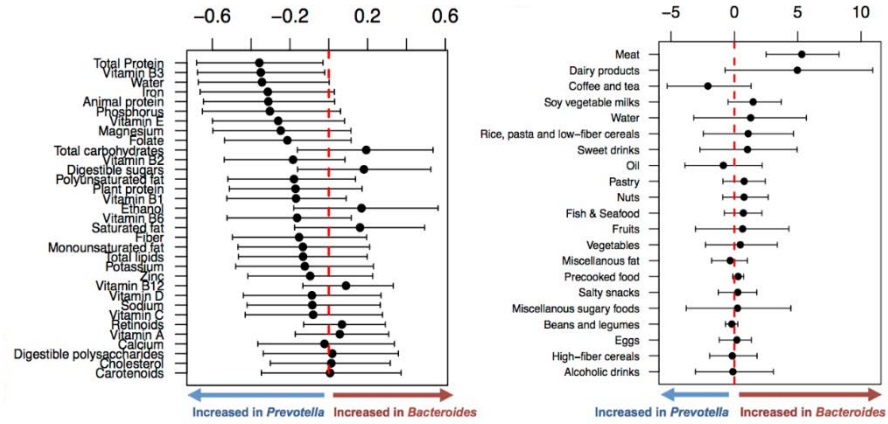
Figure 16. Main correlations between bacterial genera and nutrients / food portion consumption.



Main associations between bacterial genera, normalized amounts of nutrients (left) and food portions (right), according to a Dirichlet multinomial regression model. Positive and negative associations are shown in red and blue, respectively. Line thickness is proportional to the strength of the association.

Of all links identified, the only significant differences between groups after adjusting for multiple comparisons (FDR≤0.1) were increased consumption of meat in the cluster *Bacteroides* (Figure 17) and increased intake of dietary water in MSM.

Figure 17. Differences in nutrient and portions of food consumption between individuals enriched in *Bacteroides* and individuals enriched in *Prevotella*.



Mean and 95% confidence intervals for the differences between clusters in consumption of nutrients (left) and portions of food (right). Comparisons were significant if the 95-confidence interval did not cross 0 (dashed red line).

4.2. STUDY 2: META-HIV ACUTE

4.2.1. Baseline characteristics of the study cohort

The study included a total of 312 fecal samples collected from 49 RHI and 55 NEG individuals that provided longitudinal samples, and from two chronically HIV-1 infected cross-sectional groups: 27 CHI_ART and 71 CHI_noART. All demographic data is shown in Table 10.

Both CHI_ART and CHI_noART group participants were significantly older and had higher BMI scores than NEG and RHI groups at baseline. As expected, the NEG group showed the highest values for the $CD4^+$ T-cell counts whereas no differences were observed among the three HIV-1 infected groups. The RHI group presented the highest $CD8^+$ T-cell counts, followed by the CHI_noART and the CHI_ART group. Thus, the $CD4^+/CD8^+$ ratio was highest for the NEG group (median 1.65; IQR: 1.17, 2.17), while it ranged between 0.4 and 0.6 for all the HIV-1 infected groups. RHI

individuals had 3.5 times higher viral loads than the CHI_noART. A significant decrease in viral load in the RHI group was observed between the screening viral load (i.e. tested at first hospital visit) and that from their first follow-up visit (paired Wilcoxon rank sum test, p=3.81-05). Of note, almost all individuals reporting a positive test and treated Malaria the previous month to study enrollment belonged to the RHI and NEG groups.

Table 10. Demographics of the Mozambican dataset.

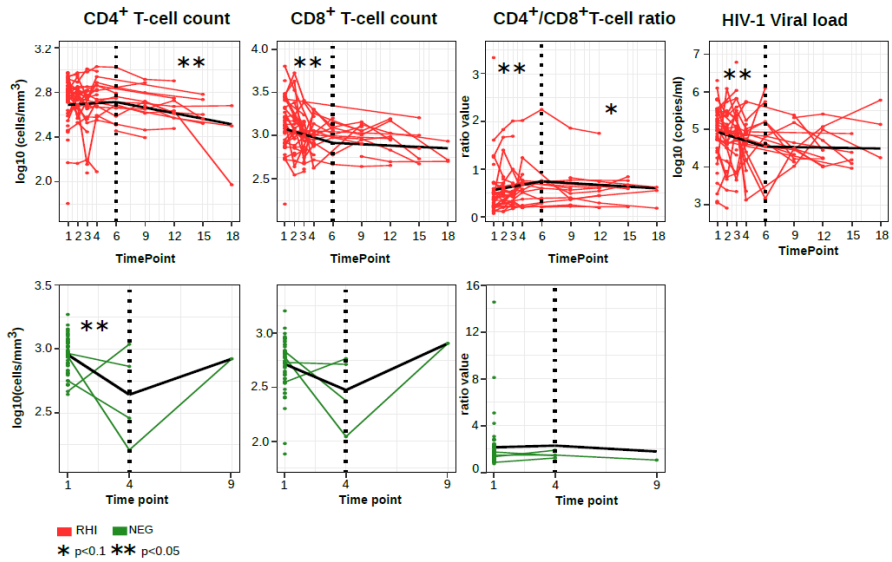
		Recent HIV-1 infection	Chronic HIV-1 infection		HIV-1 Negative	P value	
			ART-naive	On ART		RHI vs neg	Overall
N = 202		49	71	27	55		
Female Gender		32 (65%)	52 (73%)	15 (55%)	43 (78%)	0.189	0.154
Age (years)		26 (20; 30)	35 (29; 45.5)	42 (36.5; 46.5)	25 (21; 37)	0.210	<0.001
Weight (kg)		55 (49; 62)	63 (53.5; 68.5)	61 (60; 68.5)	58.5 (52; 67)	0.093	0.007
Height (cm)		160 (155; 169.7)	160 (154.2; 164)	164 (157.5; 167.5)	163.9 (160; 170.8)	0.192	0.025
BMI		21 (19.1; 23.5)	23.7 (21; 27.6)	23.3 (21.5; 25)	22.2 (18.5; 25)	0.637	<0.001
Pregnancy		3 (6.1%)	0	0	7 (12.7%)	0.730	0.003
$CD4^+$ T cell /mm3		572 (409; 677)	533 (430.5; 726)	460 (366; 594.5)	928 (741; 1142.8)	<0.001	<0.001
$CD8^+$ T cell /mm3		1229 (740; 1683)	988 (763.5; 1304)	834 (657.5; 1130)	591 (387.5; 689.7)	<0.001	<0.001
$CD4^+/CD8^+$		0.42 (0.23; 0.70)	0.56 (0.38; 0.79)	0.52 (0.33; 0.88)	1.65 (1.21; 2.24)	<0.001	<0.001
HIV-1 RNA at Timepoint 1 (copies/mL)		108220 (31898; 273650)	33400 (7288; 105200)	75 (75; 272.5)	-	-	<0.001
HIV-1 RNA at screening (copies/ml)		2752700 (179220; 15078000)	-	-	-	-	-
Time since HIV-diagnosis, days		-	1344 (977; 1737)	1814 (1463; 2302)	-	-	0.018
Time on ART, days		-	-	1257 (678; 1640)	-	-	-
Fiebig stage at screening	III	24 (49%)	-	-		-	-
	IV	7 (14.2%)	-	-		-	-
	V	7 (14.2%)	-	-		-	-
	VI	11 (22.6%)	-	-		-	-
White blood cells		5.8 (4.4; 6.7)	5.2 (4.2; 6.4)	3.7 (3.2; 5)	5.2 (4.4; 6.5)	0.413	<0.001
Hemoglobin		12.1 (10.9; 13.1)	11.8 (10.4; 12.7)	11.4 (10.8; 12.7)	12.3 (11.6; 13.2)	0.409	0.114
Hematocrite		36.9 (33.5; 39.8)	35.6 (32.7; 38.5)	34.4 (33; 38)	37.7 (34.7; 40.1)	0.320	0.044
Platelets		184.5 (140.7; 230.7)	205 (174.5; 258)	192 (173.5; 216)	226 (185; 265.5)	0.004	0.028
Hepatitis B		4 (8.1%)	2 (2.8%)	3 (11.1%)	1 (1.8%)	0.173	0.125
Syphilis		2 (4.1%)	0	1 (3.7%)	4 (7.3%)	0.682	0.089
Diarrhea, previous week		7 (14.3%)	3 (4.2%)	1 (3.7%)	4 (7.3%)	0.343	0.214
Temperature (ºC)		36.3 (36.2; 36.5)	36.4 (36.2; 36.4)	36.4 (36.2; 36.4)	36.2 (36.1; 36.5)	0.956	0.509
Fever, previous 24h		7 (14.3%)	3 (4.2%)	1 (3.7%)	3 (5.4%)	0.186	0.186
Ritchie test		2 (4.1%)	2 (2.8%)	0	1 (1.81%)	0.600	0.864
Entamoeba hystolitica		2 (4.1%)	6 (8.4%)	1 (3.7%)	2 (3.6%)	1	0.733
Giardia spp		2 (4.1%)	2 (2.8%)	0	5 (9.1%)	0.438	0.236
Cryptosporidium spp		0	2 (2.8%)	0	0	1	0.635
Clostridium difficile		2 (4.1%)	0	1 (3.7%)	1 (1.8%)	0.602	0.458
Strongyloides		1 (2.0%)	0	0	1 (1.8%)	1	0.636
Malaria, prev. month		7 (14.3%)	1 (1.4%)	0	9 (16.4%)	1	<0.001
Current Malaria test		2 (4.1%)	1 (1.4%)	0	0	-	0.071
Malaria test severity		3 (1.5; 3)	4 (4; 4)	NA	NA	-	0.071

Only first sample is considered per individual in the RHI and NEG groups. In the CHI_ART and CHI_noART groups, the only fecal sample provided is considered. Continuous variables are tested using ANOVA

*statistical test (except for Time with HIV variable which is tested using a Student T test). Categorical variables are tested using a Fisher statistical test. Significance threshold is set to p=0.05. *Malaria test was only performed in subjects reporting febrile symptoms. Malaria severity is measured in a scale 1 to 5, from light to severe.*

During the first 6 months of follow-up after HIV-1 acquisition, the RHI group showed statistically significant decreases in $CD8^+$ T-cell counts and viral load, together with a significant increase in $CD4^+/CD8^+$ ratio (Figure 18). Such trends were not observed beyond month 6. $CD4^+$ T-cell counts remained stable along the first six months and seemed to decrease after time point 6. Additionally, we observed significant decreases of pro-inflammatory cytokines along the first 6 months after HIV-1 acquisition: IL-12, IP-10, BAFF, Fas ligand, TNFR2 and PDL2. IL-17 which has been described as playing a dual role in inflammatory processes, showed a significant increase along the first 6 months, as well as IgA and IgG measurements in blood. In stool test, significant increase of PMN elastase levels along the first 6 months after HIV-1 acquisition was detected.

Figure 18. Evolution of $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ T-cell ratio and viral load in the RHI and NEG groups along follow-up.



*LMM showing longitudinal evolution of $CD4^+$ and $CD8^+$ T-cell counts and HIV-1 RNA levels in RHI (red) and NEG individuals (green). Slopes (black) were obtained using LMM. Statistically significant differences from 0 (flat slope) are shown with asterisks. P-values: *<0.1; **<0.05*

4.2.2. Recent HIV-1 infection is followed by increased fecal adenovirus shedding

HIV-1 infection was associated with increased fecal shedding of Adenovirus measured by RT-PCR (Table 11). This was observed in RHI (53% of subjects) but also in chronically HIV-1-infected individuals, regardless of whether they were ART-naïve (51%) or received ART (44%) (all p values <0.05 in comparison to the 20% of HIV-1 negative individuals). Conversely, increased shedding of Cytomegalovirus and Enterovirus was only observed in CHI_noART subjects (11% and 21%, respectively), compared to the NEG group (2% and 4%, respectively) (all p values <0.05). Human herpes viruses 6A, 6B and 8 were not detected in any individual, despite using proper extraction and positive RT-PCR controls.

Table 11. Detection of eukaryotic viruses on human feces using RT-PCR.

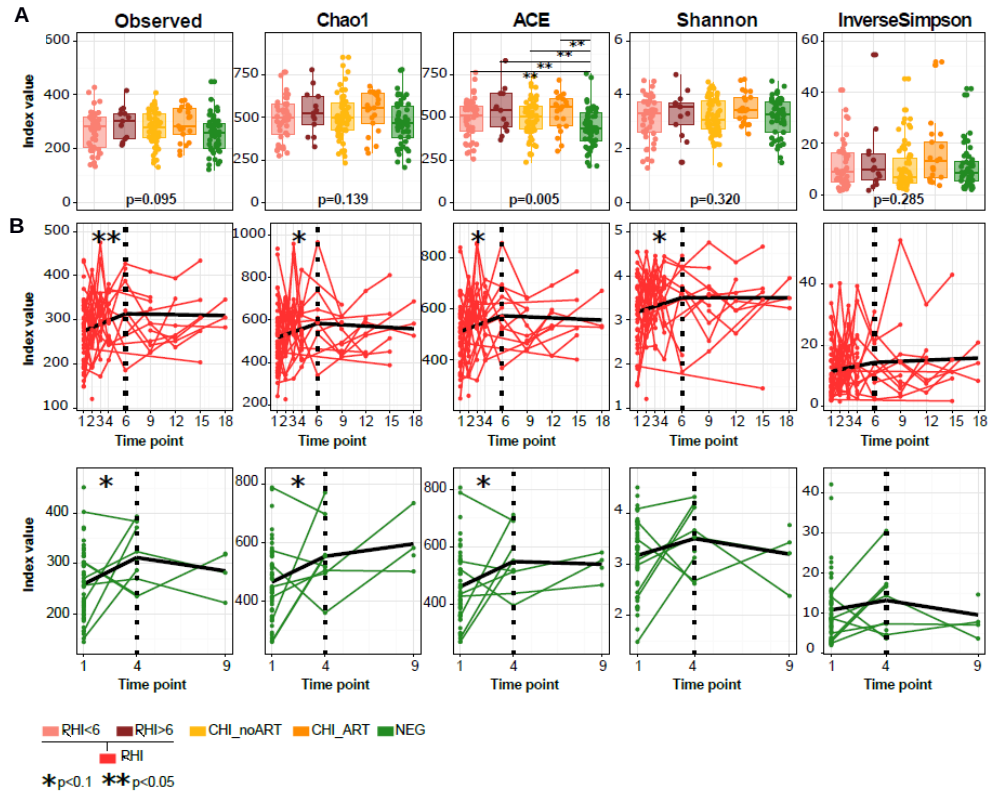
	Recent HIV-1 infection	Chronic HIV-1 infection		HIV-Negative
		ART-naive	On ART	
Adenovirus	26/49 (53.2%)**	36/71 (50.7%)**	12/27(44.4%)**	11/55 (20.0 %)
Cytomegalovirus	3/49 (6.1%)	8/71 (11.3%)*	2/27 (7.4%)	1/55 (1.8%)
Enterovirus	1/43 (2.4%)	4/19 (21.1%)*	1/25 (4.0%)	2/45 (4.4%)
Human Herpes Virus 6A, 6B & 8	0/49 (0%)	0/71 (0%)	0/71 (0%)	0/55 (0%)

*Numbers (percentage) of subjects with detectable virus in feces by qualitative commercial real-time PCR. To avoid ascertainment bias due to different follow-up between groups, only the first fecal sample available for testing was used for comparison (stringent criteria). *p <0.1; **p<0.05, Fisher's pairwise comparisons relative to HIV-1 negative.*

4.2.3. Recent HIV-1 infection is followed by transient non-HIV-1-specific changes in the gut bacteriome

Bacterial genus richness and diversity were not different between groups in cross-sectional comparisons except for the ACE richness numeric parameter, which was significantly lower in HIV-negative subjects than in the remaining groups (Figure 19A). Longitudinally, richness and diversity increased during the first 6 and 4 months in both RHI and NEG subjects, respectively, with similar slopes between the two groups (Figure 19B).

Figure 19. Cross-sectional comparison of richness and diversity ecological parameters at baseline as well as their evolution in the RHI and NEG groups.

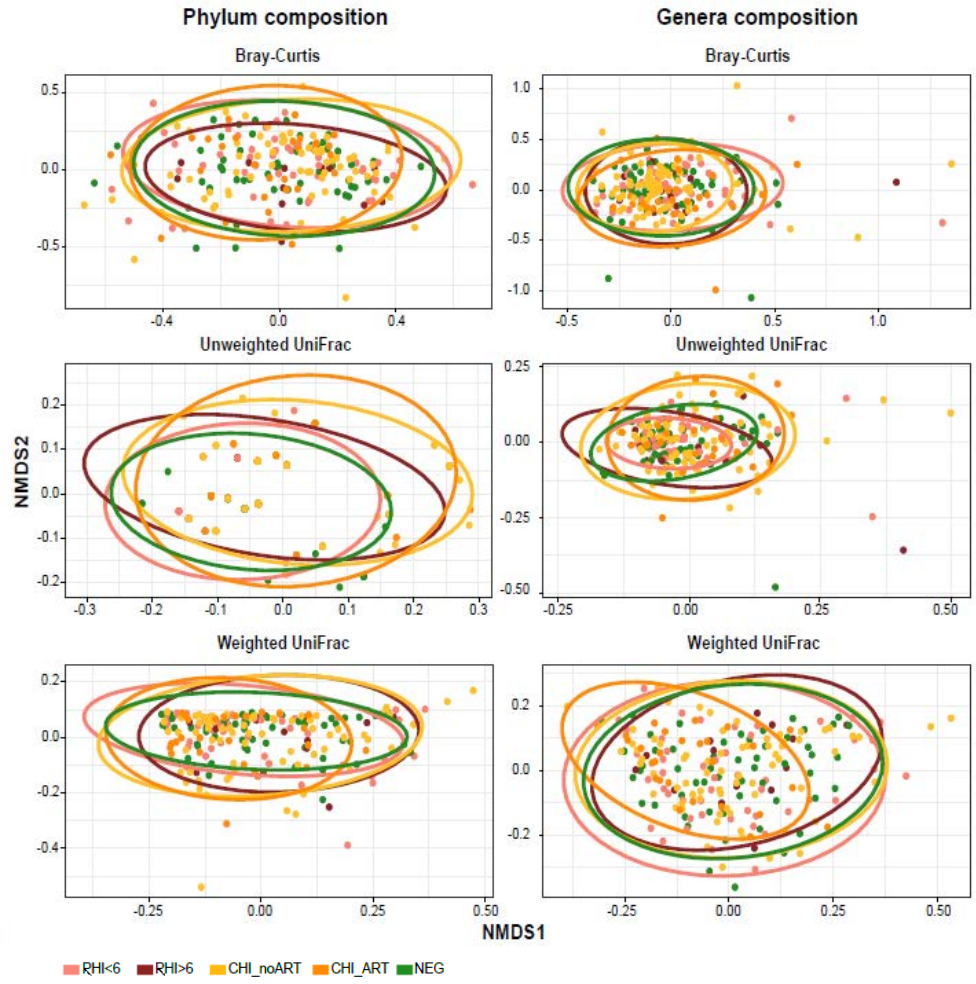


***A**, Median ± IQR values in different cross-sectional comparison groups. Kruskal-Wallis p-values are shown at the bottom of each plot. Asterisks (p-values: *<0.1; **<0.05) highlight statistically significant Tukey post-hoc pairwise differences between groups, corrected for multiple comparisons (FDR <0.05). **B**, LMM of the richness and diversity indices tested in RHI (red) and NEG (green) individuals over time. Horizontal axes show months after study enrollment. Thick black lines correspond to the modelled slope of each parameter. Vertical dashed lines show the inflexion point used for modelling in b and c. Statistically significant differences from 0 (flat slope) are shown with asterisks. P-values: *<0.1; **<0.05.*

Using NMDS on bacterial phyla and genera composition data matrices, there were no significant clustering patterns between cross-sectional comparison groups, regardless of the distance metric used (Figure 20). PAM algorithm did not support anymicrobiota clustering, and none of the metadata variables showed a significant explanatory effect on gut microbiota composition groups in PERMANOVA analysis.

However, there was a statistically significant reduction in genus beta-diversity from RHI<6 to RHI>6.

Figure 20. Two-dimensional ordination plots using bacterial phyla and genera relative abundance data matrices.

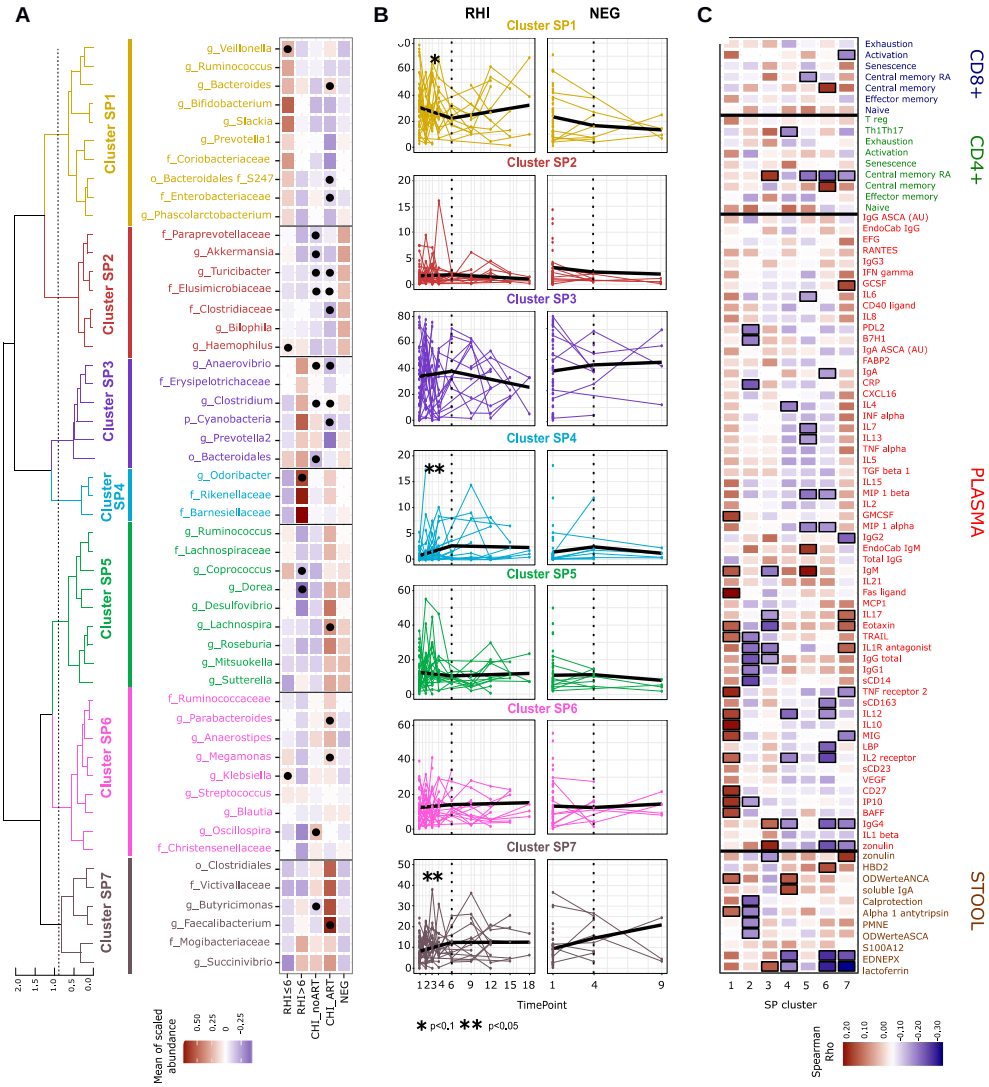


Non-metric multi-dimensional scaling plots using three different ecologic distances summarizing the 16S-rDNA gut microbiota composition at microbial phylum (left) and genera (right) taxonomic ranks.

To evaluate the longitudinal evolution of the bacterial genera, 7 co-abundant bacterial genera clusters were identified and named SP1 to SP7 (Figure 21A). Only clusters SP1, 4 and 7 showed significant longitudinal changes during follow-up on LMM, and

only in subjects with RHI (Figure 21B). Cluster SP1, which was associated with increased plasma levels of cytokines involved in TNF-mediated innate responses (IL-10, IL-12, IP-10, BAFF, CD27, IL-2 receptor, Fas ligand and TNF receptor 2), decreased during the first 6 months of follow-up (Figure 21C). Conversely, clusters SP4 and SP7, which included *Odoribacter*, Rikenellaceae unclassified and Barnesiaellaceae unclassified; and Clostridiales unclassified, *Butyricimonas*, *Faecalibacterium* and *Succinivibrio*, respectively, increased during the same period. There was no evidence, however, that the longitudinal evolution of such clusters was significantly different between RHI and NEG (Figure 21B). Clusters SP2 and SP3 did not significantly change in abundance over time but showed reduced abundance of *Akkermansia*, *Anaerovibrio*, and *Clostridium* in CHI_noART, relative to HIV-negative individuals (Figure 21A). Moreover, SP2 was negatively correlated with markers of microbial translocation (sCD14), inflammation (IP10) and gut integrity (fecal calprotectin) (Figure 21C).

Figure 21. Evolution of the co-abundance bacterial clusters in the NEG and RHI groups over follow-up and their relation to immune parameters.



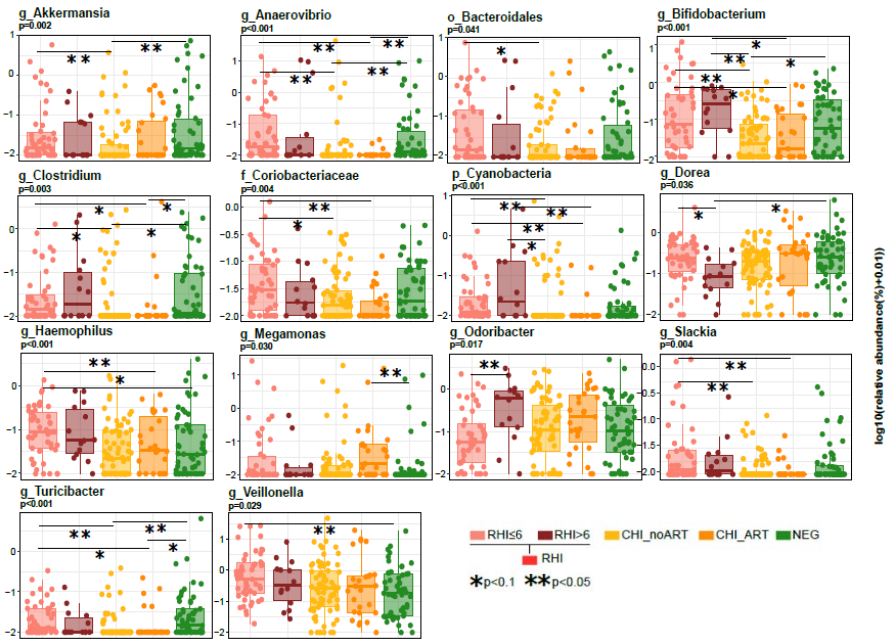
A, *Co-abundant bacterial genera clusters (SP1 to SP7) obtained using 16S rRNA sequencing. The color gradient is proportional to the mean of scaled individual relative abundance values (mean=0, standard deviation=1) per bacterial genera and study group. Dots show statistically-significant differences in genus abundance relative to NEG group.* ***B,*** *LMM of the longitudinal evolution of bacterial clusters in RHI and NEG individuals. Horizontal axes show months after study enrollment. Thick black lines correspond to the modelled slope of each bacterial cluster. Statistically significant differences from 0 (flat slope) are shown with asterisks. P-values: *<0.1; **<0.05.* ***C,*** *Spearman's correlation between bacterial clusters and: CD8^{+}*

(blue), CD4⁺ (green), blood (red), and stool (brown) immune parameters. The color gradient is proportional to the Spearman's Rho value. Black boxes highlight unadjusted statistically significant correlations (p-value <0.05).

4.2.4. An HIV-1-specific signature in the gut microbiota previously associated with chronic inflammation is observed in chronically HIV-1 infected subjects

Cross-sectional comparisons of individual bacterial genera confirmed depletion of *Akkermansia*, *Anaerovibrio*, *Bifidobacterium*, and *Clostridium* in CHI_noART relative to NEG individuals (Figure 22). ART exposure (CHI_ART) was not associated with changes in abundance of such genera, relative to CHI_noART. Individuals with RHI<6 months were enriched in *Haemophilus* and *Veillonella* relative to NEG subjects. Finally, there was an enrichment in *Odoribacter* and depletion in *Dorea* in RHI>6 months relative to RHI<6 months (Figure 22).

Figure 22. Relative abundance of bacterial genera among groups at baseline.

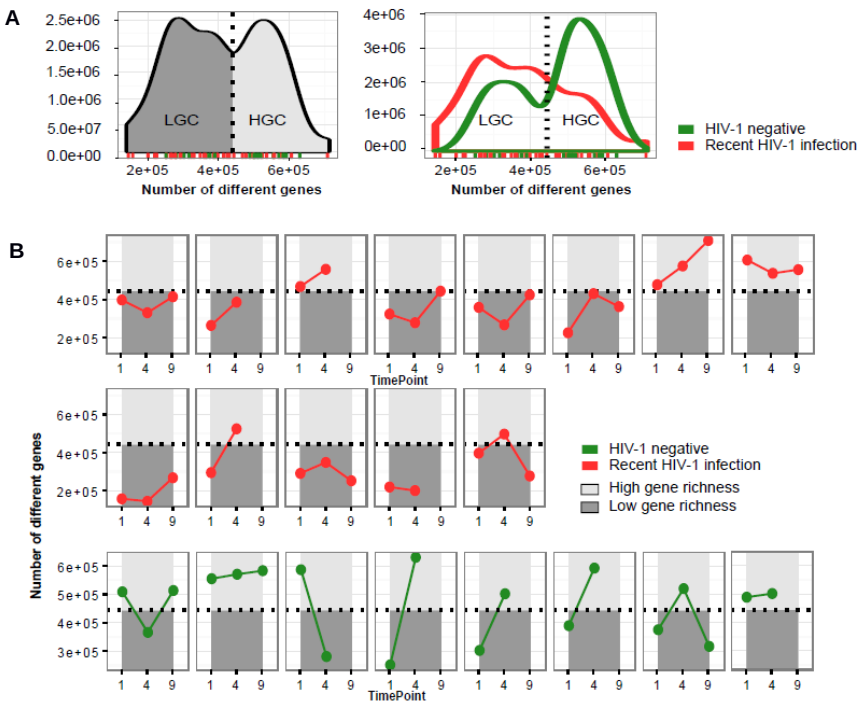


Box plots show median ±IQR relative abundance of individual bacterial genera. Bacterial genera named "unclassified" are identified by their closest taxonomic level identification. Only bacterial genera with a significantly different abundance between groups (Kruskal-Wallis p-value <0.05) are shown. Statistically significant post-hoc pairwise differences (Tukey post-hoc pairwise tests corrected for multiple comparison, FDR <0.05) are shown with asterisks.

4.2.5. HIV-1 infection is linked to reduced bacterial gene richness with potential microbial functional changes.

Like in previous publications from European subjects (Guillén et al., 2018)(Le Chatelier et al., 2013), gut microbial gene richness also followed a bimodal distribution in our Mozambican population (Figure 23A). Samples with gene counts above and below a gene richness threshold of 444.219 genes were categorized as high (HGC) and low gene counts (LGCs), respectively. Whereas 63% (22/35) of the RHI samples were included in the LGC category, only 37% (7/19) of NEG samples were LGC (Fisher's test p-value 0.003). At the first timepoint, 10/13 (76.9%) of RHI vs 4/8 (50%) of NEG subjects (Fisher's test p-value 0.172) were already LGC (Figure 23B). Eight/13 (61.5%) subjects from the RHI group but none of the NEG individuals showed low gene count values in all follow-up timepoints. Moreover, 5/8 HIV-negative subjects (62.5%) showed increases in gene richness during follow-up.

Figure 23. Microbial gene richness in RHI and NEG individuals using WGS.

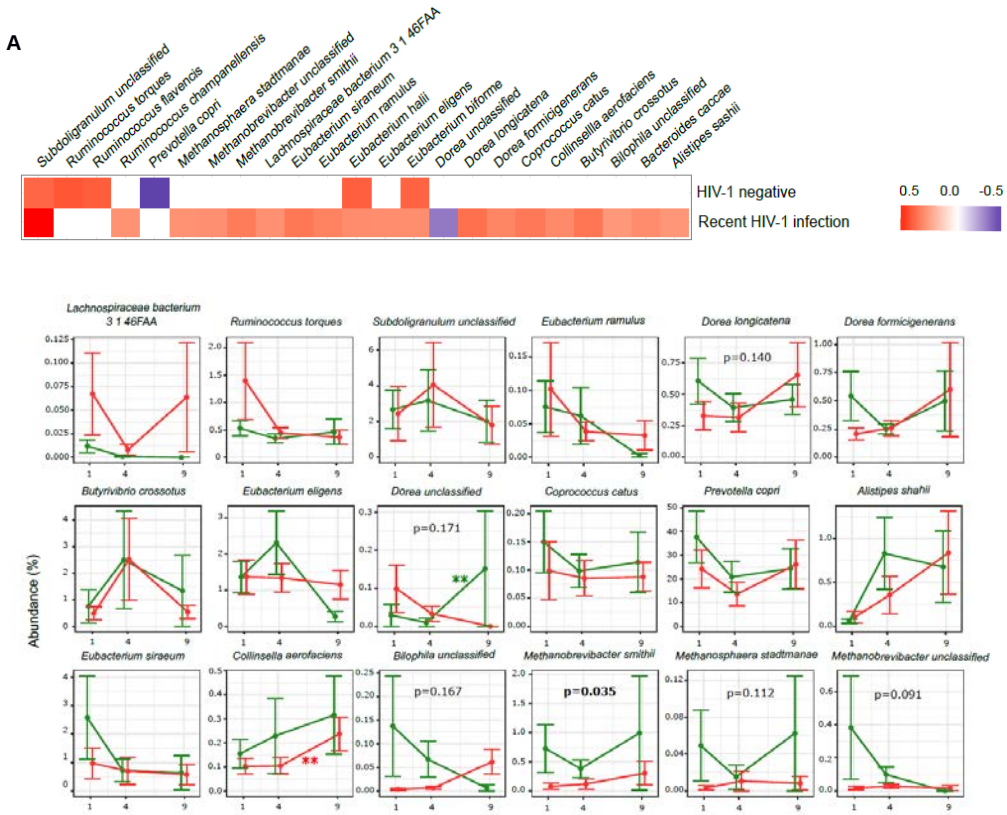


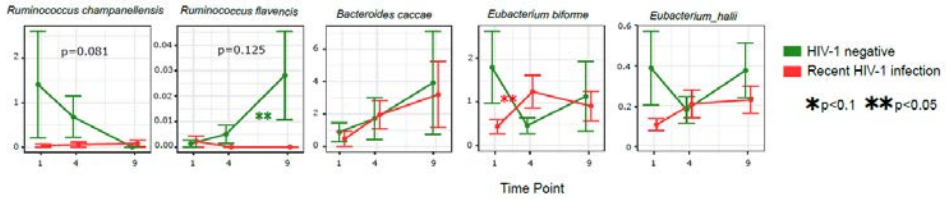
***A**, The leftmost density plot shows a bimodal distribution of all samples according to their observed gene richness value, which enables their classification into LGC and HGC. The rightmost density plot shows that HGCs are enriched in NEG, whereas RHI predominate in LGCs. **B**, longitudinal evolution of microbial gene*

richness in RHI (in red) and NEG (in green) individuals. The dark gray colored area represents the LGC zone, whereas light gray colored area represents the HGC zone.

In a longitudinal evaluation, several microbial species associated with gene richness were depleted in RHI compared NEG individuals (Figure 24A). In general, such depletion occurred already at the first time point, and in some cases (i.e., methanogenic archaea, *Ruminococcus champanellensis* and *Ruminococcus flavencis*) did not recover during the available follow-up. LMM only identified significant increases in *Eubacterium biforme* and *Collinsella aerofaciens* in RHI individuals and increases in *Dorea unclassified* and *Ruminococcus flavencis* in NEG subjects (Figure 24 B).

Figure 24. Evolution of richness-associated microbial species in RHI vs. NEG individuals using WGS.

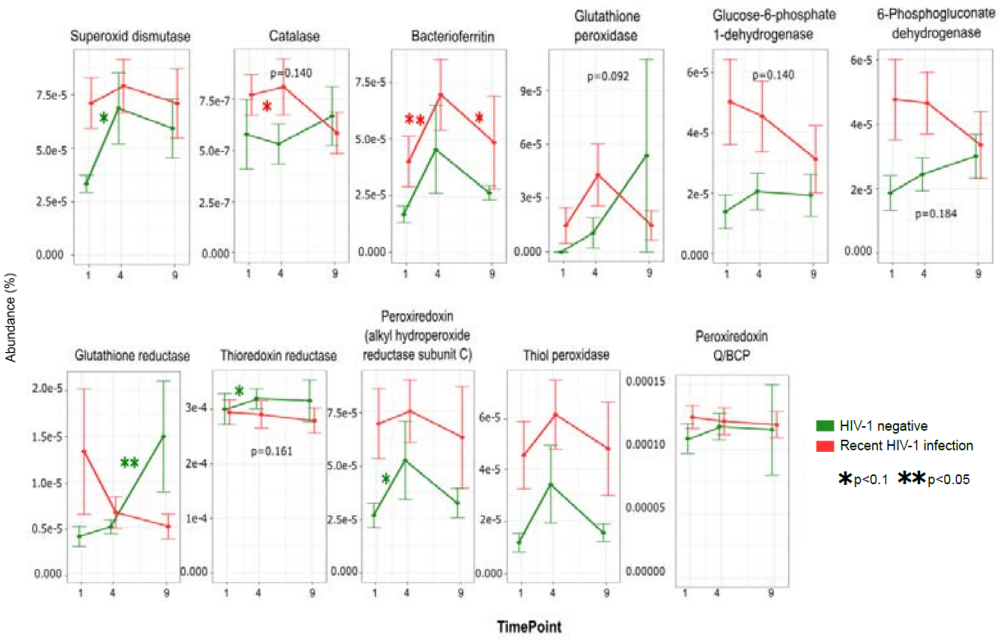




A, *Microbial species significantly (p<0.05) associated with microbial gene richness in both study groups. Color gradient shows Spearman's correlation Rho values (red is for positive, blue is for negative correlation).* **B,** *Relative abundance of richness-associated bacterial species over time (months 1, 4 and 9). P-values within each box compare month 1 to 4 area under the curves of RHI vs NEG individuals. Asterisks show slope values significantly different from 0 in LMM for each bacteria and study group.*

Consistent with previous reports (Guillén et al., 2018), subjects with RHI had higher counts of bacterial antioxidant enzymes including catalase, bacterioferritin, glucose-6-phosphate dehydrogenase or peroxiredoxin, although this shotgun analysis was underpowered to identify more granular differences (Figure 25).

Figure 25. Evolution of reactive oxygen species (ROS)-associated enzymes in recently HIV-1-infected vs. HIV-1-negative subjects.



Relative abundance of ROS-associated enzymes over time (months 1, 4 and 9). P-values within each box compare month 1 to 4 area under the curves of RHI vs. NEG subjects.

5. DISCUSSION

HIV-1 infection and the human gut microbiota richness and diversity

In agreement with previous groups reporting lower richness and diversity values in association to HIV-1 infection (Mutlu et al., 2014)(McHardy et al., 2013)(Nowak et al., 2015), results from the MetaHIV-Pheno study showed lower microbial richness and diversity in HIV-1 infected subjects compared to HIV-1 uninfected controls. More precisely, we found the lowest values in individuals with an immune-virological discordant response to ART. Individuals with an immune-virological concordant profile showed greater bacterial richness and diversity values than the previous although they did not reach those values from the HIV-1 negative control group. Individuals initiating ART during the first 6 months of HIV-1 infection, as well as ART-naïve individuals with ≥500 $CD4^+$ T-cells/mm^3, maintained similar bacterial richness and diversity values compared to the HIV-1 negative. These results support previous observations linking low bacterial richness with immune dysfunction as well as that early ART initiation might help to preserve gut microbial richness (Nowak et al., 2015). The first study analyzing the gut microbiota in association to HIV-1 infection in Mexico also reported lower microbial richness and diversity in either, long-term EFV- or PI-treated HIV-1 infected subjects (Pinto-Cardoso et al., 2017). Of note, subjects in this study started ART when their immune status dropped below <250 $CD4^+$ T-cell counts thus reinforcing that starting ART before severe immune deterioration might be crucial to maintain good bacterial richness and diversity. Monaco et al. described in a Sub-Saharan population that HIV-1 infection was associated with decreased richness and diversity and with viral-mediated decline of $CD4^+$ T-cells, but not with strong compositional differences in gut microbiota (Monaco et al., 2016). However, in our Mozambican study we did not find significant differences in richness and diversity between groups at baseline. Of note, one of the limitations of the MetaHIV-Acute study was that its design didn't include prospectively screening of HIV-1 negative subjects at risk of becoming HIV-1 infected. Thus, we missed the very early events occurring between HIV-1 infection and diagnosis, including early variations in microbiota richness, diversity and/or composition. Nonetheless, we detected significant increases in bacterial richness and diversity only during the first 6 and 4 months of follow-up in the RHI and NEG groups. Bearing this temporal limitation in mind, these

"increase—plateau" dynamics on microbial richness observed in RHI and NEG groups could be explained by a return to baseline values after a transient and unobserved early richness depletion due to an external insult. This hypothesis would then agree with the results observed by Monaco et al. linking HIV-1 infection to reduced microbial richness and diversity. In our study, the microbiota richness kinetics in RHI individuals mirrored that of $CD4^+$ T-cell counts and were inverse to the HIV-1-RNA kinetics, reinforcing the idea that our first follow-up sample might come after the initial peak of viremia typically observed in acute HIV-1 infection. However, the observation of similar microbiota dynamics in HIV-1-negative individuals suggested that such changes were not HIV-1-specific. Of note, most HIV-1 negative controls in our study were seeking medical care due to fever and/or mononucleosis-like symptoms when they were recruited into the study, and this possibly affected microbial richness as well other authors have previously reported (Xu et al., 2018).

The *Bacteroides – Prevotella* dominance in the human gut microbiota

Bacteroides to *Prevotella* shift in HIV-1 infection was not observed in any of our study cohorts. The MetaHIV-Pheno study showed that the *Prevotella*- and *Bacteroides*-dominated gut microbiota enterotypes were not linked to HIV-1 immune status but to sexual orientation (MSM vs non-MSM). Compared to non-MSM individuals, MSM individuals consistently harbored richer and more diverse microbial communities clearly dominated by genus *Prevotella*. This was, to our knowledge, the first evidence that sexual preference might have an impact on the gut microbiota composition (Noguera-Julian et al., 2016). Sexual orientation being a significant driver in the gut microbiota composition, suggested that results from previous reports describing *Bacteroides* to *Prevotella* replacement following HIV-1 infection (Mutlu et al., 2014)(Lozupone et al., 2013)(Dillon et al., 2014)(Vujkovic-Cvijin et al., 2013)(Vázquez-Castellanos et al., 2014), might be explained by enrichment of HIV-1 infected groups by MSM individuals related to HIV-1 negative control groups whose participants were mainly recruited from hospital research staff or college students and did not account for MSM factor. Since our findings in 2016, MSM-*Prevotella* association has been further supported by more recent publications (Armstrong et al., 2018)(Neff et al., 2018)(Kelley et al., 2017). Aiming to characterize the rectal mucosa immune environment and the microbiota composition in MSM individuals engaging in

condomless receptive anal intercourse (CRAI), Kelley et al. reported a distinct mucosal phenotype after unprotected anal intercourse including higher levels of mucosal $CD8^+$ T-cell proliferation, an increased pro-inflammatory cytokine production including TNFα and IFNγ, a distinct gut microbiota composition enriched for *Prevotellaceae* family members and a significantly higher expression of genes associated with cell proliferation, mucosal injury and repair, and neutrophil and Th17 cell activity (Kelley et al., 2017). Beyond enrichment of *Prevotella*, they also described enrichment of microbial metabolic pathways linked to arachidonic acid, a known intermediate of inflammatory responses. Interestingly, they hypothesized that *dysbiosis* linked to CRAI might involve increases in species able to metabolize products derived from mucosal injury. In April 2018, Neff et al. published interesting results on whether a gut microbiota with particularly pro-inflammatory bacteria, regardless of microbial translocation, was sufficient to drive high immune activation in individuals with HIV-1, including both MSM and non-MSM (Neff et al., 2018). They confirmed enrichment of *Prevotella* in the gut microbiota from MSM individuals independently of HIV-1 status. Remarkably, they reported that the gut microbiota from HIV-1 uninfected MSM individuals induced higher activation of monocytes compared to that from HIV-1 uninfected non-MSM subjects, suggesting that a MSM-associated gut microbiota might be pro-inflammatory itself, regardless of HIV-1 infection. These results agreed with previous reports where high levels of LPS in plasma of HIV-1 uninfected MSM individuals correlated with markers of innate immune activation including TNFα, IP10 and MCP-1 (Palmer et al., 2014). Meanwhile *Prevotella* has been linked to immune activation in diverse *ex vivo* studies (Vázquez-Castellanos et al., 2014)(Dillon et al., 2016b); members of the *Bacteroides* have reported anti-inflammatory properties through their stimulation of iNKT in the GALT which, in turn, increase secretion of IL-4 and IL-10 cytokines and are linked to less microbial translocation (Paquin-Proulx et al., 2017). Nonetheless, in our BCN0 cohort we did not find significant differences in immune activation, inflammation and/or microbial translocation between *Bacteroides*-rich and *Prevotella*-rich gut bacterial communities. The MetaHIV-Acute study was performed in a *Prevotella*-rich African population and we did not collect information on same-sex relationships from our Mozambican subjects. In addition, there is extensive data showing *Prevotella* predominance in

African populations (Ou et al., 2013)(De Filippo et al., 2010), so the effect of same-sex intercourse on the gut microbiota would have been hard to discern in this study.

Taxonomic changes on the gut microbiota composition linked to HVI-1 infection

Although we did not observe an HIV-1-specific gut microbiota composition in our European study cohorts (BCN and STK), we observed that the gut microbiota from recently HIV-1 infected Mozambicans experienced transient longitudinal changes after viral infection. Main changes included reductions in bacterial clusters associated with TNF-mediated innate immune responses and increases in certain butyrate-producing bacteria like *Odoribacter*. In the RHI group, weak reductions in *Enterobacteriaceae* family were also detected over the first 6 months of follow-up. Links between enrichment of *Proteobacteria* and higher levels of immune activation and inflammation have been reported both *in vitro* (Dillon et al., 2014) and *in vivo* in chronic HIV-1 infected individuals (Dinh et al., 2015)(Vujkovic-Cvijin et al., 2013)(Yang et al., 2016) and in NHPM with acute SIV infection (Glavan et al., 2016). This observation agreed with the abovementioned hypothesis that we started our follow-up in Mozambique when subject's gut microbiota was returning to baseline values after a transient *dysbiosis.* Again, such dynamics were not clear enough to be considered HIV-1-specific when also assessed in the NEG group.

The only statistically significant association between the gut microbiota and HIV-1 infection in our sub-Saharan study population was a reduction in *Akkermansia*, *Anaerovibrio*, *Bifidobacterium*, and *Clostridium* in CHI_noART individuals compared to NEG individuals. Previous reports already reported depletions in *Bifidobacterium*, and *Clostridium* in HIV-1 infected subjects (McHardy et al., 2013)(Dillon et al., 2014)(Vujkovic-Cvijin et al., 2013) so our observations support the hypothesis that reductions in these genera could contribute to chronic inflammation commonly observed in HIV-1-infected subjects. *Akkermansia* has a key role in human fat and sugar metabolism (Schneeberger et al., 2015) and promotes $CD8^+$ T-cell-mediated immune responses to anti-cancer immune modulators like PD-1 and CTLA-4 agonists (Routy et al., 2018). Defects in *Akkermansia* might thus be related to the HIV-1-associated metabolic disorders as well as to $CD8^+$ T-cell exhaustion. In our study, *Akkermansia* and *Clostridiaceae* abundance was negatively correlated with markers

of microbial translocation (sCD14), inflammation (IP10) and gut damage (fecal calprotectin). Interestingly, ART initiation was not associated with improved abundance of such microbes. Pinto-Cardoso et al. also reported lower relative abundance of key bacterial players in maintaining gut homeostasis in HIV-1-infected individuals on long-term ART compared to HIV-1 uninfected controls (Pinto-Cardoso et al., 2017). Their commensal-bacteria depletion was led by *Faecalibacterium prausnitzii* although correlation with other members of order *Clostridiales* such as family *Ruminococcaceae* were also reported. Significantly, relative abundance of such bacteria were negatively correlated to IFABP which is released into circulation after mucosal tissue damage (Funaoka et al., 2010). Nonetheless they did not find correlation between any individual taxa and sCD14, $CD4^+$ and $CD8^+$ T-cell activation, D-dimer or high sensitivity CRP protein. Limited by our MetaHIV-Acute study follow-up, we could not properly assess dynamics on these bacterial genera after acute HIV-1 infection, so we couldn't describe the exact timing of their depletion. Similarly, it would have been interesting to assess changes in their relative abundance in immune-deteriorated subjects initiating ART. In our European cross-sectional dataset, plasma levels markers of enterocyte damage, microbial translocation and systemic inflammation followed an overall predictable response across the different HIV-1 phenotypes tested with the highest levels corresponding to individuals with an immune-virological discordant profile and the late-presenters (Noguera-Julian et al., 2016). We found slight positive correlations between viral load and IL-6 (Spearman's rho=0.26, p-value=0.002) and IP10 (Spearman's rho=0.21, p=value=0.015), and negative correlations between $CD4^+$ T-cell counts and IL-6 (Spearman's rho=-0.19, p=value=0.026) and LBP (Spearman's rho=-0.23, p=value=0.0086). Nowak et al. reported that the gut microbiota composition might be linked to HIV-1 viral load since HIV-1 infected subjects with an elite controller phenotype (i.e. immune competent individuals with suppressed viral load in absence of ART) had a distinct gut microbiota that those with a viremic controller phenotype (i.e. immune competent individuals with viral load <2000 copies/ml in absence of ART) and closer to that of HIV-1 negative subjects (Nowak et al., 2015).

Other factors shaping the human gut microbiota composition

Non-HIV-1 related factors have also been reported to affect the gut microbiota. Previous studies reported that the gut microbiota bacterial composition was linked to genetic (Goodrich et al., 2014), environmental and lifestyle factors (Modi et al., 2014)(Wu et al., 2011)(Clarke et al., 2014)(David et al., 2014). Although some studies reported that *Prevotella* was linked to carbohydrate-rich diets and *Bacteroides* to fat and animal protein-rich diets (Wu et al., 2011), this was not observed in our BCN0 cohort, as we found diet as having a marginal effect on the gut microbiota composition (Noguera-Julian et al., 2016). But, we should mention that extreme diets were excluded in our BCN0 cohort since exclusion criteria included any gross dietary deviation from a regular diet or any specific regular diet such as vegetarian and low-carb. Still, Pinto-Cardoso et al. also reported no correlation between gut microbiota composition and dietary measurements in their Mexican cohort, thus reinforcing the hypothesis that other factors beyond diet might be driving gut microbiota overall composition (Pinto-Cardoso et al., 2017).

Since the original aim of the MetaHIV-Pheno study was not to elucidate the mechanisms influencing the gut microbiota composition in relation to sexual orientation, there are other many potential factors that were not studied such as if enrichment of *Prevotella* in MSM is comparable to heterosexual women engaging receptive anal intercourse, its influence on transmission of HIV-1 and/or the existence of person-to-person transmission of commensal bacteria.

Changes on the gut viral communities in relation to HIV-1 infection

Importantly, beyond bacterial changes in the gut microbiota, our Mozambican cohort showed increased Adenovirus fecal shedding in all HIV-1 infected groups compared to the NEG control group. This result is consistent with previous studies in NHPM where SIV infection was associated with expansion of the enteric virome with minimal changes in the bacteriome (Handley et al., 2012)(Handley et al., 2016). Using RT-PCR we observed that fecal Adenovirus shedding was detectable not only in more than half of RHI subjects, but also in subjects with chronic HIV-1 infection, regardless of ART-administration. These results are interesting because: 1) this Adenovirus expansion seems to occur already in hyper-acute phases of HIV-1 infection, and 2) it is persistent in ART-treated subjects with good $CD4^+$ T-cell recovery and undetectable

HIV-1 viral load. Similar findings were reported in Ugandan subjects infected by HIV-1, where an Adenovirus-dominated viral expansion was linked to $CD4^+$ T-cell counts below 200 cells/mm^3 (Monaco et al., 2016). Contrary to Adenovirus, fecal Cytomegalovirus and Enterovirus shedding was mostly observed in CHI_noART individuals, suggesting that their expansion in the human gut and fecal shedding might require more prolonged immune dysregulation. Of note, we did not detect any of the tested Human Herpesvirus in our Mozambican dataset so its link to human gut microbiota could not be assessed. Precise role of bacteriophage gut communities in HIV-1 infection is still unclear. So far, Monaco et al. did not find a link between phage richness and diversity and $CD4^+$ T-cell counts, HIV-1 infection, type of ART or bacterial richness and diversity (Monaco et al., 2016). Our RT-PCR based virome approach was not intended to detect nor quantify bacteriophages in our fecal samples, so their role could not be discussed in our studies.

Gut microbiota gene richness and changes on its functional potential in HIV-1 infection

More sensitive WGS analysis on the MetaHIV-Acute study showed that RHI individuals had lower bacterial gene richness compared to the NEG controls. This observation agreed with other studies linking low gene richness to gut-associated alterations (Le Chatelier et al., 2013)(Guillén et al., 2018). Even though this reduction in gene richness would occur alongside the hypothetic reduction in bacterial taxonomic richness and diversity before first sampling at month 1, their evolution over time seemed to differ. While we detected significant longitudinal increases in bacterial richness and taxonomy in both RHI and NEG individuals over follow-up, reductions in gene richness seemed to resolve earlier in NEG than in RHI. This difference in evolution between groups, might be explained by a chronicity-effect linked to HIV-1 infection that was absent in the external non-HIV-1 perturbations from NEG group. Most bacterial species that were positively correlated to bacterial gene richness were depleted in RHI individuals compared NEG individuals, both, at month 1 and over the first 4 months of follow-up. Significantly, species depleted in RHI have been already reported to be linked to HIV-1 infection and to RNS/ROS-oxidative responses to external insults in previous European cohorts (Guillén et al., 2018).

To summarize, our study in Barcelona and Stockholm showed a strong association between fecal microbiota composition and sexual orientation and that HIV-1 infection was only linked to reduced gut microbiota richness and diversity. The mechanisms behind the *Prevotella*-enriched gut bacterial communities in MSM individuals have not been elucidated yet and are beyond the scope of this thesis. Thus, complete assessments on socioeconomic status as well as complete and detailed assessment of sexual practices are needed to understand the role of sexual orientation on the gut microbial communities. Results from our Mozambican study led to hypothesize that HIV-1 infection might negatively impact the gut microbiota in hyper-acute phases of the infection (i.e. at least before the first month after diagnosis) and that the HIV-1 signature does not appear until chronic phases of the infection. Additionally, observation of fecal viral shedding in HIV-1 infected subjects, already in recent infection, highlighted the importance of studying other microbial communities beyond bacteria in the loss of gut homeostasis.

6. CONCLUSIONS

1. The fecal microbiota of gay men in two European cohorts from Barcelona and Stockholm was richer and more diverse and had a *Prevotella*-enriched bacterial composition compared to heterosexual individuals.
2. Some factor associated with sexual orientation was an important confounding factor in HIV-1 - gut microbiome studies.
3. HIV-1 infection was significantly and independently associated to lower bacterial richness and diversity in European cohorts, more so in subjects with an immune-virological discordant phenotype.
4. Recent HIV-1 infection in Mozambique was followed by increased fecal Adenovirus shedding and by transient, non-HIV-1 specific, changes in the gut bacteriome.
5. An HIV-1 specific signature previously associated with chronic inflammation, $CD8^+$ T-cell anergy and metabolic disorders including depletion of *Akkermansia*, *Anaerovibrio*, *Bifidobacterium*, and *Clostridium* was observed in the gut microbiota of chronically HIV-1 infected subjects in Mozambique.

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8. APPENDIX

Marc Noguera-Julian[1], Muntsa Rocafort[1], Yolanda Guillén, Javier Rivera, Maria Casadellà, Piotr Nowak, Falk Hildebrand, Georg Zeller, Mariona Parera, Rocío Bellido, Cristina Rodríguez, Jorge Carrillo, Beatriz Mothe, Josep Coll, Isabel Bravo, Carla Estany, Cristina Herrero, Jorge Saz, Guillem Sirera, Ariadna Torrela, Jordi Navarro, Manel Crespo, Christian Brander, Eugènia Negredo, Julià Blanco, Francisco Guarner, Maria Luz Calle, Peer Bork, Anders Sönnerborg, Bonaventura Clotet, Roger Paredes. 2016. **Gut Microbiota Linked to Sexual Preference and HIV Infection**. EBioMedicine, Volume 5, Pages 135:146.

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Muntsa Rocafort, Marc Noguera-Julian, Javier Rivera, Lucía Pastor, Yolanda Guillén, Jost Langhorst, Mariona Parera, Inacio Mandomando, Jorge Carrillo, Víctor Urrea, Cristina Rodríguez, Maria Casadellà, Maria Luz Calle, Bonaventura Clotet, Julià Blanco, Denise Naniche and Roger Paredes. **Evolution of the Gut Microbiome Following Acute HIV-1 Infection**. *Submitted*

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